(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 August 2013 (29.08.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/123588 A1

(51) International Patent Classification:
A61K 39/395 (2006.01) *A61P 35/00* (2006.01)
A61K 31/5377 (2006.01)

(21) International Application Number:
PCT/CA2013/000167

(22) International Filing Date:
22 February 2013 (22.02.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/601,786 22 February 2012 (22.02.2012) US

(71) Applicant: **ALETHIA BIOTHERAPEUTICS INC.** [CA/CA]; 141 President-Kennedy Avenue, Suite SB-5100, Montreal, Quebec, H2X 1Y4 (CA).

(72) Inventors: **TREMBLAY, Gilles Bernard**; 100 Denise-Lemaistre, La Prairie, Quebec, J5R 6N8 (CA). **VIAU, Elisabeth**; 6032 Pronovost, Laval, Quebec, H7H 3E2 (CA). **FILION, Mario**; 739 Maple Street, Longueuil, Quebec, J4J 4M8 (CA).

(74) Agent: **FORGET, Janique**; Alethia Biotherapeutics Inc., 141 President-Kennedy Avenue, Suite SB-5100, Montreal, Quebec, H2X 1Y4 (CA).

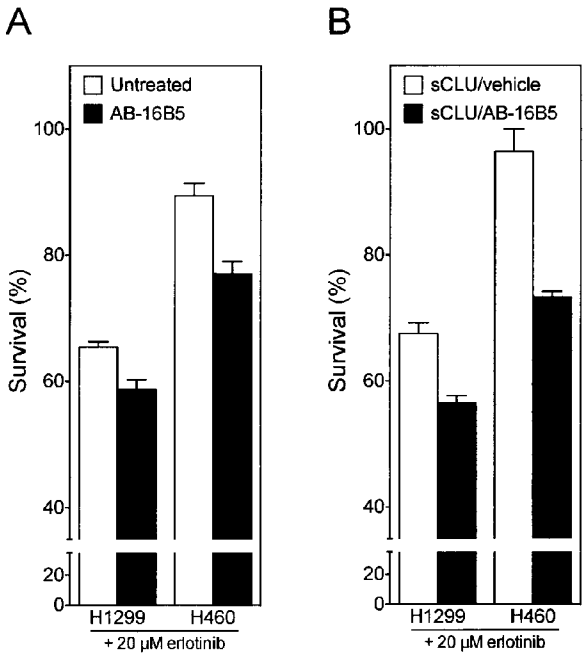
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE,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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,

[Continued on next page]

(54) Title: CO-USE OF A CLUSTERIN INHIBITOR WITH AN EGFR INHIBITOR TO TREAT CANCER

Figure 7



(57) Abstract: Epidermal growth factor receptor (EGFR) expression and phosphorylation is increased in cancer cells treated with anti-clusterin antibodies. Such treatment is also accompanied with the reappearance of an epithelial phenotype of the cancer cell, as determined by an increased E-cadherin expression at the surface of cancer cells. Clusterin inhibitors may thus induce reversal of the epithelial to mesenchymal phenotype and restore sensitivity of cancer cells to EGFR inhibitors. Combinations of a clusterin inhibitor and an EGFR as well as their use in treatment of cancer are thus provided herewith.

WO 2013/123588 A1

UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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

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

Co-Use Of A Clusterin Inhibitor With An EGFR Inhibitor To Treat Cancer

FIELD OF THE INVENTION

The invention relates to combination of a clusterin inhibitor with an inhibitor of epidermal growth factor receptor (EGFR) for use in treatment of cancer. More particularly, the present invention encompasses the use of a clusterin inhibitor, such as an anti-clusterin antibody, to restore the sensitivity of cancer cells to an EGFR inhibitor or to potentiate the effect of the EGFR inhibitor.

BACKGROUND OF THE INVENTION

Development of resistance to chemotherapy and invasion to other secondary sites are common features of solid tumor malignancies. It is well known that development of resistance to chemotherapeutic agents is caused by over-expression of proteins involved in drug and apoptosis resistance. The invasion process is also fairly well documented. Tumor invasion is caused by an increased motility of cancer cells and the expression of genes that cause degradation of the proteins in the extra-cellular matrix. There is growing evidence that resistance to chemotherapy and tumor invasion might share a common starting point through a biological process called the epithelial-to-mesenchymal transition (EMT). Recent studies have demonstrated that transforming growth factor beta (TGFβ) may be a critical mediator of EMT. Despite these advances, few therapeutic avenues are available to inhibit the development of chemo-resistance and the spread of cancer to other organs. It has also emerged in the recent literature that certain tumor cells undergoing EMT dedifferentiate and adopt stem cell-like properties (cancer stem cells or CSCs). As is the case for normal stem cells, CSCs are inherently refractory to chemotherapy and radiation therapy. Therefore, targeting a specific regulator of EMT and CSC maintenance represents a very promising therapeutic strategy to increase response to chemotherapeutic agents and to prevent recurrence of cancer.

Utilizing well characterized cell lines as models of EMT, proteins were identified that were up-regulated upon induction of EMT. One of these, a secreted protein termed clusterin (sCLU), was found to be stimulated during EMT and could, on its own, promote the EMT process (Lenferink et al., 2009). Several high-affinity antibodies were generated that interact with sCLU, which when tested in cell-based assays for their ability to block

the EMT, those antibodies that neutralized EMT all bound to the same critical amino acid sequence in the sCLU protein. This discovery demonstrated that a specific region of sCLU was responsible for mediating its EMT-promoting activity. By blocking the EMT-epitope in sCLU, the antibodies, in particular an antibody designated 16B5 could block EMT as exemplified by the maintenance of the membrane expression of the epithelial cell marker, E-cadherin, when incubated with cancer cells. Furthermore, human xenograft animal studies using prostate cancer and pancreatic cancer tumors showed that blocking the activity of tumor-associated sCLU resulted in the increased response to standard chemotherapeutic drugs such as docetaxel and gemcitabine, as measured by a significant reduction in tumor growth. Taken together, these results demonstrated that blocking EMT with an antibody capable of interacting with a specific region in sCLU resulted in tumor growth inhibition and increased response to cytotoxic drugs (see international application No. PCT/CA2006/001505 published under No. WO2007/030930 and international application No. PCT/CA2010/0001882 published under No. WO2011/063523, the entire content of which is incorporated herein by reference

Lung cancer is one of the most common cancers and a leading cause of death worldwide, with over a million cases diagnosed yearly and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers. Despite recent improvements in diagnostic and therapeutic approaches, the majority of patients are diagnosed with advanced NSCLC where the median survival remains poor (Adamo et al., 2009).

One of the most important targets in NSCLC is the epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, that is a cell membrane receptor that plays an important role in proliferation and survival of cancer cells. It is a large transmembrane glycoprotein that serves as a receptor for EGF and several additional endogenous ligands. It has three domains consisting of an extracellular region, a transmembrane domain and an intracellular tyrosine kinase (TK) domain. Functionally, ligand binding to EGFR induces receptor dimerization leading to a structural change that promotes autophosphorylation and activation of the intracellular TK domain. Consequently, EGFR activation influences multiple downstream signaling pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3'-kinase (PI3K)/Akt pathway, which influence cell proliferation, invasiveness, motility, survival and apoptosis (Shigematsu et al., 2005).

Although EGFR is ubiquitously expressed, it is often modified in tumors cells. These modifications include gene amplification, overexpression of ligands and/or receptors and activating mutations. Overexpression or dysregulation of EGFR or its primary ligands is characteristic of many solid human tumors, including lung cancer. In NSCLC, between 43 and 83% of tumors overexpress EGFR (Adamo et al., 2009). Several agents against EGFR such as monoclonal antibodies that target the extracellular domain or small molecules are able to inhibit the TK activity.

