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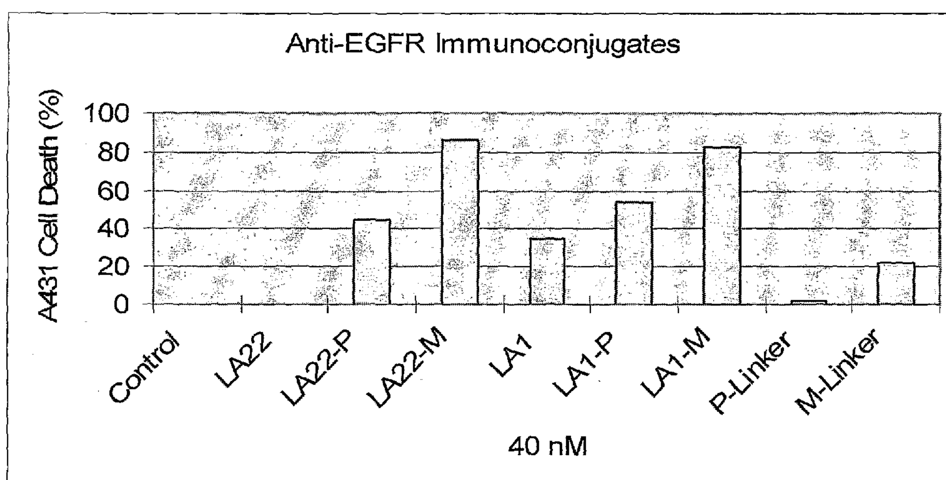
(54) **Title:** ANTIBODIES FOR THE TREATMENT OF CANCERS



(57) **Abstract:** The present invention features monoclonal antibodies LA1 or LA22 conjugated with mitomycin C, pingyangmycin or other anti-cellular agents. The present invention also features other anti-EGFR antibodies conjugated with mitomycin C or pingyangmycin. The antibodies of the present invention can be used to treat cancers, including but not limited to, those of epithelial origin, such as glioblastoma or cancer of the lung, breast, head and neck, and bladder.

WO 2006/116001 A2

ANTIBODIES FOR THE TREATMENT OF CANCERS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority from and incorporates by reference the entire disclosure of U.S. Provisional Patent Application Serial No. 60/675,094, filed April 27, 2005.

TECHNICAL FIELD

[0002] The present invention relates to anti-EGFR immunoconjugates and methods of using the same for treating cancers.

BACKGROUND

[0003] Epidermal growth factor receptor (EGFR) is a transmembrane protein involved in signaling pathways essential for cell proliferation. Over-expression of this receptor often accompanies development and growth of malignant tumors. There is increasing evidence that high expression of EGFR is correlated with aggressive tumor growth, as well as with poor clinical outcome of common cancers in humans, including breast, cervix, lung, and head and neck carcinomas (Baselga, J. *et al.*, *J. Clin. Oncol.*, 18:904-914, 2000; Nicholson, R. I. *et al.*, *Eur. J. Cancer*, 37:S9-S15, 2001). The critical role the EGFR plays in cancer has led to an extensive search for selective inhibitors of the EGFR signaling pathway. Development of monoclonal antibodies to prevent ligand binding to the EGFR is one promising strategy, which was first proposed by J. Mendelsohn in the 1980s (Ciardello, F. *et al.*, Clin. Cancer *Res.*, 7:2958-2970, 2001. Erbitux® was the first approved anti-EGFR monoclonal antibody drug with anticancer activity in the market. Besides Erbitux®, at least three other blocking monoclonal antibodies have been developed against the EGFR (Speake, G. *et al.*, *Curr. Opin. Pharmacol.*, 5:343-349, 2005).

[0004] Activation of the EGFR autocrine growth pathway in cancer cells can be attributable to several mechanisms, namely, EGFR overexpression, increased concentration of ligand(s), decreased phosphatase activity, decreased receptor turnover, and the presence of aberrant receptors. Administration of blocking anti-EGFR antibodies only is not so therapeutic to those cancers independent to ligand binding. Currently-used anticancer drugs are unable to recognize tumor cells from normal cells. The amount of an anticancer drug required to achieve a clinically effective level of cell kill often causes severe damage to

actively propagating nonmalignant cells such as cells of the gastrointestinal tract and bone marrow, resulting in a variety of undesirable side effects. This property dictates a careful balance in treatment schedules between killing the tumor cells and excessive toxicity to the patient, which creates the major obstacle in cancer chemotherapy. Such considerations often impose administration of doses that are limited in efficiency (Aboud-Pirak, E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:3778-3781, 1989). Targeted biological therapies that selectively interfere with cancer cells may improve the therapeutic efficacy without increasing treatment-related toxicity. EGFR-mediated specific tumor delivery of anticancer agents by anti-EGFR antibody-drug conjugate is an alternative strategy whose therapeutic effects are independent to ligand binding to EGFR (Aboud-Pirak, E. *et al. supra*; Mamot, C. *et al.*, *Cancer Res.* 63:3154-3161, 2003). Theoretically, conjugation of a drug to an antibody renders the drug inactive. Once at the tumor site, the conjugate binds to the surface of tumor cells and is further internalized to release the drug to kill tumor cells. Such antibody-drug conjugates are also considered as tumor-activated pro-drugs (Lambert, J. M., *Curr. Opin. Phamacol.*, 5:543-549, 2005). Based on the above, it is, therefore, very important to develop new chemotherapeutic agents with improved tumor specificity and favorable anti-cancer activities.

SUMMARY OF THE INVENTION

[0005] The present invention features additional anti-EGFR immunoconjugates and methods of using the same for suppressing the growth of tumor cells and for treating cancers of epithelial origin. In one aspect, the present invention features EGFR-binding molecules. Each molecule comprises an antigen-binding fragment of an anti-EGFR monoclonal antibody and a cytotoxic agent. In one embodiment, an EGFR-binding molecule of the present invention is a monoclonal antibody LA1 or LA22 conjugated with a cytotoxic agent.

[0006] Any type of cytotoxic agent can be conjugated with the EGFR-binding molecules. Non-limiting examples of cytotoxic agents include cytotoxic antibiotics, chemotherapeutic agents, radioisotopes, cytotoxins, or anti-cancer pro-drug activating enzymes. In one example, the cytotoxic agent being conjugated is mitomycin C or pingyangmycin.

[0007] The present invention also features humanized or engineered antibodies comprising an antigen-binding fragment of LA1 or LA22 conjugated with mitomycin C, pingyangmycin, or other anti-cellular agents.

[0008] In addition, the present invention features other anti-EGFR antibodies conjugated with mitomycin C or pingyangmycin. These antibodies can be monoclonal antibodies, human antibodies, humanized or engineered antibodies, synthetic antibodies, or single chain antibodies.

