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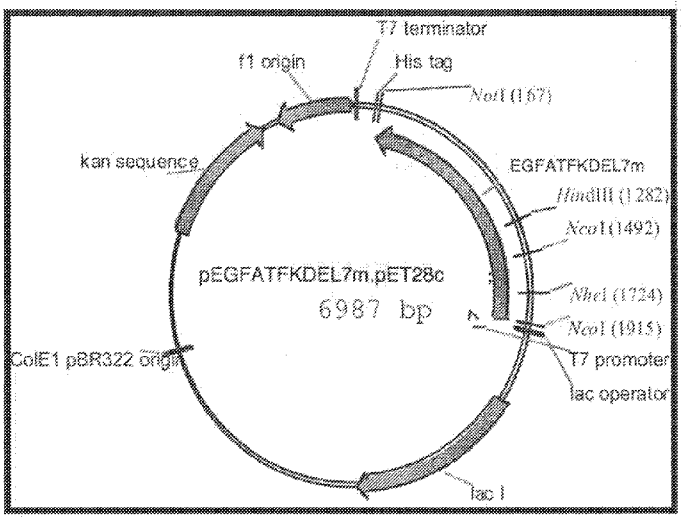
(54) Title: BISPECIFIC TARGETING REAGENTS

Fig. 1A – 1B

A.

B.



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(57) Abstract: The present disclosure features, inter alia, receptor-targeting reagents (e.g., toxic receptor-targeting reagents), which are useful in, e.g., methods of binding a receptor-targeting reagent to a cell and methods for treating a variety of disorders such as, but not limited to, cancers. Also featured are methods, compositions, and kits useful for selecting an appropriate treatment modality for a subject (e.g., a subject with a cancer) and/or treating a variety of disorders such as cell proliferative disorders.

BISPECIFIC TARGETING REAGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of priority of U.S. application serial No. 61/376,623, filed August 24, 2010 and U.S. application serial No. 61/417,385 filed November 26, 2010, which applications are herein incorporated by reference.

U.S. GOVERNMENT RIGHTS

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BACKGROUND OF THE INVENTION

Cancer is a leading cause of death in Western cultures and is generally characterized by excessive, uncontrolled cell proliferation in defiance of normal restraints on cell growth. These cancer cells can invade and colonize (metastasize) territories normally reserved for other cells.

Glioblastoma multiforme (GBM) is a common type of primary brain tumor in humans and is a very aggressive and devastating cancer, with a median survival of approximately one year. (Eramo et al., Chemotherapy resistance of glioblastoma stem cells, *Cell Death and Differentiation* (2006) 13, 1238-1241). Glioblastoma has the worst prognosis of any central nervous system malignancy. Therapy for GBM is difficult due to its biological location in the brain. Current treatments can involve chemotherapy, radiation, radiosurgery, corticosteroids, antiangiogenic therapy, and surgery. Despite the development of new surgical and radiation techniques and the use of multiple antineoplastic drugs, a cure for malignant gliomas does not exist. (Eramo et al., Chemotherapy resistance of glioblastoma stem cells, *Cell Death and Differentiation* (2006) 13, 1238-1241). Glioblastoma cells are resistant to cytotoxic agents, and the high incidence of recurrence in a very short period of time in glioblastoma patients suggests that tumorigenic cells are capable of overtaking the treatments.

Modes of cancer therapy include chemotherapy, surgery, radiation, and combinations of these treatments. While many anti-cancer agents have been developed, there remains a need for more effective therapies.

Targeted toxins are molecules that contain targeting domains that direct the molecules to target cells of interest (*e.g.*, cancer cells or immune cells mediating an inflammatory disorder) and toxic domains that inhibit the proliferation of (or kill) the target cells.

SUMMARY OF THE INVENTION

The present disclosure details the surprising discovery that bispecific, receptor-targeting reagents linked to a toxin domain were much more effective at killing cancer cells and reducing tumor burden of mice than their monospecific receptor-targeting reagent counterparts. The bispecific reagents comprise an epidermal growth factor receptor (EGFR)-binding domain and urokinase plasminogen activator receptor (uPAR)-binding domain. Thus, the bispecific receptor-targeting reagents described herein are useful, inter alia, in methods of treating a variety of proliferative disorders including cancer.

The disclosure also details the surprising discovery that the toxicity of receptor-targeting reagents administered to a subject can be decreased by mutating the toxin domain.

In some embodiments of any of the receptor-targeting reagents described herein, the uPAR-binding agent can contain, or consist of, an antibody that binds to uPA or an antigen-binding fragment thereof or a uPA polypeptide or a uPA-binding fragment thereof, such as an amino acid terminal fragment-binding domain (ATF) of uPA. In certain embodiments, the uPAR-binding agent is 135 amino acids of the amino acid terminal fragment of uPA. In certain embodiments, the uPAR-binding agent is SEQ ID NO:1 or SEQ ID NO:7. In certain embodiments, the ATF binds with high affinity to uPAR (Kd = 10^{-9} to 10^{-11}). Cubellis MV, Nolli ML, Cassani G, Blasi F. Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. J Biol Chem 1986;261:15819–22. Ellis V, Behrendt N, Dano K. Plasminogen activation by receptorbound urokinase. J Biol Chem 1991;266:12752–8.

In one aspect, the disclosure features a receptor-targeting reagent comprising: (a) a first targeting domain comprising an epidermal growth factor receptor (EGFR)-binding agent and (b) a second targeting domain comprising an ATF-binding agent, and (c) a toxin domain, wherein (a), (b) and (c) are operably linked into one molecule.

In some embodiments of any of the receptor-targeting reagents described herein, the EGFR-binding agent can contain, or consist of, an antibody that binds to the EGFR or an antigen-binding fragment thereof or an EGF polypeptide or an EGFR-binding fragment thereof.

In some embodiments of any of the receptor-targeting reagents described herein, the EGFR-binding agent can contain, or consist of: (i) an antibody that binds to the EGFR or an antigen-binding fragment thereof; (ii) an epidermal growth factor polypeptide or an EGFR-binding fragment thereof; (iii) a betacellulin polypeptide or an EGFR-binding fragment thereof; (iv) a transforming growth factor alpha polypeptide or EGFR-binding fragment thereof; (v) an amphiregulin polypeptide or EGFR-binding fragment thereof; (vi) an epiregulin polypeptide or EGFR-binding fragment thereof; or (vii) a heparin-binding EGF polypeptide or EGFR-binding fragment thereof. The antibody (*e.g.*, the antibody that binds to an EGFR) or antigen-binding

fragment can be, *e.g.*, a monoclonal antibody, a polyclonal antibody, a humanized antibody, a fully human antibody, a single chain antibody, a chimeric antibody, an F_{ab} fragment, an $F_{(ab')2}$ fragment, an F_{ab}' fragment, an F_v fragment, or an scF_v fragment. In some embodiments, (a) and (b) can be bound to each other by a covalent bond or a non-covalent bond. In some embodiments, (a) and (b) can be bound to each other by a first and second member of a binding pair. The binding pair can be streptavidin (or avidin) and biotin. In some embodiments, the receptor-targeting reagent can contain, or consist of, a fusion protein comprising (a) and (b).

In some embodiments, the receptor-targeting reagents can further comprise a toxic domain. The toxic domain can contain, or consist of, a small molecule, a radiological agent, and/or a toxic polypeptide. The toxic polypeptide can consist of, or contain, a Diphtheria toxin or a biologically active fragment thereof. The toxic polypeptide can consist of, or contain, SEQ ID NO:9 or SEQ ID NO: 10. The toxic polypeptide can consist of, or contain, a Pseudomonas exotoxin A or a biologically active fragment thereof. The toxic polypeptide can contain, or consist of, SEQ ID NO: 11 or SEQ ID NO: 12. The toxic polypeptide can be, *e.g.*, a Pseudomonas exotoxin (PE), bryodin, gelonin, α- sarcin, aspergillin, restrictocin, angiogenin, saporin, abrin, a prokaryotic ribonuclease, a eukaryotic ribonuclease, ricin, pokeweed antiviral protein (PAP), a pro-apoptotic polypeptide, a ribosomal inhibitory protein, or a biologically active fragment of any of the foregoing. The pro-apoptotic polypeptide can be, *e.g.*, Bax, Fas, Bad, Bak, Bim, Bik, Bok, Hrk, FasL, TRAIL, or TNF-alpha.

In certain embodiments, the immunogenicity of the toxic polypeptide is reduced by at least 60-90%, such as by 80%.

In some embodiments, the receptor-targeting reagents can contain a toxic domain that has been modified to reduce or prevent immunogenicity of the polypeptide in a subject. For example, the receptor-targeting reagent can contain a toxic polypeptide with the amino acid sequence depicted in SEQ ID NO: 11, wherein (i) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 432 is glycine, the arginine at position 467 is alanine, and the lysine at position 590 is serine; (ii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 332 is serine, and the arginine at position 313 is alanine; (iii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, and the arginine at position 313 is alanine; or (iv) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is

glycine, the glutamine at position 332 is a serine, and the arginine at position 313 is alanine. It is understood that a receptor-targeting reagent can contain fragment of the toxic polypeptide having the amino acid sequence of SEQ ID NO: 11 and the substitutions set forth in any one of (i)-(iv). For example, a receptor-targeting reagent can contain a toxic polypeptide having an amino acid sequence of amino acids 276-633 of SEQ ID NO:11, wherein the sequence contains the substitutions set forth in any of (i)-(iv).

In certain embodiments, the toxic polypeptide has an amino acid sequence of amino acids 276-633 of SEQ ID NO: 11 and amino acids KDEL (SEQ ID NO:15) at positions 358-361.

In some embodiments, (a), (b), or (a) and (b) can be bound to the toxic domain by a non-covalent bond or a covalent bond. The receptor-targeting reagent can contain a fusion protein comprising: the toxic domain and (a), (b), or (a) and (b). (a) and/or (b) can be bound to the toxic domain in any configuration described herein. In some embodiments, the receptor-targeting reagent can further contain one or more linker moieties. At least one of the one or more linker moieties can be a peptide linker. The peptide linker can contain, or consist of, *e.g.*, hma (SEQ ID NO:13), EASGGPE (SEQ ID NO:14), or any other linker peptides described herein.

In some embodiments, the receptor-targeting reagents can contain, or consist of, any one of SEQ ID NOS:1-17. In some embodiments, the receptor-targeting reagents can contain, or consist of, any one of SEQ ID NO: 17 or SEQ ID NO:18. Table 1 provides sequences as used herein.

Table 1

Seq ID No.	Description	Sequence
1	ATF	SNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKSKTCYEGNGHFYRGK ASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGLKP LVQECMVHDCADGK
2	DTEGFATF	MGADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYSTDNKY DAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPLMEQVGTE EFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEINFETRGKRGQDAMYEYMA QACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQY LEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILP GIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESHNLFQVVH NSYNRPAYSPGHKTQPFEASGGPENSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYI GERCQYRDLKWWELRPSGQAGAAASESLFVSNHAY SNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKSKTCYEGNGHFYRGK ASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGLKP LVQECMVHDCADGK
3	EGFATF	NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELRPSGQAGA AASESLFVSNHAY SNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKSKTCYEGNGHFYRGK ASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGLKP LVQECMVHDCADGK
4	ARL aggregation reducing linker	GSTSGSGKPGSGEGSTKG
5	polyGGGGS (SEQ ID NO: 21) sequence	GGGGSGGGGSGGGGS
6	EGF	NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR
7	full-length uPAR	MGHPPLLPLL LLLHTCVPAS WGLRCMQCKT NGDCRVEECA LGQDLCRTTI VRLWEEGEEL ELVEKSCTHS EKTNRTLSYR TGLKITSLTE VVCGLDLCNQ GNSGRAVTYS RSRYLECISC

		GSSDMSCERG RHQSLQCRSP EEQCLDVVTH WIQEGEEGRP KDDRHLRGCG YLPGCPGSNG FHNNDTFHFL KCCNTTKCNE GPILELENLP QNGRQCYSCK GNSTHGCSSE ETFLIDCRGP MNQCLVATGT HEPKNQSYMV RGCATASMCQ HAHLGDAFSM NHIDVSCCTK SGCNHPDLDVQYRSGAAPQP GPAHLSLTIT LLMTARLWGG TLLWT
8	DT leader sequence	MLVRGYVVSRKLFASILIGALLGIGAPPSAHA
9	full length DT	MLVRGYVVSRKLFASILIGALLGIGAPPSAHAGADDVVDSSKSFVMENFSSYHGTKPGYVDSI QKGIQKPKSGTQGNYDDDWKGFYSTDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKV LALKVDNAETIKKELGLSLTEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWE QAKALSVELEINFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKT KIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANY AAWAVNVAQVIDSETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVA QAIPLVGELVDIGFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTQPFLHDGYAVSWNTVEDS IIRTGFQGESGHDIKITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTF CRPKSPVYVGNGVHANLHVAFHRSSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSLFFEIK S
10	variant DT	MGADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYSTDNKY DAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPLMEQVGTE EFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEINFETRGKRGQDAMYEYMA QACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQY LEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILP GIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESIINLFQVVH NS YNRPAYSPGHKTQPF
11	full length PE	MHLIPHWIPLVASLGLLAGGSSASAAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPAIADT NGQGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQARGSWSL NWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFFVRAHESNEMQ PTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNLDDTWEG KIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLE QCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAES ERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVS FSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGF YIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIG HPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALP

		DYASQPGKPPREDLK
12	truncated PE	PEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRL V AL YLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDE AGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVE RLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYA QDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPE EEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPKDE L
13	hma	PSGQAGAAASESLFVSNHAY
14	EASGGPE linker	EASGGPE
15	KDEL	KDEL
16	signal polypeptide	MAISGVPVLGFFIIAVLMSAQESWA
17	EGFATFKDEL	MENSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELRPSGQA GAAASESLFVSNHAY SNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKSKTCYEGNGHFYRGK ASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGLKP LVQECMVHDCADGK EASGGPEPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLS WNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANA DVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAH RQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPD ARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRL ETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPKDEL
18	EGFATFKDEL 7mut	MENSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELRPSGQA GAAASESLFVSNHAYSNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKS KTCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDN RRRPWCYVQVGLKPLVQECMVHDCADGK EASGGPEPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLS WNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANA DVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAH RQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPD ARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRL ETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPKDEL
19	ATFKDEL	MASNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDKSKTCYEGNGHFYR

	GKASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGL KPLVQECMVHDCADGKEASGGPEPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCG YPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERF VRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFST RGTQNWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIA GDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPL PLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDY ASQPGKPPKDEL
20	EGFKDEL MANSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELREASGG PEPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANADVVSLT CPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEER GYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIR NGALLRVYVPRSSL PGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPT DPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPKDEL

In some embodiments, the receptor-targeting reagent can contain one or more detectable labels.

In some embodiments, the receptor-targeting reagents can bind to a cell. The cell can be, *e.g.*, a mammalian cell such as a human cell. The cell can express an uPAR and/or an EGFR. In another aspect, the disclosure features a pharmaceutical composition containing any of the receptor-targeting reagents described above and a pharmaceutically acceptable carrier.

In another aspect, the disclosure provides a nucleic acid encoding any of the polypeptides described herein (*e.g.*, any one of SEQ ID NOS: 1-18).

In another aspect, the disclosure provides a nucleic acid encoding a fusion protein comprising any of the polypeptide receptor-targeting reagents described above. For example, the nucleic acid can encode a fusion protein containing, or consisting of, any one of SEQ ID NOS: 1-18. In another example, the nucleic acid can encode a fusion protein containing, or consisting of, SEQ ID NO: 18.

In yet another aspect, the disclosure features: (i) a vector containing any of the nucleic acids described above; (ii) an expression vector containing any of the nucleic acids described above; and/or (iii) a cell containing the expression vector of (ii).

In another aspect, the disclosure provides a method of producing a fusion protein. The method includes the step of culturing a cell comprising the expression vector described immediately above under conditions suitable for expression of the fusion protein. The method can also include the step of isolating the protein from the cells or the culture medium in which the cells are cultured.

In another aspect, the disclosure features a polypeptide encoded by any of the nucleic acids described above.

In yet another aspect, the disclosure features an *in vitro* method for binding a receptor-targeting reagent to a cell, which method includes the step of contacting a cell with any of the receptor-targeting reagents described above. The method can also include the step of determining whether the cell expresses an EGFR or an uPAR. The cell can express an EGFR, and/or a uPAR.

In yet another aspect, the disclosure features an *in vitro* method for binding a receptor-targeting reagent to a cell, which method includes the step of contacting a cell with any of the receptor-targeting reagents described above. The method can also include the step of determining whether the cell expresses an EGFR and/or a uPAR. The cell can express an EGFR and/or a uPAR.

In some embodiments of any of the methods described herein, the cell can be a cancer cell such as, but not limited to, a lung cancer cell, a breast cancer cell, a colon cancer cell, a pancreatic cancer cell, a renal cancer cell, a stomach cancer cell, a liver cancer cell, a bone cancer cell, a hematological cancer cell, a neural tissue cancer cell, a melanoma cell, a thyroid cancer cell, a glioblastoma cell, an ovarian cancer cell, a testicular cancer cell, a prostate cancer cell, a cervical cancer cell, a vaginal cancer cell, a head and neck cancer cell, and a bladder cancer cell. The cell can be a carcinoma cell or a sarcoma cell (*e.g.,* an angiosarcoma cell). The cell can be a cancer stem cell. The cell can be an immune cell such as a T cell or a B cell. The cell can be, *e.g.*, a mammalian cell such as a human cell.

In yet another aspect, the disclosure features an *in vivo* method for binding a receptor-targeting reagent to a cell. The method includes the step of delivering to a subject any of the receptor-targeting reagents described above. The method can also include the step of determining whether the subject has a cancer cell. The cancer cell can be, *e.g.*, a lung cancer cell, a breast cancer cell, a colon cancer cell, a pancreatic cancer cell, a renal cancer cell, a stomach cancer cell, a liver cancer cell, a bone cancer cell, a hematological cancer cell, a neural tissue cancer cell, a melanoma cell, a thyroid cancer cell, an ovarian cancer cell, a testicular cancer cell, a prostate cancer cell, a cervical cancer cell, a glioblastoma cell, a vaginal cancer cell, a head and neck cancer cell, or a bladder cancer cell. The cancer cell can be a carcinoma cell or sarcoma cell (*e g.*, angiosarcoma). The cell can be a cancer stem cell. The cell can also be an immune cell such as a T cell or a B cell. The subject can be a mammal such as a human. The subject can be one who has, is suspected of having, or at risk of developing, an inflammatory condition such as any of those described herein. The method can also include the step of determining if one or more one or more cells of the subject's cancer express an EGFR and/or a uPAR. The method can further include the step of determining whether the receptor-targeting reagent bound to the cell or whether the receptor-targeting reagent killed a cell (or inhibited the proliferation of the cell). In embodiments where the subject is one having, suspected of having, or at risk of developing an inflammatory condition, the method can be a method of treating an inflammatory condition.

In another aspect, the disclosure features an *in vitro* method for inhibiting the growth of a cell. The method includes the step of contacting a cell with any of the receptor-targeting reagents described above, wherein contacting the cell with the receptor-targeting reagent inhibits the growth of the cell. In some embodiments, the receptor-targeting reagent can kill a cell, thus the method can be an *in vitro* method for killing a cell. The cell can be a cancer cell such as any of those described above.

In yet another aspect, the disclosure provides an *in vivo* method for treating a cancer in a subject, which method includes the step of delivering to a subject having, suspected of having, or at risk of developing, a cancer any of the receptor-targeting reagents described above. The receptor-targeting reagent can be toxic (*i.e.*, contain at least one toxic domain). Delivering can include administering the receptor-targeting reagent to the subject, *e.g.*, intravenously and/or through the use of a systemic pump.

In yet another aspect, the disclosure features a method for selecting a therapeutic agent for a mammal with cancer. The method includes the steps of: determining if one or more cancer cells of a cancer in a mammal express an uPAR or an EGFR; and if one or more of the cancer cells express an uPAR or an EGFR, selecting as a therapeutic agent for the mammal any of the receptor-targeting reagents described above (*e.g.*, any of the toxin-receptor-targeting reagents described above). In some embodiments, the method can also include the step of after determining that one or more of the cells of the cancer express an uPAR or an EGFR, delivering to the subject the selected receptor-targeting reagent.

In yet another aspect, the disclosure features a method for selecting a therapeutic agent for a mammal with cancer. The method includes the steps of: determining if one or more cancer cells of a cancer in a mammal express an uPAR or an EGFR; and if one or more of the cancer cells express an uPAR or an EGFR, selecting as a therapeutic agent for the mammal any of the receptor-targeting reagents described above (*e.g.*, any of the toxic receptor-targeting reagents described above). In some embodiments, the method can also include the step of after determining that one or more of the cells of the cancer express an uPAR or an EGFR, delivering to the subject the selected receptor-targeting reagent. In yet another aspect, the disclosure features a method for selecting a therapeutic agent for a mammal with cancer. The method includes the steps of: determining if one or more cancer cells of a cancer in a mammal express an uPAR or an EGFR; and if one or more of the cancer cells express an uPAR or an EGFR, selecting as a therapeutic agent for the mammal any of the receptor-targeting reagents described above (*e.g.*, any of the toxic receptor-targeting reagents described above). In some embodiments, the method can also include the step of after determining that one or more of the cells of the cancer express an uPAR or an EGFR, delivering to the subject the selected receptor-targeting reagent.

In another aspect, the disclosure features a method for selecting a therapeutic agent for a mammal with cancer, which method includes the step of selecting as a therapeutic agent for a mammal with cancer any of the toxic receptor-targeting reagents described above if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR. The method can also include

the step of determining if one or more of the cancer cells in a mammal express an uPAR or an EGFR.

In another aspect, the disclosure features a method for selecting a therapeutic agent for a mammal with cancer, which method includes the step of selecting as a therapeutic agent for a mammal with cancer any of the toxic receptor-targeting reagents described above if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR. The method can also include the step of determining if one or more of the cancer cells in a mammal express an uPAR or an EGFR.

In some embodiments of any of the *in vivo* methods described above, the subject can be, *e.g.*, a mammal such a human. In some embodiments, any of the *in vivo* methods described above can include the step of determining if the subject has, or is at risk of developing, a cancer. The methods can also include the step of determining if one or more cancer cells of the subject's cancer express an uPAR and/or an EGFR. The *in vivo* methods can also include the step of: (i) determining whether the receptor-targeting reagent bound to the cell (*e.g.*, a cell expressing an EGFR, an uPAR such as a cancer cell or an immune cell); and/or (ii) determining if the receptor-targeting reagent inhibited the proliferation of the cell or killed the cell to which it bound.

In yet another aspect, the disclosure features a kit comprising: any of the receptor-targeting reagents described above; and instructions for administering the receptor-targeting reagent. The kit can also include one or more pharmaceutically acceptable carriers and/or a pharmaceutically acceptable diluent.

In another aspect, the disclosure features a kit comprising: one or more reagents for detecting expression of an uPAR or an EGFR; and instructions for administering to a subject any of the receptor-targeting reagents described herein if the expression of an uPAR or an EGFR is detected.

In another aspect, the disclosure features an article of manufacture comprising: a container; and a composition contained within the container, wherein the composition comprises an active agent for treating cancer in a mammal, wherein the active agent in the composition comprises any of the toxic receptor-targeting reagents described above, and wherein the container has a label indicating that the composition is for use in treating cancer in a mammal. The label can further indicate that the composition is to be administered to the mammal if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR. The article of manufacture can also include instructions for administering the active agent to the mammal. The composition can be in liquid form, or dried or lyophilized.

In another aspect, the disclosure features an article of manufacture comprising: a container; and a composition contained within the container, wherein the composition comprises

an active agent for treating cancer in a mammal, wherein the active agent in the composition comprises any of the toxic receptor-targeting reagents described above, and wherein the container has a label indicating that the composition is for use in treating cancer in a mammal. The label can further indicate that the composition is to be administered to the mammal if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR. The article of manufacture can also include instructions for administering the active agent to the mammal. The composition can be in liquid form, or dried or lyophilized. In another aspect, the disclosure features an article of manufacture comprising: a container; and a composition contained within the container, wherein the composition comprises an active agent for treating cancer in a mammal, wherein the active agent in the composition comprises any of the toxic receptor-targeting reagents described above, and wherein the container has a label indicating that the composition is for use in treating cancer in a mammal. The label can further indicate that the composition is to be administered to the mammal if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR. The article of manufacture can also include instructions for administering the active agent to the mammal. The composition can be in liquid form, or dried or lyophilized.

As used herein, "bound" or "bound to" in the context of an interaction between two or more atoms or molecular units, refers to any covalent or non-covalent bonding of two or more atoms or molecular units (*e.g.*, two or more domains such as targeting domains or toxic domains) to each another. The chemical nature of covalent bonds (two atoms sharing one or more pairs of valence electrons) are known in the art and include, *e.g.*, disulfide bonds or peptide bonds. A non-covalent bond is a chemical bond between atoms or molecules that does not involve the sharing of pairs of valence electrons. For example, non-covalent interactions include, *e.g.*, hydrophobic interactions, hydrogen-bonding interactions, ionic bonding, Van der Waals bonding, or dipole-dipole interactions. Examples of such non-covalent interactions include antibody-antigen complexing or binding pair interactions (interactions of a first and second member of a binding pair such as the interaction between streptavidin and biotin). The term "(a) bound to (b)", where (a) and (b) are targeting domains, means (a) bound to (b) via: (i) any one of the above chemical bonds; (ii) a linker (including a binding pair); or (iii) a toxic domain. The term "(a) directly bound to (b)" means (a) bound to (b) via any of (i) or (ii), but not (iii).

"Polypeptide" and "protein" are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification.

Any of the various components (*e.g.*, domains) of the receptor-targeting reagents described herein, *e.g.*, the EGFR-binding domains, the uPAR-binding domains, or the toxic domains comprising toxic polypeptides can consist of, or include, the full-length, wild-type

forms of the polypeptides. Where mature and immature forms of a polypeptide exist, the one used in the receptor-targeting agent of the invention is preferably the mature form. For example, an EGFR-binding domain can consist of, or be, a full-length epidermal growth factor (*e.g.*, a human epidermal growth factor such as the epidermal growth factor with the amino acid sequence SEQ ID NO:6).

The disclosure also provides (i) biologically active variants and (ii) biologically active fragments or biologically active variants thereof, of the wild-type, full-length polypeptides (*e.g.*, the various polypeptide domains of receptor-targeting reagents) described herein. Biologically active variants of full-length, preferably mature, wild-type proteins or fragments of the proteins can contain additions, deletions, or substitutions. Proteins with substitutions will generally have not more than 50 (*e.g.*, not more than one, two, three, four, five, six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35, 40, or 50) conservative amino acid substitutions. A conservative substitution is the substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution. By contrast, a non-conservative substitution is a substitution of one amino acid for another with dissimilar characteristics.

Deletion variants can lack one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or noncontiguous single amino acids.

Additions (addition variants) include fusion proteins containing: (a) full-length, wild-type polypeptides or fragments thereof containing at least five amino acids; and (b) internal or terminal (C or N) irrelevant or heterologous amino acid sequences. In the context of such fusion proteins, the term "heterologous amino acid sequences" refers to an amino acid sequence other than (a). A fusion protein containing a peptide described herein and a heterologous amino acid sequence thus does not correspond in sequence to all or part of a naturally occurring protein. A heterologous sequence can be, for example a sequence used for purification of the recombinant protein (*e.g.*, FLAG, polyhistidine (*e.g.*, hexahistidine (SEQ ID NO: 22)), hemagluttanin (HA),

glutathione-S- transferase (GST), or maltose-binding protein (MBP)). Heterologous sequences can also be proteins useful as diagnostic or detectable markers, for example, luciferase, green fluorescent protein (GFP), or chloramphenicol acetyl transferase (CAT). In some embodiments, the fusion protein contains a signal sequence from another protein such as a KDEL (SEQ ID NO: 15) sequence or any other described herein. In some embodiments, the fusion protein can contain a carrier (*e.g.*, KLH) useful, *e.g.*, in eliciting an immune response (*e.g.*, for antibody generation; see below). In some embodiments, a fusion protein can contain an exogenous methionine amino acid residue. In some embodiments, the fusion protein can contain one or more linker moieties (see below). Heterologous sequences can be of varying length and in some cases can be longer sequences than the full-length target proteins to which the heterologous sequences are attached. A "fragment" as used herein, refers to a segment of the polypeptide that is shorter than a full-length, immature protein. Fragments of a protein can have terminal (carboxy or amino- terminal) and/or internal deletions. Generally, fragments of a protein will be at least four (*e.g.*, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 12, at least 15, at least 18, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, or at least 100 or more) amino acids in length. Biologically active fragments or biologically active variants of any of the targeting polypeptides or toxic polypeptides described herein have at least 25% (*e.g.*, at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even greater) of the activity of the wild-type, full-length polypeptide. In the case of a targeting polypeptide, the relevant activity is the ability of the targeting polypeptide to bind to the target of interest (*e.g.*, an EGFR receptor and/or an uPAR). In the case of a toxic polypeptide, the relevant activity is the ability to inhibit the proliferation of a cell (or kill the cell).

Depending on their intended use, the polypeptides, biologically active fragments, or biologically active variants thereof can be of any species, such as, *e.g.*, nematode, insect, plant, bird, fish, reptile, or mammal (*e.g.*, a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human). In some embodiments, biologically active fragments or biologically active variants include immunogenic and antigenic fragments of the proteins. An immunogenic fragment is one that has at least 25% (*e.g.*, at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even more) of the ability of the relevant full-length, wild-type protein to stimulate an immune response (*e.g.*, an antibody response or a cellular immune response) in an animal of interest. An antigenic fragment of a protein is one having at least 25% (*e.g.*, at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%; 98%; 99%; 99.5%, or 100% or even greater) of the ability of the relevant full-

length, wild-type protein to be recognized by an antibody specific for the protein or a T cell specific to the protein. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Preferred methods and materials are describe below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

In another aspect, the present invention provides a device comprising: a hollow fiber (HF) catheter having a proximal tip and a distal tip, wherein the HF catheter has a porosity that replicates the porosity of brain tissue, and a receptor targeting reagent as described above. In certain embodiments, the HF catheter comprises a plurality of nanoscale pores that approximate the impendence of brain interstitial spaces. In certain embodiments, the HF catheter consists of a single 380-μm-diameter hollow fiber. In certain embodiments, the HF catheter is made of polysulfone. In certain embodiments, the HF catheter comprises a sealed distal tip. In certain embodiments, the proximal tip is operably attached an infusion pump. In certain embodiments, the proximal tip is attached to tubing, and the tubing is attached to the infusion pump. In certain embodiments, the HF catheter is about 3 mm in length. In certain embodiments, the HF catheter comprises a porous material with multiple interconnected passageways. In certain embodiments, the HF catheter comprises a nominal pore size of 0.45 μm.

In another aspect, the present invention provides a method of treating an animal by contacting the animal with a device described above to administer a therapeutic amount of the receptor-targeting reagent described above. In certain embodiments, the receptor-targeting reagent is administered intracranially. In certain embodiments, the amount of receptor-targeting reagent delivered is 2 μg for each time. In certain embodiments, the flow rate is about 0.25 μl/min. In certain embodiments, the method further comprises administering irradiation therapy to the animal. In certain embodiments, the irradiation therapy is administered before, during or after administration of the polypeptide. In certain embodiments, the method further comprises administering a radiation sensitizer to the animal. In certain embodiments, the animal is a mammal. In certain embodiments, the mammal is a human. In certain embodiments, the method further comprises administering a chemotherapy. In certain embodiments, the chemotherapy is sunititib, ontak, cyclophosphamide, gemcitabine, and/or retionoic acid.

In another aspect, the present invention provides a method of treating glioblastoma multiforme in a patient in need thereof, comprising administering to the patient the receptor targeting reagent as described above.

In another aspect, the present invention provides a method of treating head and neck cancer in a patient in need thereof, comprising administering to the patient the receptor targeting reagent as described above.

In another aspect, the present invention provides a method of treating sarcoma in a patient in need thereof, comprising administering to the patient the receptor targeting reagent as described above. In certain embodiments the sarcoma is angiosarcoma.

In another aspect, the present invention provides a receptor targeting reagent as described herein for use in medical treatment or diagnosis.

In another aspect, the present invention provides for the use of a receptor targeting reagent as described herein to prepare a medicament useful for treating cancer in an animal.

In another aspect, the present invention provides a receptor targeting reagent as described herein for use in therapy.

In another aspect, the present invention provides a receptor targeting reagent as described herein for use in treating cancer.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting. Other features and advantages of the invention, *e.g.*, methods for inhibiting the proliferation of a cancer cell (or killing a cancer cell), will be apparent from the following description, from the drawings and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. (a) Construction and purification of EGFATFKDEL 7mut. EGFATFKDEL 7mut consisted of an NcoI restriction site, an ATG initiation codon, and human EGF. EGF was followed downstream by the 135-amino terminal fragment (ATF). The ligands were followed by the 7 amino-acid linker EASGGPE (SEQ ID NO: 14) and then the first 362 amino acids of the pseudomonas exotoxin 38 (PE38) molecule. Finally, the final 5 amino acids of PE38 (REDLK (SEQ ID NO: 23)), were replaced with KDEL (SEQ ID NO: 15) as an endoplasmic reticulum retention sequence and then a NotII restriction site at the 3' end of the construct. The resultant gene was spliced into the pET28c bacteria expression vector under control of an isopropyl-b-D-thiogalactopyranoside inducible T7 promoter. (b) A vector map is also included in the figure.

Figure 2. Selective activity of EGFATFKDEL against glioblastoma cells *in vitro*. (a) EGFATFKDEL was tested against U87-luc cells in a protein inhibition assay. The effects of parental EGFATFKDEL and deimmunized EGFATFKDEL 7mut were determined by analyzing 3H-leucine uptake after a72-hr incubation with targeted toxins. BIC3KDEL is an irrelevant control recognizing CD3+ T cells. Data are reported as percent control response. Each data point

represents an average of triplicate measures ± S.D. (b) EGFATFKDEL was measured against U118 glioblastoma cells (c) Bispecific EGFATFKDEL, EGFATFKDEL 7mut, monospecific EGFKDEL, ATFKDEL, and a mixture of monospecific EGFKDEL and ATFKDEL were tested against $uPAR^+$ HUVEC cells in ^3H-thymidine uptake assays to determine their ability to target tumor neovasculature. (d) A blocking assay was performed. U87 cells were incubated with 1 nM of EGFATFKDEL 7mut and 100 nM of either recombinant anti-urokinase (α-uPA), EGF, recombinant anti-pseudomonas exotoxin (α-PE), or a mixture of α-uPA and EGF. The non-specific recombinant α-Ly5.2 was included as a negative control.

Figure 3. Intracranial tumor studies. Athymic nude rats were given 4×10^5 U87-luc cells intracranially on day 0. In experiment 1 on day 3, a group of 4 rats were either untreated or treated with 2 μg EGFATFKDEL 7mut delivered by CED using the hollow fiber catheter. Drug was delivered at the same coordinates as tumor. Animals were individually imaged weekly to monitor tumor growth. Physical appearance and behavior bias of animals were monitored daily. In experiment 2, Animals were either untreated or treated with 2 μg EGFATFKDEL 7mut.

Figure 4. Survival. A) A Kaplan Mieier survival curve was drawn for animals in Experiment 2. B) In experiment 2, a group of animals were treated with control 2219KDEL 7mut that does bind U87 tumor cells. Total bioluminescence over time is plotted for both the 2219KDEL7mut group and the untreated group. Tumor grew at a similar rate in both groups indicating that the anti-brain tumor effect of EGFATFKDEL 7mut was selective.

Figure 5. Effect of EGFATFKDEL 7mut on kidney and liver enzymes. In a separate experiment, normal rats (N= 5/group) were given 2 μg EGFATFKDEL 7mut IC on the same two injection schedule. To study systemic toxicity, fourteen days after the final injection, rats were bled and alanine aminotransferase (ALT) levels and blood urea nitrogen (BUN) levels determined. A group of 5 control rats were not treated and studied in an identical manner. ALT (A) and BUN (B) levels were determined. Data are shown as mean ± standard deviation.