The status of EGFR in metastatic NSCLC and the response to chemotherapy is the subject of much debate. Despite the high proportion of tumors with increased expression of EGFR, some clinical studies have shown that this was a poor predictor of response in first-line therapy (Barr et al., 2008). Furthermore, despite a mild but significant response in patients treated first-line with cisplatin, vinorelbine and cetuximab (EGFR monoclonal antibody) compared to chemotherapy alone, there was no correlation between cetuximab and EGFR over-expression (Mirshahidi and Hsueh, 2010). Overall, an overwhelming amount of clinical results with EGRF inhibitors in NSCLC showed that the status of the receptor was not important in first-line therapy until a recent study reported results showing that patients with high EGFR expression that were treated with cetuximab and chemotherapy exhibited an increase in overall survival compared to chemotherapy alone (Pirker et al., 2012). It is clear from these results that there are other mechanisms involving EGFR in tumors that influence the response of NSCLC patients to EGFR inhibitors.

Additional characteristics of EGFR that likely influence the response of inhibitors are those that permit the binding of small molecules to the TK activity of the receptor. A few have been developed and approved for cancer indications including gefitinib and erlotinib, two small molecules that mimic ATP-binding to this region thus preventing intracellular signaling. Neither of these inhibitors was found to be active in NSCLC in first-line therapy but significant clinical responses were achieved in second- and third line settings (Mirshahidi and Hsueh, 2010). Interestingly, EGFR overexpression had no influence on patient response but it was discovered that activating mutations in EGFR and certain other genes were critical. For example, activating mutations were found to lead to significant increase in progression free survival in patients treated with gefitinib (Costanzo et al., 2011). In addition, NSCLC patients treated with erlotinib who also had mutations in a gene called KRAS, showed no response (Herbst and Sandler, 2008).

Thus, patient selection is critical for attempting to understand if they will be responders to EGFR TK inhibitors.

As described above, EMT can have a tremendous influence on the way tumors cells will respond to therapy and the ability of cancer cells to remain epithelial is critical for this response. In cell-based studies, cells that have increased expression of the epithelial cell marker, E-cadherin, are more sensitive to EGFR inhibitors (Barr et al., 2008). In agreement with these observations, there was a correlation between E-cadherin expression and sensitivity to erlotinib (Yauch et al., 2005). At the tumor level, it has been shown that restoration of E-cadherin expression increases sensitivity to EGFR inhibitors (Witta *et al.*, 2006). To date, however, the link between EMT and EGFR status in clinical trials has not been clearly examined. However, given the lack of correlation between EGFR overexpression and response to EGFR inhibitors and the influence of activating EGFR mutations on their response, it is probable that additional influences such as EMT might be directly involved in increasing the efficacy of EGFR inhibitors in NSCLC patients.

This present application provides a method of treatment with an antibody that blocks EMT by inhibiting sCLU in tumors that express EGFR. The EGFR status in these tumors might include *EGFR* gene amplifications or amplification in EGFR ligands. Furthermore, the tumors cells might include increased autocrine signaling through EGFR and EGFR protein partners. The EGFR status might also include activating mutations in EGFR that cause the receptor to exhibit increased activity. In another embodiment, the tumors might be sensitive or resistant to EGFR inhibitors, including monoclonal antibodies against EGFR or small molecule inhibitors that abrogate the activity of EGFR. Furthermore, EGFR status might also include tumors that were *a priori* negative for EGFR expression that have reacquired EGFR.

When NSCLC cell lines that express sCLU are exposed to an anti-clusterin antibody, both the expression and the activation of EGFR are increased. In parallel, by virtue of EMT inhibition by the anti-clusterin antibody, the NSCLC cell lines also show increased E-cadherin expression. Taken together, NSCLC patients treated with a clusterin inhibitor in combination with EGFR inhibitors may show an increased response to the EGFR inhibitors.

SUMMARY OF THE INVENTION

The present invention generally relates to a combination of a clusterin inhibitor and EGFR inhibitors for cancer treatment.

Methods of the present invention encompass administration of a clusterin inhibitor capable of inhibiting epithelial-to-mesenchymal transition (EMT) and an EGFR inhibitor to an individual in need. An EGFR inhibitor may also be administered separately, concurrently or sequentially with the clusterin inhibitor.

The clusterin inhibitor may be administered especially when EGFR resistance is observed, detected or suspected.

Methods of the present invention also comprise administering a clusterin inhibitor to prevent EGFR resistance or to sensitize cancer cells to an EGFR inhibitor.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Lung cancer cell lines express clusterin. This figure shows RT-PCR analysis of clusterin mRNA expression in several cell lines derived from NSCLC (upper panel). As a control, the mRNA from actin was also amplified (lower panel) to ensure equal amounts a starting material in each reaction.

Figure 2. Lung cancer cell lines express clusterin (sCLU). This figure shows the quantification of sCLU that is secreted by lung cancer cell lines. The quantities were measured with a sCLU-specific ELISA and are expressed in ng/ml.

Figure 3. h16B5 increases E-cadherin expression in lung cancer cell lines. This figure demonstrates the increase in the epithelial character of A549 NSCLC cells when incubated with the sCLU inhibitor, h16B5. The left panel shows the cells incubated with a control IgG for 48h whereas the right panel shows cells incubated with h16B5 under identical conditions.

Figure 4. Treatment of NSCLC cells with h16B5 results in increased EGFR expression. EGFR expression in the presence of control vehicle or h16B5 was measured using an EGFR-specific antibody in whole cell lysates prepared from either A549 or H226 cells.

Figure 5. Treatment of NSCLC cells with h16B5 results in increased EGFR phosphorylation. EGFR phosphorylation in the presence of control vehicle or h16B5 was measured using an phosphotyrosine-specific antibody in whole cell lysates prepared from either A549 or H226 cells.

Figure 6. Treatment of NSCLC cells with h16B5 results in increased EGFR phosphorylation. EGFR phosphorylation in the presence of control vehicle, h16B5 or a control IgG was measured using an phosphotyrosine-specific antibody in whole cell lysates prepared from either A549 or H226 cells.

Figure 7. Combination of h16B5 with erlotinib result in an enhanced anti-tumor effect. Survival of H1299 and H460 cancer cells was determined in the presence of a combination of h16B5 with erlotinib in the presence or absence of recombinant clusterin.

DETAILED DESCRIPTION OF THE INVENTION

Epithelial growth factor receptor (EGFR) is overexpressed in several types of epithelial carcinomas. The existence of EGFR genetic alterations may render tumors more susceptible to EGFR inhibitors. However, patients with initial response to EGFR tyrosine kinase inibitors (TKIs) relapse within an average of one year. Although it has been shown that deletions in EGFR exon 9 or L858R mutations results in favorable clinical outcome, secondary molecular event such as mutations T790M, L747S and/or D761Y are associated with resistance and tumor relapse. Amplification of the MET receptor and activation of IGFR signaling that both activate the PI3/AKT pathway independently of EGFR have also been shown to drive secondary resistance.

The Applicant has come to the unexpected discovery that EGFR expression and phosphorylation is increased in cancer cells treated with a clusterin inhibitor. Treatment of cancer cells with the clusterin inhibitor is also accompanied with reappearance of an epithelial phenotype of the cancer cell, as illustrated by an increased E-cadherin expression at the cell surface.

Thus, not only may clusterin inhibitors induce reversal of the EMT phenotype, they may restore sensitivity of cancer cells to EGFR inhibitors.

The present invention generally relates to a combination of a clusterin inhibitor and EGFR inhibitors for use in cancer treatment.