[0009] The EGFR-binding molecules or antibodies of the present invention can be used to kill or inhibit the growth of cancer cells. These methods comprise contacting cancer cells with an EGFR-binding molecule or antibody of the present invention.

[0010] In addition, the EGFR-binding molecules or antibodies of the present invention can be used to treat cancers. These methods comprise administrating an effective amount of an EGFR-binding molecule or antibody of the present invention to a subject in need thereof. Cancers or cancer cells amenable to the present invention include those of epithelial origin.

[0011] Furthermore, the present invention also features pharmaceutical compositions comprising an EGFR-binding molecule or antibody of the present invention.

[0012] Other features, objects, and advantages of the present invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments of the present invention, is given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The drawings are provided for illustration, not limitation.

[0014] FIGURE 1 demonstrates the cytotoxic effects of anti-EGFR monoclonal antibodies conjugated with pingyangmycin or mitomycin C on human epidermoid carcinoma cells.

[0015] FIGURE 2 illustrates the dose-dependent cytotoxic effect of monoclonal antibody LA22 conjugated with pingyangmycin or mitomycin C on human epidermoid carcinoma cells.

[0016] FIGURE 3A and 3B show the *in vitro* cytotoxic effect of LA22-MMC immunoconjugate, MMC and naked mAb LA22 on A431 and A549 cells.

[0017] FIGURE 4 shows the dose-dependent cytotoxic effect of monoclonal antibody LA1 conjugated with pingyangmycin or mitomycin C on human epidermoid carcinoma cells.

[0018] FIGURE 5 demonstrates the dose-dependent cytotoxic effect of monoclonal antibody LA1 conjugated with pingyangmycin or mitomycin C on human lung cells.

[0019] FIGURE 6 illustrates the in vivo tumor toxicity effect of LA22-MMC on female Balb/c mice. FIGURE 6A shows the treatment scheme of LA22-MMC immunoconjugate on A431 xenografts and the inhibitory effect of tumor growth *in vivo*. FIGURE 6B summarizes the tumor size and percent of tumor inhibition of each of the treatment groups (control, MMC, and LA22-MMC at 0.0032 mg/kg and 0.08 mg/kg).

[0020] FIGURE 7 shows the biodistribution of LA22-MMC immunoconjugate on tumor-bearing mice after ^{125}I-radiolabeling and immunoscintigraphy.

[0021] FIGURE 8 compares the *in vitro* cytotoxic effect of LA22-MMC immunoconjugate on human epidermoid carcinoma cells with Erbitux®-MMC and Herceptin®-MMC.

DETAILED DESCRIPTION

[0022] The present invention features anti-EGFR monoclonal antibodies LA1 or LA22 conjugated with cytotoxic or anticellular agents. Both LA1 and LA22 are commercially available from Upstate USA, Inc., Charlottesville, VA. Monoclonal antibody LA22 is also described in U.S. Patent No. 5,459,061, which is incorporated herein by reference in its entirety.

[0023] Non-limiting examples of suitable cytotoxic or anti-cellular agents include chemotherapeutic agents, radioisotopes, and cytotoxins. Specific examples of cytotoxic or anti-cellular agents include, but are not limited to, cytotoxic antibiotics (such as mitomycin C or pingyangmycin), hormones (such as steroids), anti-metabolites (such as cytosine arabinoside, fluorouracil, methotrexate or aminopterin), anthracycline, vinca alkaloids, demecolcine, etoposide, mithramycin, alkylating agents (such as chlorambucil or melphalan), doxorubicin, daunomycin, methotrexate, vinblastine, neocarzinostatin, macromycin, trenimon, α-amanitin, ricin, ricin A-chain, daunorubicin, taxol, ethiduim bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphteria toxin, Pseudomonas exotoxin (PE) A, PE40, abrin, arbrin A chain, modeccin A chain, alpha-sarcin, gelonin, mitogellin, retstrictocin, phenomycin, enomycin, α-sarcin, aspergillin, restirictocin, a ribonuclease, diphtheria toxin, pseudomonas exotoxin, curicin, crotin, calicheamicin, sapaonaria officinalis inhibitor, maytansinoids, glucocorticoid, and radioisotopes.

[0024] LA1 or LA22 monoclonal antibodies can also be conjugated to an anti-cancer pro-drug activating enzyme capable of converting a pro-drug to its active form. See, for example, U.S. Patent No. 4,975,287, which is incorporated herein by reference in its entirety.

[0025] Linking or coupling one or more cytotoxic moieties to an antibody can be achieved by a variety of mechanisms, for example, covalent binding, affinity binding, intercalation, coordinate binding and complexation. Preferred binding methods are those involving covalent binding, such as using chemical cross-linkers, natural peptides or disulfide bonds.

[0026] The covalent binding can be achieved either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent agents are useful in coupling protein molecules to other proteins, peptides or amine functions. Examples of coupling agents are carbodiimides, diisocyanates, glutaraldehyde, diazobenzenes, and hexamethylene diamines. This list is not intended to be exhaustive of the various coupling agents known in the art but, rather, is exemplary of the more common coupling agents that may be used.

[0027] In many embodiments, an antibody is first derivatized, following by attachment of the cytotoxic component to the derivatized product. As used herein, the term "derivatize" refers to the chemical modification of the antibody substrate with a suitable cross-linking agent. Examples of cross-linking agents for use in this manner include the disulfide-bond containing linkers SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate) and SMPT (4-succinimidyl-oxycarbonyl-α-methyl-α(2-pyridyldithio)toluene).

[0028] Biologically-releasable bonds can also be employed in constructing a clinically-active immunoconjugate, such that the cytotoxic moiety is capable of being released from the antibody once it has entered the target cell. Numerous types of linking constructs are known, including simply direct disulfide bond formation between sulfhydryl groups contained on amino acids such as cysteine, or otherwise introduced into respective protein structures, and disulfide linkages using available or designed linker moieties.

[0029] Numerous types of disulfide-bond containing linkers are known, which can successfully be employed to conjugate cytotoxic moieties to antibodies. Certain linkers are preferred, such as, for example, sterically hindered disulfide bond linkers, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at the site of action. Another preferred cross-linking reagent is SMPT, although other linkers such as SATA, SPDP and 2-iminothiolane may also be employed.

[0030] Once conjugated, the conjugate can be purified to remove contaminants such as unconjugated cytotoxic agents or antibodies. In many cases, it is important to remove unconjugated cytotoxic agents because of the possibility of increased toxicity. Moreover, unconjugated antibodies may be removed to avoid the possibility of competition for the antigen between conjugated and unconjugated species. Numerous purification techniques can be used to provide conjugates to a sufficient degree of purity to render them clinically useful.