Figure 6. The immunogenicity of EGFATFKDEL 7mut. The comparative ability of EGFATFKDEL and EGFATFKDEL 7mut to induce the production anti-toxin (PE38KDEL) antibodies in immunocompetent mice was determined by measuring anti-PE38(KDEL) serum IgG levels on samples of mice immunized weekly with 0.25 μg of parental EGFATFKDEL or deimmunized EGFATFKDEL 7mut (n=5 group). Animals were bled on day 75 and 90. The day 75 serum was taken after 12 immunizations and the day 90 measurement after 14 immunizations. Serum IgG anti-toxin levels were made using an indirect ELISA and quantification of antibodies was determined using a standard curve generated with M40-1 anti-PE(KDEL) antibody. Data comparisons between the EGFATFKDEL-immunized and the deimmunized EGFATFKDEL 7mut groups were significant by Student T test ($p<0.05$).

Figure 7. EGFATFKDEL 7mut activity against head and neck cancer carcinoma cells in vitro. To determine the selective activity of EGFATFKDEL 7mut against head and neck cancer cells in vitro, human UMSCC-11 cells derived from human tongue cancer were cultured with increasing concentrations of EGFATFKDEL 7mut for 72 hours and proliferation was measured by uptake of tritiatedthymidine. EGFATFKDEL 7mut inhibited proliferation while negative control anti-B cell 2219ARLKDEL 7mut did not.

Figure 8. EGFATFKDEL 7mut activity against head and neck cancer carcinoma cells in vivo. To determine the selective activity of EGFATFKDEL 7mut against head and neck cancer cells in vivo, human UMSCC-11 cells. Nude mice bearing flank tumors were treated intratumorally with drug. A) Mean tumor volumes and tumor growth is plotted over time B) Mean weights of mice are shown in each treatment group. The drug inhibited tumor growth without a loss of body weight indicating a safe and efficacious dose of drug could be given multiple times.

Figure 9. Selective activity of EGFATFKDEL against glioblastoma cells *in vitro*. (a) EGFATFKDEL was tested against U87-luc cells in a protein inhibition assay. The effects of EGFATFKDEL and T-cell targeting CD3CD3KDEL were determined by analyzing ^{3}H-leucine uptake after a 72-hr incubation with targeted toxins. Data are reported as percent control response. Each data point represents an average of triplicate measures ± S.D. (b) The activities of EGFATFKDEL, EGFATFKDEL 7mut, EGFKDEL, ATFKDEL, and CD3CD3KDEL were tested against HUVEC in ^{3}H-thymidine uptake protein inhibition assays to determine their ability to target tumor neovasculature. (c) The specificity of EGFATFKDEL was shown by testing its activity toward EGFR^{-} uPAR^{-} Raji B cells in protein inhibition assays. 2219ARLKDEL was used as a positive control. (d) A blocking assay was performed. U87 cells were incubated with 1 nM of EGFATFKDEL and 100 nM of either recombinant anti-urokinase (α-uPA), EGF, recombinant anti-pseudomonas exotoxin (α-PE), or anti-Ly5.2 (α-Ly5.2). The non-specific recombinant α-Ly5.2 was included as a negative control.

Figure 10. The ability for EGFKDEL, ATFKDEL, and EGFATFKDEL to bind to U87 cells. Percent positive values from flow cytometry analysis are graphed for U87 cells incubated 500 nM of EGFKDEL (a), ATFKDEL (a), EGFATFKDEL (a), control RFB4 (b), or control HD37 (b).

Figure 11. The immunogenicity of EGFATFKDEL 7mut (a) The immune response to EGFATFKDEL and EGFATFKDEL 7mut was determined by measuring anti-PE38KDEL serum IgG on weekly samples of mice treated with 0.25 μg of EGFATFKDEL (n=5) or EGFATFKDEL 7mut (n=5). Measurements were made using an indirect ELISA and quantification of antibodies was determined using a standard curve generated with M40-1 anti-

PE38KDEL antibody. (b) Mutated EGFATFKDEL 7mut was compared to unmutated parental EGFATFKDEL against U87 cells in a proliferation assay. ^{3}H-thymidine incorporation was measured after 72-hr incubation with targeted toxins. Data are reported as percentage of control response and measurements were made in triplicates ± S.D. CD3CD3KDEL was a negative control.

Figure 12. *In vivo* efficacy of EGFATFKDEL 7mut against U87-luc tumors. Weekly images show the luciferase activity of U87-luc tumors in individual mice during and after intratumoral treatment. Here, three representative untreated mice (a), three mice treated with control 2219ARLKDEL (b), and three mice treated with EGFATFKDEL 7mut (c) are shown. Mice were treated on days 6, 7, 8, 9, 12, 13, 14, 15, 19, 20, 21, 22, 26, 27, 28, 29, 33, 34, 35, 36, and 40. Crosses indicate mouse death. Mice given control 2219ARLKDEL received drug on the same schedule as EGFATFKDEL 7mut. On the right of the images, we graphed the total bioluminescence activity for each of the individual mice in the entire treatment group over time.

Figure 13. (a) In addition to measuring bioluminescence of U87 tumors, we measured tumor growth with calipers. Thus, tumor volume in 3 dimensions was measured for each animal in Figure 12 and the average tumor volume was plotted versus time. The stars indicate the points on the curve in which EGFATFKDEL 7mut treatment differed significantly from the untreated controls ($p<0.05$ as determined by one-way ANOVA with Tukey's test for multiple comparison). (b) Tumor growth was measured in a second similar experiment (n=6/group) and plotted similarly.

Figure 14. In the same experiment as shown in Figures 12 and 13, an extra group of mice (n=5/group) was treated with monospecific ATFKDEL. As in Figure 12 we measured total bioluminescence activity for each of the individual mice in the treatment group and graphed it over time.

Figure 15. Emma and K12 cells express cognate receptor mRNA and surface protein. RT-PCR results are shown. Cells were grown in culture prior to RNA isolation and cDNA synthesis. Different primers were used for canine and feline tumor cell lines. Emma cells expressed low and approximately equivalent levels of uPAR and EGFR. In contrast, consistent with published data showing K12 cells expressed <100,000 EGFR/cell, these cells expressed high levels of surface EGFR and low levels of surface uPAR, the latter which were comparable to those seen in Emma cells.

Figure 16. Canine HSA tumor cell lines are sensitive to EGFATFKDEL 7mut. Canine HSA cell lines (Emma, Frog, DD-1) were cultured for 72 hrs with EGFATFKDEL 7mut, negative control BIC3 (T-cell specific BLT) or without BLT (No Drug). Viability was measured in triplicate samples using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation

Assay (MTS), normalized to 100% in the absence of BLT. K12 cells, which overexpress EGFR, were used as a positive control. Data are representative of three independent experiments. Error bars represent normalized standard deviation.

Figure 17. EGFR and uPAR blockade via competitive binding with native ligand prevents LTT mediated cell death. Emma cells were incubated with unlabeled ligand at varying concentrations, as indicated above, at 4° C for 30 minutes. EGFATFKDEL 7mut was added at 0.05 nM and cells were incubated for 48 hrs. Viability was measured in duplicate samples using the MTS assay, normalized to 100% in the absence of LTT. A value of 100 represents maximal viability. Values <100 represent death due to cytotoxic effects of EGFATFKDEL 7mut. One representative experiment of three is shown for each cell line. (a) Results for Emma cells. (b) Results for K12 cells. The K12 cells showed similar results. Note, the Emma cells were more readily blocked with uPA, which correlates to the decreased uPAR levels in K12 cells shown in Figure 15.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure features, inter alia, receptor-targeting reagents (*e.g.*, toxic receptor-targeting reagents), which are useful in a variety of *in vitro*, *in vivo*, and *ex vivo* methods. For example, the receptor-targeting reagents are useful in methods of binding a receptor-targeting reagent to a cell. The receptor-targeting reagents described herein are useful in treating a variety of proliferative disorders such as, but not limited to, cancers and inflammatory disorders.

Also provided herein are methods, compositions, and kits useful for selecting an appropriate treatment modality for a subject (*e.g.*, a subject with a cancer or inflammatory disorder) and/or treating a variety of proliferative disorders.

Receptor-targeting Reagents

The disclosure features receptor-targeting reagents, which reagents contain: (a) a first targeting domain containing an epidermal growth factor receptor (EGFR)-binding agent and (b) a second targeting domain containing an urokinase plasminogen activator receptor (uPAR)-binding agent, wherein (a) is bound to (b).

The EGFR-binding agent can be any agent that selectively binds to an EGFR (*e.g.*, an EGFR/HERl/ErbBl, a HER2/ErbB2/neu, a HER3, or a HER4). For example, the EGFR- binding agent can include all, or an EGFR-binding fragment, of an antibody specific for an EGFR. The EGFR-binding agent consist of, or contain, all or part (an EGFR-binding fragment) of a natural ligand for EGFR. For example, the EGFR-binding can consist of, or contain, a natural ligand for

a HER1 such as, but not limited to, an epidermal growth factor polypeptide, a betacellulin polypeptide, a transforming growth factor alpha polypeptide, an amphiregulin polypeptide, an epiregulin polypeptide, a heparin-binding EGF polypeptide, or EGFR-binding fragment of any of the foregoing. An exemplary amino acid sequence for a full length, mature (lacking a signal peptide) human EGF polypeptide is as follows:

NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWEL R (SEQ ID NO:6).

Additional natural ligands for an EGFR suitable for the methods described herein include, but are not limited to: neuregulins (also known as heregulins, neu differentiation factors, or glial growth factors; *e.g.*, heregulin α, heregulin β) and the neuregulin-2s.

In some embodiments, the EGFR-binding agent can be a small molecule that binds to an EGFR, *e.g.*, a small molecule that binds to an EGF-binding site of an EGFR.

The receptor-targeting reagents can contain a first targeting domain containing an EGFR-binding agent and a second targeting domain containing an uPAR-binding agent. The uPAR-binding agent can be any agent that selectively binds to an uPAR (*e.g.*, a mammalian uPAR such as human uPAR). For example, the uPAR-binding agent can consist of, or contain, an antibody that binds to the uPAR or an antigen-binding fragment thereof. The uPAR binding agent can be, *e.g.*, all or part (an uPAR-binding fragment) of a natural ligand for uPAR. For example, the uPAR-binding agent can also consist of, or contain, an uPAR polypeptide or an uPAR-binding fragment thereof. An exemplary amino acid sequence for a full length, mature (lacking a signal peptide) human uPAR polypeptide (SEQ ID NO:7).

Fusion toxins of the invention also include a targeting domain that contains uPA or a portion of uPA. As used herein, "targeting domain" refers to a polypeptide or functional fragment thereof that has significant binding affinity for a target molecule on the surface of a target cell. For fusion toxins such as DTAT, the target is a cell surface receptor and the targeting domain is a ligand for the receptor. According to the present invention, the targeting domain contains uPA or any portion of uPA capable of binding to uPAR on the surfaces of target cells. The ATF of uPA is particularly useful. The ATF includes the receptor-binding domain of uPA but does not include the catalytic or internalization domains.

Fusion toxin targeting domains of the invention may include the entire ATF (*i.e.*, the amino-terminal 135 amino acids of uPA). These residues include the receptor-binding domain, which is an epidermal growth factor-like domain situated between amino acids 12 and 32, and which binds to uPAR with high affinity (K_d =0.5 nM). Alternatively, fusion toxins may contain a targeting domain that is a functional fragment of the uPA ATF.

Fusion toxin targeting domains of the present invention can have amino acid sequences that are identical to the wild-type sequence of the uPA ATF. Alternatively, a targeting domain may contain amino acid deletions, additions, or substitutions, provided that the targeting domain has at least 10% (*e.g.*, 10%, 25%, 50%, 70%, 85%, 100%, or more) of the ability of the wild-type uPA polypeptide to bind to the target molecule. Methods of comparing the relative ability of two of more molecules (*e.g.*, fusion toxins or ligands) to bind to a target cell are well known in the art. Amino acid substitutions typically will be conservative substitutions, although non-conservative substitutions are possible.

In some embodiments, the uPAR-binding agent can be a small molecule that binds to an uPAR, *e.g.*, a small molecule that binds to an uPAR-binding site of an uPAR. In certain embodiments, the uPAR-binding agent is the amino acid terminal fragment of uPA (ATF), which is 135 amino acids in length (SEQ ID NO: 1)

The EGFR- or uPAR-specific antibody (or antigen-binding fragment thereof) described above can be, *e.g.*, a monoclonal antibody, a polyclonal antibody, a humanized antibody, a fully human antibody, a single chain antibody, a chimeric antibody, or an Fab fragment, an $F(ab')_2$ fragment, an Fab' fragment, an Fv fragment, or an scFv fragment of an EGFR-specific antibody. Methods for making such antibodies are described below (see "Methods For Generating Antibodies").

In some embodiments, the receptor-targeting reagents can be toxic. That is, any of the receptor-targeting reagents described herein can further contain one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, 10 or more, 15 or more, or 20 or more) toxic domains. A toxic domain can consist of, or include, *e.g.*, a small molecule. Small molecules that are suitable for toxic domains include, *e.g.*, chemotherapeutic agents such as, but not limited to, cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, adriamycin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide, verampil, podophyllotoxin, tamoxifen, taxol, transplatinum, 5-flurouracil, vincristin, vinblastin, methotrexate, or an analog of any of the aforementioned. Where the receptor-targeting reagent contains more than one small molecule, the various small molecules can each be the same, different, or a mixture of both of the aforementioned.

The toxic domain can consist of, or contain, at least one (*e.g.*, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 15, or at least 20) radionuclide(s). The at least one radionuclide can be, *e.g.*, ^{90}Y, ^{186}Re, ^{188}Re, ^{64}Cu, ^{67}Cu, ^{212}Pb, ^{212}Bi, ^{213}Bi, ^{123}I, $^{125}1$, ^{131}I, ^{211}At, ^{32}P, ^{177}Lu, ^{47}Sc, ^{105}Rh, ^{109}Pd, ^{153}Sm, or ^{199}Au.

In some embodiments, the radionuclide atom can be can be part of a larger molecule (*e.g.*, ^{125}I in meta-[^{125}I]iodophenyl-N-hydroxysuccinimide ([^{125}I]mIPNHS), which binds via free amino groups to form meta-iodophenyl (mIP) derivatives of relevant proteins (see, *e.g.*, Rogers et al. (1997) J. Nucl. Med. 38:1221-1229), or a chelate (*e.g.*, radioactive metal atoms such as ^{99m}Tc, ^{188}Re, ^{186}Re, ^{90}Y, ^{212}Pb, ^{212}Bi, ^{64}Cu, ^{67}Cu, ^{177}Lu, ^{47}Sc, ^{105}Rh, ^{109}Pd, ^{153}Sm, ^{199}Au chelated to, for example, hydroxamic acids, DOTA, or DTPA), which are themselves part of the receptor-targeting reagent. Where the receptor-targeting reagent contains more than one radionuclide atom, the various radionuclide atoms can be either all the same radionuclide (*e.g.*, more than one of ^{90}Y), all different radionuclides, or a mixture of both of the aforementioned. The radionuclides can emit α-, β-, or γ-radiation or a combination of two or more of these types of irradiation. As is described in the accompanying Examples, the toxic domain can contain, or consist of, a toxic polypeptide. For example, the toxic polypeptide can be a Diphtheria toxin or a biologically active fragment (a toxic fragment) or variant (a toxic variant) thereof.

An exemplary amino acid sequence for a full-length Diphtheria toxin (which contains the amino-terminal leader sequence: MLVRGYVVSRKLFASILIGALLGIGAPPSAHA (SEQ ID NO:8)) is as follows:

MLVRGYVVSRKLFASILIGALLGIGAPPSAHAGADDVVDSSKSFVMENFSSYHG
TKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYSTDNKYDAAGYSVDNENPLSGKAGG
VVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPLMEQVGTEEFIKRFGDGASRVVLSL
PFAEGSSSVEYINNWEQAKALSVELEINFETRGKRGQDAMYEYMAQACAGNRVRRSV
GSSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTAL
EHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSILPGIGSVM
GIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESIINLFQVVHNS
YNRPAYSPGHKTQPFLHDGYAVSWNTVEDSIIRTGFQGESGHDIKITAENTPLPIAGVLL
PTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTFCRPKSPVYVGNGVHANLHVAFHR
SSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSLFFEIKS (SEQ ID NO:9).

An exemplary amino acid sequence for a biologically active variant of a Diphtheria toxin, which contains amino acids 33-421 of SEQ ID NO:9 (*i.e.*, it lacks the N-terminal leader sequence of SEQ ID NO:9) and an exogenous methionine at position 1, is as follows:

MGADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWK
GFYSTDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGL
SLTEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEINFE
TRGKRGQDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPI
KNKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAV
NVAQVIDSETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQA

IPLVGELVDIGFAAYNFVESIINLFQVVHNS YNRPAYSPGHKTQPF (SEQ ID NO: 10). This amino acid sequence is also herein referred to as DT390.

In some embodiments the toxic polypeptide can consist of, or contain, a Pseudomonas exotoxin A or a biologically active fragment thereof.

An exemplary amino acid sequence for a full-length Pseudomonas exotoxin A is as follows:

MHLIPHWIPLVASLGLLAGGSSASAAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPA IADTNGQGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQ ARGSWSLNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFF VRAHESNEMQPTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYL AQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHL PLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDL GEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPA DSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYH GTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGAL LRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLETILGWP LAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPREDLK (SEQ ID NO:11).

An exemplary amino acid sequence for a biologically active variant of a Pseudomonas exotoxin A, which contains amino acids 276-633 of (SEQ ID NO:11) is as follows:

PEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRL V AL YLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGT GNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTR GTQNWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGF YIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVE RLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDK EQAISALPDYASQPGKPPKDEL (SEQ ID NO: 12).

Other exemplary amino acid sequences for a biologically active variant of a Pseudomonas exotoxin include the amino acid sequence of SEQ ID NO: 11, wherein (i) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 432 is glycine, the arginine at position 467 is alanine, and the lysine at position 590 is serine; (ii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 332 is serine, and the arginine at position 313 is alanine; (iii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position

467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, and the arginine at position 313 is alanine; and (iv) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, the glutamine at position 332 is a serine, and the arginine at position 313 is alanine.

The toxic polypeptide can consist of, or contain, Pseudomonas exotoxin (PE), bryodin, gelonin, α-sarcin, aspergillin, restrictocin, angiogenin, saporin, abrin, a prokaryotic ribonuclease, a eukaryotic ribonuclease, ricin, pokeweed antiviral protein (PAP), a pro-apoptotic polypeptide, a ribosomal inhibitory protein, or a biologically active fragment of any of the foregoing.

Suitable pro-apoptotic polypeptides include, but are not limited to, Bax, Bad, Bak, Bim, Bik, Bok, Hrk, FasL, TRAIL, or TNF-α.

In some embodiments, a toxic domain can include, *e.g.*, one or more (*e.g.*, one, two, three, four, five, six, seven, or eight or more) of toxins (*e.g.*, toxic small molecules, radionuclides, or toxic polypeptides or biologically active fragments thereof) such as any of those described herein. In addition, more than one (*e.g.* two, three, four, five, six, seven, eight, nine, or ten or more) toxin or biologically active fragment of one or more (*e.g.*, one, two, three, four, five, six, seven, or eight or more) toxins can be included in a toxic domain. A toxic domain can include multiple copies or repeats of one or more toxins (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more copies of a toxin). Where repeats are included, they can be immediately adjacent to each other, separated by one or more targeting fragments, separated by a linker peptide (see below), separated by another, different, toxin.

The components (the domains, *e.g.*, the first and second targeting domains and the toxic domain) of the receptor-targeting reagents described herein can be bound to each other by covalent or non-covalent bonds. For example, the components of the receptor-targeting reagents can be bound together by a first and second member of a binding pair. That is, the first targeting domain can be bound to a first member of a binding pair and the second targeting domain can be bound to a second member of a binding pair. The binding pair can be, *e.g.*, avidin (or streptavidin) and biotin (or biocytin).

The components of the receptor-targeting reagents described herein can be bound together in a molecular complex in any of numerous configurations or conformations. For example, a first targeting domain (a), a second targeting domain (b) , and a toxic domain (c) can be bound together as: (a) bound to (b) bound to (c);

(c) bound to (a) bound to (b);

(a) bound to (c) bound to (b); (c) bound to (b) bound to (a);

(b) bound to (a) bound to (c); (b) bound to (c) bound to (a); or

(a) bound to (b), (b) bound to (c), (c) bound to (a).

The domains can be bound together by any interaction described herein. It is understood that any of the domains of the receptor targeting reagents can be bound together by a mixture of covalent and non-covalent bonds. For example, the first and second targeting domains can be bound together by a first and second member of a binding pair and a toxic domain can be bound to the first or second targeting domain (or both) by a covalent bond.

Two or more (or all) of the domains of the receptor-targeting reagents can be covalently bound together as a fusion protein. For example, the first and second targeting domains, the first or second targeting domain and the toxic domain, or the first and second targeting domains and the toxic domain can be covalently bound together as a single fusion protein.

Any of the receptor-targeting reagents described herein can include one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, or seven or more) linker moieties, which link one or more (or all) of the domains (*e.g.*, the first targeting domain, the second targeting domain, or the toxic domain) of the receptor-targeting reagent together. Such linker moieties can be useful, *e.g.*, in minimizing steric hindrance between two or more domains of a receptor-targeting reagent (*e.g.*, minimizing steric hindrance between the first and second targeting domains) or in preventing the toxic domain (if present) from interfering with the ability of one or more targeting domains from binding to a target cell. Linker moieties can include, *e.g.*, a first and second member of a binding pair or a peptide linker. Linker peptides can be one amino acid in length, but can be longer in length. For example, a linker peptide can be at least two (*e.g.*, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, or at least 55 or more) amino acids long. Linker peptides can contain any amino acids.

In some embodiments, the peptide linker can consist of, or contain, part of the human muscle aldolase (hma) protein (*e.g.*, PSGQAGAAASESLFVSNHAY (SEQ ID NO: 13)). In some embodiments, one or more peptide linkers can contain, or consist of, EASGGPE (SEQ ID NO: 14). In some embodiments, one or more peptide linkers can contain, or consist of, (i) an aggregation reducing linker (ARL) having the amino acid sequence GSTSGSGKPGSGEGSTKG (SEQ ID NO:4) and/or (ii) a mono or poly-GGGGS (SEQ ID NO: 21) sequence (*e.g.*, GGGGSGGGGSGGGGS (SEQ ID NO:5)). In embodiments where the receptor-targeting reagent is a fusion protein, the one or more linker moieties can link two or more of the domains of the fusion protein.

Exemplary linker moieties and their roles in linking two or more domains of a receptor-targeting reagent are described in the accompanying Examples.

Exemplary receptor-targeting reagents are described in the accompanying Examples and include, *e.g.*, the DTEGFATF receptor-targeting reagent comprising: a biologically active variant of Diphtheria toxin (SEQ ID NO: 10), a linker (SEQ ID NO: 14), an epidermal growth factor (SEQ ID NO:6), a linker (SEQ ID NO:13), and an ATF (SEQ ID NO:1), and with the following amino acid sequence (SEQ ID NO:2);

The EGFATF receptor-targeting reagent comprising: an epidermal growth factor (SEQ ID NO:6), a linker (SEQ ID NO: 13), and an ATF (SEQ ID NO:1), and having the following amino acid sequence SEQ ID NO:3;

The EGFATFKDEL receptor-targeting reagent comprising: an epidermal growth factor (SEQ ID NO:6), a linker (SEQ ID NO:13), an ATF (SEQ ID NO:1), a linker (SEQ ID NO: 14), a biologically active fragment of Pseudomonas toxin A (SEQ ID NO : 12), and a KDEL (SEQ ID NO: 15), and having the following amino acid sequence SEQ ID NO:17.

The EGFATFKDEL 7mut receptor-targeting reagent comprising: an epidermal growth factor (SEQ ID NO:6), a linker (SEQ ID NO:13), an ATF (SEQ ID NO:1), a linker (SEQ ID NO: 14), a biologically active fragment of Pseudomonas toxin A (SEQ ID NO : 12), and a KDEL (SEQ ID NO: 15), and having the following amino acid sequence SEQ ID NO:18.

The linker regions of each of the above exemplary receptor-targeting reagents are underlined.

Any of the receptor-targeting reagents described herein can also include one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or 10 or more) detectable labels. For example, a receptor-targeting reagent can include an enzyme (*e.g.*, horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase), a fluorescent material (*e.g.*, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, allophycocyanin (APC), or phycoerythrin), a luminescent material (*e.g.*, europium, terbium), a bioluminescent material (*e.g.*, luciferase, luciferin, or aequorin), or a radionuclide (*e.g.*, any of the radionuclides described herein).

It is understood that any of the receptor-targeting reagents described herein can contain one or more (*e.g.*, one, two, three, four, five, six, seven, eight, nine, or 10 or more) additional "targeting" domains and/or one or more (*e.g.*, one, two, three, four, five, six, seven, eight, nine, or 10 or more) additional toxic domains. The receptor-targeting reagents can contain one or more of a first targeting domain, one or more of a second targeting domain, and/or one or more of a toxic domain. For example, a receptor-targeting reagent described herein can contain two or

more EGFR-binding domains and/or two or more uPAR-binding domains. The various domains (*e.g.*, the first targeting domain, the second targeting domain, or the toxic domain) of the receptor-targeting reagents can be arranged in any orientation with respect to each other. For example, a toxic domain can be N-terminal or C-terminal to one or more (or all) targeting domains. In another example, the toxic domain can be between two targeting domains. Likewise, the targeting domains can be adjacent to each other or separated by, *e.g.*, a toxic domain and/or a linker moiety (see above).

In some embodiments, any polypeptide described herein (*e.g.*, a toxic polypeptide) can be modified in such a way as to reduce or prevent immunogenicity of the polypeptide in a subject. As used herein, a polypeptide modified to have a "reduced immunogenicity" is one that elicits less of an immune response in a given subject, or cohort of subjects, than the corresponding unmodified polypeptide. As exemplified in the working Examples, a nucleic acid encoding a polypeptide can be modified through standard molecular biology techniques (Sambrook et al., supra) such that the encoded polypeptide contains one or more substitutions effective to reduce the immunogenicity of the polypeptide in a subject. For example, a Psuedomonas exotoxin (PE) A polypeptide having SEQ ID NO: 11 can contain one or more substitutions (*e.g.*, conservative or non-conservative substitutions) such as, but not limited to: (i) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 432 is glycine, the arginine at position 467 is alanine, and the lysine at position 590 is serine; (ii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 332 is serine, and the arginine at position 313 is alanine; (iii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, and the arginine at position 313 is alanine; or (iv) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, the glutamine at position 332 is a serine, and the arginine at position 313 is alanine.

A modified polypeptide can contain at least two (*e.g.*, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 32, 35, or 40 or more) amino acid substitutions. A modified polypeptide can contain less than 20 (*e.g.*, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, nine, eight, seven, six, five, four, three, two, or one) amino acid substitutions. It is understood that a modified polypeptide can contain at least two, but less than 20 amino acid substitutions.

Suitable *in vitro* and *in vivo* methods for determining the immunogenicity of a polypeptide are known in the art and described in the working Examples. *In vitro* methods include, *e.g.*, culturing lymphoid cells (including T and B lymphocytes) obtained from a mammalian subject with a polypeptide (*e.g.*, modified or unmodified) described herein. The lymphoid cells can be from a subject pre-exposed to the polypeptide, to the protein from which the polypeptide was derived, or where the polypeptide is derived from a microorganism, to the microorganism that naturally produces the polypeptide. Alternatively, the donor of the lymphoid cells need not have been exposed to any of these entities. The cultures can be "restimulated" as often as necessary with either the polypeptide. The cultures can also be monitored at various times to ascertain what time of immune reactivity (*e.g.*, antibody production or CD4+ helper T cell activity) has occurred. In such experiments, one set of cells would be contacted with a modified polypeptide and a identical second set of cells would be contacted with the corresponding unmodified polypeptide. A decrease in the immune response generated by the modified polypeptide as compared to the immune response generated by the unmodified polypeptide is an indication that the modified polypeptide has reduced immunogenicity. In *in vivo* methods, the polypeptide itself can be administered to a subject. The polypeptides can be administered to a subject orally, transdermally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily, or injected (or infused) intravenously, subcutaneously, intramuscularly, or intraperitoneally. The polypeptide can be delivered directly to an appropriate lymphoid tissue (*e.g.* spleen, lymph node, or mucosal- associated lymphoid tissue (MALT)). In studies where human subjects are used, a polypeptide can be administered subcutaneously and the occurrence, or severity, of a "wheel and flare" can indicate the immunogenicity of a polypeptide to that subject. In some embodiments, a modified polypeptide and the unmodified polypeptide can be administered to the same subject, *e.g.*, using skin tests. Such assays test for both antibodies and pre-activated CD4+ T cells specific for the test antigen. A positive response within 12 hours is indicative of an antibody response, while a response that is optimal between 48 and 96 hours indicates the presence of CD4+ T cells that have previously been exposed to the relevant antigen. For example, a human subject can be administered subcutaneously a modified and an unmodified composition to different positions within the same region of the body, *e.g.*, to different positions on the subject's back or abdomen. In some embodiments, such as animal experiments, one animal or group of animals (*e.g.*, mice) can be administered the modified polypeptide and an identical animal or group of animals can be administered the corresponding unmodified polypeptide. As in the *in vitro* experiments, a decrease in the immune response generated by the modified polypeptide as compared to the

immune response generated by the unmodified polypeptide is an indication that the modified polypeptide has reduced immunogenicity.

Methods for measuring the level of an immune response are known in the art, set forth in the detailed description, and exemplified in the working Examples. For example, methods for determining antibody production are described in the section entitled "Methods for Generating Antibodies."

Nucleic Acids and Methods of Making the Receptor-Targeting Reagents.

Also featured are nucleic acids encoding the polypeptide receptor-targeting reagents (*e.g.*, full length polypeptide receptor-targeting reagents or polypeptide domains thereof) described herein and vectors containing the nucleic acids. The nucleic acids and vectors can be used, *e.g.*, to express the polypeptide receptor-targeting reagents in a host cell (*e.g.*, a bacterial, yeast, or mammalian cell). The nucleic acids and vectors can also be used in, *e.g.*, *ex vivo* methods of treatment as described below.

A single nucleic acid can encode an entire receptor-targeting reagent, *e.g.*, a receptor-targeting reagent fusion protein. A receptor-targeting reagent can be, in some instances, encoded by two or more (*e.g.*, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) different nucleic acids. For example, each domain of a receptor-targeting reagent can be encoded by a separate nucleic acid.

In some embodiments, the nucleic acids can be operably-linked to promoter and/or enhancer elements that direct the expression of the polypeptide receptor-targeting reagents encoded by the nucleic acids. The coding sequence for a given polypeptide receptor-targeting reagent can be contained within a single expression vector containing a nucleic acid sequence (*e.g.*, a genomic DNA sequence or a cDNA sequence) or can be contained in two or more vectors. For example, a polypeptide receptor-targeting reagent containing three different domains (*e.g.*, a first targeting domain, a second targeting domain, and a toxic domain) can be encoded by different nucleic acids that are present in three different vectors, where each vector contains, *e.g.*, the coding sequence of one domain. In the latter case, the domains encoded within the respective vectors can be designed such that they associate post-translationally within the cell in which they are produced either by covalent (*e.g.*, disulfide) bonds or non-covalent (*e.g.*, hydrophobic or ionic) interactions. Alternatively, each of the separate domains can be isolated first and then bound together (*e.g.*, chemically or enzymatically bound together) in a separate step.

Enhancers provide expression specificity in terms of time, location, and level. Unlike a promoter, an enhancer can function when located at variable distances from the transcription

initiation site, provided a promoter is present. An enhancer can also be located downstream of the transcription initiation site. To bring a coding sequence under the control of a promoter, it is necessary to position the translation initiation site of the translational reading frame of the peptide or polypeptide between one and about fifty nucleotides downstream (3') of the promoter. Promoters of interest include but are not limited to the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3 phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α mating factors, the adenoviral EIb minimal promoter, or the thymidine kinase minimal promoter.

Where a single fusion protein is encoded, the nucleic acid sequence encoding the targeting domains can be 5' of a nucleic acid encoding the toxic domain or vice versa. The two coding sequences will be in frame with each other and can be immediately adjacent to each other or separated by a linker region encoding a linker peptide which can serve, for example, to prevent steric hindrance by the toxic domain of binding of the first or second targeting domains to the surface of the target cell.

In some embodiments, the 5' end of a nucleic acid encoding a polypeptide receptor-targeting reagent (*e.g.*, a fusion protein receptor-targeting reagent) or any of the polypeptide targeting domains, polypeptide toxic domains (or toxic polypeptides therein), can include a non-native ATG "start sequence." That is an ATG sequence can be added to, *e.g.*, a nucleic acid encoding a biologically active fragment or variant of a full-length polypeptide to ensure that the protein is properly transcribed and translated. Although a leader sequence generally includes an ATG start sequence, in embodiments where it does not, the ATG sequence can be added at the 5' end of a nucleic acid encoding the leader sequence. Naturally, this will result in a non-native methionine residue amino acid in the corresponding sequence of a polypeptide receptor-targeting reagent.

Suitable methods for constructing nucleic acids and expression vectors are well known to those skilled in the art and described in, *e.g.*, Sambrook et al., Molecular Cloning: A Laboratory Manual Second Edition vol. 1, 2 and 3. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, USA, Nov. 1989; the disclosure of which is incorporated herein by reference in its entirety.

A recombinant nucleic acid can be introduced into a cell using a variety of methods, which methods can depend, at least in part, on the type of cell into which the nucleic acid is introduced. For example, bacterial cells can be transformed using methods such as electroporation or heat shock. Methods for transfecting yeast cells include, *e.g.*, the spheroplast

technique or the whole-cell lithium chloride yeast transformation method (see, *e.g.*, U.S. Patent No. 4,929,555; Hinnen et al. (1978) Proc. Nat. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163; U.S. Patent No. 4,879,231; and Sreekrishna et al. (1987) Gene 59:115, the disclosures of each of which are incorporated herein by reference in their entirety). Transfection of animal cells can feature, for example, the introduction of a vector to the cells using calcium phosphate, electroporation, heat shock, liposomes, or transfection reagents such as FUGENE® or LIPOFECTAMINE®, or by contacting naked nucleic acid vectors with the cells in solution (see, *e.g.*, Sambrook et al., supra).

Expression systems that may be used for small or large scale production of the receptor-targeting reagents described herein include, but are not limited to, microorganisms such as bacteria (for example, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing the nucleic acid molecules of the invention; yeast (for example, *Saccharomyces* and *Pichia*) transformed with recombinant yeast expression vectors containing the nucleic acid molecules of the invention; insect cell systems infected with recombinant virus expression vectors (for example, baculovirus) containing the nucleic acid molecules of the invention; plant cell systems infected with recombinant virus expression vectors (for example, cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (for example, Ti plasmid) containing fusion protein nucleotide sequences; or mammalian cell systems (for example, COS, CHO, BHK, 293, VERO, HeLa, MDCK, WB 8, and NIH 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (for example, the metallothionein promoter) or from mammalian viruses (for example, the adenovirus late promoter, a CMV promoter, an SV40 promoter, or the vaccinia virus 7.5K promoter). Also useful as host cells are primary or secondary cells obtained directly from a mammal, transfected with a plasmid vector or infected with a viral vector (*e.g.*, viral vectors such as herpes viruses, retroviruses, vaccinia viruses, attenuated vaccinia viruses, canary pox viruses, adenoviruses and adeno-associated viruses, among others; see *Ex vivo* Methods below). Following the expression of any of the receptor-targeting reagents described herein, the receptor-targeting reagents can be isolated from the cultured cells, or from the media in which the cells were cultured, using standard techniques (see Sambrook et al., supra). Methods of isolating proteins are known in the art and include, *e.g.*, liquid chromatography (*e.g.*, HPLC), affinity chromatography (*e.g.*, metal chelation or immunoaffinity chromatography), ion-exchange chromatography, hydrophobic-interaction chromatography, precipitation, or differential solubilization.