The pharmaceutical combination may particularly comprise a clusterin inhibitor in association with a pharmaceutically acceptable carrier and an EGFR inhibitor in association with a pharmaceutically acceptable carrier.

Methods of the present invention encompass administration of a clusterin inhibitor and an EGFR inhibitor to an individual in need. The EGFR inhibitor may be administered separately, concurrently or sequentially with the clusterin inhibitor.

The clusterin inhibitor may be administered especially when EGFR resistance is observed, detected or suspected.

Methods of the present invention also comprise administering a clusterin inhibitor to prevent EGFR resistance or to sensitize cancer cells to an EGFR inhibitor.

In one aspect of the invention, the clusterin inhibitor may be administered prior to the EGFR inhibitor. For example, the clusterin inhibitor may be administered from a few hours to several days or months prior to administration of the EGFR inhibitor. In another aspect of the invention, the clusterin inhibitor may also be administered at the same time (e.g., same day) or between each treatment with the EGFR inhibitor.

In accordance with the present invention, the clusterin inhibitor may be administered in multiple doses prior to administration of the EGFR inhibitor, e.g., daily, every other day, once a week, twice a week, etc.

The method may also comprise testing for reversal of the epithelial-to-mesenchymal phenotype of the cancer cells before administration of the EGFR inhibitor. The EMT status may be determined, for example, by measuring expression levels of one or more genes/protein selected from E-cadherin, RAB25, integrin beta 6, vimentin, ZEBI and SIPI.

Clusterin inhibitors may be identified by their ability to impair clusterin expression, secretion or clusterin activity.

Exemplary embodiments of clusterin inhibitors include those identified, for example, by their ability to interfere with the EMT-promoting effect of secreted clusterin (sCLU) or of TGF-β. For example, carcinoma cells (e.g., 4T1: breast carcinoma cells, DU145: prostate cancer cells, etc.) may be treated with a putative clusterin inhibitor in the

presence of TGF-β or sCLU and markers of EMT may be assessed as described below. A putative compound, which is capable of increasing the expression of epithelial markers and/or reducing the expression of mesenchymal markers, may be identified as a suitable clusterin inhibitor.

Alternatively, the motility of carcinoma cells in the presence of the putative clusterin inhibitor may be assessed. For example, carcinoma cells may be treated with a putative clusterin inhibitor in the presence of TGF-β or sCLU and a wound healing assay or a black ink motility assay may be carried out as described for example in PCT/CA2006/001505. A putative compound, which is capable of inhibiting or reducing the motility of carcinoma cells in these types of assays, may be identified as a suitable clusterin inhibitor. It is to be understood that other techniques may be used to identified suitable clusterin inhibitors.

Clusterin inhibitors particularly encompassed by the present invention include for example, anti-clusterin antibodies or antigen binding fragments thereof.

In accordance with the present invention, clusterin inhibitors include anti-clusterin antibodies or antigen binding fragment capable of inhibiting EMT (e.g., in carcinoma cells).

Commonly used molecular markers of EMT include, for example, a reduced expression of E-cadherin, cytokeratin and β-catenin (in the membrane) and/or an increased expression of Snail, Slug, Twist, ZEB1, ZEB2, N-cadherin, vimentin, a-smooth muscle actin, matrix metalloproteinases etc. (see for example, Kalluri and Weinberg, The Journal of Clinical Investigation, 119(6), p1420-1428; 2009; Fassina et al., Modern Pathology, 25; p86-99; 2012; Lee et al., JCB; 172; p973-981; 2006). An EMT phenotype may also be distinguished by an increased capacity for migration, invasion of by resistance to anoikis/apoptosis. Cells that are undergoing epithelial-to-mesenchymal transition may thus be detected by a reduction of epithelial markers and apparition of mesenchymal markers or EMT phenotypes.

Expression of markers may generally be determined by comparing their level of cellular expression (at the genetic level or at the protein level (e.g., including cell surface expression) in one state in comparison with another state. For example, the level of expression of one or more specific markers may be determined in cancerous cells in

comparison with non-cancerous cells. Alternatively, the level of expression of one or more specific markers may be determined in cancerous cells that are resistant to a EGFR inhibitor in comparison with cancerous cells that are sensitive to the EGFR inhibitor. Furthermore, level of expression of one or more specific markers may be evaluated over values that are statistically representative of controls.

Individuals who would benefit from such treatment include those having carcinoma (i.e., epithelial carcinoma) including, prostate cancer, breast cancer (e.g., triple negative or basal-like), endometrial carcinoma, ovarian carcinoma, hepatocellular carcinoma, colorectal carcinoma, head and neck carcinoma (e.g., head and neck squamous cell carcinoma), lung carcinoma (e.g., non-small cell lung cancer), pancreatic cancer, renal cell carcinoma, etc. (including advanced or metastatic forms of these cancers).

Exemplary embodiments of anti-clusterin antibodies that may be used to carry the present invention include those that are capable of binding to amino acids 421 and 443 of a C-terminal portion of a β-subunit of human clusterin. More particular embodiments of antibodies or antigen binding fragment encompassed by the present invention include those described in international application No. PCT/CA2006/001505 published under No. WO2007/030930 and international application No. PCT/CA2010/0001882 published under No. WO2011/063523.

The present invention especially encompasses antibodies and antigen binding fragment having at least one complementary determining region (CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and/or CDRL3) identical to those of the antibodies identified as 16B5, 21B12, 20E11, 16C11 and 11E2. More particularly, the present invention especially encompasses antibodies and antigen binding fragment having a light chain and/or heavy chain identical to those of identified as 16B5, 21B12, 20E11, 16C11 and 11E2 (see also PCT/CA2006/001505) or to those identified as humanized 16B5 (h16B5), humanized 21B12 (h21B12), h16B5 VL consensus 1, h16B5 VL consensus 2, h16B5 VL consensus 3, h16B5 VH consensus 1, h16B5 VH consensus 2, h16B5 VH consensus 3, h21B12 VL consensus 1, h21B12 VL consensus 2, h21B12 VL consensus 3, h21B12 VH consensus 1, h21B12 VH consensus 2 or h21B12 VH consensus 3 (see also PCT/CA2010/0001882).

The amino acid sequence of the light chain and/or heavy chain variable regions of the antibody identified as 20E11, 16C11 and 11E2 are presented in SEQ ID NOs.:62-67, where the predicted complementarity determining regions are shown in bold.

Other exemplary embodiments of antibodies and antigen binding fragments include those that can compete with the antibodies identified herein.

The invention encompasses monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies and human antibodies (isolated) as well as antigen binding fragments having the characteristics described herein. Antibodies or antigen binding fragments encompassing permutations of the light and/or heavy chains between a monoclonal, chimeric, humanized or human antibody are also encompassed herewith.

The antibodies or antigen binding fragments of the present invention may thus comprise amino acids of a human constant region and/or framework amino acids of a human antibody.

The term "antibody" refers to intact antibody, monoclonal or polyclonal antibodies. The term "antibody" also encompasses multispecific antibodies such as bispecific antibodies. Human antibodies are usually made of two light chains and two heavy chains each comprising variable regions and constant regions. The light chain variable region comprises 3 CDRs, identified herein as CDRL1, CDRL2 and CDRL3 flanked by framework regions. The heavy chain variable region comprises 3 CDRs, identified herein as CDRH1, CDRH2 and CDRH3 flanked by framework regions.