[0031] The present invention also features anti-EGFR molecules comprising the antigen-binding fragments of LA22 or LA1 monoclonal antibodies conjugated with cytotoxic or anticellular agents. These antigen-binding fragments can include or consist of the scFv, diabody, minibody, Fab, $F(ab')_2$, or Fv fragments of LA22 or LA11 monoclonal antibodies.

[0032] In one embodiment, the anti-EGFR molecules of the present invention are humanized or engineered monoclonal antibodies prepared from monoclonal antibodies LA22 or LA1. Humanized or engineered antibodies are particularly desirable for therapeutic treatment of human subjects. Humanized or engineered forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, or antigen-binding fragments thereof (such as scFv, diabody, minibody, Fab, or $F(ab')_2$), which contain minimal sequence derived from non-human immunoglobulin. Humanized or engineered antibodies are derived from human immunoglobulins in which the residues forming the complementary determining regions (CDRs) are replaced by the residues from CDRs of a non-human antibody, such as LA22 or LA1. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized or engineered antibodies may also comprise residues, which are found neither in the recipient antibody nor in the imported CDR or framework sequences. The humanized or engineered antibody can comprise at least one or two variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the constant regions are those of a human immunoglobulin consensus sequence.

[0033] In addition, humanized or engineered antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a murine antibody, is used to guide the selection of a humanized or engineered antibody recognizing the same epitope.

[0034] The methods, as described in U.S. Patent Nos. 6,639,055 and 6,794,132, both of which are incorporated herein by reference, can also be used to prepare humanized or engineered antibodies.

[0035] Moreover, the present invention features other anti-EGFR antibodies conjugated with mitomycin C, pingyangmycin, or other cytotoxic antibiotics. Antibodies suitable for this purpose include, but are not limited to, polyclonal, monoclonal, mono-specific, poly-specific, non-specific, humanized or engineered, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, or *in vitro* generated antibodies. Preferably, an antibody of the present invention binds to an epitope in the extracellular domain of EGFR and has an EGFR-binding affinity of at least 10^{-5} M^{-1}, 10^{-6} M^{-1}, 10^{-7} M^{-1}, 10^{-8} M^{-1}, 10^{-9} M^{-1}, 10^{-10} M^{-1}, or stronger. The scFv, diabody, minibody, Fab, $F(ab')_2$, or Fv fragments of these antibodies can also be used for the conjugation of mitomycin C, pingyangmycin, or other cytotoxic antibiotics.

[0036] After a sufficient amount of a purified EGFR-binding molecule has been prepared, one may desire to prepare it into a pharmaceutical composition. A pharmaceutical composition of the present invention typically includes an EGFR-binding molecule of the present invention (*e.g.*, mitomycin C- or pingyangmycin-conjugated LA22 or LA1) and a pharmacologically-acceptable carrier. As used herein, "pharmaceutically-acceptable carrier" includes any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically-active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic or prophylactic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

[0037] Administration of a pharmaceutical composition of the present invention can be by way of any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal, intratumoral, circumferentially, catheterization, or intravenous injection.

[0038] A pharmaceutical composition can also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically-acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene

glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

[0039] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In most cases, the form is sterile and fluid to the extent that easy syringability exists. It is preferably also stable under the conditions of manufacture and storage and preserved against the contaminating action of 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 polyethylene glycol, and the like), suitable mixtures thereof, or vegetable oils. 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, or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various anti-bacterial or anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal or the like. In many cases, it will be preferable to include isotonic agents, for example, sugars 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.

[0040] Sterile injectable solutions can be prepared by incorporating an EGFR-binding molecule of the present invention in the required amount in an appropriate solvent with various other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle, which contains the 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 preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0041] For oral administration, an EGFR-binding molecule of the present invention can be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, an EGFR-binding molecule may be incorporated into an anti-septic wash containing sodium borate, glycerin and potassium bicarbonate. An EGFR-binding molecule

may also be dispersed in dentifrices, including: gels, pastes, powders, or slurries. An EGFR-binding molecule may be added in a therapeutically or prophylactically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, or humectants.

[0042] The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts formed with the free amino groups of the protein or formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[0043] Upon formulation, compositions or solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically or prophylactically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution can be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see, for example, REMINGTON'S PHARMACEUTICAL SCIENCES (15th Edition), pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated.

[0044] The skilled artisan is directed to REMINGTON'S PHARMACEUTICAL SCIENCES (15th Edition), Chapter 33, in particular, pages 624-652, the entire contents of which are incorporated herein by reference. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will determine the appropriate dose for the individual subject.

[0045] The EGFR-binding molecules of the present invention can be used to kill or inhibit the growth of cancer cells. The EGFR-binding molecules of the present invention can also be used to treat cancers in patients or animals. Examples of cancers that are amenable to

the present invention include, but are not limited to, cancers of epithelial origin, such as glioblastoma, cancer of the lung, breast, head and neck, and bladder, or other epidermoid cancers.

[0046] To kill cells or inhibit cell growth, one may contact a target cell with an EGFR-binding molecule of the present invention. The EGFR-binding molecule would be provided in an amount effective to kill or inhibit proliferation of the tumor cells.

[0047] The EGFR-binding molecules can also be combined with other traditional therapies to treat cancers. Agents or factors suitable for use in a combined therapy are any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as chemotherapeutic agents, function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, *e.g.*, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), and in some cases, hydrogen peroxide.

[0048] It should be understood that the above-described embodiments and the following examples are given by way of illustration, not limitation. Various changes and modifications within the scope of the present invention will become apparent to those skilled in the art from the present description.

EXAMPLES

Materials and Methods

[0049] LA22 hybridoma (ATCC No. HB10342) cells at 2×10^4 cell density were injected to Balb/c nude mice. Monoclonal antibodies from ascites was purified using Protein G affinity agarose beads (Upstate, Lake Placid, NY). Briefly, ascites fluid was diluted 1:1 with PBS (pH 7.4) and centrifuged at 14,000g for 4 min. Supernatant was carefully collected and loaded onto a Protein G column (containing 2 mL Protein G agarose) and a 10 mL PBS was then applied to the column to wash unbound components. Bound mAb LA22 was eluted with elution buffer (50 mM glycine-HCl, pH 2.7) and transferred into PBS (pH 7.4) by Desalting Column (Pierce, Rockford, IL). Aliquots were analyzed for purity with SDS-PAGE. Concentration of mAb LA22 was determined to be at 1 mg/ml of mouse IgG, which was equivalent to an O.D. (optical density) reading of 1.40.

[0050] The A431 human epidermoid carcinoma cells, which express large quantities of EGFR, and A549 human lung adenocarcinoma cells were used for *in vitro* cytotoxicity assays. These cells were grown at 37 °C in monolayer culture with Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT).