Smaller receptor-targeting reagents (or domains thereof), *e.g.*, receptor-targeting reagents or domains having less than 200 (*e.g.*, less than 175, less than 150, less than 125, less than 100, less than 90, less than 80, less than 70, or less than 60) amino acids can be chemically synthesized by standard chemical means. In some embodiments, the isolated receptor-targeting reagents can be frozen, lyophilized, or immobilized and stored under appropriate conditions, which allow the proteins to retain activity (*e.g.*, toxic activity or the ability to bind to a cell).

Where one or more domains or agents (*e.g.*, a targeting domain or toxic agent) have been produced independently, each domain or agent can be linked to together by covalent or non-covalent bonds using methods known in the art. For example, a terminal or internal cysteine residue on one domain (or agent) can be utilized to form a disulfide bond with a terminal or internal cysteine residue on another domain or agent.

Domains or agents can also be cross-linked using any of a number of known chemical cross linkers. Examples of such chemical cross-linkers are those which link two amino acid residues via a linkage that includes a "hindered" disulfide bond. In these linkages, a disulfide bond within the cross-linking unit is protected (by hindering groups on either side of the disulfide bond) from reduction by the action, for example, of reduced glutathione or the enzyme disulfide reductase. One suitable chemical cross-linker, 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio)toluene (SMPT), forms such a linkage between two domains (or agents) utilizing a terminal lysine on one of the domains (or agents) and a terminal cysteine on the other. Heterobirunctional reagents which cross-link by a different coupling moiety on each domain (*e.g.*, each domain polypeptide) or agent (*e.g.*, a toxic small molecule or radionuclide). Thus, the coupling moiety on one domain or agent could be a cysteine residue and on the other a lysine residue. In this way, the resulting dimers will be heterodimers rather than either homodimers or a mixture of homodimers and heterodimers. Other useful cross-linkers include, without limitation, chemicals that link two amino groups (*e.g.*, N-5-Azido-2-nitrobenzoyloxysuccinimide), two sulfhydryl groups (*e.g.*, 1 ,4-Bis-maleimidobutane) an amino group and a sulfhydryl group (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester), an amino group and a carboxyl group (*e.g.*, 4-[p-azidosalicylamido]butylamine), and an amino group and a guanadium group that is present in the side chain of arginine (*e.g.*, p-azidophenyl glyoxal monohydrate).

While these cross-linking methods can involve residues ("coupling moieties") that are native to any of the domains or agents, they can also be used to cross-link non-native ("heterologous") sequences (*e.g.*, linker sequences) incorporated into the polypeptide chains. While not necessarily the case, such sequences will generally be composed of amino acids (*e.g.*, cysteine, lysine, arginine, or any N-terminal amino acid). Non-amino acid moieties include,

without limitation, carbohydrates (*e.g.*, on glycoproteins) in which, for example, vicinal diols are employed (Chamow et al. (1992) J. Biol. Chem. 267, 15916-15922). The cross-linking agent 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH), for example, can be used to cross-link a carbohydrate residue on one domain (or agent) and a sulfhydryl group on another. They can be added during, for example, chemical synthesis of a domain (or agent) or a part of the domain or agent. Alternatively, they can be added by standard recombinant nucleic acid techniques known in the art.

The heterologous coupling moieties can be positioned anywhere in a domain or agent of a fusion protein, provided that the activity of the resulting receptor-targeting reagent is not compromised. Thus, the linkage must not result in disruption of the structure of a targeting domain such that it is substantially unable to bind to the cell-surface molecule for which it is specific. Furthermore, the linkage must not result in the disruption of the structure of the toxic domain (or agent) such that it is substantially unable to inhibit the proliferation of (or kill) its respective target cell. Using standard binding and toxicity assays known to those in the art (and detailed in the accompanying Examples), candidate receptor-targeting reagents employing linkages involving different residues on the domains can be tested for their ability to bind and inhibit the proliferation of (or kill) target cells of interest. Using molecular modeling techniques, it will frequently be possible to predict regions on a targeting domain or toxic domain (or agents) that would be appropriate for the insertion of moieties by which inter-domain linkages could be formed. Thus, for example, regions predicted to be on the exterior surface of a targeting domain, but unlikely to be involved in binding to a target molecule, could be useful regions in which to an insert an appropriate moiety in the targeting domain. Similarly, regions predicted to be on exterior surface of a toxic domain (or agent), but unlikely to be involved in the toxic activity, could be useful regions in which to an insert an appropriate moiety in the toxic domain. The coupling moieties will preferably be at the termini (C or N) of the domains. They can be, as indicated above, a cysteine residue on each domain (or agent), or a cysteine on one and a lysine on the other. Where they are two cysteine residues, cross-linking can be effected by, for example, exposing the domains (or agents) to oxidizing conditions.

It can be desirable in some cases to eliminate, for example, one or more native cysteine residues in a domain or agent in order to restrict cross-linking to only non-native moieties inserted into the domains or agents. A potentially troublesome cysteine could, for example, be replaced by an alanine or a tyrosine residue. This can be done by, for example, standard recombinant techniques. Naturally, these replacements should not compromise the activity of the resulting receptor-targeting reagent.

Where more than two domains (or more than two agents) are to be joined, at least one of the domains (or agents) can have more than one cross-linking moiety. Such multimers can be constructed "sequentially," such that each domain or agent is joined to the next such that the terminal two domains (or agents) in the chain only have one residue involved in an inter-domain (or inter-agent) bond while the "internal" domains (or agents) each have two moieties involved in inter-domain bonds. Alternatively, one domain could be linked to multiple (*e.g.*, 2, 3, 4, or 5) other domains or agents.

Pharmaceutical Compositions Containing Receptor-targeting Reagents.

Any of the receptor-targeting reagents described herein can be incorporated into pharmaceutical compositions. Such compositions typically include the receptor-targeting reagent and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. A receptor-targeting reagent can be formulated as a pharmaceutical composition in the form of a syrup, an elixir, a suspension, a powder, a granule, a tablet, a capsule, a lozenge, a troche, an aqueous solution, a cream, an ointment, a lotion, a gel, an emulsion, etc. Supplementary active compounds (*e.g.*, one or more chemotherapeutic agents) can also be incorporated into the compositions.

A pharmaceutical composition is generally formulated to be compatible with its intended route of administration. Examples of routes of administration include oral, rectal, and parenteral, *e.g.*, intravenous, intramuscular, intradermal, subcutaneous, inhalation, transdermal, or transmucosal. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The compositions can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be

fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contamination by microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of contamination by microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be facilitated by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

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

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the receptor-targeting reagent can be incorporated with excipients and used in the form of tablets, troches, or capsules, *e.g.*, gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. The powders and tablets can contain from 1% to 95% (w/w) of the receptor-targeting reagent. In certain embodiments, the receptor-targeting reagent ranges from 5% to 70% (w/w). Suitable

carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the receptor-targeting reagent with encapsulating material as a carrier providing a capsule in which the receptor-targeting reagent with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration. Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents. For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

A receptor-targeting reagent suitable for topical administration can be formulated as, *e.g.*, a cream, a spray, a foam, a gel, an ointment, a salve, or a dry rub. A dry rub can be rehydrated at the site of administration. A receptor-targeting reagent can also be formulated for direct infusion into (*e.g.*, soaked into and dried) a bandage, gauze, or patch for topical administration. The receptor-targeting reagents can also be formulated in a semi-liquid, gelled, or fully-liquid state in a bandage, gauze, or patch for topical administration (see, *e.g.*, U.S. Patent No. 4,307,717, the content of which is incorporated herein by reference in its entirety).

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives.

Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the receptor-targeting reagents are formulated into ointments, salves, gels, or creams as generally known in the art. The receptor-targeting reagents can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the receptor-targeting reagents are prepared with carriers that will protect the receptor-targeting reagent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to physically discrete units suited as unitary dosages for the subject to be treated, each unit containing a predetermined quantity of receptor-targeting reagent calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Dosage units can also be accompanied by instructions for use. Any of the pharmaceutical compositions described herein can be included in a container, pack, or dispenser together with instructions for administration as described in the following section.

Methods for Generating Antibodies

Methods of making an antibody specific for an EGFR (*e.g.*, HERl, HER2, HER3, or HER4) or uPAR described herein are known in the art. For example, methods for generating antibodies or antibody fragments specific for a protein encoded by one or more genes can be generated by immunization, *e.g.*, using an animal, or by *in vitro* methods such as phage display. A polypeptide that includes all or part of a target protein (*e.g.*, all or part of an EGFR,), uPAR, or ATF) can be used to generate an antibody or antibody fragment.

A peptide can be used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse, or other mammal) with the peptide. An appropriate immunogenic preparation can contain, for example, a chemically synthesized peptide or a recombinantly expressed peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic peptide preparation induces a polyclonal anti-peptide antibody response. The term antibody as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules (*i.e.*, molecules that contain an antigen binding site that specifically bind to the peptide). An antibody that specifically binds to a peptide described herein is an antibody that binds the peptide, but does not substantially bind other molecules in a sample. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments.

The anti-peptide antibody can be a monoclonal antibody or a preparation of polyclonal antibodies. The term monoclonal antibody, as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with the peptide. A monoclonal antibody composition thus typically displays a single binding affinity for a particular peptide with which it immunoreacts.

Polyclonal anti-peptide antibodies can be prepared as described above by immunizing a suitable subject with a peptide immunogen. The anti-peptide antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized peptide. If desired, the antibody molecules directed against the peptide can be isolated from the mammal (*e.g.*, from the blood) and further purified by techniques such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-peptide antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) Nature 256:495-497, the human B cell hybridoma technique (Kozbor et al. (1983) Immunol. Today 4:72), or the EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-peptide monoclonal antibody (see, *e.g.*, Current Protocols in Immunology, supra; Galfre et al. (1977) Nature 266:55052; R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); and Lerner (1981) Yale J. Biol. Med., 54:387-402).

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-peptide antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with a peptide described herein to isolate immunoglobulin library members that bind the peptide. An anti-peptide antibody (*e.g.*, a monoclonal antibody) can be used to isolate the peptide by techniques such as affinity chromatography or immunoprecipitation. Moreover, an anti- peptide antibody can be used to detect the peptide in screening assays described herein. An antibody can optionally be coupled to a detectable label such as any of those described herein or a first or second member of a binding pair (*e.g.*, streptavidin/biotin or avidin/biotin), the second member of which can be conjugated to a detectable label.

Non-human antibodies to an EGFR or uPAR can also be produced in non-human host (*e.g.*, a rodent) and then humanized, *e.g.*, as described in US Pat. No. 6,602,503, EP 239 400, US Pat. No. 5,693,761, and US Pat. No. 6,407,213.

EP 239 400 (Winter et al.) describes altering antibodies by substitution (within a given variable region) of their CDRs for one species with those from another. CDR-substituted antibodies can be less likely to elicit an immune response in humans compared to true chimeric antibodies because the CDR-substituted antibodies contain considerably less non-human components. See Riechmann et al., 1988, Nature 332, 323-327; Verhoeyen et al., 1988, Science 239, 1534-1536. Typically, CDRs of a murine antibody are substituted into the corresponding regions in a human antibody by using recombinant nucleic acid technology to produce sequences encoding the desired substituted antibody. Human constant region gene segments of the desired isotype (*e.g.*, gamma I for CH and kappa for CL) can be added and the humanized heavy and light chain genes can be co-expressed in mammalian cells to produce soluble humanized antibody.

WO 90/07861 describes a process that includes choosing human V framework regions by computer analysis for optimal protein sequence homology to the V region framework of the original murine antibody, and modeling the tertiary structure of the murine V region to visualize framework amino acid residues that are likely to interact with the murine CDRs. These murine amino acid residues are then superimposed on the homologous human framework. See also US Pat. Nos. 5,693,762; 5,693,761; 5,585,089; and 5,530,101. Tempest et al., 1991, Biotechnology 9, 266-271 use, as standard, the V region frameworks derived from NEWM and REI heavy and light chains, respectively, for CDR-grafting without radical introduction of mouse residues. An advantage of using the Tempest et al. approach to construct NEWM and REI based humanized antibodies is that the three dimensional structures of NEWM and REI variable regions are known from x-ray crystallography and thus specific interactions between CDRs and V region framework residues can be modeled. Non-human antibodies can be modified to include substitutions that insert human immunoglobulin sequences, *e.g.*, consensus human amino acid residues at particular positions, *e.g.*, at one or more (*e.g.*, at least five, ten, twelve, or all) of the following positions: (in the framework of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 7OL, 71L, 73L, 85L, 87L, 98L, and/or (in the framework of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 6OH, 67H, 68H, 69H, 7OH, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and/or 103H (according to the Kabat numbering). See, *e.g.*, US Pat. No. 6,407,213, the disclosure of which is incorporated herein by reference in its entirety.

Fully human monoclonal antibodies that bind to an EGFR or uPAR can be produced, *e.g.*, using in vzYro-primed human splenocytes, as described by Boerner et al., 1991, J. Immunol., 147, 86-95. They may be prepared by repertoire cloning as described by Persson et al., 1991, Proc. Nat. Acad. Sci. USA, 88: 2432-2436 or by Huang and Stollar, 1991, J. Immunol.

Methods 141, 227-236; also US Pat. No. 5,798,230, the disclosures of each of which are incorporated herein by reference in their entirety. Large nonimmunized human phage display libraries may also be used to isolate high affinity antibodies that can be developed as human therapeutics using standard phage technology (see, *e.g.*, Vaughan et al, 1996; Hoogenboom et al. (1998) Immunotechnology 4:1-20; and Hoogenboom et al. (2000) Immunol Today 2:371-8; US 2003-0232333).

As used herein, an "immunoglobulin variable domain sequence" refers to an amino acid sequence that can form the structure of an immunoglobulin variable domain. For example, the sequence may include all or part of the amino acid sequence of a naturally-occurring variable domain. For example, the sequence may omit one, two or more N- or C-terminal amino acids, internal amino acids, may include one or more insertions or additional terminal amino acids, or may include other alterations. In one embodiment, a polypeptide that includes an immunoglobulin variable domain sequence can associate with another immunoglobulin variable domain sequence to form a target binding structure (or "antigen binding site"), *e.g.*, a structure that interacts with an EGFR or a uPAR.

The VH or VL chain of the antibody can further include all or part of a heavy or light chain constant region, to thereby form a heavy or light immunoglobulin chain, respectively. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains. The heavy and light immunoglobulin chains can be connected by disulfide bonds. The heavy chain constant region typically includes three constant domains, CHl, CH2 and CH3. The light chain constant region typically includes a CL domain. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (CIq) of the classical complement system.

One or more regions of an antibody can be human, effectively human, or humanized. For example, one or more of the variable regions can be human or effectively human. For example, one or more of the CDRs, *e.g.*, heavy chain (HC) CDRl, HC CDR2, HC CDR3, light chain (LC) CDRl , LC CDR2, and LC CDR3, can be human. Each of the light chain CDRs can be human. HC CDR3 can be human. One or more of the framework regions (FR) can be human, *e.g.*, FRl, FR2, FR3, and FR4 of the HC or LC. In some embodiments, all the framework regions are human, *e.g.*, derived from a human somatic cell, *e.g.*, a hematopoietic cell that produces immunoglobulins or a non-hematopoietic cell. In one embodiment, the human sequences are germline sequences, *e.g.*, encoded by a germline nucleic acid. One or more of the constant regions can be human, effectively human, or humanized. In another embodiment, at least 70, 75,

80, 85, 90, 92, 95, or 98% of the framework regions (*e.g.*, FRl, FR2, and FR3, collectively, or FRl, FR2, FR3, and FR4, collectively) or the entire antibody can be human, effectively human, or humanized. For example, FRl, FR2, and FR3 collectively can be at least 70, 75, 80, 85, 90, 92, 95, 98, or 99% identical to a human sequence encoded by a human germline segment.

An "effectively human" immunoglobulin variable region is an immunoglobulin variable region that includes a sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. An "effectively human" antibody is an antibody that includes a sufficient number of human amino acid positions such that the antibody does not elicit an immunogenic response in a normal human. A "humanized" immunoglobulin variable region is an immunoglobulin variable region that is modified such that the modified form elicits less of an immune response in a human than does the non-modified form, *e.g.*, is modified to include a sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. Descriptions of "humanized" immunoglobulins include, for example, US Pat. No. 6,407,213 and US Pat. No. 5,693,762, the disclosures of each of which are incorporated herein by reference in their entirety. In some cases, humanized immunoglobulins can include a non-human amino acid at one or more framework amino acid positions.

All or part of an antibody can be encoded by an immunoglobulin gene or a segment thereof. Exemplary human immunoglobulin genes include the kappa, lambda, alpha (IgAl and IgA2), gamma (IgGl, IgG2, IgG3, IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin "light chains" (about 25 kDa or 214 amino acids) are encoded by a variable region gene at the NH2 -terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH-terminus. Full-length immunoglobulin "heavy chains" (about 50 kDa or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, *e.g.*, gamma (encoding about 330 amino acids).

The term "antigen-binding fragment" of a full length antibody refers to one or more fragments of a full-length antibody that retain the ability to specifically bind to a target of interest (*i.e.*, an EGFR, an IL13R, or an IL4R). Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include: (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CHl domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CHl domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody; (v) a dAb fragment (Ward et al., (1989)

Nature 341 :544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) that retains functionality. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules known as single chain Fv (scFv). See *e.g.*, Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883, the disclosures of each of which are incorporated herein by reference in their entirety.

Methods for Binding a Receptor-Targeting Reagent to a Cell

The present disclosure provides a variety of *in vitro*, *in vivo*, and *ex vivo* methods for binding a receptor-targeting reagent to a cell. Where the receptor-targeting reagent is immunotoxic (*i.e.*, the receptor-targeting reagent contains one or more toxic domains), the methods can be used to inhibit the proliferation of (or kill) a cell (*e.g.*, a cancer cell or an immune cell mediating an inflammatory disorder). In embodiments where the receptor-targeting reagents are detectably labeled, such reagents can be useful for detecting the presence of a cell (*e.g.*, a cancer cell or an immune cell mediating an inflammatory disorder) expressing one or more of an EGFR, an IL13R, or an IL4R. Thus, the *in vivo* or *ex vivo* methods are useful, inter alia, in the treatment and/or diagnosis of cancers or inflammatory disorders, which conditions include any of the cancers or inflammatory disorders (*e.g.*, autoimmune diseases) described herein. In some embodiments, the methods for binding a receptor-targeting reagent to a cell can also be, *e.g.*, methods for contacting a receptor-targeting reagent to a cell, methods for killing a cell, methods for inhibiting the proliferation of a cell, or methods for detecting the presence or absence of a cell.

***In vitro* Methods For Binding a Receptor-targeting Reagent to a Cell.**

Provided herein are *in vitro* methods for binding a receptor-targeting reagent to a cell. The method is useful, for example, in studies evaluating the efficacy of an immunotoxic receptor-targeting reagent at inhibiting the proliferation of (or killing) cancer cells in culture or in diagnostic assays for identifying one or more cells expressing certain receptors (*e.g.*, an EGFR and/or uPAR). For example, a detectably-labeled receptor-targeting reagent can be contacted to a cell sample obtained from a subject to determine if one or more cells of the cell sample express an EGFR and/or uPAR. The methods can also serve as a "positive control" in assays to identify compounds having similar activity (*e.g.*, similar ability to bind to a cell or where the receptor-targeting reagent is toxic).

The *in vitro* methods of binding a receptor-targeting reagent to a cell include the step of contacting a cell with any of the receptor-targeting reagents described herein. Methods for

contacting a cell with a receptor-targeting reagent described herein are detailed in the accompanying Examples. For example, adherent cells can be plated on solid support matrix (*e.g.*, a plastic tissue culture plate, or a multi-well (96 or 386-well) tissue culture plate) and grown in appropriate medium such as DMEM or RPMI medium. After seeding the cells on the solid support, the receptor-targeting reagents can be added to medium in which the cells are cultured (at various concentrations) and incubated with the cells (for varying amounts of time) under conditions that allow for the binding of the receptor-targeting reagent to the cell to occur. The method can also, optionally, include the step of determining if the cell expresses an EGFR and/or uPAR. Expression can be mRNA or protein expression of an EGFR and/or uPAR. Suitable methods of detecting protein or mRNA expression are well known to those of skill in the art and described, *e.g.*, in Sambrook et al. (supra). These methods can include, for example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE)/western blotting techniques using antibodies specific for the target protein (see above under "Methods of Generating Antibodies"), or RT-PCR or northern blotting techniques for detection of mRNA expression. The method can also, optionally, include the step of, after contacting the receptor-targeting reagent with the cell, determining if the receptor-targeting reagent bound to the cell. For example, a receptor-targeting reagent can be detectably-labeled as described above and after contacting the cell with the detectably-labeled receptor-targeting reagent, the binding of the reagent to the cell can be detected by detecting the presence of the detectable label. Alternatively, after contacting the cell with a non detectably-labeled receptor-targeting reagent, the binding of the receptor-targeting reagent to the cell can be detected by contacting the receptor-targeting reagent with a detectably-labeled antibody that specifically binds to the receptor-targeting reagent.

Methods of detecting and/or for quantifying a detectable label depend on the nature of the label and are known in the art. Examples of detectors useful for detecting a detectable label include, without limitation, x-ray film, radioactivity counters, scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers.

Suitable methods for determining if the receptor-targeting reagent bound to the cell are also detailed in the accompanying Examples.

The cells can express one or more of an EGFR (*e.g.*, a HERl, a HER2, a HER3, or a HER4) and/or a uPAR. The cells include both prokaryotic (*e.g.*, bacterial cells) and eukaryotic cells. Eukaryotic cells can include, for example, fungus (*e.g.*, yeast), insect, plant, fish, reptile, and mammalian cells (*e.g.*, mouse, rat, rabbit, guinea pig, dog, cat, pig, horse, goat, cow, whale, monkey, or human). The cells can be normal, transformed, or malignant and of any histological type, *e.g.*, without limitation, epithelial cells, fibroblasts, lymphoid cells,

macrophages/monocytes, granulocytes, keratinocytes, or muscle cells. Cancer cells can be cancer stem cells. Cancer cells can include cells from cancers such as, but not limited to, lung cancer, breast cancer, colon cancer, pancreatic cancer, renal cancer, stomach cancer, liver cancer, bone cancer, hematological cancer, neural tissue cancer (*e.g.*, glioblastoma), melanoma, thyroid cancer, ovarian cancer, testicular cancer, prostate cancer, cervical cancer, vaginal cancer, head and neck cancer, or bladder cancer. Cancer cells can include cells from cancers such as, carcinomas and sarcomas (*e.g.,* angiosarcoma). Suitable cell lines include those recited in the accompanying Examples, *e.g.*, glioblastoma, carcinoma, sarcoma, or prostate cancer cell lines.

In embodiments where the *in vitro* method includes contacting a cell with a toxic receptor-targeting reagent (such as any of those described herein), the methods can also be methods of inhibiting the proliferation of (or killing) a cell such as a cancer cell or an immune cell mediating an inflammatory disorder. The method can, optionally, include the step of determining if the toxic receptor-targeting reagent killed the cell (or inhibited the proliferation of the cell). Generally, cells can be killed, *e.g.*, through necrosis (cells swell and break open) or through programmed cell death (apoptosis). Methods for determining whether a cell is killed are known in the art and described in the accompanying Examples. For example, the number of viable cells in a cell population remaining after contact with a toxic receptor-targeting reagent are compared to the number of viable cells in a control cell population that were not contacted with the reagent.

One method for determining the viability of a cell (or a population of cells) is trypan blue exclusion analysis. For example, cells from a well of a tissue culture dish can be trypsinized from the plate, washed, stained with a dye (*e.g.*, typan blue), and counted using a microscope or mechanical cell counter (*e.g.*, Beckman-Coulter Zl™ Series COULTER COUNTER® Cell and Particle Counter). Since dyes like trypan blue are only taken up by dead or dying cells, this method allows for discrimination (*i.e.*, blue or white cell) between viable and non-viable cells in a population.

Another method for determining the viability of a cell (or a population of cells) is a metabolic assay, for example, an MTT-metabolic assay (*e.g.*, an MTT-metabolic assay from Invitrogen, USA). MTT Diphenyltetrazolium Bromide, is a tetrazolium salt (yellowish) that is cleaved to formazan crystals by the succinate dehydrogenase system which belongs to the mitochondrial respiratory chain, and is only active in viable cells. The mitochondrial succinate dehydrogenase reduces the MTT crystals into purple formazan in the presence of an electron coupling reagent. Following the treatment of the cells with a compound, the cells are exposed to the MTT reagent and the more viable cells are present in a well, the more formazan dye is produced. Extent of formazan dye can be measured, for example, using a spectrophotometer.

Other commonly used methods of detecting increased cell death include monitoring DNA synthesis in a population of cells (*i.e.*, a reduction in the amount of DNA synthesis in a population of cells). Cells grown, for example, in the presence or absence of a toxic receptor-targeting reagent are also treated with a nucleotide analog that can incorporate into the DNA of the cell upon cell division. Examples of such nucleotide analogs include, for example, BrdU or ^{3}H-thymidine. In each case, the amount of label incorporated into the cells (grown in the presence and absence of a given inhibitory agent) is quantified, and the amount of label incorporation is directly proportional to the amount of remaining viable cells in the cell population. In this context, cell viability (*e.g.*, cancer cell viability) can be decreased by at least 10% (*e.g.*, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% or more) relative to the cell viability in the absence of the toxic receptor-targeting reagent.

It is understood that the above methods can also be used to determine if a receptor-targeting reagent has inhibited the growth of a target cell.

Comparisons of the extent of apoptosis between cells cultured with and without a toxic receptor-targeting reagent can be accomplished by measuring a host of indicators, for example, DNA fragmentation, caspase activity, loss of mitochondrial membrane potential, increased production of reactive oxygen species (ROS), intracellular acidification, chromatin condensation, phosphatidyl serine levels at the cell surface, or an increased cell permeability.

DNA fragmentation can be measured, *e.g.*, by with the TUNEL assay (terminal deoxynucleotide transferase dUTP nick end labeling). Commercial versions of the assay are widely available, for example, APO-BrdUTM TUNEL Assay Kit (Invitrogen), APO-DIRECTTM Kit (BD-Biosciences-Pharmingen) and ApoAlertTM DNA fragmentation Assay Kit (Clontech).

Caspase activity can be measured via fiuorogenic, chromogenic, and luminescent substrates specific for a given caspase (*e.g.*, Caspase 3 or Caspase 9). Commercial kits are available for a variety of caspases such as caspase 3, caspase 7, caspase 8, and caspase 9 (see BD-Pharmingen or Invitrogen).

Loss of mitochondrial membrane potential can be measured with fluorescent dyes that selectively accumulate in various compartments of the mitochondria based on their integrity and functionality. One non-limiting example of such a dye is Mitotracker Red (Invitrogen).

Production of reactive oxygen species can be monitored with fluorescent dyes such as H2DCFDA.

Chromatin condensation can be measured with dyes such as Hoechst 33342 or propidium iodide. Phosphotidyl serine (PS) levels can be measured at the cell surface. For example, Annexin V having a high affinity for PS, can be used to as a probe for PS on a cell surface. Numerous commercially available assay kits are suitable for such measurements (see BD-Biosciences Pharmingen).

***In vivo* Methods For Binding a Receptor-targeting Reagent to a Cell.**

Also featured are *in vivo* methods for binding a receptor-targeting reagent to a cell. The method includes the step of delivering to a subject any of the receptor-targeting reagents described herein. The subject can be any mammal, *e.g.*, a human (*e.g.*, a human patient) or a non-human primate (*e.g.*, chimpanzee, baboon, or monkey), mouse, rat, rabbit, guinea pig, gerbil, hamster, horse, a type of livestock (*e.g.*, cow, pig, sheep, or goat), a dog, cat, or a whale. The subject can be one having, suspected of having, or at risk of developing a cancer or an inflammatory disorder.

Where the receptor-targeting reagent is toxic (*i.e.*, the receptor-targeting reagent contains one or more toxic domains), the *in vivo* methods for binding a receptor-targeting reagent to a cell can be methods of inhibiting the proliferation of (or killing) a cancer cell.

Generally, the receptor-targeting reagents delivered to the subject will be suspended in a pharmaceutically-acceptable carrier (*e.g.*, physiological saline) and administered orally, rectally, or parenterally, *e.g.*, injected intravenously, subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily (as described above). The receptor-targeting reagents can also be delivered directly to cells or a tissue (*e.g.*, tumor cells). Where the receptor-targeting reagents are toxic, the methods can be used to kill tumor cells or immune cells mediating an inflammatory disorder or, *e.g.*, to kill any residual tumor cells in a tumor bed following surgical resection of a tumor.

The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable dosages are in the range of 0.0001 mg/kg - 100 mg/kg. Wide variations in the needed dosage are to be expected in view of the variety of receptor-targeting reagents and the differing efficiencies of various routes of administration. For example, oral administration may require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art.

Administrations can be single or multiple (*e.g.*, 2-, 3-, 4-, 6-, 8-, 10-, 20-, 50- 100-, 150-, or more fold). Encapsulation of the receptor-targeting reagent in a suitable delivery vehicle (*e.g.*,

polymeric microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery. Alternatively, a polynucleotide containing a nucleic acid sequence encoding the receptor- targeting reagent can be delivered to appropriate cells in a mammal. Expression of the coding sequence can be directed to any cell in the body of the subject. However, expression will preferably be directed to the target cells themselves or in some instances, in the vicinity of the cells whose viability it is desired to decrease. This can be achieved by, for example, the use of polymeric, biodegradable microparticle or microcapsule delivery devices known in the art. Another way to achieve uptake of the nucleic acid is using liposomes, prepared by standard methods. The vectors can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific or tumor-specific antibodies. Alternatively, one can prepare a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells (Cristiano et al. (1995), J. Mol. Med. 73:479, the disclosure of which is incorporated herein by reference in its entirety). Alternatively, tissue specific targeting can be achieved by the use of tissue-specific transcriptional regulatory elements (TRE) which are known in the art. Delivery of "naked DNA" (*i.e.*, without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site is another means to achieve *in vivo* expression.

Polynucleotides can be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles that are suitable for administration to a human, *e.g.*, physiological saline or liposomes. A therapeutically effective amount is an amount of the polynucleotide that is capable of producing a medically desirable result (*e.g.*, decreased proliferation of cancer cells) in a treated subject. As is well known in the medical arts, the dosage for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for administration of polynucleotide is from approximately 10 to approximately 10^{12} copies of the polynucleotide molecule. This dose can be repeatedly administered, as needed. Schedules and co-administration can be any of those described herein (see, for example, "Methods of Treatment").

In some embodiments, the *in vivo* methods can include the step of determining whether a subject has a cancer or an inflammatory disorder. Where the subject has (or is determined to have) a cancer or an inflammatory disorder, the methods can include the step of determining if one or more cells of the subject's cancer or one or more immune cells mediating the subject's inflammatory condition express an EGFR and/or uPAR. Methods for determining expression of an EGFR, an IL13R, or an IL4R are described above.

Any of the receptor-targeting reagents can also, in some instances, be co-administered with one or more additional therapies or therapeutic agents such as chemotherapeutic agents.

Methods for co-administration and exemplary additional therapies and therapeutic agents that can be co-administered with any of the receptor-targeting reagents described herein are detailed below.

Where the receptor-targeting reagent delivered to the subject is detectably-labeled, the *in vivo* methods can be used to detect the presence of a cell, *e.g.*, a cell expressing an EGFR and/or a uPAR. That is, any of the detectably-labeled receptor-targeting reagents described herein can be used as probes, *e.g.*, to guide surgery or detect a disease. For example, an area suspected of containing cancer cells (*e.g.*, a primary tumor or microscopic metastases) can be exposed to a receptor-targeting reagent capable of binding to the cell (through an EGFR and/or a uPAR). Thus, all cancer cells to which the receptor-targeting reagent binds will be differentiated from the non-cancer cells and can aid in the treatment of the cancer (*e.g.*, surgical removal of the cancer or targeted chemotherapy). In another example, cells detectably labeled by a receptor-targeting reagent described herein can be isolated away from non-labeled cells. For example, certain types or populations of cells (*e.g.*, B cell or T cell populations (*e.g.*, B cell or T cell populations mediating an inflammatory disorder) or stem cell populations) can be detected and isolated from non-detectably labeled cells. Detectably labeled cells can also be visualized *in vivo* to determine, *e.g.*, their localization. *In vivo* methods of detecting the receptor- targeting reagents depend of course on the nature of the detectable label and can include, *e.g.*, bioluminescence imaging, micro positron emission tomography/single photon emission commuted tomography, magnetic resonance imaging, and intravital microscopy (see, *e.g.*, Dustin (2003) Arthritis Res. Ther. 5:165-171, the disclosure of which is incorporated herein by reference in its entirety).

Further description of suitable *in vivo* methods (*e.g.*, methods of treatment using the receptor-targeting reagents described herein) can be found under "Methods of Treatment."

***Ex vivo* Methods for Binding a Receptor-targeting Reagent to a Cell.**

An *ex vivo* strategy can involve transfecting or transducing cells obtained from the subject to be treated (or another subject) with a polynucleotide encoding a receptor-targeting reagent (*e.g.*, a toxic receptor-targeting reagent) that, *e.g.*, is capable of binding to a target cell or killing a target cell. The transfected or transduced cells are then administered to the subject. The cells can be any of a wide range of types including, without limitation, hemopoietic cells (*e.g.*, bone marrow cells, macrophages, monocytes, dendritic cells, T cells, or B cells), fibroblasts, epithelial cells, endothelial cells, keratinocytes, or muscle cells. Such cells act as a source of the receptor-targeting reagent for as long as they survive in the subject. Alternatively, tumor cells or

inflammatory cells (*e.g.*, immune cells), preferably obtained from the subject (autologous) but potentially from a subject of the same species other than the subject (allogeneic), can be transfected or transformed by a vector encoding the receptor-targeting reagent. The tumor cells, preferably treated with an agent (*e.g.*, ionizing irradiation) that ablates their proliferative capacity, are then introduced into the subject, where they secrete the receptor- targeting reagent.

The *ex vivo* methods include the steps of harvesting cells from a subject, culturing the cells, transducing them with an expression vector, and maintaining the cells under conditions suitable for expression of the receptor-targeting reagent. These methods are known in the art of molecular biology. The transduction step is accomplished by any standard means used for *ex vivo* gene therapy, including calcium phosphate, lipofection, electroporation, viral infection, and biolistic gene transfer (also see above). Alternatively, liposomes or polymeric microparticles can be used. Cells that have been successfully transduced can be selected, for example, for expression of the coding sequence or of a drug resistance gene. The cells may then be lethally irradiated (if desired) and injected or implanted into the same or another subject.

In some embodiments, the *ex vivo* methods can be used to purge a cell population from a mixture of cells. For example, a mixture of cells (*e.g.*, bone marrow or any other stem cell population) obtained from a subject with cancer (*e.g.*, any of the cancers described herein) can be purged of any cancer cells therein. The mixture of cells can be contacted with a toxic receptor-targeting reagent described herein to kill cancer cells contained therein. Following the killing of the cancer cells, the mixture of cells can be returned to the subject, *e.g.*, after the subject has been treated with a chemotherapeutic agent.

Diseases Treatable by a Therapy Comprising a Receptor-targeting Reagent

The receptor-targeting reagents (*e.g.*, the toxic receptor-targeting reagents) described herein can be used to treat a variety of proliferative disorders and/or inflammatory disorders. Proliferative disorders include, *e.g.*, cancers, certain immune disorders (such as inflammatory disorders, or warts). Examples of some specific disorders that can be treated (or in some instances prevented) by the administration of one or more receptor-targeting reagents are reviewed in the following sections.