The term "antigen-binding fragment", as used herein, refers to one or more fragments of an antibody that retain the ability to bind to an antigen (e.g., KAAG1, secreted form of KAAG1 or variants thereof). It has been shown that the antigen-binding function of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR), e.g., V_H

CDR3. Furthermore, although the two domains of the Fv fragment, V_L and V_H, 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 polypeptide chain in which the V_L and V_H 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). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding fragment" of an antibody. Furthermore, the antigen-binding fragments include binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide (such as a heavy chain variable region, a light chain variable region, or a heavy chain variable region fused to a light chain variable region via a linker peptide) that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. The hinge region may be modified by replacing one or more cysteine residues with serine residues so as to prevent dimerization. Such binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

A typical antigen binding site is comprised of the variable regions formed by the pairing of a light chain immunoglobulin and a heavy chain immunoglobulin. The structure of the antibody variable regions is very consistent and exhibits very similar structures. These variable regions are typically comprised of relatively homologous framework regions (FR) interspaced with three hypervariable regions termed Complementarity Determining Regions (CDRs). The overall binding activity of the antigen binding fragment is often dictated by the sequence of the CDRs. The FRs often play a role in the proper positioning and alignment in three dimensions of the CDRs for optimal antigen binding.

Antibodies and/or antigen binding fragments of the present invention may originate, for example, from a mouse, a rat or any other mammal or from other sources such as through recombinant DNA technologies.

Other clusterin inhibitor may include for example and without limitation siRNAs (e.g., targeting clusterin RNA) and antisenses (e.g., targeting clusterin RNA).

Exemplary embodiments of antisense include for example, those described in U.S. Patent No. 7,569,551 (the entire content of which is incorporated herein by reference) and especially include OGX-011 (a.k.a., custirsen sodium, OncoGenex, see also SEQ ID NO.:61).

Exemplary embodiment of EGFR inhibitors include tyrosine kinase inhibitors such as, for example, gefitinib, erlotinib, imatinib, lapatinib or semazinib. Other exemplary embodiments of EGFR inhibitors include for example, monoclonal antibodies such as cetuximab, panitumumab, nimotuzumab, or metuzumab.

Other individuals who would benefit from treatment with the pharmaceutical combinations of the present invention include those that have a tumor which is resistant to one or more EGFR inhibitors. Such individuals may be selected prior to administration of the pharmaceutical combination.

EGFR resistance may be determined by evaluating clinical parameters such as tumor relapse or by measuring molecular markers of resistance, e.g., mutations, amplifications in EGFR or in the EGFR pathway (RAS/MAPK, phospholipase C, phosphatidylinositol 3-kinase/AKT, SRC/FAK pathways, etc.) and/or EMT markers.

Testing for resistance to an EGFR inhibitor may thus includes determining the presence of mutation in EGFR (e.g., mutation in the tyrosine kinase domain, truncating mutations, insertions), determining EGFR amplification.

As used herein "pharmaceutically acceptable carrier" or "pharmaceutical carrier" are known in the art and include, but are not limited to, 0.01-0.1 M or 0.05 M phosphate buffer or 0.8 % saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's orfixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

The experiments described herein were carried out with a humanized form of 16B5 (h16B5).

EXAMPLES

Example 1

NSCLC cell lines express sCLU

We wished to determine if clusterin was expressed in cell lines derived from NSCLC tumors. As a first step, total RNA was prepared from each cell line and used as a template to prepare cDNA with random primed oligonucleotides. RT-PCR was carried out using methods known to those skilled in the art with human clusterin gene-specific oligonucleotides. The 5'-primer (ogs1788) is encoded by the sequence shown in SEQ ID NO:57 and the 3'-primer (ogs1721) is encoded by the sequence shown in SEQ ID NO:58. The PCR product is 1412 bp in length. As a control for the amount of total RNA in each reaction, a parallel RT-PCR reaction was performed with oligonucleotides specific for the human house-keeping gene actin. The 5'-primer (ogs387) is encoded by the sequence shown in SEQ ID NO:59 and the 3'-primer (ogs965) is encoded by the sequence shown in SEQ ID NO:60. The PCR product is 746 bp in length. As shown in Figure 1, all NSCLC cell lines analyzed except for one, contained mRNA encoding clusterin, which was detected at different levels. As expected, actin was present in all RNA samples indicating that there was an approximately equal amount of starting total RNA in each RT-PCR reaction. The cell lines used in this analysis were: lane 1, A549; lane 2, EKVX; lane 3, HOP-62; lane 4, HOP-92; lane 5, H322M; lane 6, H226; lane 7, H23; lane 8, H460; and lane 9, H522.

In parallel, we determined if sCLU was secreted by the NSCLC cell lines. A549, H226, H292, H460 and H1299 cells were purchased from ATCC (Manassas, VA) and cultured according to the manufacturer's instructions. Following several days in culture and when the cells reached confluence, the conditioned medium from each cell line was collected for analysis. A commercial ELISA kit (BioVendor LLC, Candler, NC) designed to measure human clusterin was obtained and the analysis was conducted according to the manufacturer's instructions. As shown in the table of Figure 2, all five media samples contained sCLU levels that ranged from 13.6 ng/ml to greater than 100 ng/ml, with the exception of the H226 cell line which had levels below 10 ng/ml.

Taken together, these results show that cancer cell lines derived from patients with NSCLC secrete sCLU in abundance and have the potential of responding to antibodies, such as h16B5, that inhibit the EMT-inducing activity of sCLU.

EXAMPLE 2

Incubation of NSCLC cells with h16B5 leads to an increase in the expression of the epithelial cell marker, E-cadherin

We also examined the expression of E-cadherin by monitoring its expression on the surface of A549 cells using immunofluorescence. Briefly, the cells were seeded on coverslips and incubated with either a control IgG or h16B5 at 10 μg/ml for 48h. Following this incubation, the cells were fixed with paraformaldehyde and incubated with a mouse anti-human E-cadherin antibody (manufacturer) for 1h. After washing, the E-cadherin stained cells were incubated with a secondary antibody conjugated to Rhodamine Red-X. The slides were mounted and specific E-cadherin staining was visualized by fluorescence microscopy. As shown in Figure 3, A549 cells have low expression of E-cadherin on their cell surface and incubation for 48h with a non-specific IgG did not increase the level of this protein (see left panel). By contrast, incubation of the cells with h16B5 caused a marked increase in the intensity of E-cadherin staining on the surface of A549 cells (see right panel). This indicates that the epithelial character of the cells is very high.

EXAMPLE 3

Inhibition of sCLU with h16B5 causes an increase in EGFR expression and phosphorylation in NSCLC cells

In this example, we examine if inhibiting EMT using h16B5 resulted in any effect on the status of EGFR in NSCLC cell lines. In order to address this question, conditions were optimized to measure the expression and phosphorylation of the receptor in these cells. EGFR is known to be phosphorylated very rapidly following exposure to its ligands, including EGF. In parallel with this phosphorylation, the receptor is internalized and recycled and its presence is lost from the cell surface. Two cell lines, A549 and H226, which secreted relatively high levels of sCLU were selected for this analysis. The cells were seeded in multi-well plates and treated for 48h with 10 μg/ml h16B5, 10 μg/ml

control IgG or the vehicle, PBS. Following this incubation, the cells were treated with EGF (10 ng/ml) and the cells were harvested at different times and converted to lysates. These lysates were electrophoresed by SDS-PAGE, the proteins transferred to a nylon membrane and used in Western blots to examine the expression or phosphorylation of EGFR. To measure the expression of EGFR, a commercial antibody designated clone 1005 (Santa Cruz, Biotech, Santa Cruz, CA) was used whereas the phosphorylation of EGFR was monitored with an anti-phosphotyrosine antibody designated clone 4G10 (Millipore, Etobicoke, ON). As shown in Figure 4, A549 cells that were treated with the vehicle control for 48h followed by EGFR displayed a decrease in EGFR expression, as expected, which is rapidly restored after 10 min (upper panel: compare lane 1 with lane 2). By contrast, A549 cells treated with h16B5 anti-sCLU antibody protected the cells from the EGF-induced degradation of the receptor (upper panel: compare lane 5 with lane 6). To control for the amount of protein in each lane, the membrane was re-blotted with an anti-actin antibody (clone AC-15, Sigma, Oakville, ON) which was expressed equally among the different samples separated on the gel. The other NSCLC cell line H226 was examined using the same approach (lower panel) and the results that were observed were similar to those observed with A549 cells. In this case, the EGF-induced degradation of the EGFR was even more pronounced when the cells were treated with the control for 48h (lower panel, lanes 1 – 4). In the case of the cells treated with h16B5, no decrease in EGFR was seen (lower panel, lanes 5 – 8). This again showed that inhibition of sCLU with an antibody results in the maintenance of EGFR expression. Thus, these findings suggest that the cells would display increased sensitivity to EGFR inhibitors.