[0051] The principle used to conjugate small molecule anticancer drug mitomycin (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) or pingyangmycin (PYM) to anti-EGFR mAb LA22 or LA1 involved the activation of the amino group of the mAb with *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP), followed by disulfide exchange with iminothiolane-modified mAb LA22 (Fig. 1). See Knoll, K. *et al.*, *Cancer Res.*, 60:6089-6094, 2000. For activation, MMC (10 mg in 5 mL PBS) was reacted with 0.9 mL of SPDP (Pierce, Rockford, IL) stock (10 mg in 1 mL DMF) at 4°C for 6 h. The resulting solution was stored in small aliquots at -80°C until further use. mAb LA22 (5.5 mg in 3 mL of PBS) or mAb LA1 was reduced by adding 46 µL of 2-iminothiolane (4.4 mg dissolved in 1 mL DMF) for 1 h under nitrogen. The reduced antibody was purified by size exclusion chromatography using a desalting column (Pierce, Rockford, IL), that was pre-equilibrated with PBS. The conjugation of activated MMC (M_r: 137) or activated PMC with thiolated mAb LA22 (M_r: ~150 K) or thiolated mAb LA1, respectively, was done at room temperature for 1 h under nitrogen using a 40:1 molar ratio. Excess-free MMC-SPDP or PMC-SPDP was removed using a desalting column (Pierce, Rockford, IL), also pre-equilibrated with PBS. LA22-MMC (or LA22-PMC) or LA1-MMC (or LA1-PMC) products were filter-sterilized using 0.2 µm Supor membrane (Pall, Ann Arbor, MI). The dosage of the LA22-MMC (or LA22-PMC) or LA1-MMC (or LA1-PMC) conjugates for cytotoxicity and/or *in vivo* experiments was calculated according to the total amount of MMC or PMC added. Aliquots of the final immunoconjugates products were stored at 4 °C prior to use.

Example 1

[0052] A431 cells, a human epidermoid carcinoma cell line (ATCC No. CRL-1551), were seeded at 2,000 cells/well of 96-well plate in DMEM-10% FBS. Four hours later, either control medium, mAb LA22 (40 nM), LA22-PYM immunoconjugate (LA22-P, 40 nM), LA22-MMC immunoconjugate (LA22-M, 40 nM), mAb LA1 (40 nM), LA1-PYM immunoconjugate (LA1-P, 40 nM), LA1-MMC immunoconjugate (LA1-M, 40 nM), PYM-SPDP (P-Linker, 40 nM), or MMC-SPDP (M-Linker, 40 nM) were added to the cell cultures. Four days later, the cell numbers in each well were determined by MTT assay (Mosmann, J.

Immunol. Methods, 65:55-63 (1983). The numbers were expressed as the percentage of the cell death (FIGURE 1).

[0053] As shown in FIGURE 1, no significant cell death was observed with anti-EGFR mAb LA22 alone at 40 nM, while LA22-PYM and LA22-MMC led to 42% and 84% cell death, respectively. In the same dosage (40 nM), anti-EGFR mAb LA1 alone induced 35% of cell death. Enhanced cell killing activities were observed with LA1-PYM and LA1-MMC immunoconjugates.

Example 2

[0054] To evaluate the concentration dependency of the above-mentioned *in vitro* cytotoxicity of LA22-antibiotic immunoconjugates, various amounts of LA22, LA22-PYM, and LA22-MMC were added to A431 cell cultures 4 hours after the cells were seeded at 2,000 cells/well of 96-well plate in DMEM-10% FBS. Four days later, the cell numbers in each well were determined by MTT assay. The numbers were expressed as the percentage of the cell death (FIGURE 2). FIGURE 2 illustrates that anti-EGFR mAb LA22-PYM or LA22-MMC induced the death of A431 human cancer cells in a dose-dependent manner.

[0055] Another LA22-MMC cytotoxicity assay was performed with additional controls. The A431 cells, as well as human lung adenocarcinoma A549 cells (ATCC No. CCL-185) were plated (2×10^3 cells/well, 100 μL/well) in 96-well cell culture plates (Corning, Corning, NY) in the presence of DMEM medium and 10% FBS for 4 h at 37 °C, 5% CO_2. MMC, naked mAb LA22 or LA22-MMC immunoconjugate in serum-free DMEM medium (100 μL) were added onto the individual wells and incubated for 4 days at 37°C and 5% CO_2. The supernatants were discarded first. For dead cells control, 100 μL of cold 95% ethanol was added into the well for 1 min then discarded. MTT solutions (USB, Cleveland, OH; 10% in DMEM medium) were added (100 μL/well) and incubated for 4 h at 37°C. The supernatants were discarded and DMSO was added (100 μL/well) to dissolve the precipitates. Optical density was read at 570 nm using a microplate reader (Bio-Rad, Hercules, CA).

[0056] The above-mentioned inhibition of A431/A549 *in vitro* cell growth was used as a measure for drug activity *in vitro* and was determined by exposing cultured cells (2×10^3 cells/well) for 4 days at 37°C with MMC, LA22-MMC or LA22. The tested MMC concentrations ranged from 0.03 μg/mL to 30 μg/mL. The dosage of the LA22-MMC conjugate for cytotoxicity was calculated according to the total amount of MMC added in chemical coupling reaction. Because the efficiency of chemical coupling reactions was not

100%, theoretically the real MMC concentration linked to LA22 was lower than indicated concentration. The dosage of LA22 was the same as LA22-MMC. These results indicated that MMC conjugated LA22 were less potent than the free MMC alone, but more potent than naked mAb LA22 (see FIGURES 3A and 3B). Non-specific mouse IgG (as a negative control) was labeled with MMC and added into A431 cells to evaluate its cytotoxicity on cell growth *in vitro*. The result showed that non-specific mouse IgG-MMC immunoconjugates did not kill cultured cells as efficiently as LA22-MMC, which proved that the cytotoxicity of LA22-MMC can be mediated via LA22-EGFR binding (data not included).

Example 3

[0057] Likewise, an *in vitro* cytotoxicity assay employing LA1-antibiotic immunoconjugates was similarly evaluated. A431 cells were seeded at 2,000 cells/well of 96-well plate in DMEM-10% FBS. 4 hr later, various amounts of LA1, LA1-PYM, or LA1-MMC were added to the cell cultures. Four days late, the cell numbers in each well were determined by MTT assay. The numbers were expressed as the percentage of the cell death (FIGURE 4). As demonstrated in FIGURE 3, mAb LA1 and its immunoconjugates induced the death of A431 human cancer cells in a dose-dependent manner. Conjugation with either pingyangmycin or mitomycin C significantly increased the cell killing potencies of mAb LA1.