Cancer

Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system). Cancer can affect people at all ages, but risk tends to increase

with age. Types of cancer can include, *e.g.*, carcinomas and sarcomas (*e.g.*, angiosarcoma). Types of cancers can also include, *e.g.*, lung cancer, breast cancer, colon cancer, pancreatic cancer, renal cancer, stomach cancer, liver cancer, bone cancer, hematological cancer, neural tissue cancer (*e.g.*, glioblastoma such as glioblastoma multiforme), melanoma, thyroid cancer, ovarian cancer, testicular cancer, prostate cancer, cervical cancer, vaginal cancer, head and neck cancer, or bladder cancer.

As used herein, a subject "at risk of developing a cancer" is a subject that has a predisposition to develop a cancer, *i.e.*, a genetic predisposition to develop cancer such as a mutation in a tumor suppressor gene (*e.g.*, mutation in BRCAl, p53, RB, or APC) or has been exposed to conditions that can result in cancer. Thus, a subject can also be one "at risk of developing a cancer" when the subject has been exposed to mutagenic or carcinogenic levels of certain compounds (*e.g.*, carcinogenic compounds in cigarette smoke such as acrolein, arsenic, benzene, benz{a}anthracene, benzo{a}pyrene, polonium-210 (Radon), urethane, or vinyl chloride). Moreover, the subject can be "at risk of developing a cancer" when the subject has been exposed to, *e.g.*, large doses of ultraviolet light or X-irradiation, or exposed (*e.g.*, infected) to a tumor-causing/associated virus such as papillomavirus, Epstein-Barr virus, hepatitis B virus, or human T-cell leukemia-lymphoma virus. From the above it will be clear that subjects "at risk of developing a cancer" are not all the subjects within a species of interest.

A subject "suspected of having a cancer" is one having one or more symptoms of a cancer. Symptoms of cancer are well-known to those of skill in the art and include, without limitation, breast lumps, nipple changes, breast cysts, breast pain, weight loss, weakness, excessive fatigue, difficulty eating, loss of appetite, chronic cough, worsening breathlessness, coughing up blood, blood in the urine, blood in stool, nausea, vomiting, liver metastases, lung metastases, bone metastases, abdominal fullness, bloating, fluid in peritoneal cavity, vaginal bleeding, constipation, abdominal distension, perforation of colon, acute peritonitis (infection, fever, pain), pain, vomiting blood, heavy sweating, fever, high blood pressure, anemia, diarrhea, jaundice, dizziness, chills, muscle spasms, colon metastases, lung metastases, bladder metastases, liver metastases, bone metastases, kidney metastases, and pancreas metastases, difficulty swallowing, and the like.

In addition to the administration of one or more receptor-targeting reagents described herein, a cancer can also be treated by chemotherapeutic agents, ionizing radiation, immunotherapy agents, or hyperthermotherapy agents. Chemotherapeutic agents include, but are not limited to, cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, adriamycin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide,

verampil, podophyllotoxin, tamoxifen, taxol, transplatinum, 5-flurouracil, vincristin, vinblastin, and methotrexate. As described above, any of these non-receptor-targeting reagent therapies can be co-administered (administered in a combination therapy regimen) with any of the receptor-targeting reagents described herein (see below under "Methods of Treatment").

Inflammatory Disorders

An "inflammatory disorder," as used herein, refers to a process in which one or more substances (*e.g.*, substances not naturally occurring in the subject), via the action of white blood cells (*e.g.*, B cells, T cells, macrophages, monocytes, or dendritic cells) inappropriately trigger a pathological response, *e.g.*, a pathological immune response. Accordingly, such immune cells involved in the inflammatory response are referred to as "inflammatory cells." The inappropriately triggered inflammatory response can be one where no foreign substance (*e.g.*, an antigen, a virus, a bacterium, a fungus) is present in or on the subject. The inappropriately triggered response can be one where a self-component (*e.g.*, a self-antigen) is targeted (*e.g.*, an autoimmune disorder such as multiple sclerosis) by the inflammatory cells. The inappropriately triggered response can also be a response that is inappropriate in magnitude or duration, *e.g.*, anaphylaxis. Thus, the inappropriately targeted response can be due to the presence of a microbial infection (*e.g.*, viral, bacterial, or fungal). Types of inflammatory disorders (*e.g.*, autoimmune disease) can include, but are not limited to, osteoarthritis, rheumatoid arthritis (RA), spondyloarthropathies, POEMS syndrome, Crohn's disease, graft- versus host disease, multicentric Castleman's disease, systemic lupus erythematosus (SLE), multiple sclerosis (MS), muscular dystrophy (MD), insulin-dependent diabetes mellitus (IDDM), dermatomyositis, polymyositis, inflammatory neuropathies such as Guillain Barre syndrome, vasculitis such as Wegener's granulomatosus, polyarteritis nodosa, polymyalgia rheumatica, temporal arteritis, Sjogren's syndrome, Bechet's disease, Churg-Strauss syndrome, or Takayasu's arteritis. Also included in inflammatory disorders are certain types of allergies such as rhinitis, sinusitis, urticaria, hives, angioedema, atopic dermatitis, food allergies (*e.g.*, a nut allergy), drug allergies (*e.g.*, penicillin), insect allergies (*e.g.*, allergy to a bee sting), or mastocytosis. Inflammatory disorders can also include ulcerative colitis and asthma.

A subject "at risk of developing an inflammatory disorder" refers to a subject with a family history of one or more inflammatory disorders (*e.g.*, a genetic predisposition to one or more inflammatory disorders) or one exposed to one or more inflammation-inducing conditions. For example, a subject can have been exposed to a viral or bacterial superantigen such as, but not limited to, *Staphylococcal* enterotoxins (SEs), a *Streptococcus pyogenes* exotoxin (SPE), a *Staphylococcus aureus* toxic shock-syndrome toxin (TSST-I), a *Streptococcal* mitogenic exotoxin (SME) and a *Streptococcal* superantigen (SSA). From the above it will be clear that

subjects "at risk of developing an inflammatory disorder" are not all the subjects within a species of interest.

A subject "suspected of having an inflammatory disorder" is one who presents with one or more symptoms of an inflammatory disorder. Symptoms of inflammatory disorders are well known in the art and include, but are not limited to, redness, swelling (*e.g.*, swollen joints), joints that are warm to the touch, joint pain, stiffness, loss of joint function, fever, chills, fatigue, loss of energy, headaches, loss of appetite, muscle stiffness, insomnia, itchiness, stuffy nose, sneezing, coughing, one or more neurologic symptoms such as dizziness, seizures, or pain.

In addition to the administration of one or more altered receptor-targeting reagents described herein, an inflammatory disorder can also be treated by non-steroidal antiinflammatory drug (NSAID), a disease-modifying anti-rheumatic drug (DMARD), a biological response modifier, or a corticosteroid. Biological response modifiers include, *e.g.*, an anti-TNF agent (*e.g.*, a soluble TNF receptor or an antibody specific for TNF such as adulimumab, infliximab, or etanercept). As described above, any of these non-receptor-targeting reagent therapies can be co-administered (administered in a combination therapy regimen) with any of the receptor-targeting reagents described herein (see below under "Methods of Treatment").

Methods for Selecting an Appropriate Therapeutic Modality for a Subject

Also provided herein are methods for selecting an appropriate therapeutic modality for a subject (*e.g.*, a human such as one having a cancer or inflammatory disorder), which methods are useful to, *e.g.*, medical professionals in effectively and appropriately treating subjects having disorders such as cancers or inflammatory disorders. The method can include the step of selecting as a therapeutic agent for the subject having cancer or an inflammatory disorder any of the receptor-targeting reagents described herein (*e.g.*, any of the toxic receptor-targeting reagents described herein) if one or more cancer cells of the subject's cancer, or one or more immune cells mediating a subject's inflammatory disorder, express an uPAR or an EGFR. The method can also include the step of determining if one or more cancer cells of the subject's cancer, or one or more immune cells mediating a subject's inflammatory disorder, express an EGFR or an uPAR. Methods for determining whether a cell expresses an EGFR or an uPAR are described above.

In some instances where a medical practitioner selects for a subject a therapy comprising a toxic receptor-targeting reagent, the practitioner may also select a therapy comprising a non-toxic receptor-targeting reagent. For example, the medical professional can select a first therapy comprising a non-toxic receptor-targeting reagent and a second therapy comprising a toxic

receptor-targeting reagent. It is understood that two different therapies (*e.g.*, a therapy comprising a toxic receptor-targeting reagent and a therapy comprising a non-toxic receptor-targeting reagent) can be selected for a subject by different medical professionals. For example, one medical professional can select for a subject a first therapy comprising a toxic receptor-targeting reagent and a second medical professional can select for the subject a therapy comprising a non-toxic receptor-targeting reagent.

In accordance with the methods described herein, any medical practitioner (*e.g.*, a doctor or a nurse) can select an appropriate therapeutic modality for the subject so identified as having one or more cancer cells (or immune cells mediating an inflammatory disorder) that express an EGFR or an uPAR. Selecting a therapy for a subject can be, *e.g.*: (i) writing a prescription for a medicament; (ii) giving (but not necessarily administering) a medicament to a subject (*e.g.*, handing a sample of a prescription medication to a patient while the patient is at the physician's office); (iii) communication (verbal, written (other than a prescription), or electronic (email, post to a secure site)) to the patient of the suggested or recommended therapeutic modality (*e.g.*, an immunotoxic receptor-targeting reagent described herein); or (iv) identifying a suitable therapeutic modality for a subject and disseminating the information to other medical personnel, *e.g.*, by way of patient record. The latter (iv) can be useful in a case where, *e.g.*, more than one therapeutic agent are to be administered to a patient by different medical practitioners. It is understood that an electronic communication can be, *e.g.*, one stored on a computer or other electronic media such as a DVD, CD, or floppy disk) or in a written (*e.g.*, printed) form.

After selecting an appropriate therapeutic modality for a subject, a medical practitioner (*e.g.*, a doctor or a nurse) can administer the appropriate therapeutic modality to the subject (*e.g.*, any of the receptor-targeting reagents described herein). Methods of administering a receptor-targeting reagent (*e.g.*, a toxic receptor-targeting reagent) to a mammal are described below and in the accompanying Examples.

Methods of Treatment

Administration of a receptor-targeting reagent described herein or pharmaceutical composition thereof can be systemic or local. As described above, pharmaceutical compositions can be formulated such that they are suitable for parenteral and/or non-parenteral administration. Specific administration modalities include subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, intrathecal, oral, rectal, buccal, topical, nasal, ophthalmic, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic, vaginal, and intra-uterine administration.

Administration can be by periodic injections of a bolus of the pharmaceutical composition or can be uninterrupted or continuous by intravenous or intraperitoneal administration from a reservoir which is external (*e.g.*, an IV bag) or internal (*e.g.*, a bioerodable implant, a bioartificial organ, or a colony of implanted receptor-targeting reagent production cells). See, *e.g.*, U.S. Pat. Nos. 4,407,957, 5,798,113, and 5,800,828, each incorporated herein by reference in their entirety. Administration of a pharmaceutical composition can be achieved using suitable delivery means such as: a pump (see, *e.g.*, Annals of Pharmacotherapy, 27:912 (1993); Cancer, 41 :1270 (1993); Cancer Research, 44:1698 (1984), incorporated herein by reference in its entirety); microencapsulation (see, *e.g.*, U.S. Pat. Nos. 4,352,883; 4,353,888; and 5,084,350, herein incorporated by reference in its entirety); continuous release polymer implants (see, *e.g.*, Sabel, U.S. Pat. No. 4,883,666, incorporated herein by reference in its entirety); macroencapsulation (see, *e.g.*, U.S. Pat. Nos. 5,284,761, 5,158,881, 4,976,859 and 4,968,733 and published PCT patent applications WO92/19195, WO 95/05452, the disclosures of each of which are incorporated herein by reference in their entirety); injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation. Therapeutically effective amounts of a pharmaceutical composition can be administered to a subject in need thereof in a dosage regimen ascertainable by one of skill in the art. For example, a composition can be administered to the subject, *e.g.*, systemically at a dosage from 0.001 μg/kg to 10,000 μg/kg body weight of the subject, per dose. In another example, the dosage is from 1 μg/kg to 100 μg/kg body weight of the subject, per dose. In another example, the dosage is from 1 μg/kg to 30 μg/kg body weight of the subject, per dose, *e.g.*, from 3 μg/kg to 10 μg/kg body weight of the subject, per dose.

In order to optimize therapeutic efficacy, a receptor-targeting reagent (*e.g.*, a toxic receptor-targeting reagent) can be first administered at different dosing regimens. The unit dose and regimen depend on factors that include, *e.g.*, the species of mammal, its immune status, the body weight of the mammal. Typically, levels of a receptor-targeting reagent in a tissue can be monitored using appropriate screening assays as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen.

The frequency of dosing for a receptor-targeting reagent is within the skills and clinical judgment of medical practitioners (*e.g.*, doctors or nurses). Typically, the administration regime is established by clinical trials which may establish optimal administration parameters. However, the practitioner may vary such administration regimes according to the subject's age, health, weight, sex and medical status. The frequency of dosing can be varied depending on whether the treatment is prophylactic or therapeutic.

Toxicity and therapeutic efficacy of such receptor-targeting reagents (*e.g.*, toxic receptor-targeting reagents) or pharmaceutical compositions thereof can be determined by known pharmaceutical procedures in, for example, cell cultures or experimental animals. These procedures can be used, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Pharmaceutical compositions that exhibit high therapeutic indices are preferred. While pharmaceutical compositions that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to normal cells (*e.g.*, non-target cells) and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in appropriate subjects (*e.g.*, human patients). The dosage of such pharmaceutical compositions lies generally within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a pharmaceutical composition used as described herein (*e.g.*, for treating a proliferative disorder in a subject), the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the pharmaceutical composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography. Methods for determining an IC_{50} for a receptor-targeting reagent in cell culture are detailed in the accompanying Examples.

As defined herein, a "therapeutically effective amount" of a receptor-targeting reagent is an amount of the reagent that is capable of producing a medically desirable result (*e.g.*, amelioration of one or more symptoms of a proliferative disorder, decreased proliferation of cancer cells or immune cells mediating an inflammatory disorder, or a decrease in one or more side-effects associated with a toxic therapy) in a treated subject. A therapeutically effective amount of a receptor-targeting reagent (*i.e.*, an effective dosage) includes milligram, microgram, nanogram, or picogram amounts of the reagent per kilogram of subject or sample weight (*e.g.*, about 1 nanogram per kilogram to about 500 micrograms per kilogram, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram).

The subject can be any of those described herein, *e.g.*, a mammal such as a human. A receptor-targeting reagent or pharmaceutical composition thereof described herein can be administered to a subject as a combination therapy with another treatment, *e.g.*, a treatment for a proliferative disorder (*e.g.*, a cancer or an inflammatory disorder). For example, the combination therapy can include administering to the subject (*e.g.*, a human patient) one or more additional agents that provide a therapeutic benefit to the subject who has, or is at risk of developing, (or suspected of having) a proliferative disorder. Thus, the receptor-targeting reagent or pharmaceutical composition and the one or more additional agents can be administered at the same time. Alternatively, the receptor-targeting reagent can be administered first in time and the one or more additional agents administered second in time. The one or more additional agents can be administered first in time and the receptor-targeting reagent administered second in time. The receptor-targeting reagent can replace or augment a previously or currently administered therapy. For example, upon treating a subject with a receptor-targeting reagent, administration of the one or more additional agents can cease or diminish, *e.g.*, be administered at lower levels. Administration of the previous therapy can also be maintained. In some instances, a previous therapy can be maintained until the level of the receptor-targeting reagent (*e.g.*, the dosage or schedule) reaches a level sufficient to provide a therapeutic effect. The two therapies can be administered in combination.

It will be appreciated that in instances where a previous therapy is particularly toxic, administration of a receptor-targeting reagent can be used to offset and/or lessen the amount of the previously therapy to a level sufficient to give the same or improved therapeutic benefit, but without the same level of toxicity. Of course, prior to administering to the subject a toxic receptor-targeting reagent, a medical practitioner can administer to the subject a non-toxic receptor-targeting reagent. In some instances, when the subject is administered a receptor-targeting reagent or pharmaceutical composition of the invention the first therapy is halted. The subject can be monitored for a first pre-selected result, *e.g.*, an improvement in one or more symptoms of a proliferative disorder such as any of those described herein (*e.g.*, see above). In some cases, where the first pre-selected result is observed, treatment with the receptor-targeting reagent is decreased or halted. The subject can then be monitored for a second pre-selected result after treatment with the receptor-targeting reagent is halted, *e.g.*, a worsening of a symptom of a proliferative disorder. When the second pre-selected result is observed, administration of the receptor-targeting reagent to the subject can be reinstated or increased, or administration of the first therapy is reinstated, or the subject is administered both a receptor-targeting reagent and first therapy, or an increased amount of the receptor-targeting reagent and the first therapeutic regimen. The receptor-targeting reagent can also be administered with a treatment for one or

more symptoms of a disease (*e.g.*, proliferative disorder). For example, the receptor-targeting reagent can be co-administered (*e.g.*, at the same time or by any combination regimen described above) with, *e.g.*, a pain medication.

Thus, the present compounds may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compounds may be applied in pure form, *i.e.*, when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid

carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions that can be used to deliver the compounds of the present invention to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compounds of the present invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) of the present invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

In general, however, a suitable dose will be in the range of from about 0.1 to about 100 mg/kg, *e.g.*, from about 10 to about 75 mg/kg of body weight per day, such as 3 to about 50 mg per kilogram body weight of the recipient per day, preferably in the range of 6 to 90 mg/kg/day, most preferably in the range of 15 to 60 mg/kg/day.

The compound is conveniently administered in unit dosage form; for example, containing 5 to 1000 mg, conveniently 10 to 750 mg, most conveniently, 50 to 500 mg of active ingredient per unit dosage form.

Ideally, the active ingredient should be administered to achieve peak plasma concentrations of the active compound of from about 0.5 to about 75 μM, preferably, about 1 to 50 μM, most preferably, about 2 to about 30 μM. This may be achieved, for example, by the intravenous injection of a 0.05 to 5% solution of the active ingredient, optionally in saline, or orally administered as a bolus containing about 1-100 mg of the active ingredient. Desirable blood levels may be maintained by continuous infusion to provide about 0.01-5.0 mg/kg/hr or by intermittent infusions containing about 0.4-15 mg/kg of the active ingredient(s).

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced

administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

Kits and Articles of Manufacture

Also provided herein are kits containing one or more of any of the receptor-targeting reagents described herein and, optionally, instructions for administering the one or more receptor-targeting reagents to a subject (*e.g.*, a human or any of the subjects described herein). The subject can have, or be suspected of having, a cancer or an inflammatory disorder. The kits can also, optionally, include one or more pharmaceutically acceptable carriers or diluents. Also featured are kits useful for detecting expression an EGFR and/or an uPAR. The kits can contain one or more reagents for detecting expression of an EGFR and/or an uPAR; and instructions for administering any of the receptor-targeting reagents described herein (*e.g.*, the toxic receptor-targeting reagents described herein) if the expression of an EGFR and/or an uPAR is detected.

The kits can optionally include, *e.g.*, a control sample that is known to contain (positive control), or not to contain (negative control), an EGFR and/or an uPAR mRNA or protein. In some embodiments, the kits can include one or more reagents for processing a sample (*e.g.*, a cell sample). For example, a kit can include reagents for isolating mRNA or protein from a sample and/or reagents for amplifying isolated mRNA (*e.g.*, reverse transcriptase, primers for reverse transcription or PCR amplification, or dNTPs) and/or detecting protein expression (*e.g.*, one or more antibodies specific for an EGFR and/or an uPAR).

The disclosure also provides an article of manufacture containing: a container and a composition contained within the container. The composition is an active agent for treating cancer (or an inflammatory disorder) in a mammal. The active agent in the composition can contain, or consist of, any of the toxic receptor-targeting reagents described herein and the container can have a label indicating that the composition is for use in treating cancer (or an inflammatory disorder) in a mammal. The label can further indicate that the composition is to be administered to the mammal if one or more cancer cells of the mammal's cancer (or one or more immune cells mediating an inflammatory disorder) express an IL13R, an IL4R, or an EGFR. The article of manufacture can also contain instructions for administering the composition (*e.g.*, the rehydrated composition) to the mammal.

In some embodiments, the composition can be dried or lyophilized. The composition can be ready to administer without need for rehydration or further formulation.

Device for Administration of bispecific targeted toxin

In another aspect, the present invention provides a device comprising: a hollow fiber (HF) catheter having a proximal tip and a distal tip, wherein the HF catheter has a porosity that replicates the porosity of brain tissue, and a receptor targeting reagent as described above. In certain embodiments, the HF catheter comprises a plurality of nanoscale pores that approximate the impendence of brain interstitial spaces. In certain embodiments, the HF catheter consists of a single 380-μm-diameter hollow fiber. In certain embodiments, the HF catheter is made of polysulfone. In certain embodiments, the HF catheter comprises a sealed distal tip. In certain embodiments, the proximal tip is operably attached an infusion pump. In certain embodiments, the proximal tip is attached to tubing, and the tubing is attached to the infusion pump. In certain embodiments, the HF catheter is about 3 mm in length. In certain embodiments, the HF catheter comprises a porous material with multiple interconnected passageways. In certain embodiments, the HF catheter comprises a nominal pore size of 0.45 μm.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

Purpose: 1) To genetically design a bispecific targeted toxin that can simultaneously target overexpressed markers on glioma and the tumor vasculature. 2) To mutate certain amino acids to reduce the immunogenicity of this new drug. 3) To determine whether the drug is able to effectively reduce human brain tumors in an aggressive rat xenograft model using a novel hollow fiber catheter delivery system.

Methods: A new bispecific ligand directed toxin (BLT) was created in which two human cytokines EGF (targeting overexpressed EGFR) and ATF (targeting receptor, uPAR) were cloned onto the same single chain molecule with truncated pseudomonas exotoxin with a terminal KDEL sequence (SEQ ID NO: 15). Site-specific mutagenesis was used to mutate amino acids in 7 key epitopic toxin regions that dictate B cell generation of neutralizing anti-toxin antibodies in order to deimmunize the drug called EGFATFKDEL 7mut. Bioassays were used to determine whether mutation reduced potency and ELISA studies were performed to determine whether anti-toxin antibodies were reduced. Aggressive brain tumors established intracranially (IC) in nude rats with human U87 glioma genetically marked with a firefly luciferase reporter gene were treated steriotactically with two intracranial injections of deimmunized EGFATFKDEL using convection enhanced delivery (CED). Drug was administered through a novel hollow fiber catheter to reduce drug backflow upon delivery.

Results: *In vitro*, EGFATFKDEL 7mut selectively killed the human glioblastoma cell line U87-luc, as well as cultured human endothelial cells in the form of the HUVEC cell line.

Deimmunization did not reduce drug activity. *In vivo*, when rats with brain tumors were treated IC with drug using CED and a novel hollow fiber catheter to reduce backflow, there were significant tumor reductions in 2 experiments ($p<0.01$). Some rats survived with tumor free status until 130 days post tumor inoculation. An irrelevant BLT control did not protect establishing specificity. The maximal tolerated dose was established at 2 μg/injection or 8.0 μg/kg and studies indicated that this dose was non-toxic. Anti-toxin antibodies were reduced at least 90%.

Conclusions: First, this study indicates that the BLT framework is highly effective for simultaneously targeting glioma and its neovasculature. Second, in rodent CED studies, newly developed hollow fiber catheters that limit backflow are highly effective for drug delivery. Third, by mutating critical amino acids, we can reduce the threat of interference of neutralizing antibodies that are generated against the drug. These studies address some of the most urgent limitations in the targeted toxin field.

Introduction

Patients with glioblastoma multiforme (GBM), the most common primary intracranial malignancy have a poor prognosis[16]. Mean overall survival time after conventional treatment is less than 2 years and recurrences are frequent[35]. Targeted toxins are a class of biological drugs that have been considered for glioma therapy because they can be directly delivered to the tumor avoiding the problems associated with systemic delivery. They consist of a catalytic toxin directed by a single selective ligand recognizing a tumor associated cytokine receptor. Truncated pseudomonas exotoxin (PE38) was chosen for these studies due to its irreversible catalytic activity[8]. The toxin catalyzes ADP ribosylation of elongation factor 2 (EF-2) leading to irreversible inhibition of protein synthesis and subsequent cell death.

Bispecific ligand directed toxins (BLTs) have been synthesized by linking a truncated toxin to two well-established targeting ligands on the tumor cell with the goal of increasing targeting capability. We and other investigators have previously examined the feasibility of targeting toxins with dual ligands[3,30,31]. These BLTs have increased anti-tumor activity compared to their monospecific counterparts or a mixture of the two, thus indicating an advantage of including both ligands on the same single chain molecule[32,34,37]. In this study, we have designed a different type of BLT capable of simultaneously targeting the cancer cell and its developing neovasculature.

EGF receptor (EGFR) promotes proliferation, migration, and invasion of malignant cells and is over expressed on glioblastoma cells. Targeted toxins have been developed to target EGFR and appear promising in several studies[4,10,12]. Patients with high-grade gliomas have been studied and show intra- and inter-tumoral heterogeneity in EGFR in immunohistochemical analysis[11].

Urokinase plasminogen activator (uPA) is associated with a specific urokinase receptor (uPAR) which is over-expressed on numerous human cancer cells. Additionally, uPAR is found on the endothelial neovasculature that sustains tumors' high metabolic demand[2,5,13,23,39]. The uPA binding region has been identified consisting of a 135 amino acid terminal fragment (ATF) which binds with high affinity to uPAR[27].

One major problem with targeted toxins in the treatment of solid tumors is toxin immunogenicity[6,40]. Multiple treatments with drug are necessary to obtain tumor regression and this results in the generation of anti-toxin antibodies that block drug efficacy[18,40]. Onda and Pastan recently mapped seven major immunodominant epitopes in PE38 that can be mutated without loss of catalytic activity[18]. Thus, we used this advancement to produce a new deimmunized anti-glioblastoma biological drug called EGFATFKDEL 7mut.

A second major problem with targeted toxins in the IC treatment of GBM is drug delivery. All clinical studies have been performed using CED and conventional single port catheters with one opening at the tip that ensure a point source of delivery, but resultant backflow up the catheter shaft adversely affect this design. A solution to this problem would be a catheter with porosity that replicates the porosity of brain tissue. Thus, we are testing novel hollow fiber (HF) catheters that have millions of nanoscale pores that approximate the impendence of brain interstitial spaces. Our previous work shows this catheter design significantly increases distribution capability compared with conventional single port catheters[19].

In this Example, we study the effectiveness of EGFATFKDEL 7mut which is designed to simultaneously target tumor cells and their neovasculature while reducing the anti-toxin response in an intracranial brain tumor model. Since recent clinical studies with IC delivery of targeted toxins revealed problems with backflow, we studied CED of drug combined with the use of HF catheters in an *in vivo* intracranial IC model that accounts for the blood-brain barrier. U87-luc is a highly aggressive human brain tumor that is genetically marked with a firefly luciferase reporter gene permitting us to follow BLT- related tumor regression in real time using bioluminescent imaging[20]. The use of this new drug and delivery in this novel manner is advantageous for glioma therapy.

Materials and Methods

Construction of targeted toxins. DNA shuffling and DNA cloning techniques were used to assemble the genes encoding the single chain BLT EGFATFKDEL 7mut which consisted of an NcoI restriction site, an ATG initiation codon, then human EGF. EGF was followed downstream by the 135-amino terminal fragment (ATF) from uPA. EGF and ATF were spliced with a 20 amino-acid segment of human muscle aldolase (HMA). The 7 amino-acid linker

EASGGPE (SEQ ID NO: 14) was then followed by the first 362 amino acids of the pseudomonas exotoxin (PE) molecule. Finally, the final 5 amino acids of PE (REDLK (SEQ ID NO: 23)), were replaced with KDEL (SEQ ID NO: 15) as an endoplasmic reticulum retention sequence and then a NotII restriction site at the 3' end of the construct. The HMA segment was incorporated into the molecule as a flexible, non-immunogenic linker[38]. The use of the ATF gene fragment was previously described by our group[27,28,36,]. The resultant gene was spliced into the pET28c bacteria expression vector under control of an isopropyl-b-D-thiogalactopyranoside inducible T7 promoter (Figure 1). Standard DNA sequencing analysis verified that the gene was in sequence and had been cloned in frame. An EGFATFKDEL molecule with decreased immunogenicity called EGFATFKDEL 7mut was prepared using the QuickChange Site-Directed Mutagenesis Kit (Stratagene. La Jolla CA) to alter eight amino acids including: R490A, R513A, R467A, E548S, K590S, R432G, Q332S, R313A and confirmed by DNA sequencing. Genes for monospecific targeted toxins EGFKDEL and ATFKDEL were created using the same techniques. CD3CD3KDEL, a bispecific toxin-targeting T-cell surface marker CD3, was made by replacing the DT390 portion of the DT390CD3CD3 molecule described previously with PE38(KDEL)[38]. 2219ARLKDEL, a control BLT which combines V_H and V_L regions (scFV) for anti-CD22 and anti-CD19, was produced as described previously[37].

Protein Expression and purification. Protein was expressed and purified from inclusion bodies using a Novagen pET expression system (Novagen, Madison WI) as previously described[21]. Refolded proteins were purified by fast protein liquid chromatography ion exchange chromatography (Q sepharose Fast Flow, Sigma). SDS-PAGE analysis was performed and the fusion proteins stained with Commasie Brilliant Blue to determine purity.

Cell Culture. The human glioblastoma cell lines U87MG and U118MG were obtained from American Type Culture Collection (ATCC, Rockville MD). A U87MG subline, U87-luc with a luciferase reporter gene was used for *in vivo* experiments[20]. Cells were grown as previously described[21]. HUVEC (human Umbilical Vein Endothelial Cells) were cultured in Medium -199 1x Earle's Salts with same supplements. Cells were grown as monolayers and incubated in a humidified 37°C atmosphere containing 5% CO_2. Only cells with viability >95%, as determined by trypan blue exclusion, were used for experiments. Reported gene transfected U87-luc cells were maintained with additional 10 μg of Blastocidin (InvivoGen, San Diego, CA).

Bioassays to measure cell proliferation. To determine the effect of EGFATFKDEL on tumor cells, proliferation assays measuring ^{3}H-thymidine incorporation were performed. Cells (10^4/well) were plated in a 96-well flat-bottomed plate and incubated overnight at 37°C with 5% CO_2. The next day, BLT in varying concentrations were added to wells in triplicate. Incubation at 37°C and 5% CO_2 continued for 72 hours. [Methyl-^{3}H]-thymidine (GE Healthcare, UK) was

added (1 μCi per well) for the final 8 hours of incubation. Plates were frozen and cells were then harvested onto a glass fiber filter, washed, dried, and counted using standard scintillation methods. Data from proliferation assays are reported as percentage of control counts.

Determining immunogenicity of deimmunized EGFATFKDEL 7mut. Mouse immunization studies were used to determine whether mutated EGFATFKDEL 7mut elicited less of an immune response than non-mutated parental EGFATFKDEL. The assay has been previously reported[21]. Briefly, female Balb/c mice (n=5/group) were injected intraperitoneally once weekly with 0.25 μg of either EGFATFKDEL or EGFATFKDEL 7mut for 90 days. Each week, five days after injection, the mice were bled (facial vein collection) to obtain serum. Serum from each mouse was isolated using centrifugation and frozen. Levels of anti-PE38KDEL IgG in each serum sample was measured using ELISA. Briefly, 5 mg of purified recombinant PE38KDEL was added to a microtiter plate and adhered overnight. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was added to each well. After washing, *o*-Phenylenediamine dihydrochloride substrate was added to each well and absorbance measured. Quantification of actual anti-PE38KDEL IgG present in each sample was determined by comparing the absorbance values in each well to a standard curve prepared using M40-1 monoclonal anti-PE38KDEL antibody generously provided by Dr. Robert Kreitman (NIH, Bethesda, MD).

U87-luc Intracranial model. For intracranial (IC) injection, an athymic nu/nu rat was anesthetized and placed in a Kopf sterotactic head frame as previously described[20]. The scalp was swabbed with betadine and a midline incision was made with a scalpel with the rat immobilized. A burr hole was placed on 0.5 mm anterior, 2.5 mm lateral from sagittal midline located bregma. A Hamilton syringe catheter was used with single port catheters (26 g) to deliver U87-luc tumor cells to a 3.5 mm depth from skull surface to about the middle of Caudate-putamen (Coordinates - AP: +0.5 mm, ML: +2.5 mm, DV: 3.5mm from bregma). After 10^5 of U87/luc cells IC inoculation, tumor growth was evaluated by bioluminescent imaging. For IC injection of drug, injections were performed by micro infusion pump using a hollow fiber catheter delivered to the same coordinates except depth (6 mm from surface) because of structure difference of hollow fiber catheters. Hollow fiber catheters (Twin Star Medical, St. Paul, MN) consisted of a single 380-μm-diameter hollow fiber made of polysulfone with a sealed distal tip. The proximal hollow fiber was attached to tubing that allows handling of the hollow fiber and its attachment to an infusion pump. The exposed hollow fiber was 3 mm in length. The wall of the hollow fiber was composed of a porous material with multiple interconnected passageways. The nominal pore size was 0.45 μm[19]. Amount of drug delivered was 2ug for each time and flow rate was 0.25μl/min, total 7.5μl delivered directly into tumor inoculated IC location.

In vivo Imaging of Intracranial Tumors. The effect of intracranial administration of EGFATFKDEL 7mut against the U87-luc xenografts model was measured by using bioluminescent imaging. Rats were imaged every week to monitor the level of luciferase activity after intracranial xenograft. D-Luciferin used for bioluminescent imaging the tumor cells which has been shown to cross the blood-brain barrier[1]. Images were captured using Xenogen Ivis 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation, Hopkington MA). Prior to imaging, rats were anesthetized with inhalation of isoflurane gas. All rats received 500 µl of a 30 mg/ml D-luciferin aqueous solution (Gold Biotechnology, St. Louis MO) IP injection 10 minutes before imaging to provide a substrate for the luciferase enzyme. All images represent 5 minutes exposure time and all regions of interest (ROI) are expressed in units of photons/sec/cm^2/sr.

Histology. Rats cured from efficacy experiments were sacrificed after bioluminescent imaging for histology evaluation. For histology, myocardial perfusion was performed and then brains were removed and embedded in OCT compound (Miles, Elkhark, IN), snap frozen in liquid nitrogen, and stored at –80°C until sectioned. Sections were cut, thawed, and mounted on glass slides and fixed for 5 minutes in acetone. Slides were stained with hematoxylin and eosin (H and E).

Efficacy of EGFATFKDEL 7mut on U87-luc Brain Tumors In vivo. Nu/Nu rats were anesthetized with IP injection of ketamine cocktail and stereotaxically inoculated with 10^5 U87-luc cells on day 0. The EGFATFKDEL 7mut group was given 2ug drug in 7.5 µls of PBS by CED (micro infusion pump, flow rate: 0.25µls/ml) as described[20]. Negative control groups received either PBS or irrelevant control 2219ARLKDEL 7mut in an identical manner.

In experiment 1, drug delivery schedule was at day 7 and 14. Imaging was done at day 5, 12, 19, 26, 33. In experiment 2, drug was delivered at day 4 and 11. Bioluminescent image was captured at day 3, 10, 17, 24, 31, 38, 57, 64, 74, 88, 102 and 130. Each experiment EGFATFKDEL 7mut treated group was 5 and PBS treated group was 4.

Blood and Serum Studies. Normal Fisher rats (n=3/group) were given two micro infusion pump injections of EGFATFKDEL 7mut on days 0 and 7. Individual serum samples were obtained via a facial vein bleeding at day 21 and 28. Blood was centrifuged and frozen at -80°C. Serum samples were analyzed on a Kodak ETA-CHEM 950 by the Clinical Chemistry Laboratory, Fairview University Medical Center (Minneapolis, MN). BUN assays were read spectrophotometrically at 670 nm as a measure of renal activity[26]. In the ALT assay, the oxidation of NADH was used to measure ALT activity at 340 nm as a measure of hepatic activity. The number of red blood cells, white blood cells and hemoglobin per unit volume were determined.