The activation of EGFR in these cells was also examined. In this instance, the membranes were blotted with an antibody that interacts only with the tyrosine-phosphorylated form of the receptor. In A549 cells, no phosphorylation of EGFR was seen in the absence of EGF (see Figure 5, upper panel, lane 1 and lane 5) as expected. However, when the cells were treated with EGF, the appearance of phosphorylated tyrosine residues was detected, even after 2 min (upper panel, lane 2 and lane 6). The cells treated with h16B5 for 48h showed a higher amount of phosphorylated tyrosine residues on the EGFR compared to the cells that were left untreated. This difference in the phosphorylation of EGFR was reproduced in the H226 cells (see Figure 5, lower panel, compare lanes 2 – 4 with lanes 6 – 8). As an additional control, the A549 cells

were also treated with a control antibody for 48h prior to being exposed to EGF (Figure 6). Again, the change in the amount of phosphorylation of EGFR was only seen in the cells that were treated with h16B5 (compare lanes 2, 3 (control) and 8, 9 (IgG) with lanes 5, 6 (h16B5)).

Taken together our results show that blocking EMT in lung cancer cells influences the EGFR status in these cells. In particular, inhibition of sCLU EMT-inducing activity with a monoclonal antibody, such as h16B5, is one of the mechanisms by which the EGFR status in these cancer cells is altered. We showed that the lung cancer cells that were treated with h16B5 have increased expression of E-cadherin and are thus more epithelial. The combination of increased EGFR sensitivity with inhibition of EMT by blocking sCLU with an antibody, is expected to increase the efficacy of EGFR inhibitors. Finally, any cancer cells that express EGFR and undergo EMT, are expected to respond to an inhibitor of sCLU, such as a monoclonal antibody, in a similar manner to the lung cancer cells.

EXAMPLE 4

A method for increasing the sensitivity of cancer cells to EGFR inhibitors in the presence of h16B5

Inhibiting EMT in cancer cells by blocking sCLU with an anti-clusterin antibody leads to increased EGFR expression on the surface of the cells or increased EGFR phosphorylation or both. Thus inhibitors of EGFR are expected to have increased efficacy under conditions where EMT is inhibited with clusterin inhibitors.

For example, cancer cell lines are seeded in multiwell plates and when close to confluence, cells are treated with an anti-clusterin antibody (e.g., h16B5) to inhibit EMT. It may be useful to induce EMT, *a priori*, with known inducers such as sCLU, TGFβ, ligands of EGFR such as EGF or other similar molecules.

The EGFR inhibitor (e.g., monoclonal antibodies that block ligand binding to the receptor or that prevent the dimerization of EGFR, TK inhibitors, etc.) is also added to the wells either together with the anti-clusterin antibody or later (e.g., a few hours later). In some instance the EGFR inhibitor may be added to the wells prior to the anti-clusterin antibody.

The EGFR inhibitor may be added at different concentrations ranging from one fmol/L to one hundred micromol/L. To determine if the cytotoxicity of the EGFR inhibitors is increased when sCLU is inhibited with h16B5, the number of cells remaining is determined using standard protocols such as proliferation assays, invasion assays, apoptosis assays or migration assays. The cancer cells appropriate for this assay include EGFR inhibitor-resistant cancer cells or cancer cells that express wild type EGFR, EGFR containing activating mutations, EGFR gene amplifications and other situations where the status of EGFR might be altered.

The clusterin inhibitor and EGFR inhibitor combination may also be tested *in vivo* in well established models of cancer. For example, human cancer cell lines that express EGFR are injected in immunocompromised mice and allowed to grow until tumor xenografts are implanted. The animals are treated with an anti-clusterin antibody (e.g., 16B5, h16B5 or else) to block EMT in combination with a EGFR inhibitor (administered concurrently or sequentially). The growth of the tumors is monitored by various methods including direct size measurements with instruments such as a calliper. Other methods used to measure tumor growth might include fluorescence or bioluminescence in the case where the tumor cells are genetically modified to express fluorescent or bioluminescent molecules. In another instance, the tumors growth could be monitored using positron emission tomography (PET) or computed tomography (CT) scanning approaches. In these assays, The clusterin inhibitor and the EGFR inhibitor may be administered repeatedly by different routes including intravenous, sub-cutaneous, intra-muscular, intra-tumoral or orally. Typical doses would range from 1 microgram/kg to 100 mg/kg.

Based on the application of this method, it will be possible to demonstrate that treatment of an agent that blocks EMT, such as 16B5, h16B5 or else, in combination with an inhibitor of EGFR will result in an enhanced anti-tumor effect compared with either agent administered separately.

In this example, we demonstrate that the response of cancer cells to EGFR inhibitors can be increased when administered in combination with an inhibitor of sCLU, such as h16B5. Two NCSLC cell lines, H1299 and H460, which are known to express wild type EGFR (Akashi et al., 2008), were treated with either sCLU (0.25 μg/ml), TGFβ (2 nM) or the combination of the two proteins for 48h to stimulate EMT. Following this induction, the cells were treated with erlotinib (20 μM) for 96h and the number of cells was

determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). As a control, additional cells were not induced with sCLU or TGFβ. As shown in Figure 7A, H1299 cells were more sensitive to erlotinib than the H460 cells. Combination of erlotinib with h16B5 resulted in a further decrease in the cell number (10.2% and 13.8% less H1299 and H460 cells, respectively) indicating that blocking the activity of endogenously expressed sCLU (see Figure 2) enhanced the ability of erlotinib to kill EGFR-positive lung cancer cells. We also compared the response to erlotinib in cells that were induced to undergo an EMT with either sCLU or TGFβ. As shown in Figure 7B, stimulation of EMT did not change the responsiveness of the cells to erlotinib. However, the decrease in cell number was larger in the presence of h16B5 under these conditions compared to cells not undergoing EMT (16.1% and 24.0% for H1299 and H460 cells, respectively). This indicates that the increase of erlotinib-response is greater in cells when EMT is inhibited. It was noteworthy to observe that the increase in erlotinib responsiveness was greater in H460 cells, which are more resistant to this EGFR inhibitor. This last result illustrates the potential of sCLU inhibition with h16B5 as a strategy to treat lung cancer patients who become resistant to EGFR inhibitors.

EXAMPLE 5

Pharmaceutical combination for use in other cancer indications

The breast cancer cell line MDA-MB-231, which is known to be triple negative (i.e., lack of expression of the estrogen and progesterone receptors, and absence of HER2 - expression) or basal-like was treated with an anti-clusterin antibody in combination with gefitinib or with gefitinib alone. Cell growth was measured over a period of several days by standard assays.

This experiment shows that the combination of the anti-clusterin antibody with gefitinib is more effective at inhibiting tumor cell growth that gefitinib alone (data not shown).

Other clusterin inhibitors and/or EGFR inhibitors combination may be tested using similar techniques as those described in Examples 1-5.