Example 4

[0058] Another *in vitro* cytotoxicity assay using LA1 immunoconjugates was further evaluated using human lung cancer A549 cell line (ATCC No. CCL-185). A549 cells were seeded at 2,000 cells/well of 96-well plate in DMEM-10% FBS. Four hours later, various amounts of LA1, LA1-MMC, or MMC-SPDP were added to the cell cultures. Four days later, the cell numbers in each well were determined by MTT assay. The numbers were expressed as the percentage of the cell death (FIGURE 5). LA1-MMC induced the death of A549 human lung cancer cells in a dose-dependent manner. No significant cell death was observed with mAb LA1 alone. Conjugation with mitomycin C significantly increased the cell killing potency of LA1.

Example 5

[0059] To test for the effect of LA22-MMC on *in vivo* tumor toxicity, five week-old female BALB/c mice with a body weight of approximately 20 g were used. Xenografts of

A431 cell lines were produced by injecting 2×10^6 tumor cells (resuspended in 0.2 mL PBS) subcutaneously into the right front leg of mice on day 0. The mice were grouped (5 mice per group) and administered intraperitoneally with PBS, MMC, naked antibodies or immunoconjugates with indicated concentrations on day 1, day 5 and day 9. Tumor size was measured three times per week and tumor volume was calculated according to the following equation: $tumor\ size = width^2 \times length/2$. At the end of the experiment (day 21), the mice were sacrificed and tumors were removed and weighed.

[0060] As illustrated in FIGURE 6A, mice treated with LA22-MMC (0.08 mg/kg) inhibited tumor growth *in vivo* when compared with control untreated mice. Although MMC (0.08 mg/kg) injection was potent to inhibit tumor growth, the efficiency was much lower than LA22-MMC (0.08 mg/kg), which suggested the great advantage of LA22-mediated specific tumor targeting. Low dosage administration (0.0032 mg/kg) of LA22-MMC was not potent as the high-dosage of LA22-MMC administration (0.08 mg/kg), which suggested the dose-dependent effect of LA22-MMC immunoconjugate. Considering the comparative big errors drawn by measuring tumors, mice were sacrificed and the tumors were weighed (FIGURE 6B). There were no detectable tumors in 2 mice injected with LA22-MMC (0.08 mg/kg). The average tumor size of LA22-MMC (0.08 mg/kg) treatment group was much lower than the other groups. The tumors shrank to 15% comparing with the control group (PBS injected) while the tumors only shrank to 60% after three MMC injections (0.08 mg/kg). These results showed that LA22-MMC was potent to inhibit tumor growth *in vivo*.

Example 6

[0061] To show the biodistribution of LA22-MMC immunoconjugate, five (5)-week old female BALB/c nude mice, kept under specific pathogen-free condition, were implanted subcutaneously with A431 cells (5×10^6) or A549 cells (5×10^6) at their right upper flanks. When the tumors reached ~1.0 cm in mean diameter, the tumor-bearing mice were subjected to immunoscintigraphy. To a vial coated with 40 μg Iodogen (Sigma-Aldrich, St. Louis, MO), 100 μg of LA-22-MMC antibody (0.76 mg/mL, in 132 μL) and 100 MBq of $Na^{125}I$ (Beijing Atom High Tech., Beijing, China) in 0.2M, pH 7.4 phosphate buffer were added. After 10 min of incubation at room temperature, the reaction mixture was purified by a PD-10 column (Amersham, Piscataway, NJ). Labeling yield and radiochemical purity were measured by ITLC (85% methanol as mobile phase) on a radio-thin layer scanner (Bioscan AR2000, Washington, DC), with more than 85% and 97%, respectively. The nude mice-

bearing A431/A549 xenografts were injected intravenously with 17.6 MBq (~20 μg, 0.3 mL) of ^{125}I-LA22-MMC, and placed prone on a two-head γ-camera (E.CAM, Siemens, Munich, Germany) equipped with a parallel-hole, low-energy, high-resolution collimator. The posterior images were acquired at different time points post-injection for the visual observation of *in vivo* distribution of LA22-MMC, and stored digitally in a 128 x 128 matrix. The acquisition count limits were set at 1000 K.

[0062] The imaging results from 4h to 7 days are shown on FIGURE 7. ^{125}I labeled LA22-MMC first distributed in the circulation system and blood-rich tissues such as liver or heart (4h imaging); gradually the labeled immunoconjugates enriched on the tumor sites (1 day to 4 days). After 4 days, LA22-MMC mainly enriched on the tumor sites.

Example 7

[0063] Erbitux® and Herceptin®, two commercially-available mAb drugs for cancer therapy, target EGFR and HER2, respectively. Both EGFR and HER2 belong to the ErbB receptor family (Mendelsohn, J. *et al.*, *Oncogene*, 19:6550-7565, 2000). To compare LA22-MMC with Erbitux® and Herceptin®, an *in vitro* cytotoxicity assay using the MTT method was conducted. The conjugation of MMC with Erbitux® (ImClone, New York, NY) or Herceptin® (Genentech, San Francisco, CA) or mouse IgG was done as described above.

[0064] Erbitux® and Herceptin® were labeled with MMC and evaluated for their cytotoxicities. A431 (FIGURE 8A) or A549 (FIGURE 8B) cells were seeded into 96-well cell culture plates. Naked antibodies or immunoconjugates were added to wells and incubated for 4 days at 37°C and 5% CO_2. Viable cells were measured using the MTT assay. Using similar antibody concentrations, LA22-MMC prodrug killed A431 and A549 cells more efficiently than Erbitux®-MMC or Herceptin®-MMC (see FIGURES 8A & 8B) .

[0065] The foregoing description of the present invention provides illustration and description, but is not intended to be exhaustive or to limit the invention to the precise one disclosed. Modifications and variations are possible consistent with the above teachings or may be acquired from practice of the invention. Thus, it is noted that the scope of the invention is defined by the claims and their equivalents.

What is claimed is:

1. An EGFR-binding molecule comprising an antigen-binding fragment of an anti-EGFR monoclonal antibody and a cytotoxic agent, wherein said anti-EGFR monoclonal antibody is selected from the group consisting of LA1 and LA22.

2. The EGFR-binding molecule of claim 1, comprising said monoclonal antibody conjugated with said cytotoxic agent.

3. An EGFR-binding molecule according to any one of claims 1 or 2, wherein said cytotoxic agent is a cytotoxic antibiotic.

4. The EGFR-binding molecule of claim 3, wherein said cytotoxic antibiotic is selected from the group consisting of mitomycin C and pingyangmycin.

5. The EGFR-binding molecule of claim 4, wherein said EGFR-binding molecule is an engineered antibody comprising an antigen-binding fragment of LA1 or LA22.

6. An EGFR-binding molecule according to any one of claims 1 or 2, wherein said cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a radioisotope, a cytotoxin, and an anticancer pro-drug activating enzyme.

7. A method for killing or inhibiting the growth of cancer cells, comprising contacting said cells with an EGFR-binding molecule according to any one of claims 1-6.