Statistical analyses. Survival curves were created using the Prism 4 software package (Graphpad Software, San Diego CA) using the product limit method of Kaplan and Meier and then compared using the logrank test. Body weight was analyzed by groupwise comparisons of continuous data by Student's t-test. Probability (p) values < 0.05 were considered significant.

Results

Purification and bioassays. EGFATFKDEL 7mut was synthesized and purified. SDSPAGE gel analysis showed a molecular weight of 63144 kDa which was in accordance to its expected molecular weight and a purity of 95% (not shown). In order to determine if drug could selectively kill glioblastoma cells *in vitro*, U87 cells were cultured with EGFATFKDEL. The drug selectively killed U87 with an IC_{50} of 0.007 nM (Figure 2A). Control CD3CD3KDEL which targets T cells and is not reactive with glioblastoma cells did not inhibit activity. Importantly, EGFATFKDEL 7mut showed similar activity with an IC_{50} of 0.001nM. In Figure 2B, EGFATFKDEL inhibited a second glioblastoma U-118, while the control targeted toxin did not. Flow studies show higher levels of EGFR expression on U-118 which could explain its greater drug susceptibility (data not shown). In Figure 2C, to investigate whether or not the uPAR targeting was effective, we tested EGFATFKDEL on human umbilical vein endothelial cells (HUVECs). These cells bear similarity to cells in the tumor neovasculature and over-express uPAR but not EGFR. EGFATFKDEL and EGFATFKDEL 7mut had the highest activity against HUVECs with IC_{50} values of 0.05 and 0.02 nM, respectively. The bispecific BLT was more effective than its monospecific counterparts against HUVEC cells (Figure 2C) and against U87 cells (not shown). In Figure 2D, we investigated the specificity of the drug. Anti-urokinase antibody (α-uPA), EGF, and anti-pseudomonas exotoxin antibody (α-PE) were incubated with a known cytotoxic concentration of EGFATFKDEL 7mut (1 nM) to block killing. All of these blocking agents inhibited the ability of EGFATFKDEL 7mut to kill U87 cells. In contrast, anti-Ly5.2 (α-Ly5.2), an irrelevant control antibody, did not block confirming that both EGF and ATF ligands are active on the EGFATFKDEL 7mut molecule.

U87-luc intracranial tumor model. Rats were injected IC with 10^5 U87-luc cells injected at coordinates (AP: +0.5 mm, ML: +2.5 mm, DV: 3.5mm from bregma). Bioluminescent signals in untreated animals progressively increased over time and most rats were dead by day 30. The Maximal Tolerated Dose (MTD) of EGFATFKDEL 7mut was determined at 2 μg/injection or 8ug/kg. (MTD) was determined in groups of rats (n=4/group) by treating rats twice (one week apart) with micropump infusions of 0.5, 1, 2, 4, or 8 μg drug. The MTD was determined at 2 μg/injection or 8ug/kg (not shown). Weight loss is frequently used as an indicator of toxicity. At the doses of 0.5, 1, or 2 μg, there was no weight loss or mortality. One of 4 animals died at the 4

μg dose level and the rest lost at least 10% of their body weight. All animals died at the 8 μg dose level. An additional 15 animals tolerated drug at the MTD without weight loss or mortality.

To test the ability of EGFATFKDEL 7mut to inhibit tumor growth *in vivo*, U87-luc cells were injected IC into rats and then drug was delivered to the same coordinates on day 7 and 14 (experiment 1), and day 4 and 11 (experiment 2) respectively (Figure 3). Experiment 1 shows a short-term experiment. Tumors had markedly progressed in 3 of 4 control rats by day 12. A group of treated rats showed delayed tumor growth and the experiment was terminated when 2 of the rats in this group showed signs of relapse. A second experiment showed similar results since control rats all developed tumor by day 10. EGFATFKDEL 7mut treatment delayed tumor development in 4 of 5 of animals. Two of the rats were long-term disease free survivors that showed no evidence of tumor on histologic examination. Figure 4A shows a Kaplan-Meier survival curve shows increased survival time in treated rats as compared to controls.

Figure 4B (also from experiment 2) shows a group of rats treated with a negative control targeted toxin that did not bind U87 cells. It did not prevent tumor development. Monospecific targeted toxins EGFKDEL and ATFKDEL were also tested *in vivo* against U87 cell in a flank tumor model (not shown). EGFKDEL was too toxic and ATFKDEL did not prevent tumor development. Together, these data showed that the BLT was selective and highly efficacious against intracranial tumor.

Toxicity. To determine liver toxicity, alanine transaminase (ALT) enzyme levels were measured in normal rats given the same drug regimen IC (Figure 5A). Serum ALT measurements showed no significant change. Blood urea nitrogen (BUN) levels were also measured simultaneously from the same animals as a measure of renal toxicity without any significant changes as compared to untreated controls (Figure 5B). Treatment did not affect the hematopoietic system since red blood cell, white blood cell, platelet or hemoglobin levels were unchanged (not shown) . There was no weight loss as a result of treatment (not shown). Together, these studies show that an efficacious dose of 2.0 μg EGFATFKDEL 7mut did not damage major organ systems and appears safe for brain tumor therapy.

Deimmunized EGFATFKDEL. To determine whether EGFATFKDEL had been deimmunized, groups of immunocompetent BALB/c mice were immunized weekly with 0.25 μg of either mutated EGFATFKDEL 7mut or non-mutated parental drug. Animals were immunized intraperitoneally over the period of 90 days. Serum samples were obtained weekly and analyzed using ELISA to detect anti-PE38(KDEL) IgG. Figure 8 shows statistical differences between the anti-toxin responses of the two groups ($P<0.05$). After 12 injections (day 75), the EGFATFKDEL 7mut group showed minimal serum anti-toxin levels, while the EGFATFKDEL group had an

average anti-PE38(KDEL) response of 6,000 μg/ml. Similar results were obtained after two more immunizations (totaling 14) on day 90.

Histology. In order to verify that long-term survivors were tumor free, rat brains from animals R15 and R17 were sectioned on day 130 (Figure 3). The brain was sectioned every 60 um, and tissue was examined for the presence of brain tumor (20 slides/animal). Figure 3 shows representative striatal sections from both rats. The needle track is visible on the section from R15. Both sections are healthy and tumor free.

Discussion

This Example contributes new information to the field of intracranial glioma therapy in three ways. First, it investigates a new BLT designed for dual targeting glioblastoma cells and their expanding neovasculature in an intracranial CED model. Second, in rodent CED studies, drugs are most commonly administered using single port catheters. In these studies, newly developed hollow fiber catheters were used. Treatment resulted in impressive anti-glioma effects observed in a sophisticated animal model in which rat brain tumor growth can be monitored in real time using bioluminescent imaging. Studies shows that the combination of drug and catheter were remarkably effective against small gliomas resulting in some long-term disease free survivors. Third, this study shows that BLT was deimmunized by mutating critical amino acids. A major problem in the targeted toxin field is the induction of the antibody response that diminishes drug efficacy. The data here show an impressive reduction in the development of serum anti-toxin antibodies in mice given mutated, deimmunized drug compared to non-mutated parental control drug. Toxicity data indicated that IC treatment did not damage liver, kidney or hematologic cells. The decision to study these toxicities was based on the fact that these are the most common toxicities of this class of drug[32,33,38] and endothelial cell damage might predispose to vascular injury.

Expansion of neovasculature (angiogenesis) is highly accelerated in tumors[7] and other investigators have designed targeted toxins to disrupt this complex process involving endothelial cells, stromal cells, and factors from the extracellular matrix. For example, Ramakrishnan reported an anti-endothelial cell targeted toxin prepared by chemically linking recombinant vascular endothelial growth factor and a truncated diphtheria toxin molecule[26]. These studies showed that treatment of HUVEC or human microvascular endothelial cells with drug resulted in a selective, dose-dependent inhibition of growth while control free toxin or a mixture of VEGF and the toxin had minimal effect. Targeting uPAR is a good choice for disrupting angiogenesis since uPAR is found on the endothelial vasculature[2]. We targeted uPAR with ATF, the truncated binding domain of urokinase that binds uPAR with high affinity. uPAR, a tumor neo-antigen, is

expressed on the expanding vasculature upon which the glioma is dependent. Also, in an immunohistochemical study of uPAR expression of 65 central nervous system tumors, uPAR was expressed on most and there was a significant positive correlation (P = 0.0006) between tumor grade and staining intensity, suggesting a correlation with anaplastic change and propensity to tumor invasion[29]. There is an advantage of also targeting EGFR since its commonly overexpressed on human glioblastoma and carcinoma as determined by analysis of patient tumor tissue[11]. We believe that effects will be magnified because EGFATFKDEL directly attacks the two most important cell types in the tumor: vascular cells and tumor cells. The data in this manuscript supports this since we showed that EGFATFKDEL 7mut is highly effective in a rat xenograft model in which brain tumors were induced by the injection of the human glioblastoma line U87. Tumor-free survivors were observed beyond day 100, despite the fact that treatment ended on day 11, indicating some durable drug responses.

Delivery may be the most urgent problem in the field and our delivery methods need urgent improvement. CED is a direct intraparenchymal infusion procedure driving infusate over large areas in the brain using bulk flow delivery via catheters. Clinical radiographic responses have been observed with targeted toxins delivered in this manner[22,24], but CED has suffered setbacks due to catheter design. Conventional intracranial delivery is performed with single port catheters with one opening at the tip. The problem occurs when interstitial tumor pressures force the drug back along the track of the delivery needle resulting in backflow and uncontrolled spread into the cerebral ventricles or leptomeningeal space. As a solution to this problem we used hollow fiber catheters (HF) in these studies. HF catheters have small tubules with semi permeable walls that contain millions of nanoscale pores[19]. Because HFs provide a large surface area, and consequently a large volumetric flow at low velocity densities, HF catheters can provide improved tissue distribution of therapeutic agents. HF catheters can produce uniform delivery along the length of the catheter, and therefore surface area is not a limiting factor. A large surface area will allow clinically relevant flow rates at low flow velocity. We have shown that the porosity of the HF catheters replicates the porosity of brain tissue[19]. With a more homogenous interstitial flow, distribution is enhanced. This approach with these same HF catheters has already shown to have clinical value in removing fluid and reducing edema[15].

Their enzymatic nature renders targeted toxins among the most-effective single cell killers. However, they are immunogenic and host exposure results in anti-toxin antibodies that limits their effectiveness. In glioblastoma studies with TT, the development of IgG antitoxin (PE) was observed in a surprising 73% of CED patients in phase 1 clinical studies where patients received intracranial IL-13 spliced to the same non mutated PE38 used in our studies[9,25]. Knowing this and knowing that we can now reduce the anti-toxin response at least 80%, we are

ethically obligated to use deimmunized toxin in future trials. Recently, investigators observed and identified the presence of 7 major epitopic groups and 13 subgroups responsible for pseudomonas exotoxin (PE) antigen recognition by B cells[17,18]. Eight amino acids were mutated in each of the 7 groups and found to reduce the immune response to toxin. The discovery that only a limited number of epitopes are responsible for generating an anti-PE response is a major advancement since the mutations have minimal effect on the catalytic activity of the fusion toxin. To address this issue, we used mutated EGFATFKDEL to deimmunize the toxin moiety. *In vitro* studies revealed that the mutated form was still just as effective as the non-mutated parent molecule. When tested in mice, the immunogenicity of the molecule was reduced at least 80%. Studies of serum from patients given multiple injections of a PE-based toxin revealed that antibodies from these patients bind to the same epitopes as recognized by mice supporting the fact that mice are an acceptable model for human immunogenicity[14]. Studies indicate that this approach works well for other targeted toxins as well[21,33].

The drug had higher activity against U118 compared to U87. This could be explained by 1) a greater number of receptors on U118, 2) the drug is internalized quicker into U118, or 3) once the drug gains entry, it is delivered into the cytosolic compartment faster. Studies are underway to investigate the mechanism of BLT activity.

In summary, these studies show for the first time that when EGFATFKDEL 7mut is administered directly into the cranium, it is highly efficacious. *In vitro*, bispecific drug kills both glioblastoma cell lines and endothelial cell lines. *In vivo*, usage of HF catheters combined with this drug resulted in effective drug delivery and long-term disease free survivors in an IC tumor model. We also have studied an *in vivo* flank tumor model and found that neither monospecific EGFKDEL, ATFKDEL, or a mixture of both work as well as the bispecific EGFATFKDEL 7mut. ELISA studies show that deimmunized toxin does effectively reduce the level of serum anti-toxin antibodies.

Conclusions

Many believe that directly targeting glioblastoma with targeted toxins delivered intracranially would be a highly effective therapeutic approach. These studies were designed to address some of the most notable obstacles to this therapy. First, we genetically engineered a new bispecific drug framework that is highly effective for simultaneously targeting glioma and its neovasculature. *In vitro*, we showed the drug selectively targets overexpressed EGFR as well as tumor endothelial cells. Second, in rodent CED studies, we showed that newly developed hollow fiber catheters that limit backflow are highly effective for delivery and cause the regression of human brain tumors genetically tagged with a luciferase reporter gene. Third, we showed that by

mutating critical amino acids, we can reduce the threat of interference of neutralizing antibodies that are generated against the drug. Continuing studies of this promising approach are warranted.

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EXAMPLE 2

Head and Neck Cancer Data

Figure 7. Drug activity against head and neck cancer carcinoma cells in vitro. To determine the selective activity of EGFATFKDEL 7mut against head and neck cancer cells in vitro, human UMSCC-11 cells derived from human tongue cancer were cultured with increasing concentrations of EGFATFKDEL 7mut for 72 hours and proliferation was measured by uptake of tritiatedthymidine. Flow cytometry studies indicate that these cells express high levels of EGFR and IL-4R. Cells (105) were plated in a 96-well flat-bottom plate in RPMI supplemented

with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin. Drug in varying concentrations was added to triplicate wells containing cells. The plates were incubated at 37°C, 5% CO2 for 72 hr. Cells were then incubated with one μCi [methyl-3H]-thymidine (GE Healthcare, UK) per well for eight hours and harvested onto glass fiber filters, washed, dried and counted for ten minutes in a standard scintillation counter. Data were analyzed using Prism 4 (GraphPad Software, Inc) and were expressed as a percentage of control response where control response is untreated cells. Data are shown as mean + standard deviation (SD). The mean values of untreated cells were 49,196 ± 16,278 cpm/20,000 cells. In the experiment, cultured cells were also treated identically with a negative control targeted toxin 2219ARLKDEL 7mut. This targeted toxin simultaneously targets CD22 and CD19 expressed on human B lymphocytes which are not expressed on UMSCC-11 cells. EGFATFKDEL 7mut was selective since it inhibited UMSCC-11 cells, but 2219ARLKDEL 7mut did not. (2219ARLKDEL 7mut has been previously published (Vallera DA, Oh S, Chen H, Shu Y, Frankel AE. Bioengineering a unique deimmunizedbispecific targeted toxin that simultaneously recognizes human CD22 and CD19 receptors in a mouse model of B-cell metastases. Mol Cancer Ther. 2010 Jun;9(6):1872-83.)

Figure 8. Effect of intratumoral administration of drug on mice given head and neck cancer by subcutaneous injection of UMSCC-11 cells in nude mice. Male nu/nu mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. All animals were housed in microisolator cages to minimize the potential of contaminating virus transmission. For flank tumor studies, mice were injected in the left flank with $4x10^6$ cells. Once palpable tumors had formed, mice were divided into groups and treated with multiple injections of drug. Drug was administered by intratumoral injection using 3/10 cc syringes with 29 gauge needles. All treatments were given in a 100 µl volume of sterile PBS. Tumor size was measured using a digital caliper, and volume was determined as a product of length, width, and height. A) Average tumor growth of a group of 6 mice treated with EGFATFKDEL 7mut. Treatment was with 2 ug of drug on days 7, 8, 14, 15, 16, 17, 21, 22, 23, 24, 28, 29, 30, 31, and 35. Tumor growth was inhibited in the group of mice given drug, but not in a group of 5 non-treated control mice. B) Average body weights of these same mice measured over time indicating that multiple dose treatment did not induce weight loss.

EXAMPLE 3

A Novel Bispecific Ligand-directed Toxin Designed to Simultaneously Target EGFR on Human Glioblastoma Cells and uPAR on Tumor Neovasculature

A bispecific ligand-directed toxin (BLT), called EGFATFKDEL, consisting of human epidermal growth factor, a fragment of urokinase, and truncated pseudomonas exotoxin (PE38) was assembled in order to target human glioblastoma. Immunogenicity was reduced by mutating seven immunodominant B-cell epitopes on the PE38 molecule to create a new agent, EGFATFKDEL 7mut. *In vitro*, the drug selectively killed several human glioblastoma cell lines. EGFATFKDEL is our first BLT designed to simultaneously target EGFR on solid tumors and uPAR on the tumor neovasculature. *In vitro* assays revealed that the agent is effective against glioblastoma cell lines as well as human umbilical vein endothelial cells (HUVEC). Additionally, the bispecific drug displayed enhanced binding to overexpressed epidermal growth factor receptor and urokinase receptor when compared to similar monospecific drugs, EGFKDEL and ATFKDEL. *In vivo*, an aggressive human glioblastoma cell line was genetically marked with a firefly luciferase reporter gene and administered to the flanks of nude mice. Treatment with intratumoral injections of

EGFATFKDEL 7mut eradicated small tumors in over half of the treated mice, which survived with tumor free status at least 100 days post tumor inoculation. ATFKDEL, which only targets the tumor neovasculature, prevented tumor growth but did not result in tumor-free mice. Specificity was shown by treating with an irrelevant BLT control which did not protect mice. Finally, immunization experiments in immunocompetent mice revealed significantly reduced neutralizing antibody production in EGFATFKDEL 7mut treated groups. Thus, EGFATFKDEL 7mut is an effective drug for glioblastoma therapy in this murine model and warrants further study.

Key words: immunotoxin, pseudomonas exotoxin, glioblastoma, xenograft model, EGF

Introduction

Glioblastoma multiforme (GBM) is the most common of all primary intracranial malignancies [1]. While newer chemotherapy agents have shown promise in GBM treatment, the long-term prognosis remains very poor with median survival rates under two years [1]. Biological agents, such as targeted toxins are attractive for GBM treatment because glioblastoma rarely metastasizes outside of the cranium. Consequently, targeted toxins may be applicable to GBM through direct administration to tumors via convection enhanced delivery (CED), a method that utilizes catheters and pumps to deliver drugs directly to the tumor site [2-4].

Targeted toxins consist of a catalytic protein toxin directed by a ligand recognizing over-expressed markers on tumor cells [5]. Our group is investigating bispecific ligand directed toxins (BLTs) which link two different tumor selective ligands to a truncated toxin [6-9]. BLTs have been shown to increase targeting capability and drug potency while reducing toxicity [6].

We synthesized a novel BLT, EGFATFKDEL, designed to simultaneously target epidermal growth factor receptors (EGFR) expressed on glioblastoma and urokinase receptors (uPAR) expressed on tumor neovasculature (and glioblastoma cells). The targeting ligands were linked to a truncated pseudomonas exotoxin A (PE38), which has been demonstrated to possess potent anticancer activity [10]. By inhibiting protein synthesis, fewer than 1000 molecules of PE38 delivered to the cytosol are sufficient to bring a complete anti-tumor response [11]. PE38 was modified by adding a Lys-Asp-Glu-Leu (KDEL) C-terminus signal (SEQ ID NO: 15) to produce PE38KDEL, which prevents luminal endoplasmic reticulum (ER) protein secretion and provides enhanced anticancer activity [12].

EGFR is overexpressed in 40 to 50% of GBM cases [1]. Activation of EGFR promotes processes responsible for tumor growth and progression, including proliferation, angiogenesis, invasion, metastasis, and inhibition of apoptosis, making it a popular target for anti-cancer therapy [13]. EGFR-directed therapies have been developed that show promise in preclinical trials [14-17].Urokinase plasminogen activator (uPA) is associated with a specific urokinase receptor (uPAR) which is over-expressed on numerous human cancer cells. Additionally, uPAR

is found on the endothelial neovasculature that sustains tumors' high metabolic demand [18-22]. Here, we use an amino terminal fragment (ATF) of uPA, which has been shown to bind with high affinity to uPAR [23]. Targeting of both these over-expressed receptors allows us to destroy both solid tumors and the tumor-associated neovasculature using EGFATFKDEL.

The efficacy of ligand-directed toxins and other biologicals in the treatment of solid tumors has been impaired by problems with immunogenicity [24]. Use of targeted toxins requires multiple treatments which results in the generation of antibodies that are mainly produced against the toxin portion of the drug [25,26]. Onda and Pastan recently mapped seven major immunodominant epitopes in PE38 that can be mutated without loss of catalytic activity [25]. We combined this advancement in toxin "deimmunization" with our observation of enhanced BLT activity to produce a new anti-glioblastoma biological drug called EGFATFKDEL 7mut.

In this paper, we study EGFATFKDEL 7mut designed to simultaneously target tumor cells and their neovasculature while reducing the anti-toxin response. The drug shows potent anti-glioblastoma effects *in vitro* and *in vivo* in a bioluminescence luciferase reporter gene mouse model in which tumor burden is imaged in real time.

Materials and Methods

Construction of EGFATFKDEL and EGFATFKDEL 7mut

Synthesis and assembly of hybrid genes encoding the single-chain EGFATFKDEL was accomplished using DNA-shuffling and DNA cloning techniques. The fully assembled fusion gene (from 5' end to 3' end) consisted of an NcoI restriction site, an ATG initiation codon, the genes for human EGF, the downstream 135-amino terminal fragment (ATF) from uPA linked by a 20 amino-acid segment of human muscle aldolase (HMA), the 7 amino-acid EASGGPE linker (SEQ ID NO: 14), the first 362 amino acids of the pseudomonas exotoxin (PE) molecule with KDEL (SEQ ID NO: 15) replacing the REDLK (SEQ ID NO: 23) at the C terminus, and a NotII restriction site at the 3' end of the construct. The HMA segment was incorporated into the molecule as a flexible, non-immunogenic linker [27]. The use of the ATF gene fragment was previously described by our laboratory [23,28]. The resultant 1748 bp NcoI/NotII fragment gene was spliced into the pET28c bacteria expression vector under control of an isopropyl-b-D-thiogalactopyranoside inducible T7 promoter. DNA sequencing analysis (Biomedical Genomics Center, University of Minnesota) was used to verify that the gene was correct in sequence and had been cloned in frame. To create an EGFATFKDEL molecule with decreased immunogenicity (Figure 1A), eight amino acids representing the seven major epitopes on PE38 were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene. La Jolla CA) [25]. The following amino acids were altered: R490A, R513A, R467A, E548S, K590S, R432G,

Q332S, R313A and confirmed by DNA sequencing. Genes for monospecific targeted toxins splicing PE38KDEL to human EGF (EGFKDEL) and mutated uPA fragment (ATFKDEL) were created using the same techniques. CD3CD3KDEL, a bispecific immunotoxin-targeting T-cell surface marker CD3, was made by replacing the DT_{390} portion of the CD3CD3 (Bic3) molecule described previously with PE38KDEL [29]. 2219ARLKDEL, a BLT which combines VH and VL regions (sFv) for anti-CD22 and anti-CD19, was produced as described previously [7].

Isolation of inclusion bodies, refolding and purification

Proteins were produced as described previously with some minor modifications to improve yield and purity [30]. Solubilization of partially purified inclusion bodies was carried out in guanidine hypochloride plus 50 mM dithierythritol in the refolding buffer to decrease protein aggregation. In addition, the purity of protein isolated from the ion exchange column was further enhanced using an FPLC and Supradex 200 size exclusion column (Sigma, Ronconcoma, NY, USA). This modified protocol resulted in a yield of 5–10 mg of protein per liter of culture and a final product with >95% purity.

Cell Culture

The human glioblastoma cell lines U87 MG, U118 MG, U373 MG, and T98G were derived from patients with GBM and were obtained from American Type Culture Collection (ATCC, Rockville MD). Human umbilical vein endothelial cells (HUVECs) were obtained from Dr. S. Ramakrishnan (University of Minnesota). Raji cancerous B cells, derived from human Burkitt's Lymphoma, were used as a control cell line. Cells were grown in either DMEM (U87) or RPMI-1640 media (Raji, U118, U373, and T98) supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. U87-luc cells, which were stably transfected with vectors containing both the firefly luciferase (luc) and blasticidin resistance genes, were maintained in DMEM with the supplements above and blasticidin. HUVECs were maintained in Medium 199 containing epidermal cell growth media supplement (Invitrogen-Gibco, Carlsbad CA), 15% heat-inactivated fetal bovine serum, and the antibiotics listed above. All carcinoma cells were grown as monolayers and Raji cells in suspension using culture flasks. Cell cultures were incubated in a humidified 37°C atmosphere containing 5% CO_2. When adherent cells were 80–90% confluent, they were passaged using trypsin-EDTA for detachment. Only cells with viability >95%, as determined by trypan blue exclusion, were used for experiments.

Flow cytometry analysis of EGFATFKDEL

To measure binding to U87 cells, EGFATFKDEL, EGFKDEL, ATFKDEL, RFB4 (a negative control monoclonal antibody binding to CD22 on B cells), and HD37 (a negative control monoclonal antibody binding to CD19 on B cells) were labeled with fluorescein

isothiocyanate (FITC) as described previously [7, 8]. FITC-labeled proteins at 500 nM were incubated with $5x10^5$ cells in 200 µl of PBS + 2% FBS on ice for 1hr to allow binding. Following incubation, cells were washed three times and binding was measured using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). The percent of positive cells was determined by gating control cells which were not incubated with targeted toxin.

Bioassays to measure cell proliferation

To determine the ability for EGFATFKDEL to inhibit cells *in vitro*, proliferation assays measuring ^{3}H-thymidine incorporation were used [31]. CD3CD3KDEL, a PE38KDEL-containing molecule which targets CD3 on T cells, was often used as a negative control. Briefly, cells (10^4/well) were plated out in a 96-well flat-bottomed plate and incubated overnight at 37°C with 5% CO_2. The next day, targeted toxins in varying concentrations were added to wells in triplicate. Raji cells (10^4/well) were plated and targeted toxins were immediately added at varying concentrations. Plates were incubated at 37°C and 5% CO_2 for 72 h. [Methyl-^{3}H]-thymidine (GE Healthcare, UK) was added (1 µCi per well) for the final 8 h of incubation. Plates were frozen to detach cells and cells were harvested onto a glass fiber filter, washed, dried, and counted using standard scintillation methods. Background counts in untreated wells ranged from <10 cpm to 500 cpm. Data from proliferation assays are reported as percentage of control counts. Results from bioassays were always reproduced. Protein synthesis inhibition assays measuring the incorporation of radioactive leucine were used in some experiments. The assays were similar, except cells were incubated in leucine-free medium. In order to prove that cells were actually killed and not just rendered static, EGFATFKDEL 7mut was tested for its ability to kill U87 cells in vital stain assays using trypan blue staining. These studies confirmed that concentrations of drug that fully inhibited in proliferation assays actually killed the cells (not shown).

Blocking studies were conducted to test the specificity of EGFATFKDEL 7mut. Briefly, EGF or anti-uPA (American Diagnostica, Stamford CT) was added to the media containing 0.1 nM EGFATFKDEL 7mut at a final concentration of 100 nM. Anti-Pseudomonas exotoxin A (Sigma-Aldrich, St. Louis MO) was obtained as whole rabbit serum and added to medium to reach a final dilution of 1:10,000. Resulting mixtures were added to wells containing U87 cells and proliferation was measured as described above. The mouse leukocyte specific antibody anti-Ly5.2 was included as a negative control [32].

Determining immunogenicity of deimmunized EGFATFKDEL 7mut

Mouse immunization studies were used to determine whether mutated EGFATFKDEL 7mut elicited less of an immune response than the unmutated parental EGFATFKDEL molecule. Female Balb/c or C57BL/6 mice (n=5/group) were injected intraperitoneally once weekly with

0.25 μg of either EGFATFKDEL or EGFATFKDEL 7mut for 104 days. Each week, five days after injection, the mice were bled (facial vein collection) to obtain serum. Serum from each mouse was isolated using centrifugation and frozen. The amount of anti-PE38KDEL IgG in each serum sample was measured using indirect ELISA. Briefly, 5 mg of purified recombinant PE38KDEL was added to each well of a 96-well microtiter plate and adhered overnight at 4°C. Unbound protein was washed away with PBS-T and blocking was performed for 1 h with 5% milk/PBS-T. Serum samples were diluted in 1:10,000 and 100 μl of each was added to appropriate wells in triplicate. Following 3 h incubation, each well was washed 3 times with PBS-T. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was added to each well for a 2 h room temperature incubation. After washing, o-Phenylenediamine dihydrochloride substrate was added to each well. After 30 min, the absorbance at 490 nm was measured using a microplate reader. Quantification of actual anti-PE38KDEL IgG present in each sample was determined by comparing the absorbance values in each well to a standard curve prepared using M40-1 monoclonal anti-PE38KDEL antibody from Dr. Robert Kreitman (NIH, Bethesda, MD).

***In vivo* efficacy studies of EGFATFKDEL 7mut against U-87 flank tumors**

Male nu/nu mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. All animals were housed in microisolator cages to minimize the potential of contaminating virus transmission.

For flank tumor studies, mice were injected with 3 x 10^6 U87-luc cells. Once tumors reached approximately 0.03 cm^3 (day 6), mice were divided into groups and treated with EGFATFKDEL 7mut, ATFKDEL or 2219ARLKDEL. Mice in treated groups were given 2 μg of targeted toxin four times a week for a total of about 5 weeks. All targeted toxins were administered by intratumoral injection in 100 μl volume of sterile saline. Drug was delivered in this volume so that it could be injected in 3 different directions. Backflow can be a problem, especially when injecting small tumors with high intrastitial pressures. Because of the anti-angiogenic nature of the drug, we reasoned that even if a full dose was not given entirely intratumorally, it would still affect the tissue immediately surrounding the tumor. Tumor size was measured using a digital caliper, and volume was determined as a product of length, width, and height. Treatment-related toxicity was monitored by measuring animal weight.

Mice were imaged in real time and images were captured using Xenogen Ivis imaging system (Xenogen Corporation, Hopkington MA) and analyzed with IGOR Pro 4.09a software

(WaveMetrics Inc., Portaland OR). Before imaging, mice were anaesthetized using isofluorane gas. All mice received 100 µl of a 30mg/ml^{-1} D-luciferin aqueous solution (Gold Biotechnology, St Louis MO) as a substrate for luciferase 10 min before imaging. All images represent a 5 min exposure time and all regions of interest are expressed in units of photons/sec/cm^2/sr. About 10^5 photons/sec/cm^2/sr is the background for luciferase imaging.

Statistical analyses

All statistical analysis was performed using Prism 4 (Graphpad Software, San Diego CA). Groupwise comparisons of single data points were made by Student's *t*-tests or one-way ANOVA with Tukey's multiple comparison tests. P-values <0.05 were considered significant.

Results

***In vitro* efficacy of EGFATFKDEL against U87 cells**

To determine selective cytotoxicity against human glioblastoma, EGFATFKDEL was tested for its ability to inhibit the protein synthesis of U87 and U87-luc cells *in vitro*. Figure 9a shows that EGFATFKDEL markedly inhibited U87-luc cells in a dose dependent fashion with an inhibitory concentration 50% (IC_{50}) value of 0.027 nM. The negative control CD3CD3KDEL had minimal effect, confirming the specificity of EGFATFKDEL to EGFR$^+$ uPAR$^+$ GBM cells.

***In vitro* efficacy of EGFATFKDEL against endothelial cell lines**

EGFATFKDEL is designed to target both solid GBM tumors and their associated endothelial neovasculature. To investigate whether or not the uPAR targeting was effective, we tested our bispecific drug on human umbilical vein endothelial cells (HUVECs). These cells bear similarity to cells in the tumor neovasculature and over-express uPAR but not EGFR. HUVECs were over 166-fold more sensitive to the uPAR-targeting since the IC_{50} of the monospecific ATFKDEL was 0.092 nM, whereas the IC_{50} of monospecific EGFKDEL was 15.31 nM (Figure 9b). However, bispecific EGFATFKDEL had the highest activity with an IC_{50} of 0.012 nM. Our laboratory has shown that other bispecific targeted toxins are consistently more toxic than their monospecific counterparts [8,9,27]. The BLT also had greater activity than an equimolar mixture of monospecific ATFKDEL and EGFKDEL indicating an advantage of combining both ligands on the same single chain molecule.

***In vitro* specificity of EGFATFKDEL**

To further demonstrate the specificity of EGFATFKDEL, EGFATFKDEL was incubated with EGFR$^-$ uPAR$^-$ Raji B cell cells (Figure 9c). Raji cells were not affected by EGFATFKDEL, EGFKDEL, or ATFKDEL. In contrast, 2219ARLKDEL, a bispecific toxin targeting both CD22 and CD19 expressed on Raji cells [7], killed the cells as anticipated (IC_{50}=0.015 nM).

To determine that both high affinity cytokines, EGF and ATF, on the EGFATFKDEL 7mut molecule bound to their appropriate receptors, anti-urokinase antibody (α-uPA), EGF, and anti-pseudomonas exotoxin antibody (α-PE) were incubated with a known cytotoxic concentration of EGFATFKDEL 7mut in attempt to block killing (Figure 9d). All of these blocking agents inhibited the ability of EGFATFKDEL 7mut to kill U87 cells. In contrast, anti-Ly5.2 (α-Ly5.2), an irrelevant control antibody, did not block EGFATFKDEL 7mut activity. These results confirm that both EGF and ATF ligands are active on the EGFATFKDEL 7mut molecule.

Binding of EGFATFKDEL to U87 Cells

The efficacy of all targeted toxins is dependent on cell surface binding and internalization. Thus, we measured ligand-mediated cell binding of EGFATFKDEL, EGFKDEL, ATFKDEL, and the negative controls, HD37 and RFB4, on U87 cells in Figure 10. All of the targeted toxins were FITC-labeled, incubated with U87 cells at 500 nM, and analyzed using flow cytometry. The percentage of positive cells was 22.8 and 34.8% with monospecific EGFKDEL-FITC and ATFKDEL-FITC, respectively. In contrast, the same cells reacted with bispecific EGFATFKDEL-FITC were 58% positive, indicating superior binding of the BLT. The mean fluorescent intensity (MFI) values for EGFATFKDEL, EGFKDEL and ATFKDEL were 30.5, 18.4 and 19.6, respectively. Cells stained with anti-B cell negative control HD37-FITC or RFB4-FITC showed little binding ability.

Reducing the immunogenicity of EGFATFKDEL

To determine whether EGFATFKDEL had been successfully deimmunized, groups of immunocompetent BALB/c mice were immunized weekly with 0.25 μg of either non-mutated EGFATFKDEL or mutated EGFATFKDEL 7mut. Mice were used because the same seven major epitopes are recognized in mice and humans [33]. Animals were immunized i.p. over the period of 62 days. Serum samples were obtained weekly and analyzed using ELISA to detect anti-PE38KDEL IgG. The results of the immunization experiment are summarized in Figure 11a and show statistical differences between the anti-toxin responses of the two groups. After nine injections (day 62), the EGFATFKDEL 7mut group showed no immune response, while the EGFATFKDEL group had an average anti-PE38KDEL response of 5,664 μg/ml. A similar evaluation of C57BL/6 mice showed the same results (not shown).

***In vitro* comparison of EGFATFKDEL and EGFATFKDEL 7mut**

To verify that EGFATFKDEL retained activity after the mutation, the mutated and non-mutated drugs were compared *in vitro* against the U87 cell line. Mutated EGFATFKDEL 7mut consistently had greater activity than parental EGFATFKDEL. Figure 11b shows the IC_{50} of EGFATFKDEL 7mut as 0.007 nM and the IC_{50} of EGFATFKDEL as 0.044 nM. A slight

increase in toxicity was also observed in HUVEC cells where the IC_{50} of EGFATFKDEL 7mut was 0.0017 nM compared to 0.012 nM in the parental EGFATFKDEL (Figure 9b). Similar increases in activity have been observed in other mutant PE38KDEL-containing drug studies but the cause is unknown [34]. Control CD3CD3KDEL was not toxic.