CLAIMS

1. A pharmaceutical combination comprising a clusterin inhibitor and an EGFR inhibitor.

2. The pharmaceutical combination of claim 1, wherein the clusterin inhibitor is capable of inhibiting epithelial-to-mesenchymal transition.

3. The pharmaceutical combination of claim 1 or 2, wherein the clusterin inhibitor is an anti-clusterin antibody or an antigen binding fragment thereof.

4. The pharmaceutical combination of claim 3, wherein the anti-clusterin antibody or antigen binding fragment binds to amino acids 421 to 443 of a C-terminal portion of a β-subunit of human clusterin.

5. The pharmaceutical combination of any one of claims 1 to 4, wherein the EGFR inhibitor is a tyrosine kinase inhibitor or an anti-EGFR antibody or antigen binding fragment thereof.

6. The pharmaceutical combination of claim 5, wherein the tyrosine kinase inhibitor is gefitinib, erlotinib, imatinib, lapatinib or semazinib.

7. The pharmaceutical combination of claim 5, wherein the tyrosine kinase inhibitor is gefitinib.

8. The pharmaceutical combination of claim 5, wherein the antibody is cetuximab, panitumumab, nimotuzumab, or metuzumab.

9. A method of treating carcinoma comprising administering a clusterin inhibitor and an EGFR inhibitor to an individual having or suspected of having carcinoma.

10. The method of claim 9, wherein the clusterin inhibitor is capable of inhibiting epithelial-to-mesenchymal transition (EMT).

11. The method of claim 9 or 10, wherein the clusterin inhibitor is an anti-clusterin antibody or an antigen binding fragment thereof.

12. The method of claim 11, wherein the anti-clusterin antibody or antigen binding fragment binds to amino acids 421 to 443 of a C-terminal portion of a β-subunit of human clusterin.

13. The method of any one of claims 9 to 12, wherein the EGFR inhibitor is a tyrosine kinase inhibitor or an anti-EGFR antibody or antigen binding fragment thereof.

14. The method of claim 13, wherein the tyrosine kinase inhibitor is gefitinib, erlotinib, imatinib, lapatinib or semazinib.

15. The method of claim 13, wherein the tyrosine kinase inhibitor is gefitinib.

16. The method of claim 13, wherein the antibody is cetuximab, panitumumab, nimotuzumab, or metuzumab.

17. The method of claim 9, wherein the anti-clusterin inhibitor and the EGFR inhibitor are administered sequentially.

18. The method of claim 16, wherein the anti-clusterin inhibitor is administered prior to the EGFR inhibitor.

19. The method of claim 9, wherein the anti-clusterin inhibitor is administered concurrently with the EGFR inhibitor.

20. The method of claim 9, comprising testing for reversal of an epithelial-to-mesenchymal phenotype of carcinoma cells before administration of the EGFR inhibitor.

21. The method of any one of claims 9 to 20, wherein the carcinoma is prostate carcinoma, breast carcinoma, endometrial carcinoma, ovarian carcinoma, hepatocellular carcinoma, colorectal carcinoma, head and neck carcinoma, lung carcinoma, pancreatic cancer or renal cell carcinoma.

22. The method of claim 21, wherein the breast carcinoma is triple negative breast cancer or basal-like breast cancer.

23. The method of claim 21, wherein the lung carcinoma is non-small cell lung cancer.

24. The method of any one of claims 9 to 23, wherein the carcinoma comprises cells characterized as being resistant to an EGFR inhibitor.

25. The method of any one of claims 9 to 23, wherein the carcinoma comprises cells expressing EMT markers.

26. The method of any one of claims 9 to 25, wherein the antibody or antigen binding fragment thereof comprises a complementary determining region as set forth in SEQ ID NO.:1, SEQ ID NO.:2, SEQ ID NO.:3, SEQ ID NO.:4, SEQ ID NO.:5, SEQ ID NO.:6, SEQ ID NO.:45, SEQ ID NO.:46, SEQ ID NO.:47, SEQ ID NO.:48, SEQ ID NO.:49 or SEQ ID NO.:50.

27. The method of claim 26, wherein the antibody or antigen binding region comprises a CDRH1 as set forth in SEQ ID NO.: 1, a CDRH2 as set forth in SEQ ID NO.:2 and a CDRH3 as set forth in SEQ ID NO.:3.

28. The method of claim 26, wherein the antibody or antigen binding region comprises a CDRH1 as set forth in SEQ ID NO.: 45, a CDRH2 as set forth in SEQ ID NO.:46 and a CDRH3 as set forth in SEQ ID NO.:47.

29. The method of claim 27 or 28, wherein the antibody or antigen binding region comprises a CDRL1 as set forth in SEQ ID NO.:4, a CDRL2 as set forth in SEQ ID NO.:5, a CDRL3 as set forth in SEQ ID NO.:6.

30. The method of claim 27 or 28, wherein the antibody or antigen binding region comprises a CDRL1 as set forth in SEQ ID NO.:48, a CDRL2 as set forth in SEQ ID NO.:49 and a CDRL3 as set forth in SEQ ID NO.:50.

31. Use of the pharmaceutical combination of any one of claims 1 to 9 for the treatment of carcinoma.

32. Use of the pharmaceutical combination of any one of claims 1 to 9 in the manufacture of a medicament for the treatment of carcinoma.