8. The method of claim 7, wherein said cancer cells are epidermoid cancer cells.

9. The method of claim 7, wherein said cancer cells are lung cancer cells.

10. A method for treating a cancer in a subject of interest, comprising administrating an effective amount of an EGFR-binding molecule according to any one of claims 1-6 to said subject of interest.

11. A pharmaceutical composition comprising an EGFR-binding molecule according to any one of claims 1-6.

12. An anti-EGFR antibody conjugated with mitomycin C or pingyangmycin.

13. The antibody of claim 12, wherein said antibody is a monoclonal antibody.

14. An antibody according to any one of claims 12 or 13, wherein said antibody is an engineered antibody or fragments of antibody.

15. A method for killing or inhibiting the growth of cancer cells, comprising contacting said cells with an antibody according to any one of claims 12-14.

16. A method for treating a cancer in a patient of interest, comprising administrating an effective amount of an antibody according to any one of claims 12-14 to said patient of interest.

17. A pharmaceutical composition comprising an EGFR-binding molecule according to any one of claims 12-14.

Figure 1

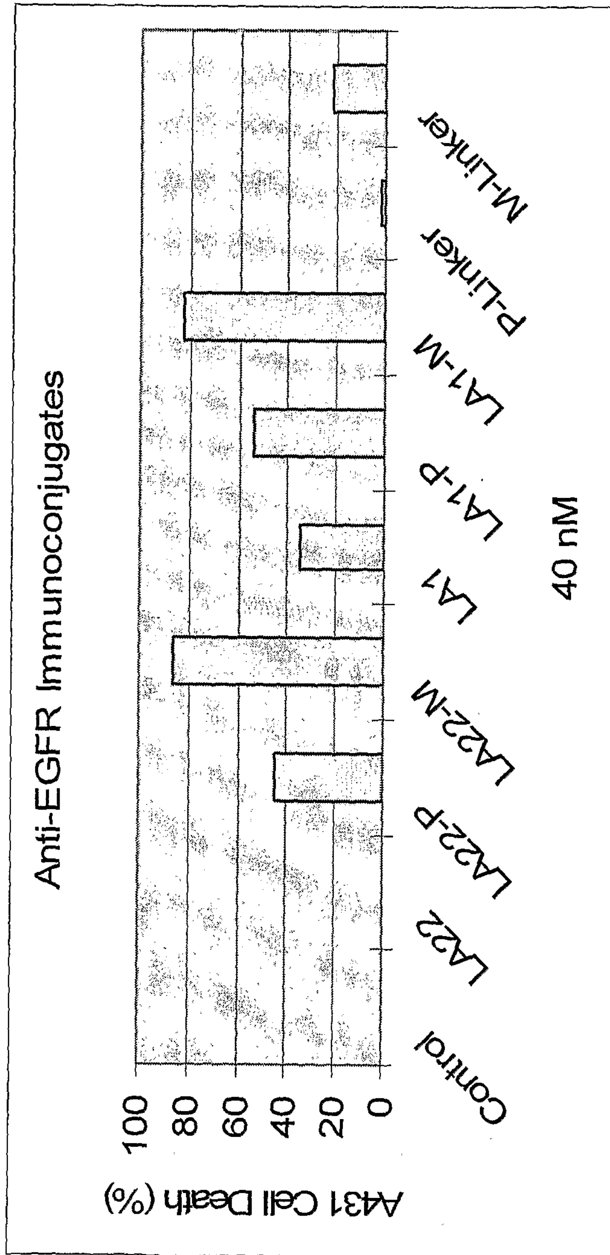


Figure 2

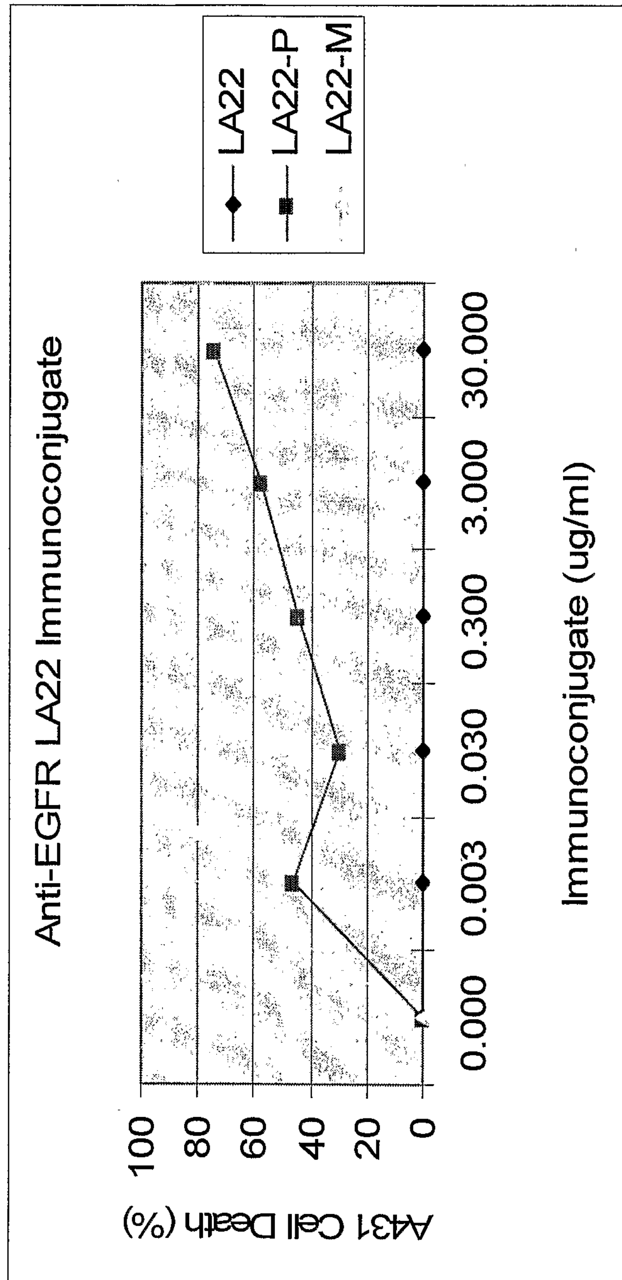
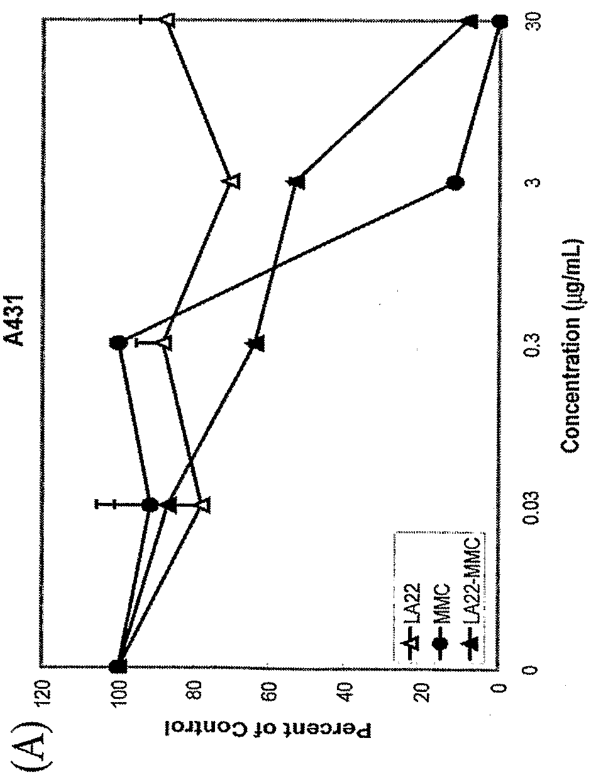
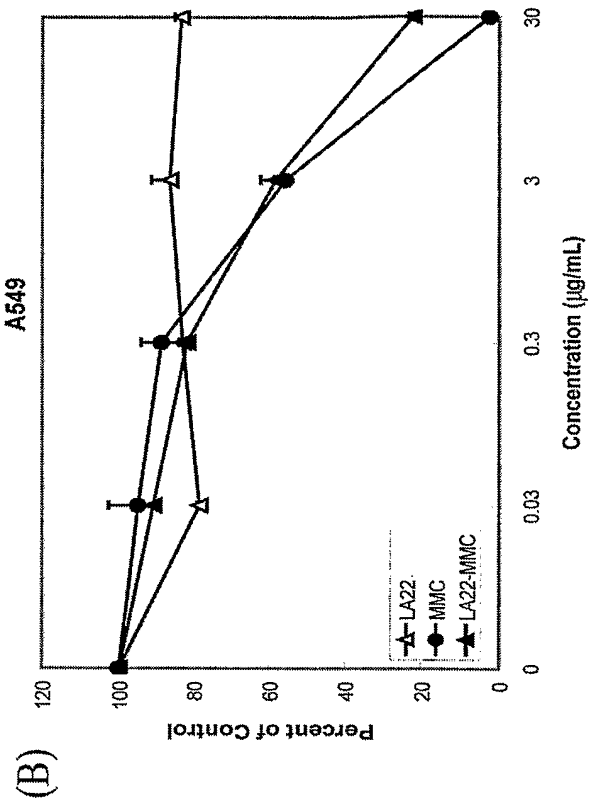