The effect of EGFATFKDEL 7mut is shown against other glioblastoma cell lines including U118, U373, and T98 in Table 2. The bispecific drug was effective against all of the glioblastoma cell lines tested and one head and neck cancer cell line, UMSCC-11B, but not against Raji (Figure 9c).

Table 2

The effect of EGFATFKDEL on the activity of various glioblastoma cell lines

Cell line	Cancer type	EGFATFKDEL 7mut IC_{50} (nM)
U87	Glioblastoma	6.5×10^{-3}
U87-luc	Glioblastoma	7.9×10^{-3}
U118	Glioblastoma	4.8×10^{-8}
U373	Glioblastoma	$<1.0 \times 10^{-8}$
T98	Glioblastoma	9.17×10^{-8}
UMSCC-11B	Head & neck squamous cell carcinoma	3.2×10^{-5}
Raji	B cell lymphoma	>100

***In vivo* efficacy of EGFATFKDEL 7mut against U87-luc flank tumors**

To study the *in vivo* activity of EGFATFKDEL 7mut, we used a bioluminescence luciferase reporter gene mouse model with U87-luc cells in athymic mice. U87-luc cells stably express firefly luciferase without affecting cell growth. This model enables real-time imaging of flank tumors. U87-luc tumors were grown on each mouse's flank. Mice were treated with 2 μg of EGFATFKDEL 7mut (n=7), or 2219ARLKDEL (n=3) four consecutive days each week. This was called one course of treatment and 5 courses were given over a 5 week period (day 6 through 40). A third group of mice was untreated (n=5). Our treatment regimen was designed from our other previously published flank tumor models with BLT [8,9]. Treatment was continued in the hopes of maintaining tumor-free mice and to potentially yield long-term survivors. Also, 20 treatments were given to show that mice survive despite continued treatment. An image of luciferase activity for each mouse was obtained weekly. Figure 12a, b, and c show the images for representative mice in different treatment groups, and demonstrates that EGFATFKDEL 7mut decreased tumor-associated bioluminescence compared to untreated mice or mice treated with control 2219ARLKDEL.

In addition to bioluminescence, tumor volumes in these mice were measured two times per week with calipers. The tumor volumes of EGFATFKDEL 7mut-treated mice were

markedly reduced compared to the controls. These tumor volumes were significantly different when compared with the untreated group at days 26, 29, 33, and 36 ($p<0.05$, Figure 13a). In the long-term, even after 110 days, four out of the eight EGFATFKDEL 7mut treated mice were tumor-free. One of the tumor-free mice died on day 55 from rapid weight loss and potentially toxicity. Another tumor-free mouse died on day 99 from unknown causes. In a second experiment, EGFATFKDEL 7mut again had potent anti-tumor activity (Figure 13b). However, differences were not significant in this experiment due to variances in tumor sizes (n=6/group).

In order to determine whether similar anti-tumor responses would be observed with monospecific drugs, groups of mice in the same experiment as Figure 13 were treated with EGFKDEL and ATFKDEL at similar drug concentrations as EGFATFKDEL. Figure 14 shows the animals treated with ATFKDEL and 4 of 5 of these animals did not elicit an anti-tumor response, but bioluminescence measurements remained static. All EGFKDEL treated animals died. These data indicate that the monospecific forms are not as effective at comparable drug concentrations.

Discussion

The original contribution of this research is the development of EGFATFKDEL 7mut, a promising new anti-glioblastoma agent with potential for clinical development. Our laboratory has designed and published BLTs which simultaneously bind to dual independent receptors on the surface of tumor cells [6,8,9,27,29,30,35]. However, EGFATFKDEL is the first targeted toxin that simultaneously attacks tumor cells and the blood supply that feeds them. Angiogenesis, or generation of neovasculature, is a complex process which involves several elements including endothelial cells, stromal cells, and factors from the extracellular matrix. There has been tremendous interest in drugs which target the neovasculature [36]. For example, bevacizumab and other anti-angiogenic drugs currently in clinical trials have demonstrated that the vasculature can be successfully targeted with antibodies and other neovasculature targeting strategies [36]. Although angiogenesis inhibitors possess impressive potential, their success has been limited mostly by the fact that tumor regressions are often only partial [36]. Since EGFATFKDEL directly attacks the most prominent cells in the tumor (vascular cells and tumor cells), we believe that effects will be magnified because the toxic effect of PE is catalytic and irreversible. Our *in vitro* data with HUVECs shows that our drug can potentially impact tumor neovasculature. While we acknowledge that HUVECs are not an optimal model of tumor neovasculature, our studies show that the ATF portion of our drug was effectively targeting uPAR on endothelial cells. Our *in vivo* data supports this because we have demonstrated that EGFATFKDEL 7mut is highly effective in a mouse model in which flank tumors were induced

by the injection of the human glioblastoma line U87. Tumor-free survivors were observed beyond day 100, despite the fact that treatment ended on day 40, indicating that drug responses were durable.

Other properties that set EGFATFKDEL 7mut apart from other biological targeted toxins are its toxin modifications. First, the KDEL sequence (SEQ ID NO: 15) was added to the c-terminus to enhance potency. The KDEL (SEQ ID NO: 15) modification does not necessarily result in an increased therapeutic window and it is possible that the advantage of increased potency may be negated by enhanced toxicity. To address this issue KDEL(SEQ ID NO: 15)-modified EGFATF 7mut could be compared to non-KDEL (SEQ ID NO: 15) modified EGFATF 7mut in future studies.

Another, more important toxin modification was the "deimmunization" of EGFATFKDEL 7mut. Kreitman et al. showed that the clinical efficacy of treatment with targeted toxins against solid tumors hinges on the ability to give multiple treatments or sustained treatment which enables the drug to penetrate a solid tumor [37]. Toxins may be administered locally to treat tumors in sensitive organs, but targeted toxins must still be used repeatedly or via sustained delivery to achieve positive results. The major problem with this is that neutralizing antibodies will be generated that significantly reduce efficacy over time. To address this issue, investigators used an expansive library of anti-PE monoclonal antibodies to epitope map prominent molecular regions which elicited the strongest antibody response. Fortunately, the immunogenic regions of PE were mapped in seven distinct epitotic areas, and not distributed throughout the molecule. We constructed our PE-based BLT and mutated key amino acids in each of the 7 regions without compromising toxin activity. The immune response to the resultant second-generation drug was reduced by 80-90% in a validated mouse model [34].

Our immunization experiments are designed to evoke gradual immune responses by administering low concentrations of drug on a weekly basis. Initial responses are mild, but they grow exponentially, as seen in Figure 11. Responses likely could be expedited by using a more aggressive immunization regimen employing greater dosages and more immunizations. However, from other studies we know that neutralizing antibodies are present when anti-toxin levels reach about 500 μg/ml. Consequently, analysis of early and low antibody production is important. In our experiments, anti-toxin levels in some mice treated with the parental drug exceeded this threshold after only four injections (day 26). After eight injections, none of the mice treated with the mutant drug had reached 20% of this threshold.

Another concern of our mouse immunogenicity studies is that major histocompatibility complex (MHC) haplotypes differ in their presentation of peptide fragments in the MHC groove and a different haplotype might present different peptides. In other words, basic immunology

dictates that a danger of mutating B cell recognizing regions of the toxin is that peptides regarded as immunogenic by one MHC polymorphism, may not be regarded as immunogenic by a different polymorphism. Thus, we used two mice strains, BALB/c and C57BL/6, with entirely different MHC haplotypes. We observed significant anti-toxin reductions in both strains, indicating that the strength of mutating all 7 regions was enough to overcome differences in MHC polymorphisms.

A final concern of the immunogenicity studies is whether mice are useful for studying human anti-toxin responses. Kreitman and Pastan have treated over 300 patients with non-mutated PE targeted toxins [33]. Nagata studied the antibodies in patients with high titers of anti-PE antibodies and found they bind to the same seven epitopes which regulate toxin B cell recognition in mice indicating that mice are a useful model for human anti-PE responses [33].

In this paper, we showed that in a mouse flank tumor model, EGFATFKDEL 7mut was impressively effective against GBM tumors. Although flank tumor models are useful for determining drug efficacy against vascularized tumors, they are not ideal. For our study, drug was injected directly into small, palpable, and established tumors because targeted toxins have been vigorously pursued for IC therapy in which they are delivered directly into the tumor. A more sophisticated model would use controlled IC delivery via CED in which drug is pumped through a catheter directly into the brain tumor [2-4]. This is an established model in our laboratory and these studies are underway [38]. Another delivery option that will require further exploration is systemic delivery. Due to its vascular reactivity, the drug may be highly effective systemically because of its ability to disrupt the BBB. Additionally, treatment in conjunction with hyperosmolar mannitol may enhance the drug's BBB disruptive capabilities.

We have noticed that toxin mutation (deimmunization) increases the activity of EGFATFKDEL 7mut, but not other BLT [6]. Onda et al. noted that the certain toxin mutations enhanced the activity of one targeted toxin as well [34]. In these instances, different toxin mutation combinations were compared, but only one ligand was explored. Several explanations are possible, but we favor binding change as a possible reason. Binding has a major impact on the activity of targeted toxins [39]. Any mutation can affect positions of alpha helices, beta strands, and turns. This consequentially impacts configuration and these shifts can improve binding. Sequence variances can also affect refolding quality which can also affect binding. This could explain why mutation of EGFATFKDEL enhances activity, but mutation of EGF4KDEL (a PE38KDEL, EGF and interleukin-4 containing targeted toxin) does not [35].

Regarding binding, our flow cytometry data indicated that the bispecific drug was superior to its monospecific counterparts because it bound better. These data correlated with our *in vivo* studies which showed that the monospecific forms were not as effective at dosages

comparable to the bispecific drug. ATFKDEL did not induce complete remissions in 4 of 5 or 80% of the mice, and EGFKDEL killed all of the mice. We have observed that monospecific EGFKDEL is at least a log more toxic than bispecific EGFATFKDEL.

In summary, we have shown that EGFATFKDEL 7mut is effective in inducing complete remissions against GBM tumors. In vitro, the drug is effective in the picomolar range against tumor cells and against HUVEC cells which proves that it binds vascular cells. Attempts at deimmunization of the drug have been successful with a reduction of 80-90% anti-toxin antibodies.

Example 3 References

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EXAMPLE 4

Ability of a ligand targeted toxin containing epidermal growth factor and urokinase to kill chemoresistant canine hemangiosarcoma and hemanigospheres

Ligand targeted toxins (LTTs) have the potential to overcome intrinsic or acquired resistance of cancer cells to conventional cytotoxic agents. Here, we hypothesized that EGFATFKDEL 7mut, a bispecific LTT consisting of a deimmunized *Pseudomonas* exotoxin conjugated to epidermal growth factor and urokinase, would efficiently target and kill canine hemangiosarcoma cells (HSA), as a model of a highly chemotherapy resistant tumor, as well as cultured hemangiospheres as surrogates for cancer stem cells (CSC). EGFATFKDEL 7mut resulted in cytotoxicity against three HSA cell lines (Emma, Frog, and DD-1) when used at a pharmaceutically relevant concentration of 100 nM, and the cytotoxicity was dependent on specific ligand-receptor interactions (Figure 16). Together, our results support the use of these toxins to treat chemoresistant tumors such as sarcomas, and including those that conform to the cancer stem cell (hierarchical) model, as well as the use of companion animals with cancer for further translational development of these cytotoxic molecules.

Introduction

Despite numerous recent advancements in cancer therapy, failure of conventional treatments to result in complete disease remission frequently occurs. Although current chemotherapeutic treatments may result in prolonged survival, tumor recurrence and disease progression represent a major cause of mortality in many cancer patients. Bispecific ligand targeted toxins (BLT) have the potential to overcome intrinsic or acquired resistance of cancer cells to conventional cytotoxic agents. The ligands are designed to bind specific receptors that are uniquely or highly expressed by tumor cells as compared to normal cells. This improves tumor specificity by directing the ligands towards the tumor, thus reducing the toxic side effects associated with cancer chemotherapy and radiation therapy. Additionally, the conjugated toxins have high potency, killing tumor cells at picomolar concentrations, with as few as 1000 molecules of PE toxin delivered to the cytosol resulting in a complete anti-tumor response (Kreitman et al., *Cancer Res.*, 58(5), 968-975 (1998)). In fact, previous work has shown that BLTs exhibit potent and specific activity against human breast cancer, brain cancer, and blood-derived tumors with acceptable safety profiles in laboratory animals.

In this study, we tested a BLT named EGFATFKDEL 7mut, designed to simultaneously target the epidermal growth factor receptor (EGFR), upregulated in a variety of human and animal cancers, and the urokinase receptor (uPAR), expressed on sarcomas as well as endothelial cells and tumor vasculature. EGF and uPA were conjugated to a truncated Pseudomonas exotoxin A (PE38), shown previously to have potent anticancer activity via inhibition of protein synthesis (Pastan et al., *Annu. Rev. Biochem.*, 61, 331-354 (1992)). To enhance potency, PE38 was then modified by adding a Lys-Asp-Glu-Leu (KDEL) (SEQ ID NO: 15) C- terminus signal to prevent luminal endoplasmic reticulum protein secretion. Finally, the toxin was deimmunized via mutation of seven B-cell epitope encoding sequences, identified by Onda and Pastan, to permit multiple in vivo treatments without generating an anti-toxin response (*J Immunol.*, 177, 8822-8834, (2006)).

EGFATFKDEL 7mut makes a promising potential chemotherapeutic agent due to EGFRs frequent upregulation in epithelial cancers and uPARs additional ability to target sarcomas and tumor neovasculature. Canine hemangiosarcoma cells (HSA), which are derived from endothelial cell tumors, express uPAR, as determined by positive hits on a gene expression array platform (GEO series record GSE15086), as well as EGFR. Additionally, canine HSA shows resistance against several chemotherapeutic agents, being resistant to 90% of compounds in a LOPAC drug screen (unpublished data). Thus, canine HSA serves as a model of tumor neovasculature, as well as a highly resistant sarcoma. Furthermore, it has been documented that one property of cancer stem cells (CSC) is greater resistance to chemotherapeutic drugs

compared to non-CSCs. Therefore, in order to further examine the effectiveness of EGFATFKDEL 7mut to overcome resistance, non-adherent tumor spheroids from the canine HSA cell line SB were used as surrogates for cancer stem cell (CSC) enrichment.

In this paper, we examine the cytotoxicity of EGFATFKDEL 7mut against HSA cell lines, as well as CSCs, as a model of a highly chemoresistant sarcoma and tumor neovasculature. EGFATFKDEL 7mut resulted in cytotoxicity against these cell lines at pharmacologically relevant concentrations, supporting its future treatment potential, as well as the use of companion animals with cancer for further translational development of these cytotoxic molecules.

Results

K12 and Emma cells express EGFR and uPAR

Since EGFATFKDEL 7mut requires binding to EGFR and uPAR, RT-PCR was performed on each cell line to validate cognate receptor mRNA receptor expression. Gene expression array data from our lab showed HSA cells express uPAR, and Thamm et al. also reported expression of EGFR mRNA by these cells in 2006. **Figure 15** shows that Emma and K12 cells indeed express EGFR and uPAR mRNA.

Highly chemoresistant canine HSA tumor cell lines are sensitive to a BLT targeted by EGF and uPA

To determine *in vitro* BLT cytotoxicity against canine HSA cells, EGFATFKDEL 7mut was tested against Emma, Frog, and DD1 cell lines (Figure 16). EGFATFKDEL 7mut showed significant cytotoxicity against HSA cell lines when used at a pharmacologically relevant concentration of 100 nM. As expected, the toxin was also effective against a highly chemosensitive control feline mammary carcinoma cell line, K12, which overexpresses EGFR. The negative control, BIC 3 (a T-cell specific toxin) had no effect.

EGFATFKDEL 7mut binding to cognate EGFR and uPAR induces cytotoxicity.

To verify that the BLT was acting through cognate receptors, we examined the effect of blocking receptor interactions with ligand on cytotoxicity. We used a single concentration to assess the effects of blocking receptor-ligand interactions using anti-ligand antibodies or excess ligand as competitive inhibitors. At the concentration used, EGFATFKDEL 7mut killed >99% of K12 cells and ~75% of the Emma HSA cells. **Table 3** shows that in Emma HSA cells, blocking the EGF ligand portion of the EGFATFKDEL 7mut with anti-EGF antibody or the uPA portion with anti-uPA antibody abrogated cell death. In contrast, blocking the EGF portion with anti-EGF antibody eliminated cytotoxicity of EGFATFKDEL 7mut against K12 cells, but blocking the uPA portion with anti-uPA antibody led to incomplete inhibition of cytotoxicity. An irrelevant antibody had no effect (α-Ly5.2) on either cell line.

Table 3. Blocking EGF and uPA inhibits LTT cytotoxicity. Emma and K12 cells were incubated with 0.1 nM EGFATFKDEL 7mut in the presence of 50ug/ml of blocking antibodies against EGF, uPA, or Ly5.2 (irrelevant isotype control) as indicated. Viability was measured in duplicate samples using the MTS assay, normalized to 100% in the absence of LTT. A value of 100 represents maximal viability. Values <100 represent death due to cytotoxic effects of EGFATFKDEL 7mut. One representative experiment of three is shown for each cell line.

Blocking Condition	% Dead	
	Emma	K12
No Toxin	0	0
Anti EGF	0	0
Anti uPA	2	65
No Antibody	74	98
Anti Ly5.2	63	100

Similarly, **Figure 17a** shows that in Emma HSA cells, blocking receptor-toxin interactions with both uPA and EGF ligands (each present at 1,000-fold excess of the toxin concentration) had the greatest effect to restore cell growth, blocking 60% of EGFATFKDEL 7mut cytotoxicity. At the same 1,000-fold excess, uPA alone prevented almost 40% of cell death, whereas EGF was minimally effective. In contrast, K12 cells do not show recovery in response to blocking either single ligand (**Figure 17b**). Both EGF and uPA ligands were each required to be present at 1,000-fold excess in order to abrogate ~70% of cell death.

In order to assess if CSCs were sensitive to EGFATFKDEL 7mut, cytotoxicity assays were performed on SBS and SBM and compared to values previously attained for Emma and K12. The inhibitory concentrations (IC50) are summarized in **Table 4** below. As shown, the sensitivity of SBM (.03 nM) was approximately equivalent to Emma (0.04 nM) and 800 times that of SBS (25 nM).

Cell Line	IC50 (nM)
SBS	25
SBM	0.03
Emma	0.04
K12	0.003

Table 4. EGFATFKDEL 7mut kills canine HSA CSCs and non-CSCs at picomolar to low nanomolar concentrations. A dose response cytotoxicity assay was carried out for 72 hours on Emma, SB cells grown as a monolayer (SBM) and SB non-adherent tumor spheroids (SBS). Viability was measured in duplicate samples using the MTS assay, normalized to 100% in the absence of LTT. A value of 100 represents maximal viability. Values <100 represent death due to cytotoxic effects of EGFATFKDEL 7mut. IC_{50} values were extrapolated from the average of

two experiments and represent the concentration required to reduce cell viability by 50% compared to untreated controls.

Discussion

In this study we sought to determine whether a BLT, consisting of a deimmunized pseudomonas exotoxin conjugated to EGF and uPAR would induce cytotoxicity of highly chemoresistant sarcoma cells. Although rare in humans, sarcomas are extremely aggressive and highly refractory to conventional therapies. Therefore, an unmet medical need exists in which new treatments are desperately needed.

BLTs comprise a promising novel treatment in cancer therapy, with potential for sarcoma treatment. Several of these have been developed and published, including EGFATFKDEL 7mut, which was shown to effectively target tumors that overexpress EGFR, in vitro and in vivo. Moreover, the uPA ligand enhanced the therapeutic benefit of the BLT by targeting uPARs in tumor vasculature. Therefore, we hypothesized this BLT would have high activity against sarcomas, which unlike many carcinomas, express both EGFR and uPAR.

In contrast to humans, where sarcomas make up less than 2% of diagnosed cancers, these tumors are commonly diagnosed in domestic animals. Thus, this provides an abundant source of samples with high comparative value. Specifically, our group and others have shown that soft tissue sarcomas and bone sarcomas of dogs share remarkable similarities with their human counterparts at the molecular level. Given the paucity of viable human reagents, canine tumors can be leveraged as a resource to study important questions that would be challenging to address in humans. Additionally, it allows us and to overcome a major obstacle in contemporary therapeutic development - the lack of translational fidelity from laboratory animal models to patients with naturally occurring disease.

Therefore, in order to test our hypothesis, we selected canine hemangiosarcoma because it is molecularly similar to idiopathic angiosarcoma in human patients and is also representative of a prototypical chemoresistant tumor for which there are limited chemotherapeutic treatment options. Moreover, although EGFATFKDEL 7mut is effective against HUVEC cells, canine HSA serves as an additional model of tumor neovasculature, as the structure resembles the tortuous, leaky, and disorganized vessels seen in tumors. For a control tumor, we chose to use a feline mammary adenocarcinoma because it exhibits EGFR overexpression.

Our data show that each of canine hemangiosarcoma cell lines tested were susceptible to EGFATFKDEL 7mut cytotoxicity, and this sensitivity was mediated by specific interactions between the ligands in the BLT and the cognate receptors on the cells. Furthermore, the BLT

effectively killed hemangiospheres, which are enriched for cells with cancer stem cell properties, and show even greater chemoresistance than cells maintained in routine monolayer cultures. Importantly, the drug was capable of killing CSCs at a therapeutically feasible concentration. Furthermore, we believe that the effects of EGFATFKDEL 7mut will be amplified in vivo through the additional effect of targeting tumor neovasculature, as well as, differentiated cancer cells.

Consistent with previous data suggesting CSCs have increased mechanisms of resistance compared to non-CSCs, hemangiospheres were less sensitive to the EGFATFKDEL 7mut. These differences might be due to upregulation of anti-apoptotic activity or ABC efflux channel proteins.

The observation that blocking either ligand with antibodies abrogated cell death in HSA cells could be explained by two non-mutually exclusive possibilities. First, steric hindrance from the anti-EGF antibodies reduced the binding affinity of EGFATFKDEL 7mut to HSA cells, and second, interactions of the EGF component in the BLT with EGFR on the HSA cell surface might stabilize binding and enhance internalization of the toxin.

The dynamics of receptor recycling also may play a role in binding, internalization and sensitivity to the toxin. In K12 cells, up to 1,000-fold molar excess of either ligand alone was insufficient to prevent the cytotoxic effects of EGFATFKDEL 7mut, but the same amount of both ligand competitors used concurrently effectively spared up to 70% of the cells. This suggests that at nanomolar concentrations, the toxin can use either receptor to achieve internalization and promote cell death of K12 cells. In HSA cells, 1,000-fold excess of uPA alone, but not EGF alone, was sufficient to inhibit up to ~40% of EGFATFKDEL 7mut cytotoxicity. In these cells, the combination of both ligand competitors at 1000-fold excess, could inhibit 60% of EGFATFKDEL 7mut cytotoxicity. This is consistent with the interpretation that the cytotoxic effects of EGFATFKDEL 7mut require binding to, and presumably internalization by uPAR, but the interactions of the toxin with EGFR possibly stabilize and augment binding and internalization of the toxin in HSA cells.

Additionally, previous data from our group show that in vivo administration of EGFATFKDEL 7mut did not show significant side effects at the doses needed to see anti-tumor effects. Therefore, we believe the intrinsic benefit of killing tumors expressing low levels of uPAR will outweigh potential side effects and expand the range of tumors able to be treated with EGFATFKDEL 7mut.

In summary, our data support the potential for the future clinical development and treatment of sarcomas with EGFATFKDEL 7mut. We have shown that EGFATFKDEL 7mut combats several problems commonly seen in therapeutic development: potency, off target side

effects, and chemoresistance. Additionally, beyond killing tumors intrinsically expressing uPAR, the effect of EGFATFKDEL 7mut may be amplified in vivo by targeting tumor neovasculature. These findings indicate the EGFATFKDEL 7mut is a promising potential therapeutic agent for the treatment of sarcomas that have failed conventional therapy, potentially by reducing or eliminating CSCs. Moreover, our data also support the use of companion animals with naturally occurring sarcomas as models for clinical translation of this compound.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (*i.e.*, meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. A receptor-targeting reagent comprising: (a) a first targeting domain comprising an epidermal growth factor receptor (EGFR)-binding agent and (b) a second targeting domain comprising a urokinase plasminogen activator receptor (uPAR)-binding agent, wherein (a) is bound to (b).

2. The receptor-targeting reagent of claim 1, further comprising a toxic domain, wherein the receptor-targeting reagent is toxic.

3. The receptor-targeting reagent of claim 2, wherein the toxic domain comprises a small molecule or a radiological agent.

4. The receptor-targeting reagent of claim 2, wherein the toxic domain comprises a toxic polypeptide.

5. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide is Diphtheria toxin or a biologically active fragment thereof.

6. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide comprises SEQ ID NO:9 or SEQ ID NO: 10.

7. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide consists of SEQ ID NO:9 or SEQ ID NO: 10.

8. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide comprises a Pseudomonas exotoxin A or a biologically active fragment thereof.

9. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide consists of a Pseudomonas exotoxin A or a biologically active fragment thereof.

10. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide comprises SEQ ID NO: 11 or SEQ ID NO: 12.

11. The receptor-targeting reagent of claim 10, wherein the immunogenicity of toxic polypeptide is reduced by at least 80%.

12. The receptor-targeting reagent of claim 10, wherein the toxic polypeptide comprises SEQ ID NO: 11, except that

(i) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 432 is glycine, the arginine at position 467 is alanine, and the lysine at position 590 is serine;

(ii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 332 is serine, and the arginine at position 313 is alanine;

(iii) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, and the arginine at position 313 is alanine; or (iv) the arginine at position 490 is alanine, the arginine at position 513 is alanine, the arginine at position 467 is glycine, the glutamic acid at position 548 is serine, the lysine at position 590 is serine, the glutamine at position 432 is glycine, the glutamine at position 332 is a serine, and the arginine at position 313 is alanine.

13. The receptor-targeting reagent of claim 4, wherein the toxic polypeptide is selected from the group consisting of Pseudomonas exotoxin (PE), bryodin, gelonin, α-sarcin, aspergillin, restrictocin, angiogenin, saporin, abrin, a prokaryotic ribonuclease, a eukaryotic ribonuclease, ricin, pokeweed antiviral protein (PAP), a pro-apoptotic polypeptide, a ribosomal inhibitory protein, and a biologically active fragment of any of the foregoing.

14. The receptor-targeting reagent of claim 13, wherein the pro-apoptotic polypeptide is Bax, Fas, Bad, Bak, Bim, Bik, Bok, Hrk, FasL, TRAIL, TNF-alpha.

15. The receptor-targeting reagent of any one of claims 4-14, wherein the toxic polypeptide comprises a KDEL amino acid sequence (SEQ ID NO: 15).

16. The receptor-targeting reagent of any one of claims 1-15, wherein the uPAR-binding agent comprises an antibody that binds to the uPAR or an antigen-binding fragment thereof.

17. The receptor-targeting reagent of any one of claims 1-16, wherein the uPAR-binding agent comprises an uPAR polypeptide or an uPAR-binding fragment thereof.

18. The receptor-targeting reagent of claim 17, wherein the uPAR-binding fragment is a 135 amino acid terminal fragment (ATF) binding domain of uPA.

19. The receptor-targeting reagent of claim 17, wherein the uPAR-binding fragment is SEQ ID NO:1.

20. The receptor-targeting reagent of claim 18, wherein the ATF binds with high affinity to uPAR.

21. The receptor-targeting reagent of any one of claims 1-20, wherein the EGFR-binding agent comprises an antibody that binds to the EGFR or an antigen-binding fragment thereof.

22. The receptor-targeting reagent of any one of claims 1-21, wherein the EGFR-binding agent comprises an epidermal growth factor polypeptide or an EGFR-binding fragment thereof.

23. The receptor-targeting reagent of any one of claims 1-21, wherein the EGFR-binding agent comprises a betacellulin polypeptide, transforming growth factor alpha polypeptide, an amphiregulin polypeptide, an epiregulin polypeptide, a heparin-binding EGF polypeptide, or an EGFR-binding fragment of any of the foregoing.

24. The receptor-targeting reagent of any one of claims 1-23, wherein (a) and (b) are bound to each other by a covalent bond.

25. The receptor-targeting reagent of any one of claims 1-23, wherein (a) and (b) are bound to each other by a non-covalent bond.

26. The receptor-targeting reagent of claim 25, wherein (a) and (b) are bound to each other by a first and second member of a binding pair.

27. The receptor-targeting reagent of claim 26, wherein the binding pair is streptavidin and biotin.

28. The receptor-targeting reagent of any one of claims 2-27, wherein (a), (b), or (a) and (b) are bound to the toxic domain by a non-covalent bond.

29. The receptor-targeting reagent of any one of claims 2-27, (a), (b), or (a) and (b) are bound to the toxic domain by a covalent bond.

30. The receptor-targeting reagent of any one of claims 1-29, wherein the receptor-targeting reagent comprises a fusion protein comprising (a) and (b).

31. The receptor-targeting reagent of any one of claims 1-27, 29, or 30, wherein the receptor-targeting reagent comprises a fusion protein comprising: the toxic domain and (a), (b), or (a) and (b).

32. The receptor-targeting reagent of any one of claims 1-31, further comprising one or more linker moieties.

33. The receptor-targeting reagent of claim 32, wherein at least one of the one or more linker moieties is a peptide.

34. The receptor-targeting reagent of claim 32 or 33, wherein the one or more linker moieties comprise SEQ ID NO: 13 or SEQ ID NO: 14.

35. The receptor-targeting reagent of any one of claims 1-34, wherein the receptor-targeting reagent comprises SEQ ID NO: 17 or SEQ ID NO: 18.

36. A pharmaceutical composition comprising the receptor-targeting reagent of any one of claims 1-35 and a pharmaceutically acceptable carrier.

37. A nucleic acid encoding the fusion protein of the receptor-targeting reagent of any one of claims 30-36.

38. The nucleic acid of claim 37, wherein the nucleic acid encodes a fusion protein comprising any of SEQ ID NO: 18.

39. The nucleic acid of claim 37 or 38, wherein the nucleic acid encodes a fusion protein consisting of any of SEQ ID NO: 18.

40. A vector comprising the nucleic acid of any one of claims 37-39.

41. An expression vector comprising the nucleic acid of any one of claims 37-39.

42. A cell comprising the vector of claim 41.

43. A method of producing a fusion protein, the method comprising culturing the cell of claim 42 under conditions suitable for expression of the fusion protein; and isolating the protein from the cells or the culture medium in which the cells are cultured.

44. The method of claim 43, further comprising isolating the protein from the cells or the culture medium in which the cells are cultured.

45. An *in vitro* method for binding a receptor-targeting reagent to a cell, the method comprising contacting a cell with a receptor-targeting reagent of any one of claims 1-35.

46. The method of claim 45, further comprising determining whether the cell expresses an EGFR or a uPAR.

47. The method of claim 45 or 46, wherein the cell expresses an EGFR.

48. The method of any one of claims 45-47, wherein the cell expresses an uPAR.

49. The method of any one of claims 45-48, wherein the cell is a cancer cell.

50. The method of claim 49, wherein the cancer cell is a cancer stem cell.

51. The method of claim 49, wherein the cancer cell is selected from the group consisting of a lung cancer cell, a breast cancer cell, a colon cancer cell, a pancreatic cancer cell, a renal cancer cell, a stomach cancer cell, a liver cancer cell, a bone cancer cell, a hematological cancer cell, a neural tissue cancer cell, a melanoma cell, a thyroid cancer cell, an ovarian cancer cell, a testicular cancer cell, a prostate cancer cell, a cervical cancer cell, a vaginal cancer cell, a head and neck cancer cell, and a bladder cancer cell.

52. The method of claim 49, wherein the cancer cell is a glioblastoma cell.

53. The method of claim 49, wherein the cancer cell is a sarcoma cell.

54. The method of claim 53, wherein the sarcoma cell is an angiosarcoma cell.

55. The method of any one of claims 45-54, wherein the cell is a mammalian cell.

56. The method of claim 55, wherein the cell is a human cell.

57. An *in vivo* method for binding a receptor-targeting reagent to a cell, the method comprising delivering to a subject the receptor-targeting reagent of any one of claims 1-35.

58. The method of claim 57, wherein the subject is a mammal.

59. The method of claim 58, wherein the subject is a human.

60. The method of any one of claims 57-59, further comprising determining whether the subject has a cancer cell.

61. The method of any one of claims 57-60, wherein the subject has a cancer cell.

62. The method of claim 60 or 61, wherein the cancer cell is a lung cancer cell, a breast cancer cell, a colon cancer cell, a pancreatic cancer cell, a renal cancer cell, a stomach cancer cell, a liver cancer cell, a bone cancer cell, a hematological cancer cell, a neural tissue cancer cell, a melanoma cell, a thyroid cancer cell, an ovarian cancer cell, a testicular cancer cell, a prostate cancer cell, a cervical cancer cell, a vaginal cancer cell, a head and neck cancer cell, or a bladder cancer cell.

63. The method of any one of claims 60-62, wherein the cancer cell is a glioblastoma cell.

64. The method of claim 60 or 61, wherein the cancer cell is a cancer stem cell.

65. The method of any one of claims 60-61, wherein the cancer cell is a sarcoma cell.

66. The method of claim 65, wherein the sarcoma cell is an angiosarcoma cell.

67. The method of any one of claims 60-66, further comprising determining if one or more of the cancer cells express an EGFR or a uPAR.

68. An *in vitro* method for killing a cell, the method comprising contacting a cell with the receptor-targeting reagent of any one of claims 1-35, wherein contacting the cell with the receptor-targeting reagent kills the cell.

69. An *in vivo* method for treating a cancer in a subject, the method comprising delivering to a subject having, suspected of having, or at risk of developing, a cancer the receptor-targeting reagent of any one of claims 1-35.

70. The method of claim 69, wherein the delivering comprises administering the receptor-targeting reagent to the subject.

71. The method of claim 69 or 70, wherein the delivering comprises administering the pharmaceutical receptor-targeting reagent to the subject intravenously.

72. The method of any one of claims 70-71, wherein the receptor-targeting reagent is administered to the subject using a systemic pump.

73. A method for decreasing a toxic side-effect of a toxic therapy in a subject, the method comprising, prior to delivering to a subject the toxic receptor targeting reagent of any one of claims 2-35, delivering to the subject a receptor-targeting reagent comprising: (a) an epidermal growth factor receptor (EGFR)-binding agent and (b) a urokinase plasminogen activator receptor (uPAR)-binding agent, wherein (a) is bound to (b), wherein the receptor-targeting reagent does not comprise a toxic domain.

74. A method for treating a cancer, the method comprising: delivering to a subject with cancer a receptor-targeting reagent comprising: (a) an epidermal growth factor receptor (EGFR)-binding agent and (b) a urokinase plasminogen activator receptor (uPAR)-binding agent, wherein (a) is bound to (b), wherein the receptor-targeting reagent does not comprise a toxic domain; and delivering to the subject the toxic receptor targeting reagent of any one of claims 2-35.

75. A method for selecting a therapeutic agent for a mammal with cancer, the method comprising: determining if one or more cancer cells of a cancer in a mammal express an uPAR or an EGFR; and if one or more of the cancer cells express an uPAR or an EGFR, selecting as a therapeutic agent for the mammal the receptor binding agent of any one of claims 1-35.

76. The method of claim 75, further comprising, after determining that one or more of the cells of the cancer express an uPAR or an EGFR, delivering to the subject the selected receptor-targeting reagent.

77. A method for selecting a therapeutic agent for a mammal with cancer, the method comprising: selecting as a therapeutic agent for a mammal with cancer the receptor binding agent of any one of claims 1-35 if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR.