33. A kit comprising a clusterin inhibitor and an EGFR inhibitor.

34. The kit of claim 33, wherein the clusterin inhibitor is an antibody or an antigen binding fragment thereof.

Figure 1

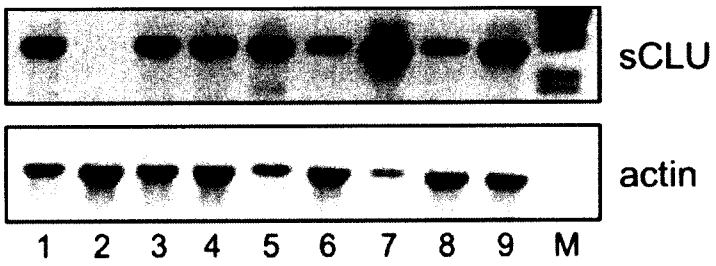


Figure 2

Cell line	[sCLU] (ng/ml)
A549	71.9
H226	<10
H292	40.8
H460	103
H1299	13.6

Figure 3

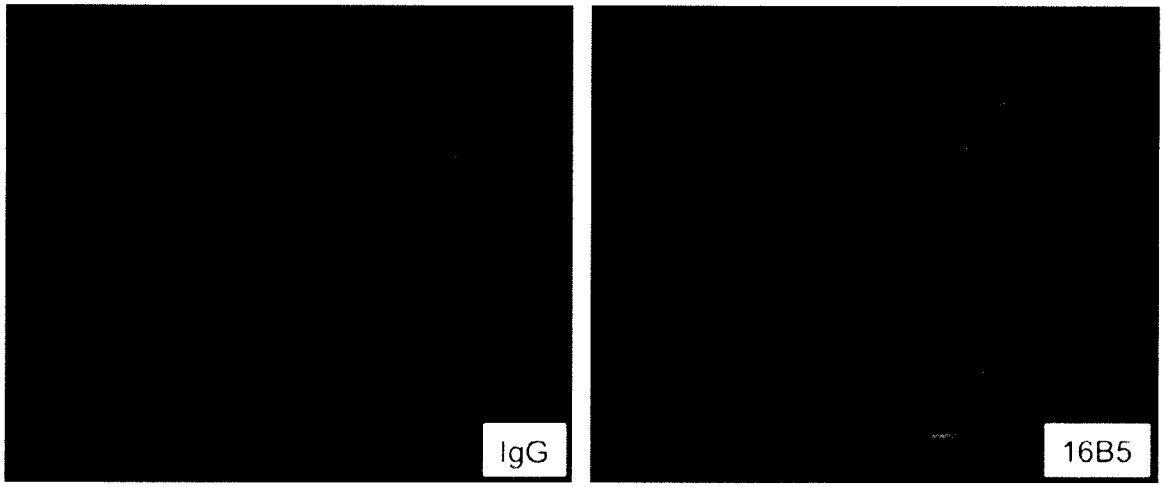
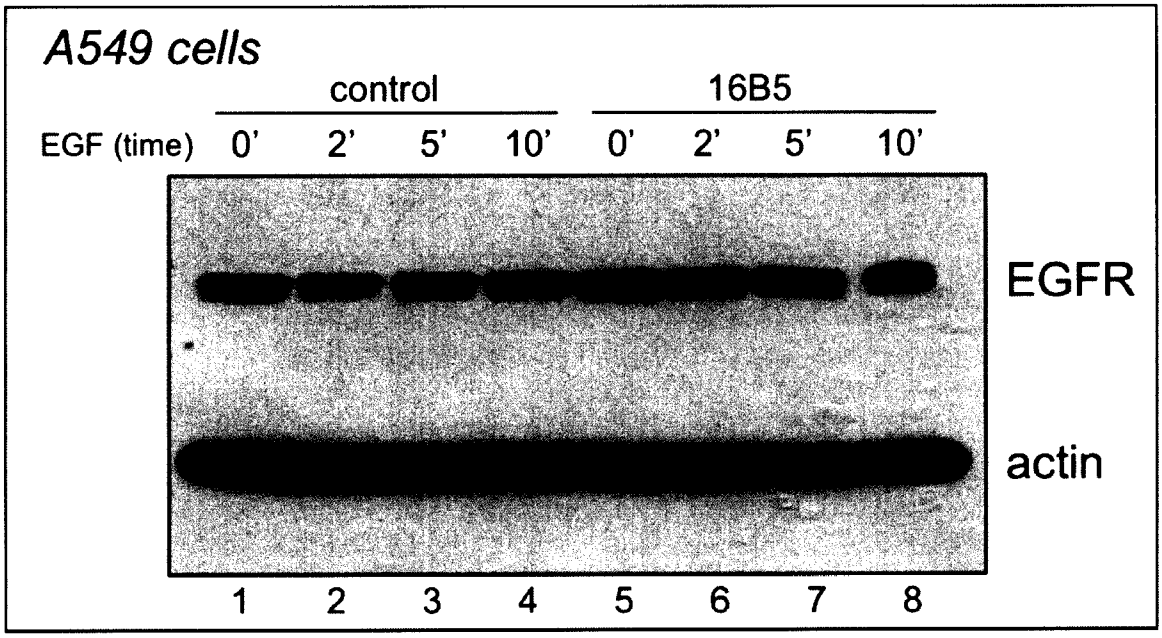


Figure 4


A549 cells
control
16B5
EGF (time)
0'
2'
5'
10'
0'
2'
5'
10'
EGFR
actin
1
2
3
4
5
6
7
8

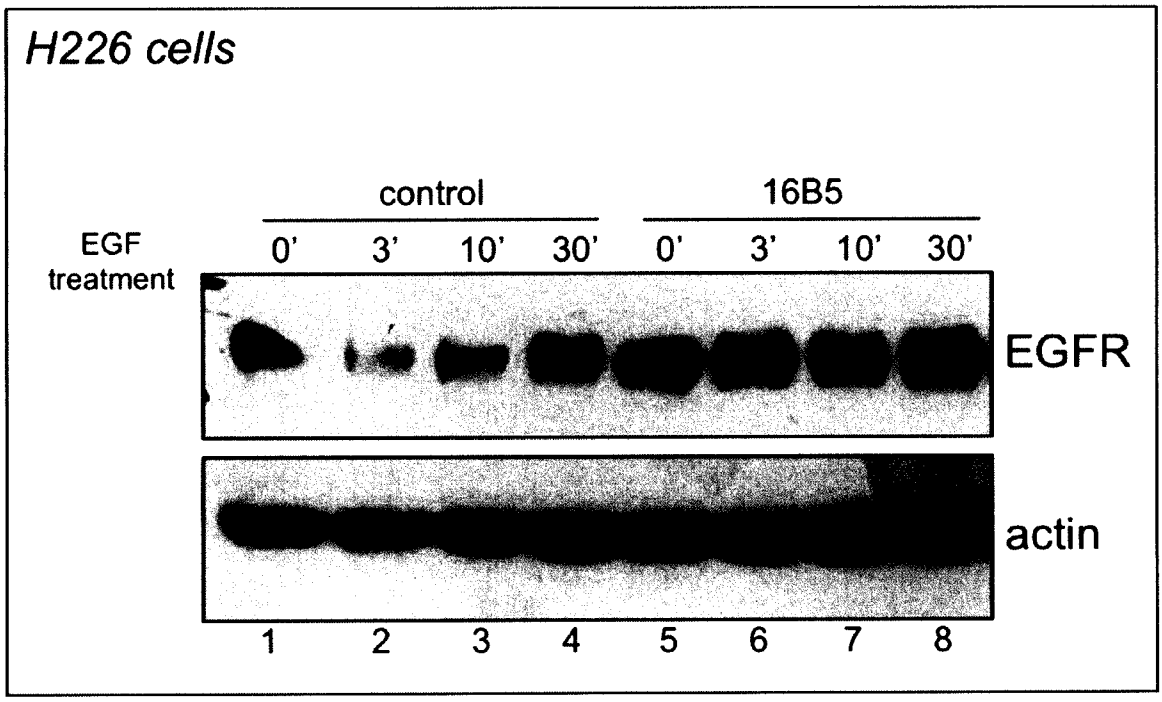
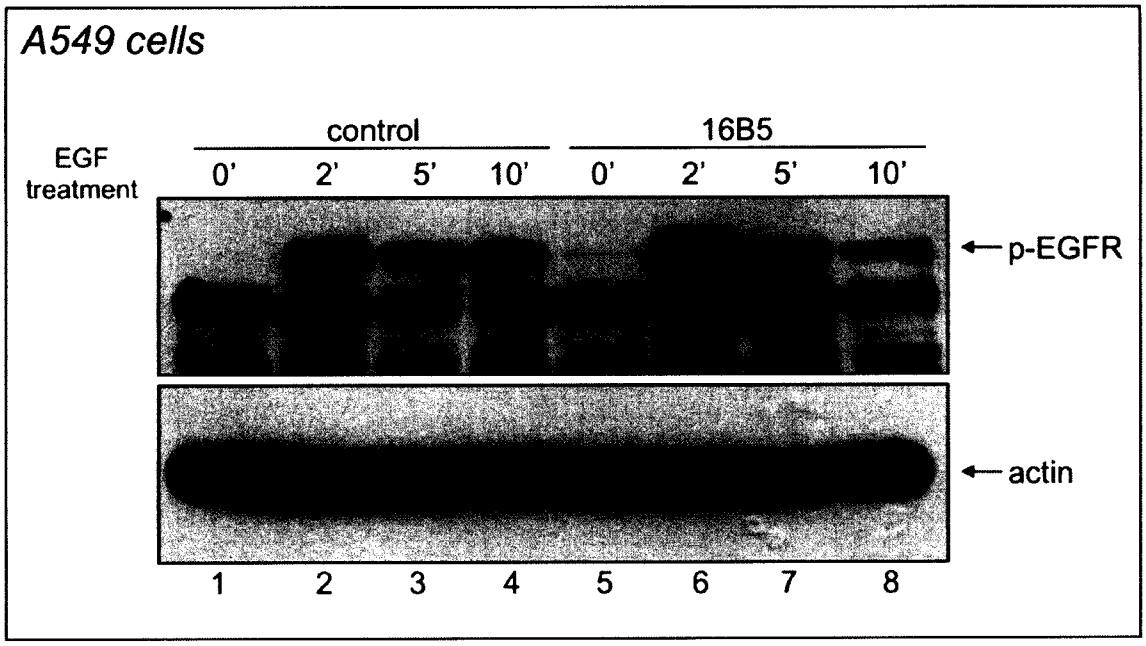