Figure 3

(A)



(B)



(C)

Figure 4

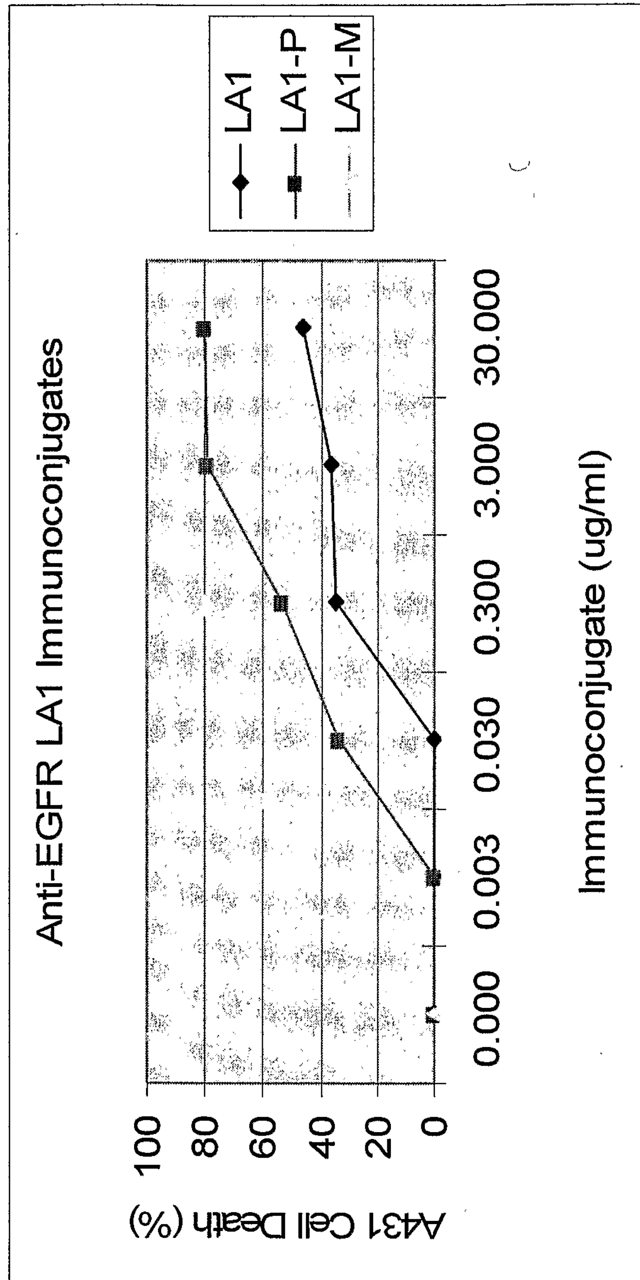


Figure 5

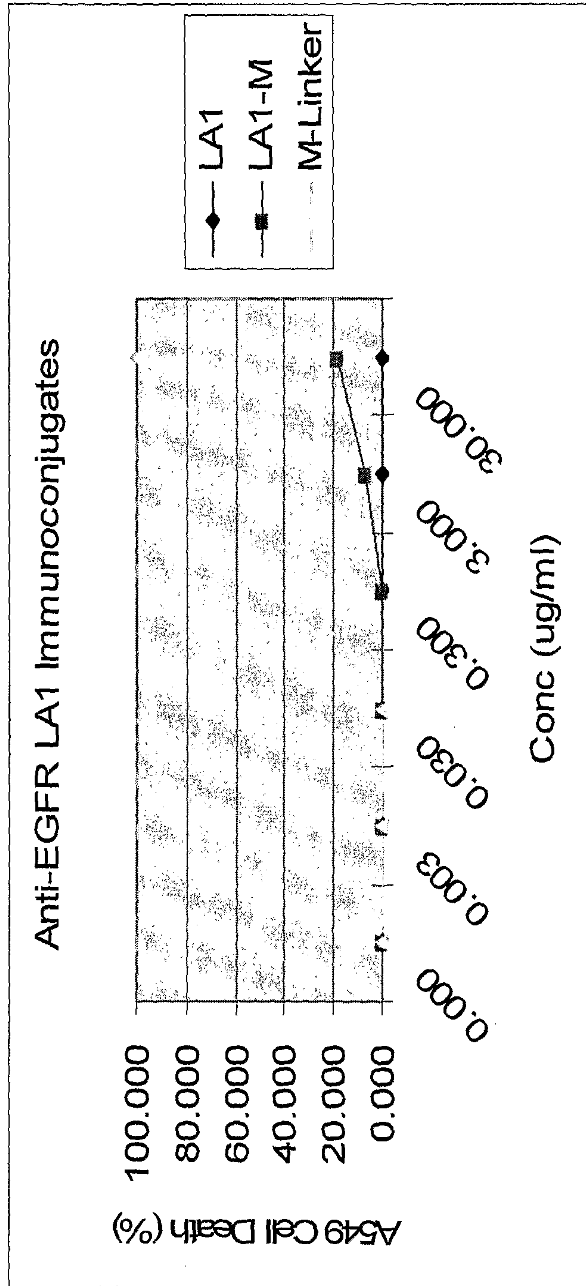
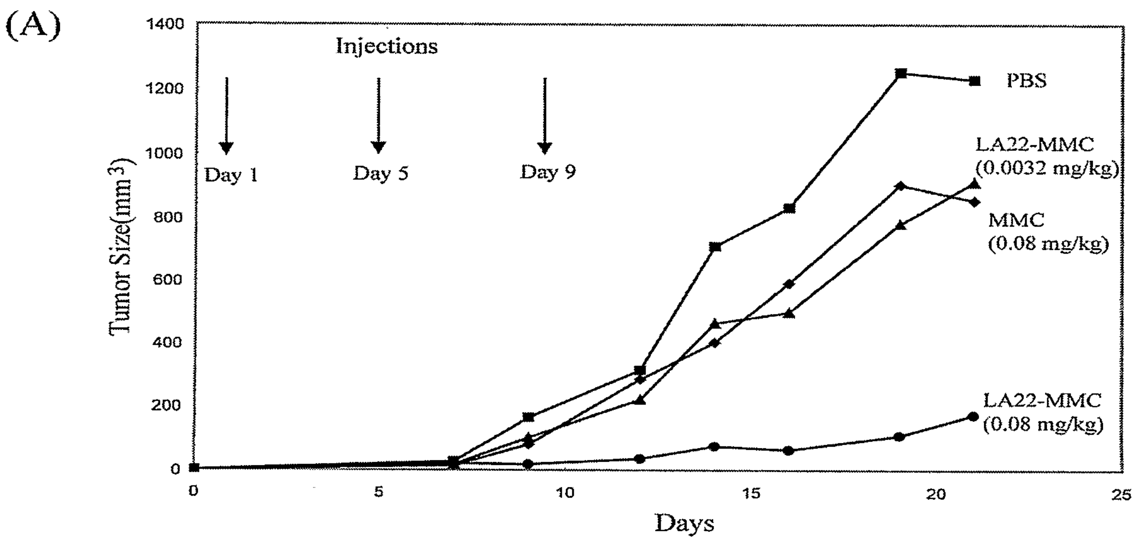


Figure 6

(A)



(B)

Injection	Tumor Size±SD (g)	Inhibition (%)