78. The method of claim 77, further comprising determining if one or more of the cancer cells in a mammal express an uPAR or an EGFR.

79. A kit comprising: the receptor-targeting reagent of any one of claims 1-35; and instructions for administering the receptor-targeting reagent.

80. The kit of claim 79, further comprising one or more pharmaceutically acceptable carriers.

81. The kit of claim 80, further comprising a pharmaceutically acceptable diluent.

82. A kit comprising: one or more reagents for detecting expression of an uPAR or an EGFR; and instructions for administering the receptor-targeting reagent of an any one of claims 1-35 if the expression of an uPAR or an EGFR is detected.

83. An article of manufacture comprising: a container; and a composition contained within the container, wherein the composition comprises an active agent for treating cancer in a mammal, wherein the active agent in the composition comprises a toxic receptor-targeting reagent of any one of claims 2-35, and wherein the container has a label indicating that the composition is for use in treating cancer in a mammal.

84. The article of manufacture of claim 83, wherein the label further indicates that the composition is to be administered to the mammal if one or more cancer cells of the mammal's cancer express an uPAR or an EGFR.

85. The article of manufacture of claim 83 or 84, further comprising instructions for administering the active agent to the mammal.

86. The article of manufacture of any one of claims 83-85, wherein the composition is dried or lyophilized.

87. A method of treating glioblastoma multiforme in a patient in need thereof, comprising administering to the patient the receptor targeting reagent of any one of claims 1-35.

88. A method of treating head and neck cancer in a patient in need thereof, comprising administering to the patient the receptor targeting reagent of any one of claims 1-35.

89. A method of treating sarcoma in a patient in need thereof, comprising administering to the patient the receptor targeting reagent of any one of claims 1-35.

90. The method of claim 89, wherein the sarcoma is angiosarcoma.

91. A receptor targeting reagent as described by any one of claims 1-35 for use in medical treatment or diagnosis.

92. The use of a receptor targeting reagent as described in any one of claims 1-35 to prepare a medicament useful for treating cancer in an animal.

93. A receptor targeting reagent according to any one of claims 1-35 for use in therapy.

94. A receptor targeting reagent as described in any one of claims 1-35 for use in treating cancer.

Fig. 1A – 1B

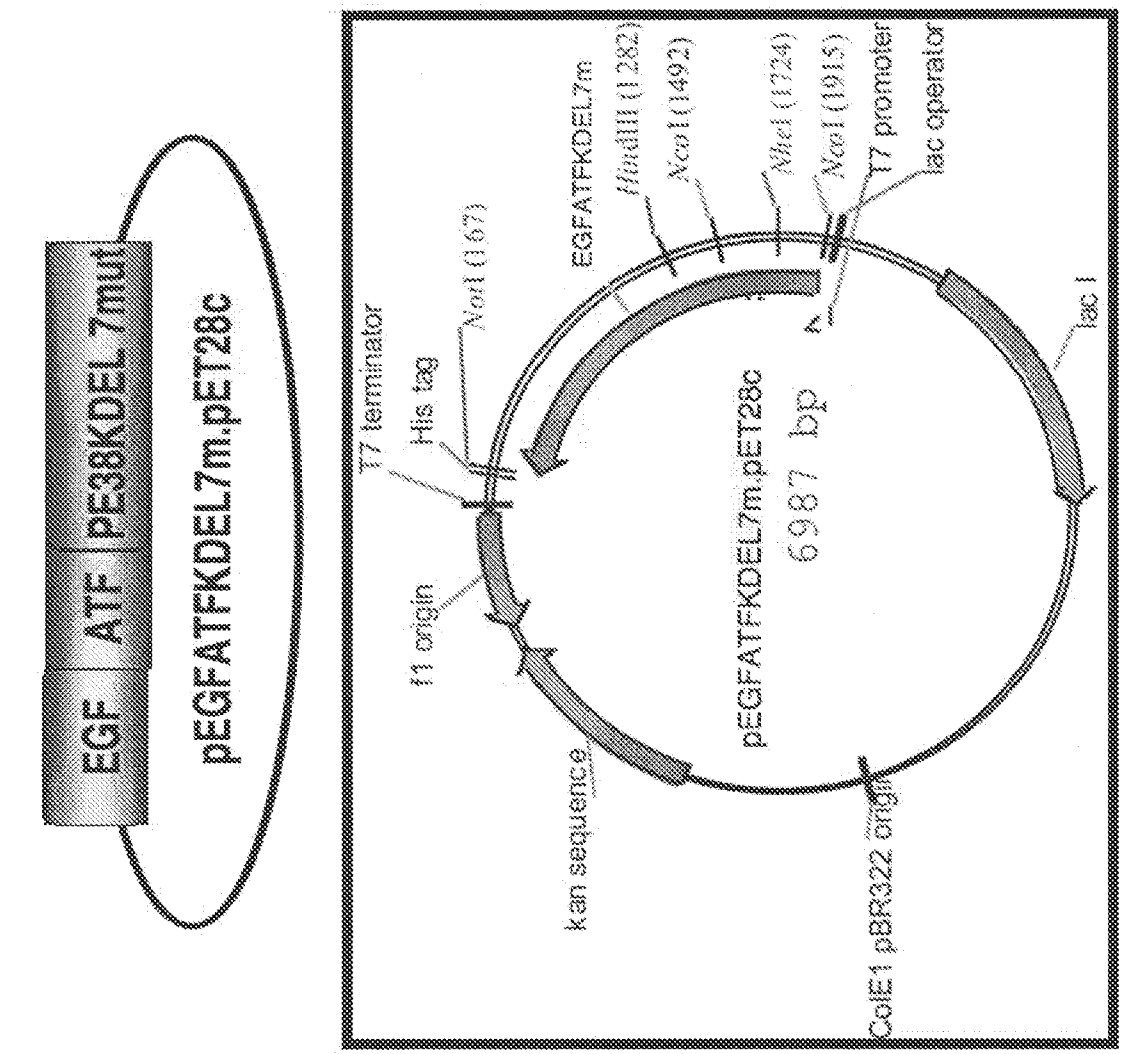

A.
EGF
ATF
PE38KDEL 7mut
pEGFATFKDEL7m.pET28c
B.
T7 terminator
His tag
NcoI (167)
EGFATFKDEL7m
HindIII (1282)
NcoI (1492)
NheI (1724)
NcoI (1915)
T7 promoter
lac operator
lac I
f1 origin
pEGFATFKDEL7m.pET28c
6987 bp
kan sequence
ColE1 pBR322 origin

Fig. 2A – 2D

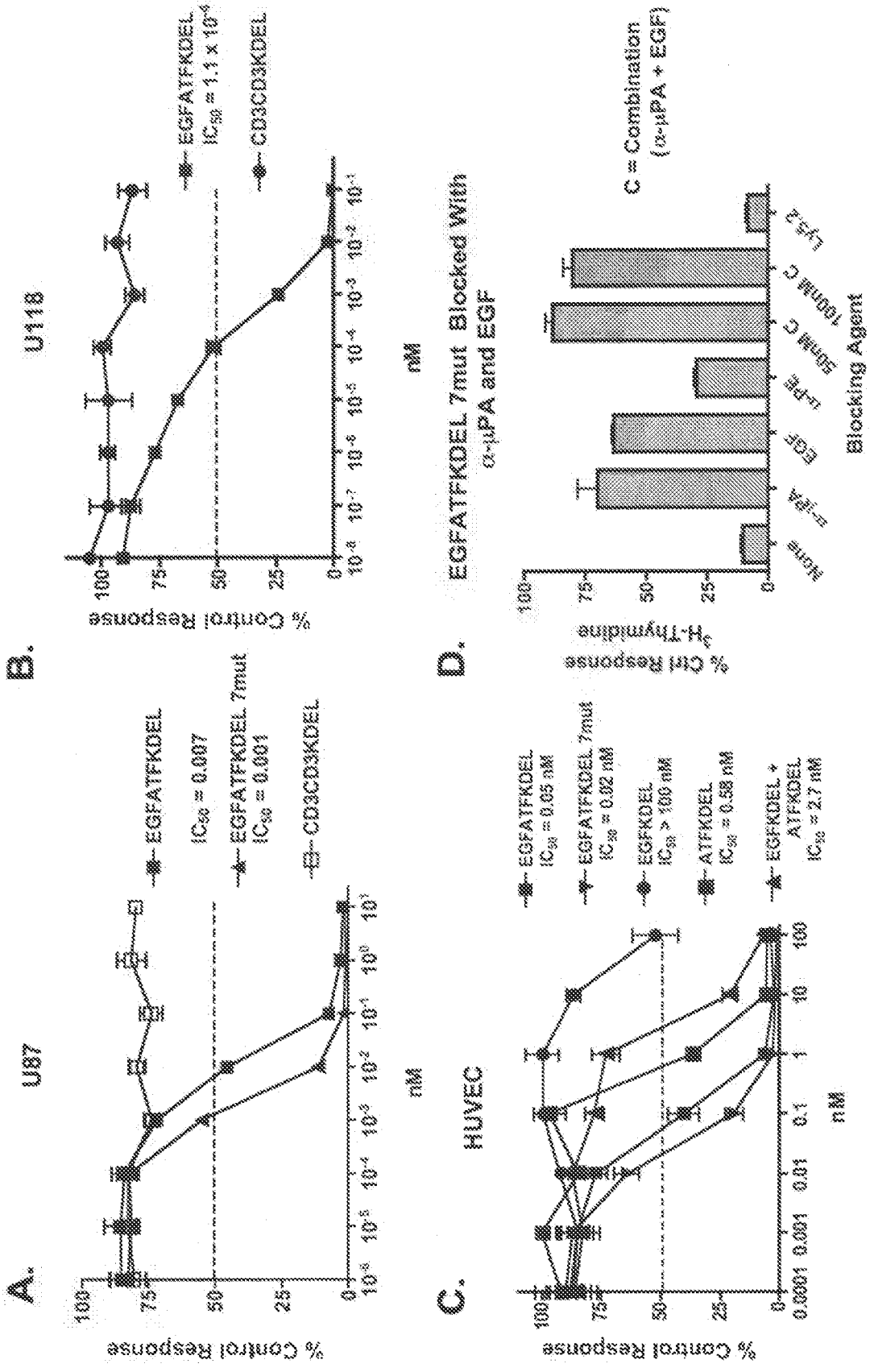
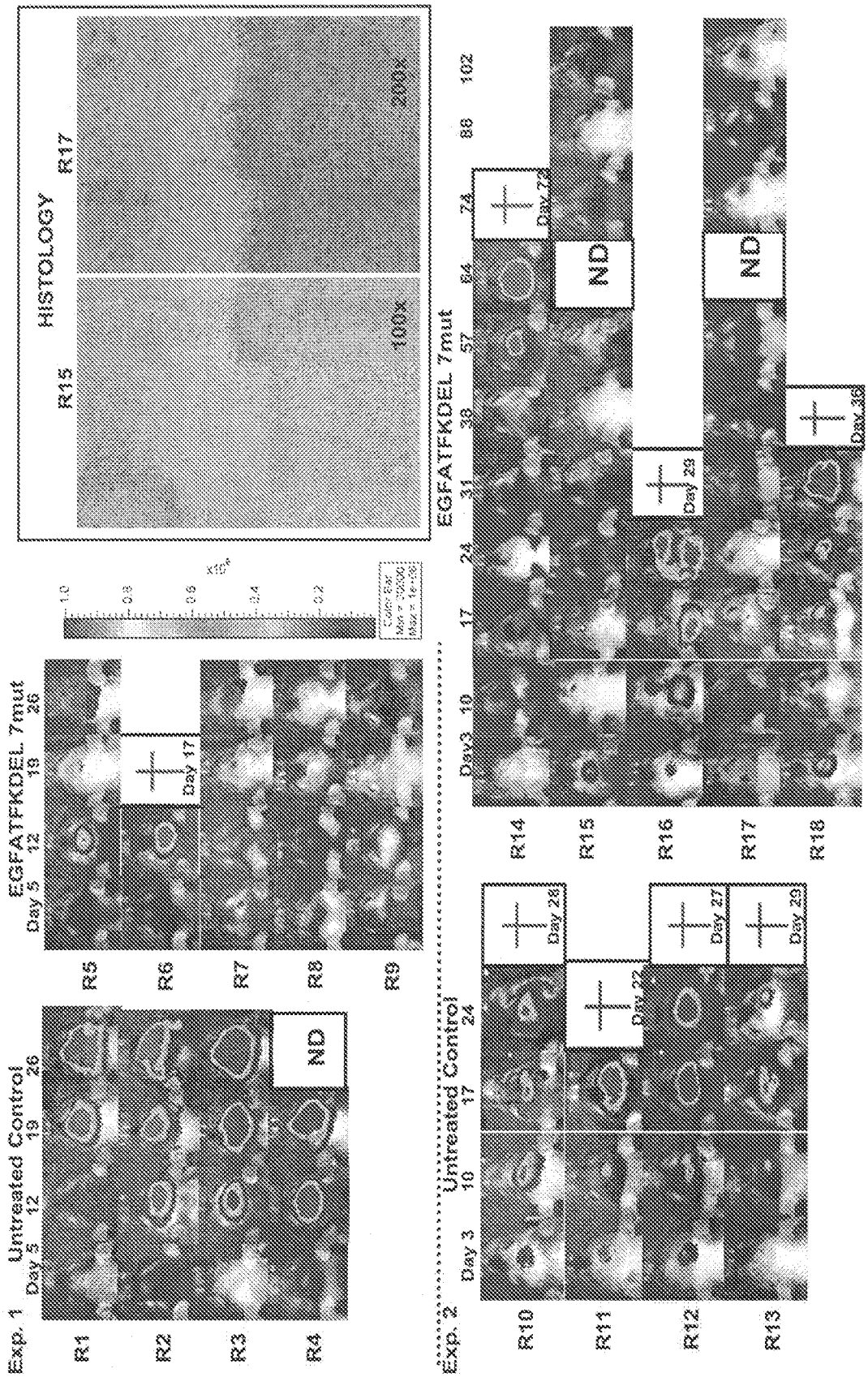

A.
U87
% Control Response
nM
EGFATFKDEL
IC50 = 0.007
EGFATFKDEL 7mut
IC50 = 0.001
CD3CD3KDEL
B.
U118
% Control Response
nM
EGFATFKDEL
IC50 = 1.1 x 10-4
CD3CD3KDEL
C.
HUVEC
% Control Response
nM
EGFATFKDEL
IC50 = 0.05 nM
EGFATFKDEL 7mut
IC50 = 0.02 nM
EGFKDEL
IC50 > 100 nM
ATFKDEL
IC50 = 0.58 nM
EGFKDEL + ATFKDEL
IC50 = 2.7 nM
D.
EGFATFKDEL 7mut Blocked With
α-μPA and EGF
% Ctrl Response
3H-Thymidine
C = Combination (α-μPA + EGF)
None
α-μPA
EGF
u-PE
50nM C
100nM C
Lys-2
Blocking Agent

Fig. 3


Exp. 1
Untreated Control
Day 5 12 19 26
EGFATFKDEL 7mut
Day 5 12 19 26
Day 17
R1
R2
R3
R4
R5
R6
R7
R8
R9
ND
HISTOLOGY
R15
R17
100x
200x
Exp. 2
Untreated Control
Day 3 10 17 24
EGFATFKDEL 7mut
Day3 10 17 24 31 38 57 64 74 88 102
Day 22
Day 27
Day 29
Day 28
Day 29
Day 36
Day 28
R10
R11
R12
R13
R14
R15
R16
R17
R18
ND
ND

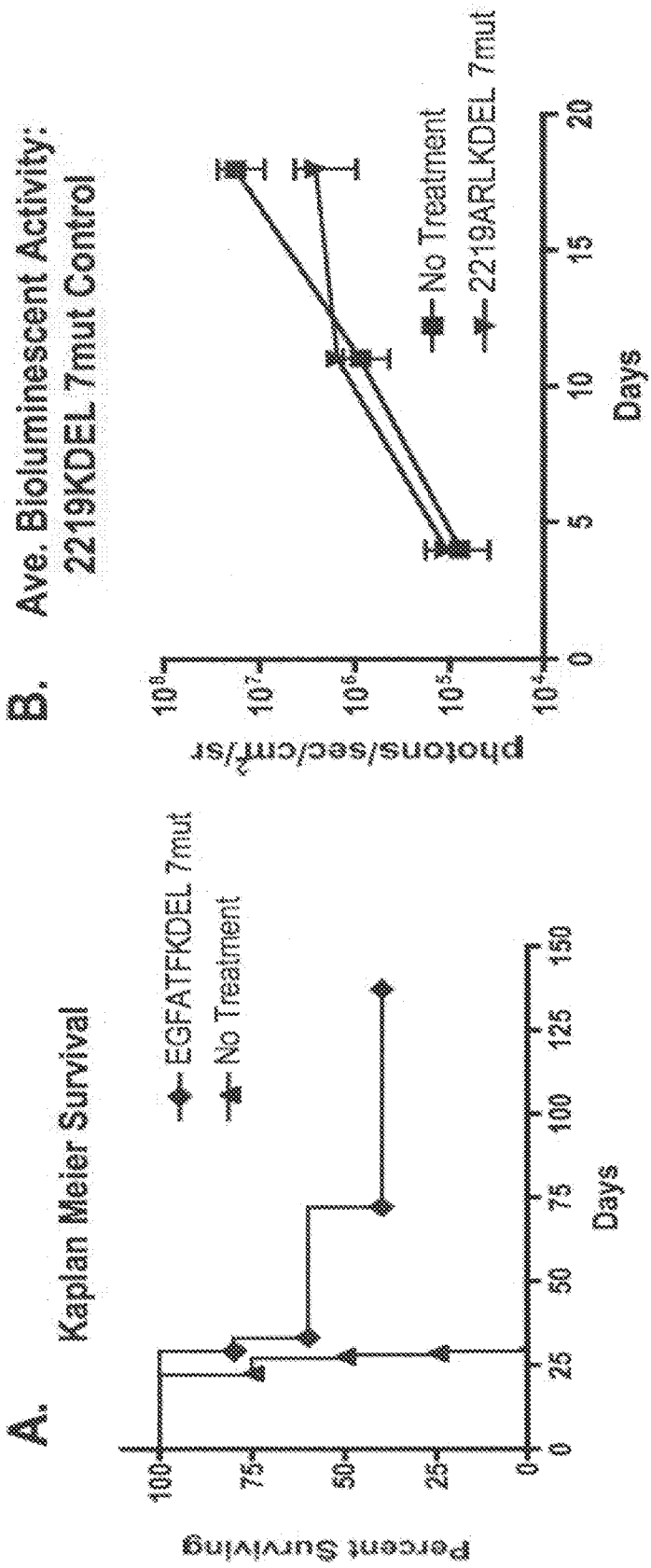

A.
Kaplan Meier Survival
EGFATFKDEL 7mut
No Treatment
Percent Surviving
100
75
50
25
0
0
25
50
75
100
125
150
Days
B.
Ave. Bioluminescent Activity:
2219KDEL 7mut Control
No Treatment
2219ARLKDEL 7mut
photons/sec/cm²/sr
10⁸
10⁷
10⁶
10⁵
10⁴
0
5
10
15
20
Days

Fig. 4A – 4B

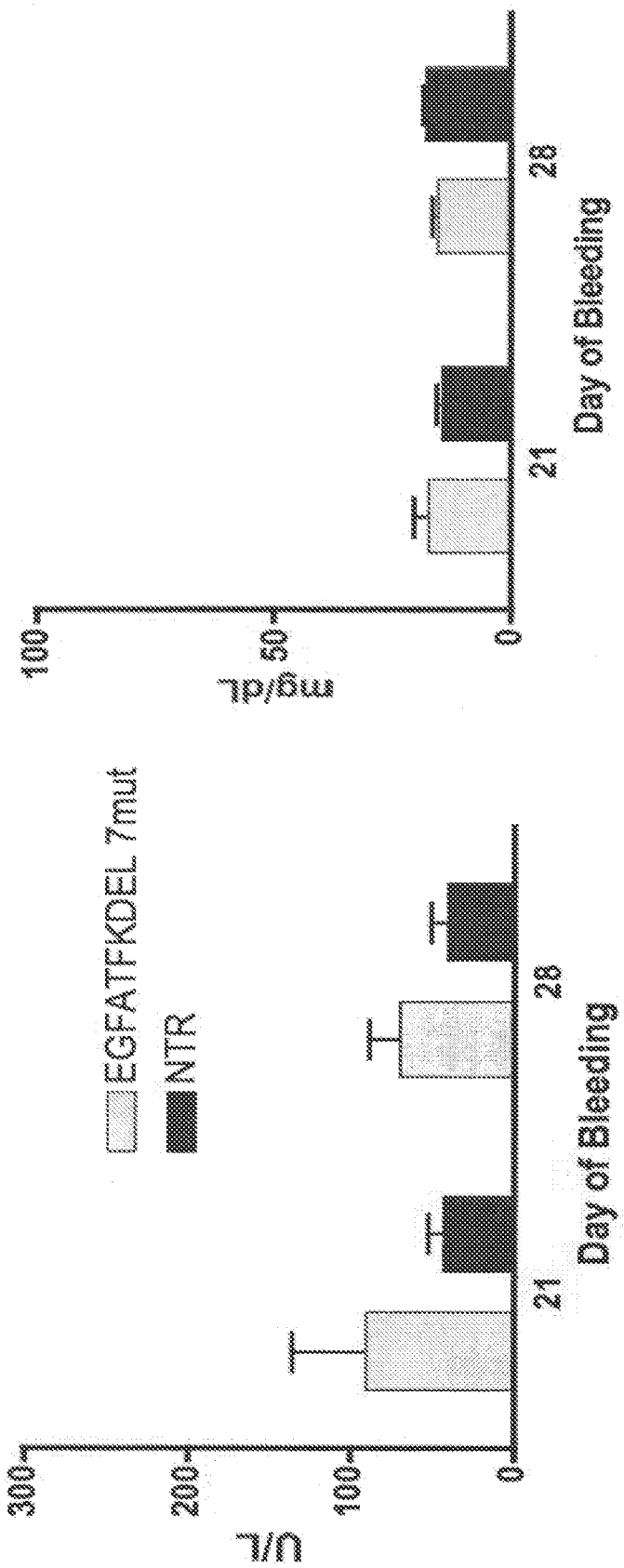

A. ALT
B. BUN
EGFATFKDEL 7mut
NTR
U/L
mg/dL
300
200
100
0
100
50
0
21
28
Day of Bleeding
21
28
Day of Bleeding

Fig. 5A – 5B

Fig. 6

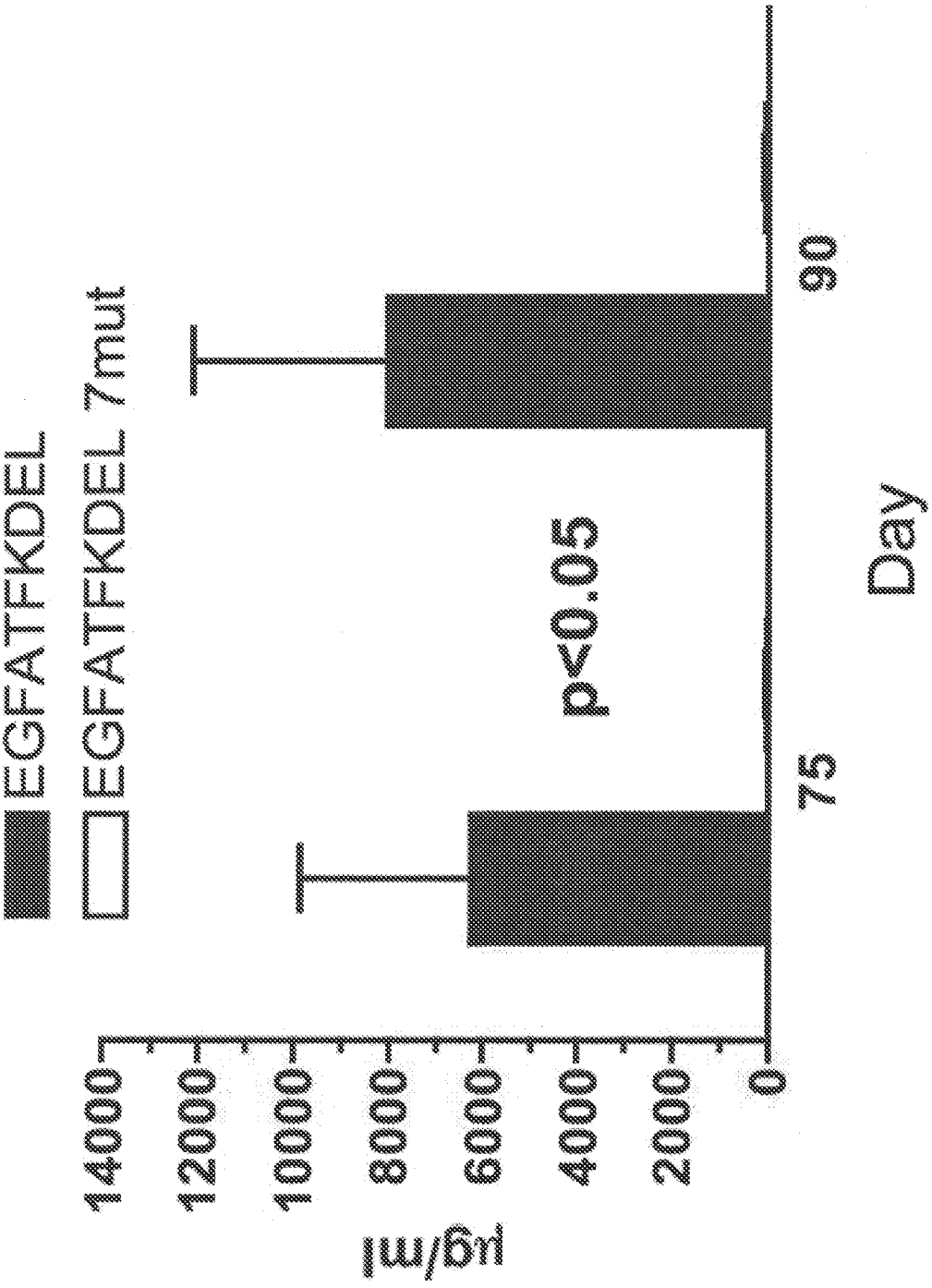
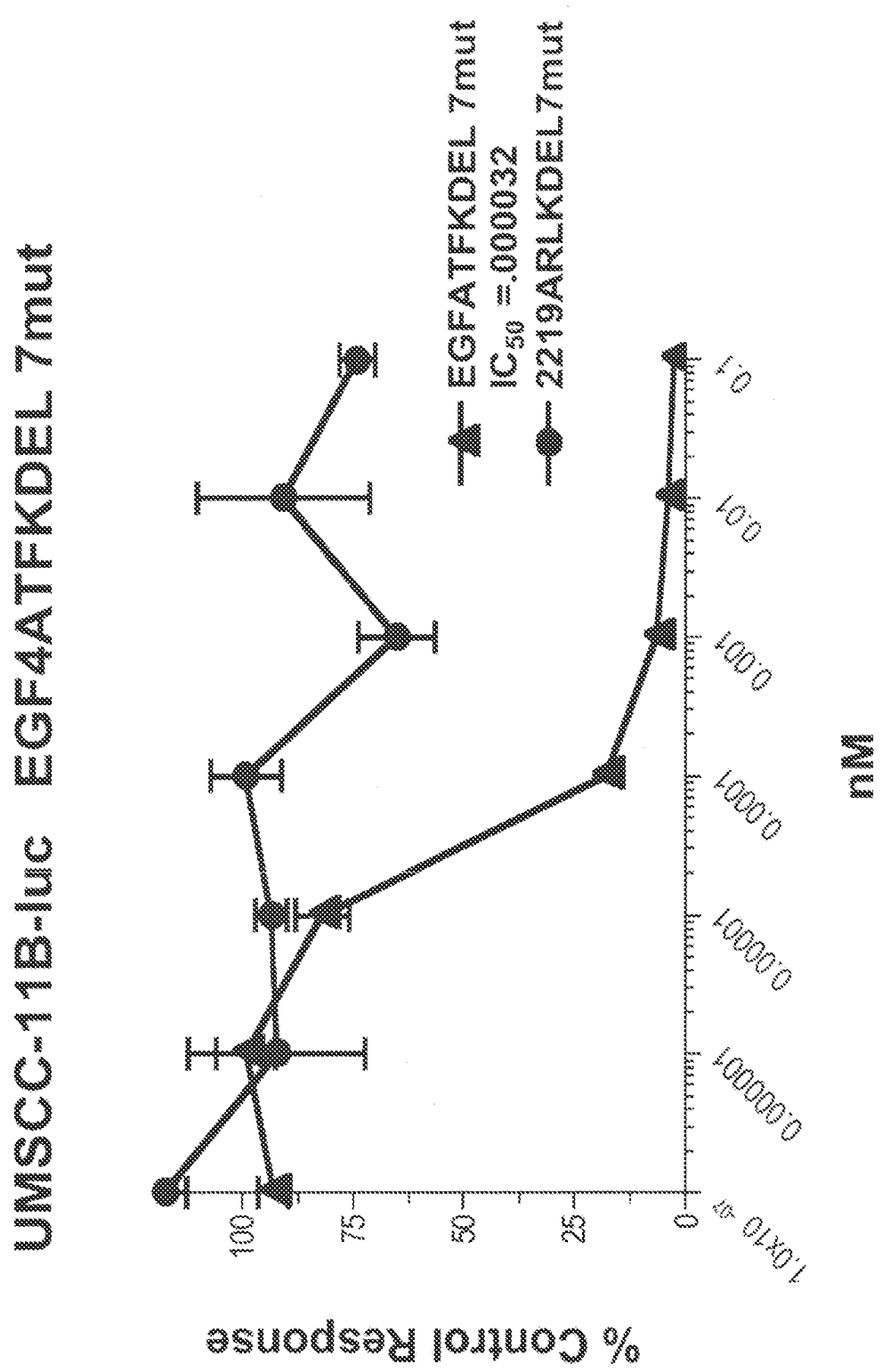

EGFATFKDEL
EGFATFKDEL 7mut
p<0.05
14000
12000
10000
8000
6000
4000
2000
0
µg/ml
75
90
Day

Fig. 7


UMSCC-11B-luc EGF4ATFKDEL 7mut
EGFATFKDEL 7mut
IC50 =.000032
2219ARLKDEL7mut
% Control Response
100
75
50
25
0
1.0X10 -8
.0001
.001
.01
.1
1000000
100000
10000
1000
100
10
1
nM

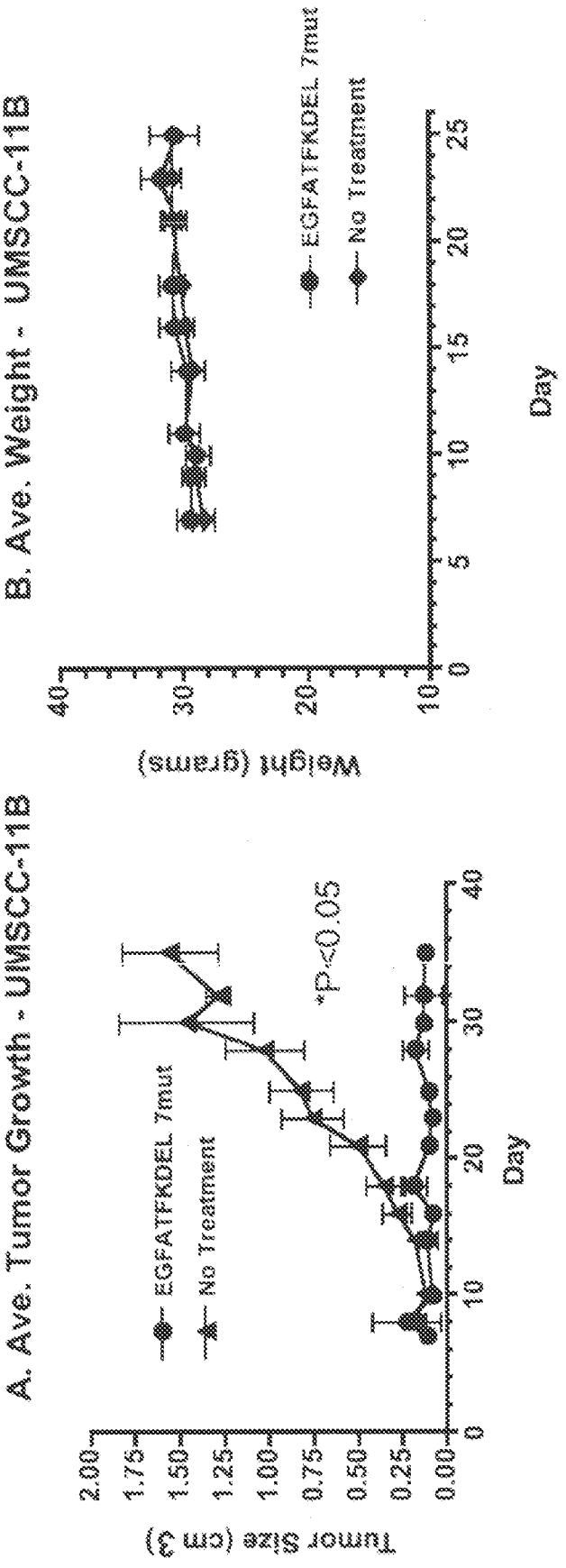
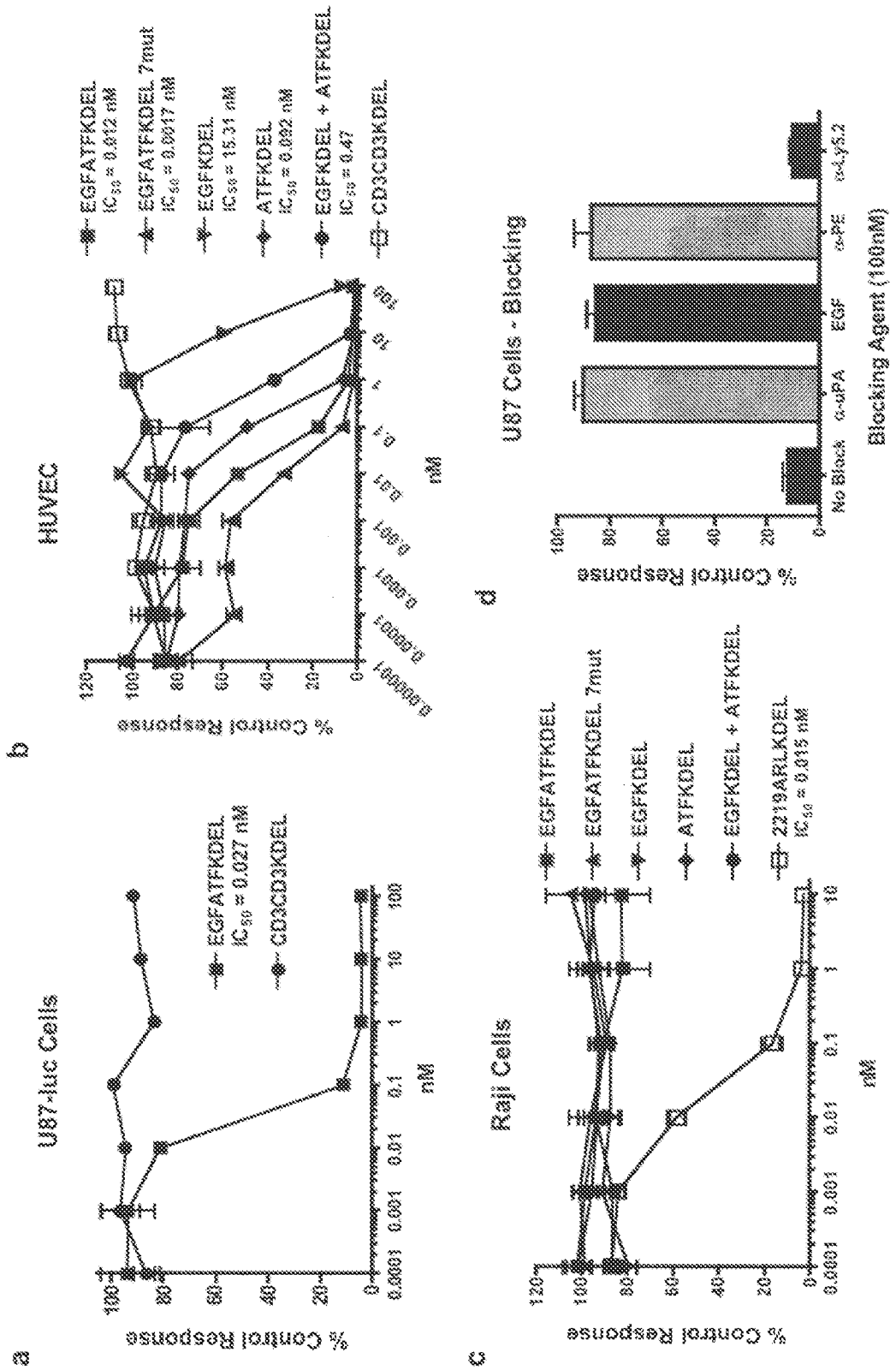