H226 cells
control
16B5
EGF treatment
0'
3'
10'
30'
0'
3'
10'
30'
EGFR
actin
1
2
3
4
5
6
7
8

Figure 5


A549 cells
control
16B5
EGF treatment
0' 2' 5' 10' 0' 2' 5' 10'
p-EGFR
actin
1 2 3 4 5 6 7 8

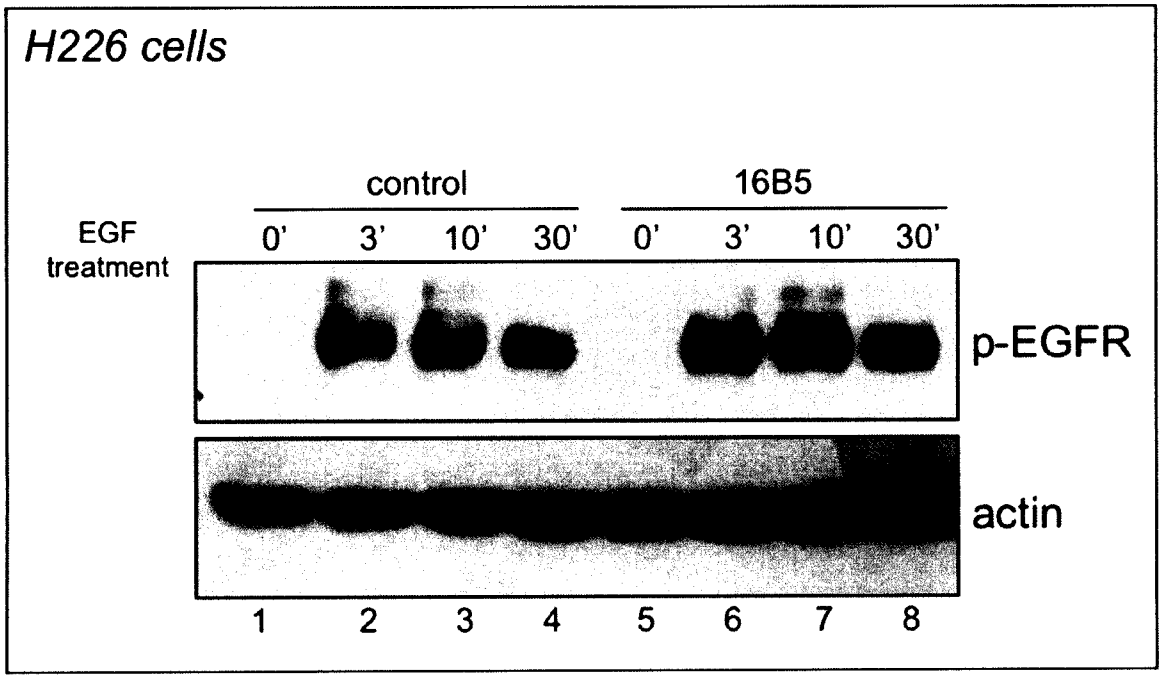

H226 cells
control
16B5
EGF treatment
0' 3' 10' 30' 0' 3' 10' 30'
p-EGFR
actin
1 2 3 4 5 6 7 8

Figure 6

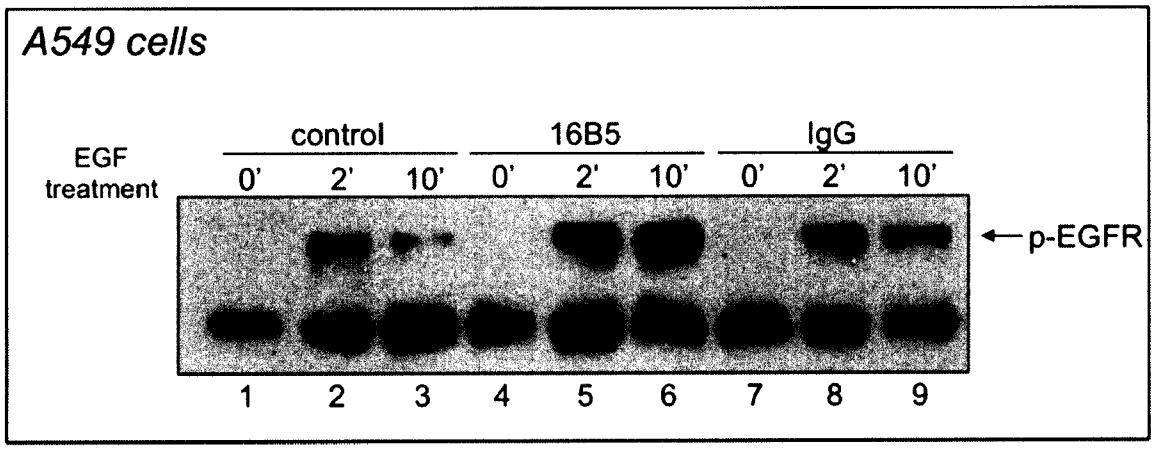
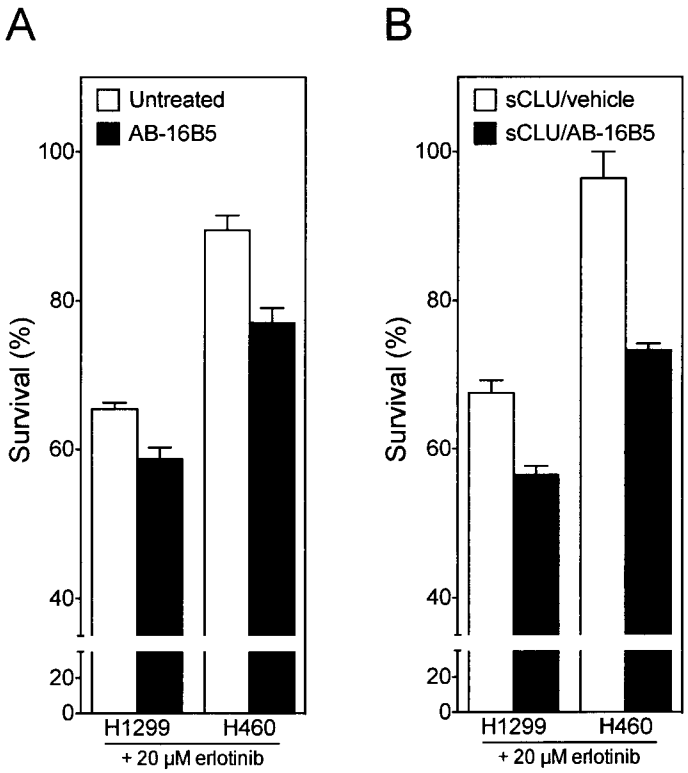

A549 cells
EGF treatment
control
16B5
IgG
0'
2'
10'
0'
2'
10'
0'
2'
10'
p-EGFR
1
2
3
4
5
6
7
8
9

Figure 7


A
Untreated
AB-16B5
Survival (%)
100
80
60
40
20
0
H1299
H460
+ 20 μM erlotinib
B
sCLU/vehicle
sCLU/AB-16B5
Survival (%)
100
80
60
40
20
0
H1299
H460
+ 20 μM erlotinib