PBS (Control)	0.87±0.36	0.00%
MMC (0.08 mg/kg)	0.60±0.26	30.40%
LA22-MMC (0.0032mg/kg)	0.76±0.56	12.14%
LA22-MMC (0.08mg/kg)	0.15±0.16	82.66%

Figure 7

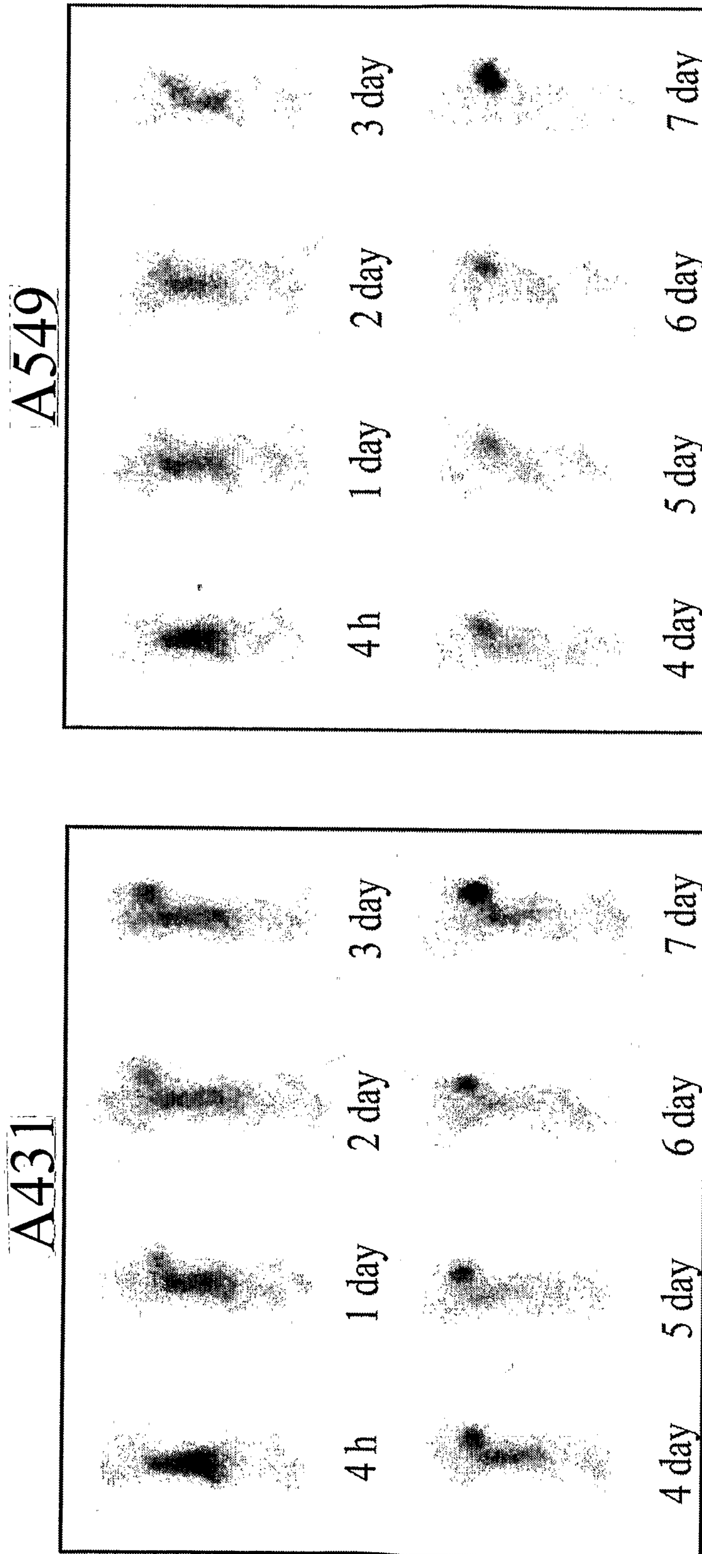


Figure 8