A. Ave. Tumor Growth - UMSCC-11B
Tumor Size (cm 3)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Day
EGFATFKDEL 7mut
No Treatment
*P<0.05
B. Ave. Weight - UMSCC-11B
Weight (grams)
40
30
20
10
0
5
10
15
20
25
Day
EGFATFKDEL 7mut
No Treatment

Fig. 8A – 8B

Fig. 9A – 9D


a
U87-luc Cells
EGFATFKDEL
IC50 = 0.027 nM
CD3CD3KDEL
% Control Response
nM
b
HUVEC
EGFATFKDEL
IC50 = 0.012 nM
EGFATFKDEL 7mut
IC50 = 0.0017 nM
EGFKDEL
IC50 = 15.31 nM
ATFKDEL
IC50 = 0.092 nM
EGFKDEL + ATFKDEL
IC50 = 0.47
CD3CD3KDEL
% Control Response
nM
c
Raji Cells
EGFATFKDEL
EGFATFKDEL 7mut
EGFKDEL
ATFKDEL
EGFKDEL + ATFKDEL
2219ARLKDEL
IC50 = 0.015 nM
% Control Response
nM
d
U87 Cells - Blocking
% Control Response
No Block
α-uPA
EGF
α-PE
α-LY5.2
Blocking Agent (100nM)

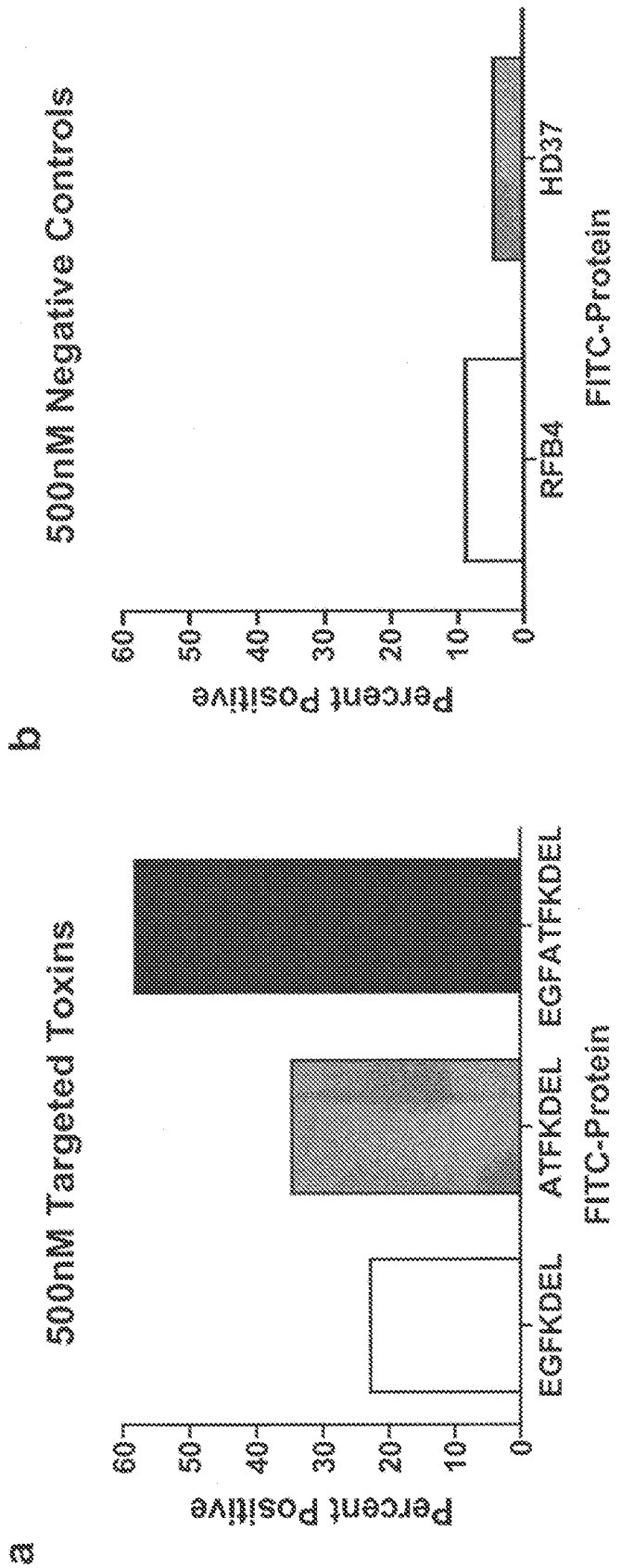

a
500nM Targeted Toxins
Percent Positive
0
10
20
30
40
50
60
EGFKDEL
ATFKDEL
EGFATFKDEL
FITC-Protein
b
500nM Negative Controls
Percent Positive
0
10
20
30
40
50
60
RFB4
HD37
FITC-Protein

Fig. 10A – 10B

Fig. 11A-11B

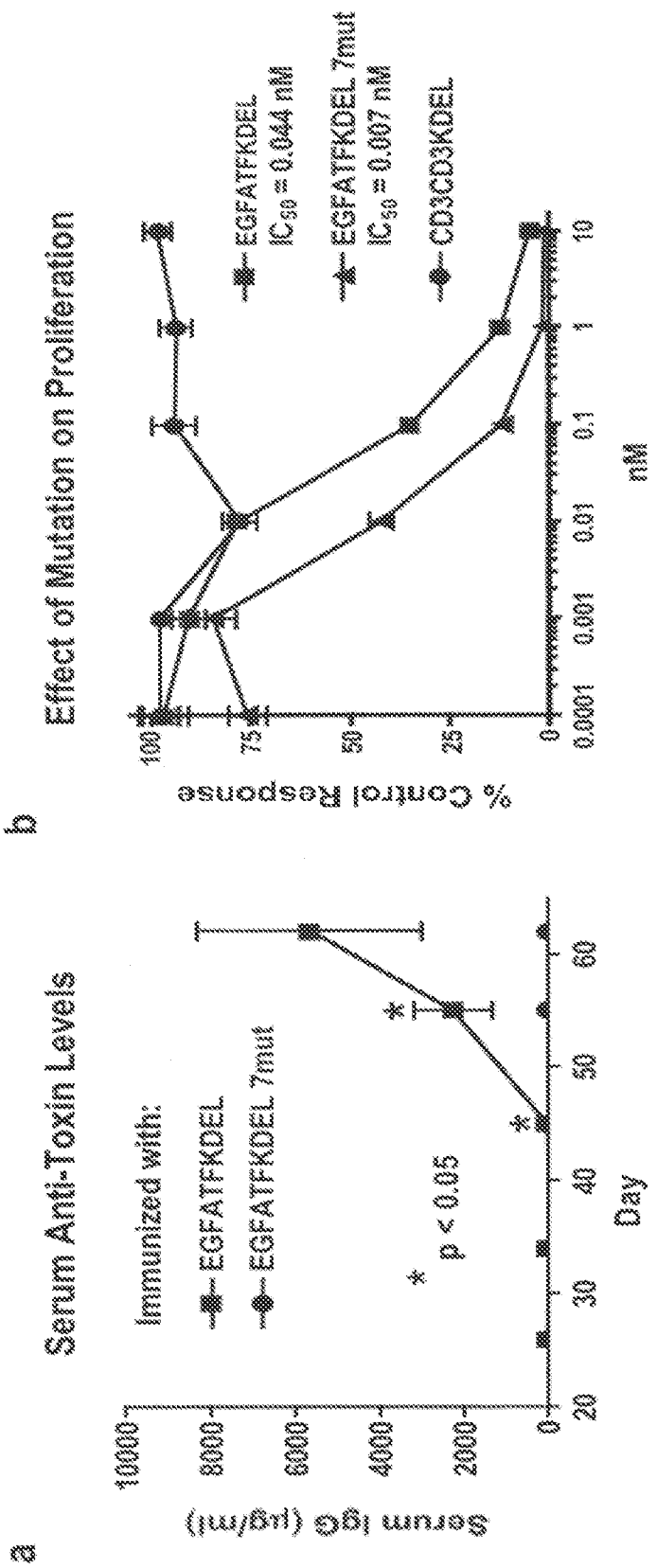
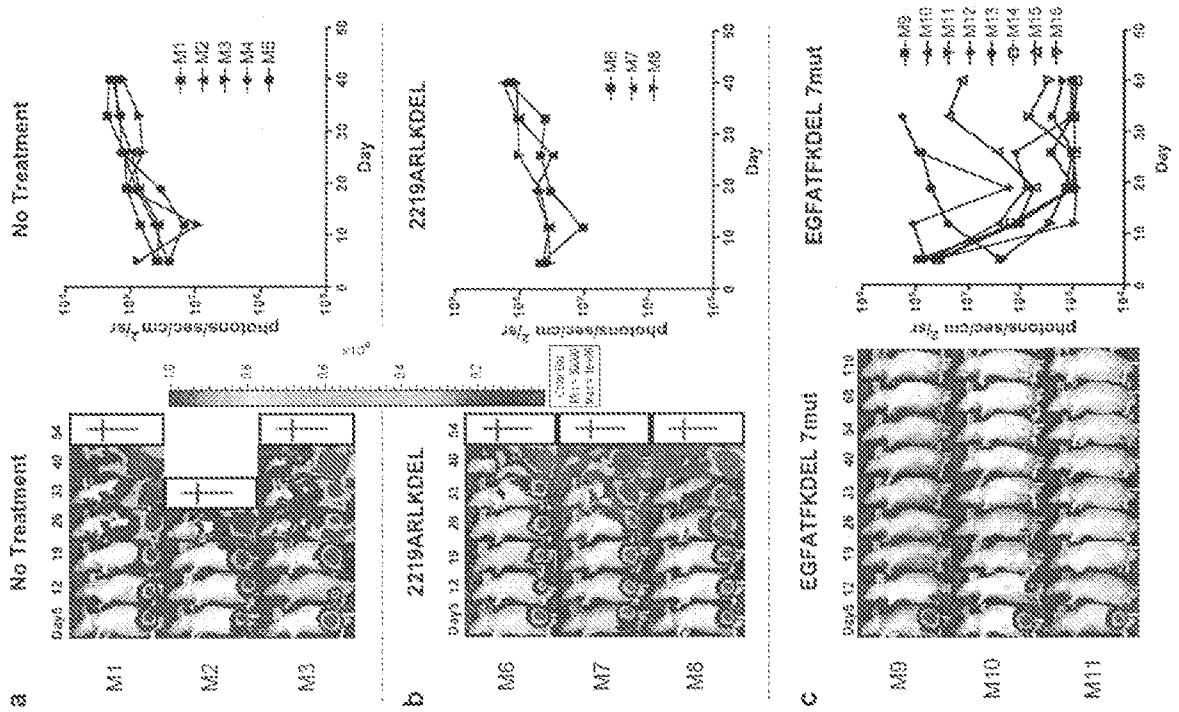

a
Serum Anti-Toxin Levels
Immunized with:
EGFATFKDEL
EGFATFKDEL 7mut
Serum IgG (µg/ml)
10000
8000
6000
4000
2000
0
20
30
40
50
60
Day
* p < 0.05
b
Effect of Mutation on Proliferation
EGFATFKDEL
IC50 = 0.044 nM
EGFATFKDEL 7mut
IC50 = 0.007 nM
CD3CD3KDEL
% Control Response
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
nM

Fig. 12A – 12C


a
No Treatment
M1
M2
M3
b
2219ARLKDEL
M6
M7
M8
c
EGFATFKDEL 7mut
M9
M10
M11
Day
Day6 12 19 26 33 40 54
Day6 12 19 26 33 40 54 68 110
photons/sec/cm²/sr
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14
M15
M16

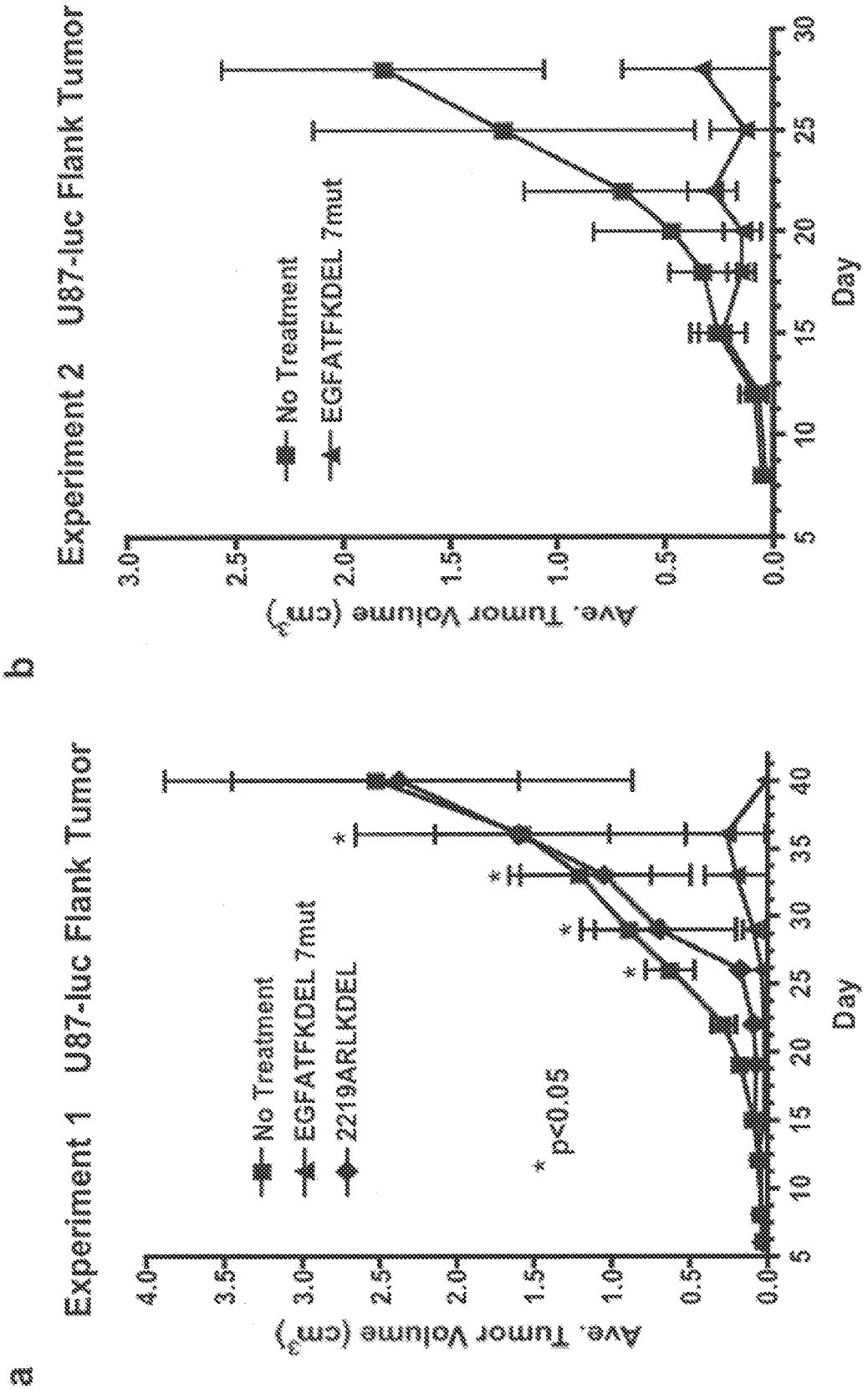

a
Experiment 1 U87-luc Flank Tumor
Ave. Tumor Volume (cm³)
Day
No Treatment
EGFATFKDEL 7mut
2219ARLKDEL
* p<0.05
b
Experiment 2 U87-luc Flank Tumor
Ave. Tumor Volume (cm³)
Day
No Treatment
EGFATFKDEL 7mut

Fig. 13A – 13B

Fig. 14

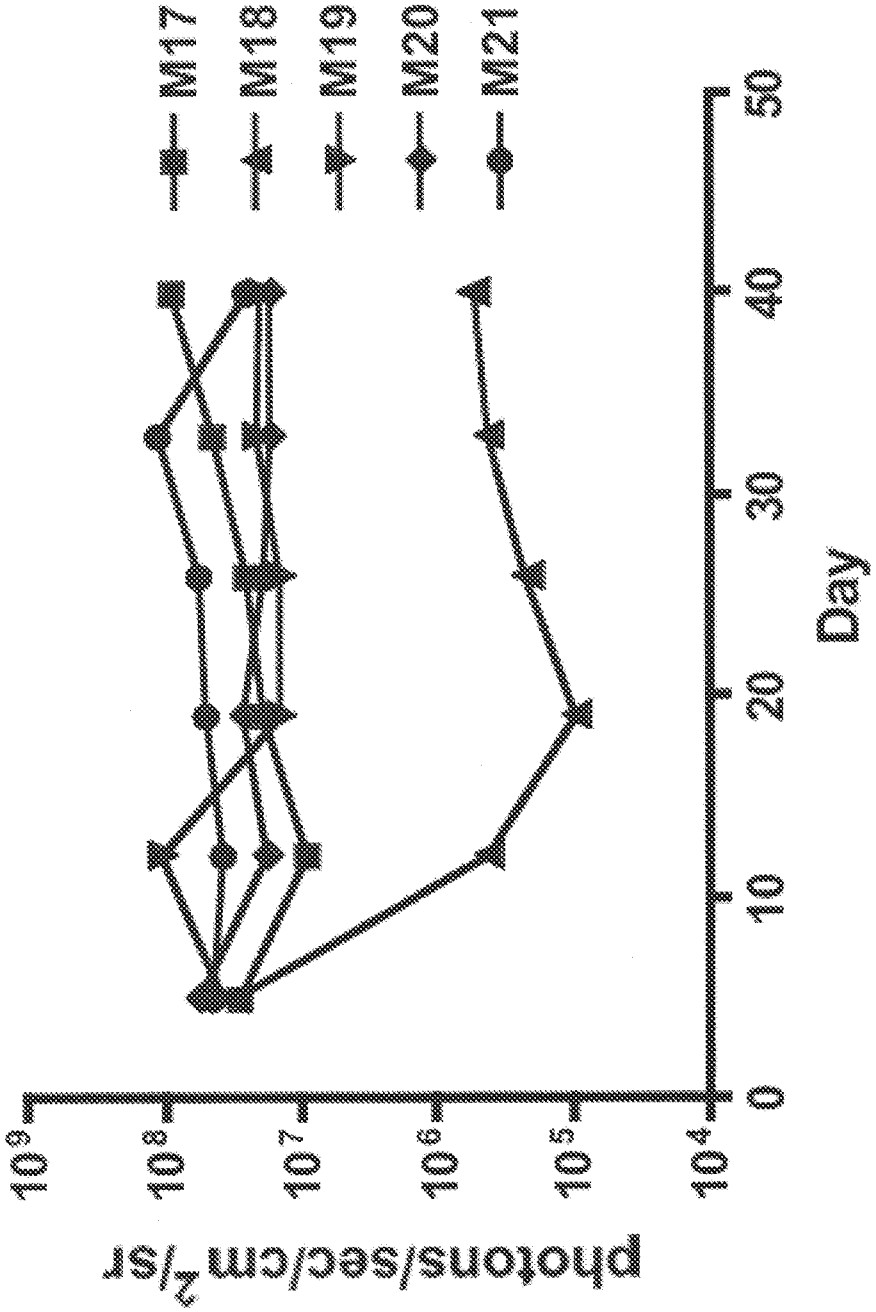

ATFKDEL
M17
M18
M19
M20
M21
photons/sec/cm²/sr
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
0
10
20
30
40
50
Day

Fig. 15

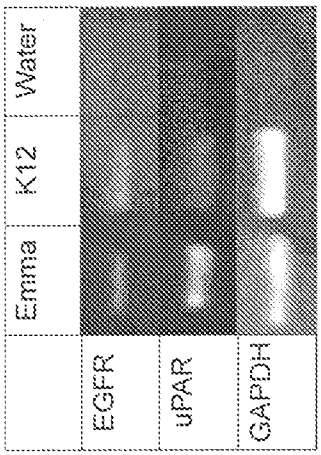


Fig. 16

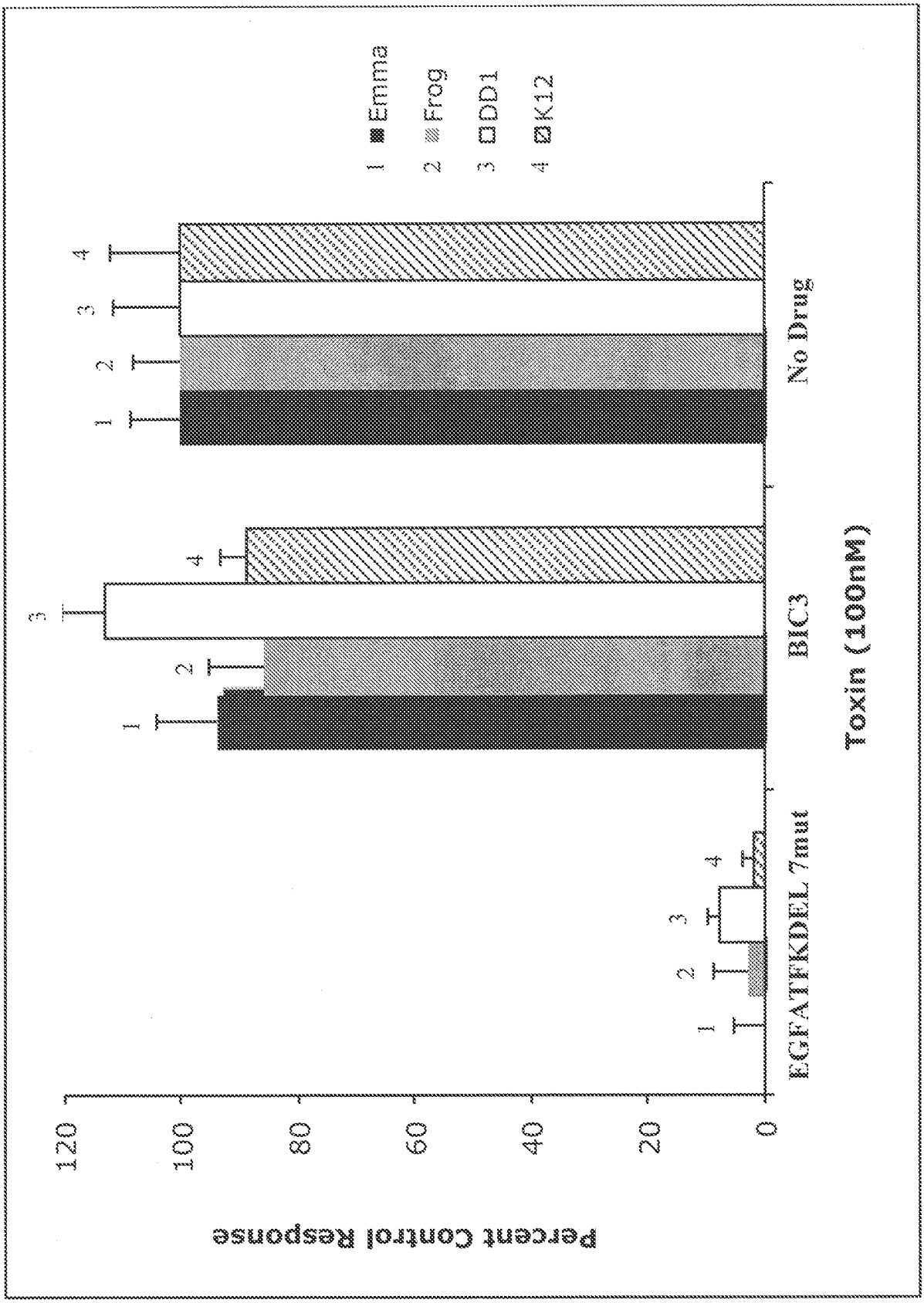

Percent Control Response
120
100
80
60
40
20
0
1 Emma
2 Frog
3 DD1
4 K12
EGFATFKDEL 7mut
BIC3
No Drug
Toxin (100nM)

Fig. 17A

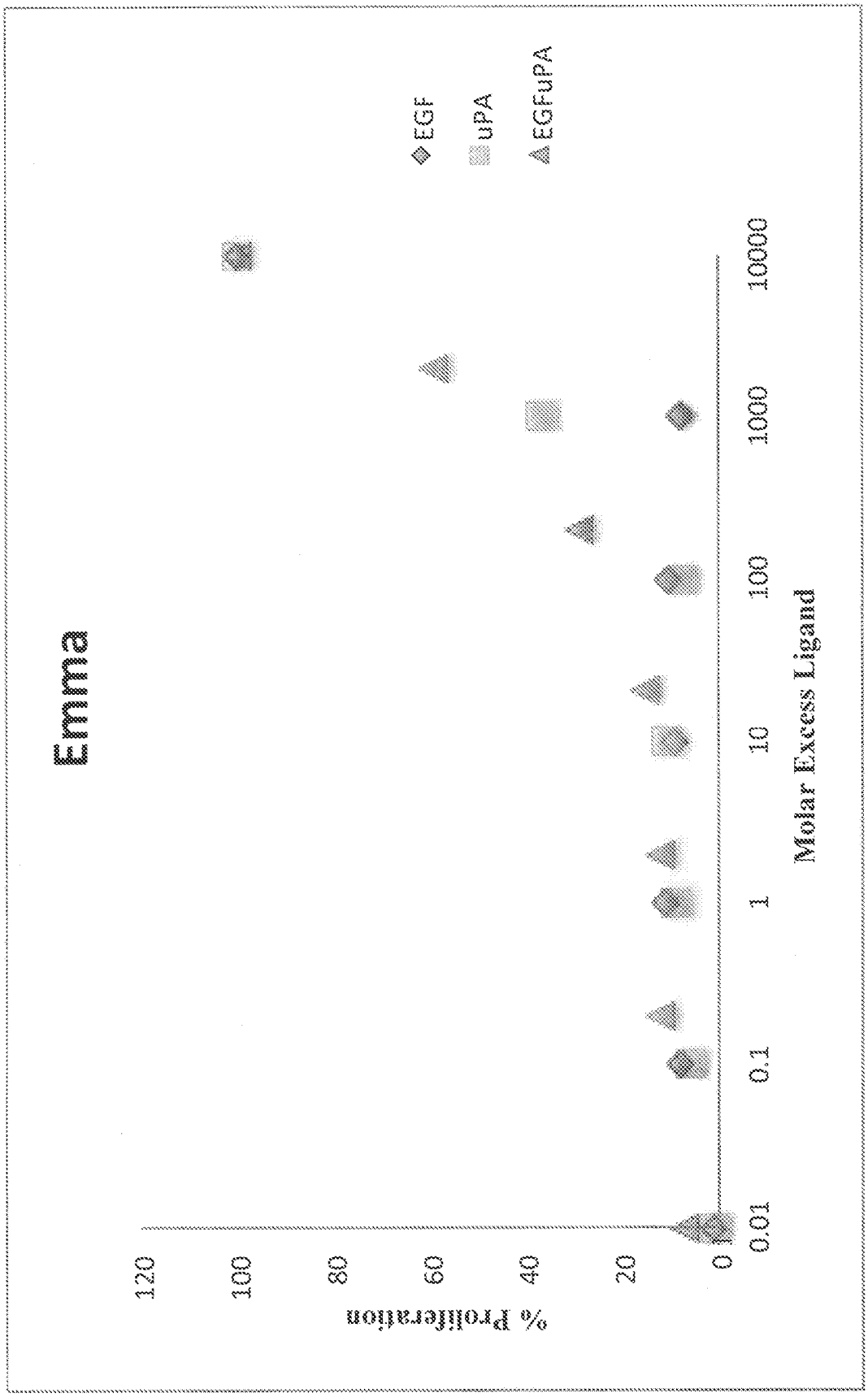
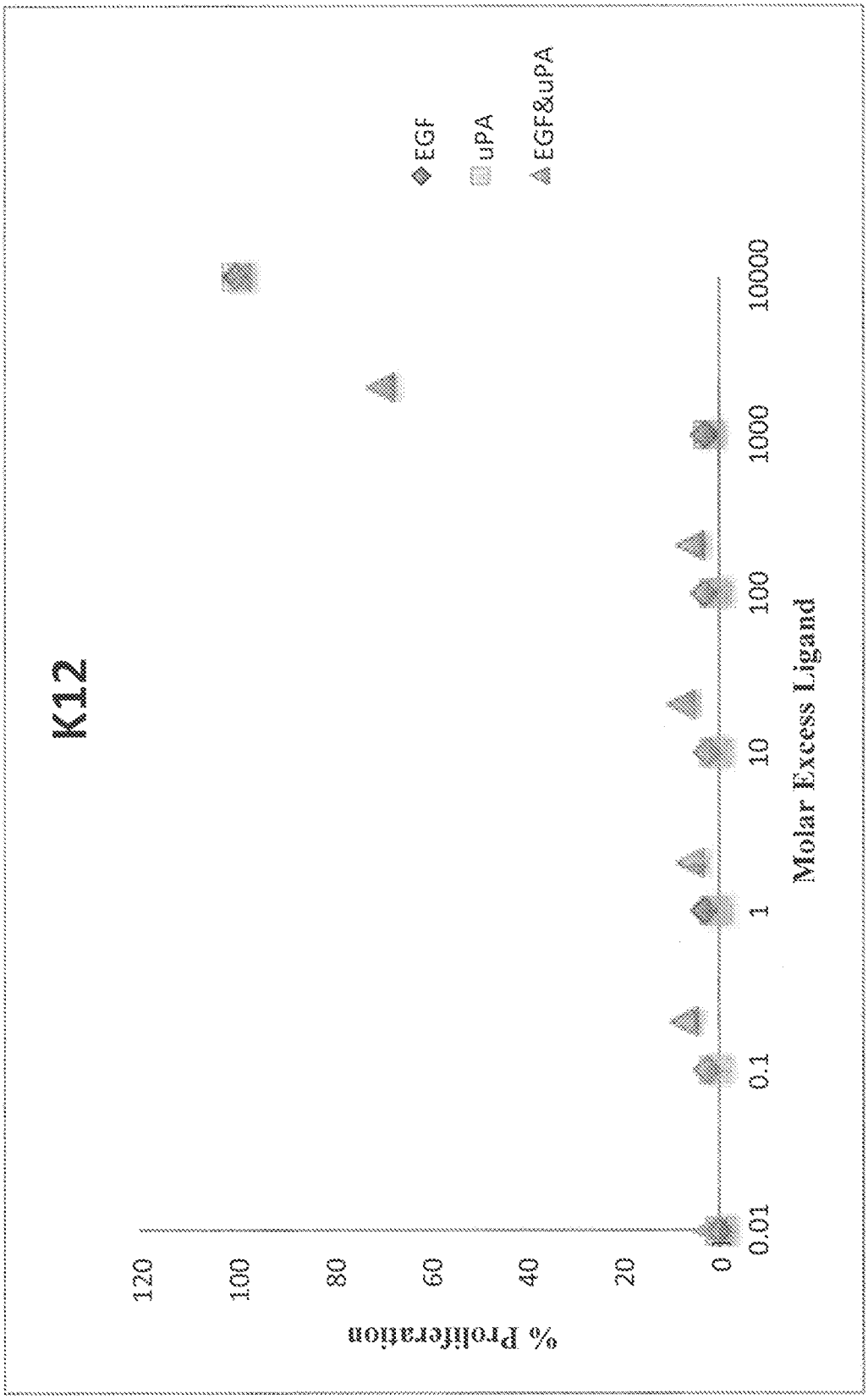

Emma
EGF
uPA
EGFuPA
% Proliferation
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Molar Excess Ligand

Fig.17B


K12
EGF
uPA
EGF&uPA
% Proliferation
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Molar Excess Ligand

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/048997

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/485 C12N9/72 C12N15/62 C12N15/58 C12N15/12
A61K47/48 C07K16/28
ADD.
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)
C07K C12N A61K

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 practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALTER A. HALL ET AL: "Efficacy of antiangiogenic targeted toxins against glioblastoma multiforme", NEUROSURGICAL FOCUS, vol. 20, no. 4, 1 April 2006 (2006-04-01), page E23, XP55012360, ISSN: 1092-0684, DOI: 10.3171/foc.2006.20.4.15 cited in the application	1,2,4-7, 17-20, 22,24, 29-34, 36,37, 40-52, 55-63, 68-70, 79-81, 83-85, 87,91-94
Y	abstract; figure 1 page 2, column 1, line 7, paragraph 1 - last line	3,8-16, 21,23, 25-28, 35,38, 39,53, 54, 64-67, 71-78,

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document 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	Date of mailing of the international search report
21 November 2011	28/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wiame, Ilse

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/048997

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
		82,86, 88-90

Y	WO 2009/029601 A2 (UNIV MINNESOTA [US]; VALLERA DANIEL A [US]) 5 March 2009 (2009-03-05)	3,8-16, 21,23, 25-28, 35,38, 39,53, 54, 64-67, 71-78, 82,86, 88-90
	page 31, line 8 - line 21 page 32, line 16 - line 30 abstract; claims page 34, paragraph 1; example 20	

A	EDWARD RUSTAMZADEH ET AL: "Immunotoxin pharmacokinetics: a comparison of the anti-glioblastoma bi-specific fusion protein (DTAT13) to DTAT and DTIL13", JOURNAL OF NEURO-ONCOLOGY, vol. 77, no. 3, 13 May 2006 (2006-05-13), pages 257-266, XP55012228, ISSN: 0167-594X, DOI: 10.1007/s11060-005-9051-7 cited in the application abstract; figure 1 page 257, column 2, last paragraph - page 258, column 1, line 6 page 265, column 1, line 3 - line 14	1-94

X,P	ALEXANDER K TSAI ET AL: "A novel bispecific ligand-directed toxin designed to simultaneously target EGFR on human glioblastoma cells and uPAR on tumor neovasculature", JOURNAL OF NEURO-ONCOLOGY, KLUWER ACADEMIC PUBLISHERS, BO, vol. 103, no. 2, 10 September 2010 (2010-09-10), pages 255-266, XP019905334, ISSN: 1573-7373, DOI: 10.1007/S11060-010-0392-5 the whole document	1-94

	-/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/048997

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	OH SEUNGUK ET AL: "Evaluation of a bispecific biological drug designed to simultaneously target glioblastoma and its neovasculature in the brain", JOURNAL OF NEUROSURGERY, AMERICAN ASSOCIATION OF NEUROLOGICAL SURGEONS, US, vol. 114, no. 6, 1 June 2011 (2011-06-01), pages 1662-1671, XP008145396, ISSN: 0022-3085, DOI: 10.3171/2010.11.JNS101214 the whole document -----	1-94

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/048997

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/048997

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009029601 A2	05-03-2009	CA 2697529 A1	05-03-2009
		EP 2197909 A2	23-06-2010
		JP 2010536386 A	02-12-2010
		US 2011091460 A1	21-04-2011
		WO 2009029601 A2	05-03-2009