ANTIBODIES AGAINST EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND USES THEREOF

Background

The natural immune system has evolved to make antibodies for efficient neutralization of pathogens. Natural antibody preparations isolated from immunized animals are polyclonal in origin, and exhibit immunodominance as compared to individual antibodies, which are restricted to one or a few epitopes of a particular antigen. Neutralizing antibodies are able to block a biological function of the antigen to which they bind. Mixtures of neutralizing antibodies may achieve neutralization that is greater than any individual antibody in the mixture.

Such results have been achieved by combining two or more neutralizing antibodies against the epidermal growth factor receptor, EGFR (ErbB1). Antibodies that bind to and inhibit EGFR have proven to provide useful anti-cancer benefits and are of great medical and commercial value. Particular combinations of pairs of antagonistic, yet non-competitive, anti-EGFR antibodies resulted in downregulation of EGFR which was faster and more effective than application of either antibody alone (Friedman *et al.* (2005) PNAS 102:1915-1920). The combination of two cross-competitive (*i.e.*, competitive with each other for binding to antigen) anti-EGFR antibodies has shown to be non-synergistic. It is possible that binding of a plurality of antibodies to distinct epitopes of EGFR forms lattices of complexed receptors on cell surfaces, leading to more efficient internalization and degradation than obtained with antibodies targeting a single epitope. The combination of a particular pair of anti-EGFR receptor antibodies have also been reported to result in additive and in some cases synergistic, antitumor activity *in vivo* (Perera *et al.* (2005) Clin Cancer Res 11:6390-6399). Monoclonal antibody 806, raised against the mutant de2-7 EGFR, combined with antagonistic antibody 528 displayed significantly higher anti-tumor activity in a glioma xenograft model than treatment with either antibody alone. The mechanism of the synergistic anti-tumor activity was shown to be associated with rapid downregulation of EGFR, which was not induced by treatment with the individual antibodies. Similarly EGFR phosphorylation was greatly reduced in the presence of another pair of anti-EGFR antibodies, cetuximab and EMD55900 (Kamat *et al.* (2008) Cancer Biol Ther 7:726-33).

Certain combinations of antibodies targeting the related receptor, ErbB2, have also been shown to function in synergy (Friedman *et al.* (2005). Trastuzumab combined with pertuzumab inhibited the survival of BT474 breast cancer cells at doses in which individual antibodies are ineffective (Nahta *et al.* (2004) Cancer Res 64:2343-2346). In another study three non-competitive anti-ErbB2 antibodies demonstrated far more effective *in vitro* killing of BT474 cells in combination than individually and similar results were obtained in a BT474 *in vivo* xenograft model (Spiridon *et al.* (2002) Clin Cancer Res 8:1699-701).

Other evidence that combining more than one antibody may enhance the growth suppressive (*e.g.*, cytotoxic) effect of antibodies on tumor cells has been reported. For example, monoclonal antibodies to the tumor antigen 17-1A were combined, tumor cell lysis was studied, and it was found that monoclonal antibodies, as well as combinations of competing antibodies, were ineffective, whereas combinations of two or more non-competing antibodies resulted in complete tumor cell lysis.

Accordingly, additional approaches and methods for producing combinatorial action so as to enhance the responsiveness of tumors to anti-EGFR antibody combinations are still needed, including combinations that enhance signaling inhibition and combinations that provide more effective cytostatic or cytotoxic outcomes.

Summary

Novel monoclonal antibodies that bind to EGFR and inhibit various EGFR functions are provided herein. These antibodies, when combined with each other or with other anti-ErbB receptor antibodies (*e.g.*, other anti-EGFR antibodies), are capable of exhibiting a synergistic or additive therapeutic effect compared to the administration of each antibody alone. These antibodies, particularly when administered in combinations as herein provided, are useful for treating a variety of disorders (*e.g.*, cancers) associated with EGFR-mediated cellular signaling. Accordingly, isolated combinations of novel monoclonal antibodies that bind to EGFR and inhibit various EGFR functions are also provided herein. Uses of these antibodies for diagnostic and therapeutic purposes are also provided, as are uses of the antibody combinations herein disclosed.

In one embodiment, monoclonal antibodies that bind EGFR and combinations of such antibodies are provided. These antibodies and combinations exhibit one or more of the following properties:

(a) inhibition of AKT or ERK phosphorylation, *e.g.*, EGFR-dependant AKT or ERK phosphorylation, as measured in a cell-based assay;

(b) inhibition of the growth of cells expressing EGFR;

(c) inhibition of EGF ligand binding to EGFR (*e.g.*, inhibition of binding of one or more ligands that bind EGFR, including EGF, transforming growth factor (TGF), or amphiregulin);

(d) inhibition of EGFR dimerization; or

(e) downregulation of EGFR on cell surfaces (*e.g.*, by internalization and recycling of the receptor, and/or internalization and degradation of the receptor).

In a particular embodiment, the antibodies (*i.e.*, when combined) bind non-overlapping epitopes, as determined using a surface plasmon resonance assay (*e.g.*, BIACORE) or FACS, or other such assays. In another particular embodiment, the antibodies additively or synergistically provide at least one of the functional properties described above, *i.e.*,

(a) inhibition of AKT or ERK phosphorylation, *e.g.*, EGFR-dependant AKT or ERK phosphorylation, as measured in a cell-based assay;

(b) inhibition of the growth of cells expressing EGFR;

(c) inhibition of ligand binding to EGFR (*e.g.*, inhibition of binding of one or more ligands that bind EGFR, including EGF, transforming growth factor (TGF), or amphiregulin);

(d) inhibition of EGFR dimerization; or

(e) downregulation of EGFR on cell surfaces (*e.g.*, by internalization and recycling of the receptor, and/or internalization and degradation of the receptor).

In another embodiment, the individual monoclonal antibodies inhibit the actions of EGFR ligands, yet do not cross-compete (*i.e.*, they bind to distinct epitopes).

Particular anti-EGFR monoclonal antibodies provided herein include those that comprise a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:1-11 or a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 12-17 and 19-22. Antibodies herein provided also include those that comprise a heavy and a light chain variable region comprising the following amino acid sequences:

TABLE I

*	Heavy Chain		Light Chain
va	SEQ ID NO:9	and	SEQ ID NO:20;

*	TABLE I Heavy Chain		Light Chain
vb	SEQ ID NO:10	and	SEQ ID NO:21;
vc	SEQ ID NO:11	and	SEQ ID NO:22;
vd	SEQ ID NO:1	and	SEQ ID NO:12;
ve	SEQ ID NO:2	and	SEQ ID NO:13;
vf	SEQ ID NO:3	and	SEQ ID NO:14;
vg	SEQ ID NO:4	and	SEQ ID NO:15;
vh	SEQ ID NO:5	and	SEQ ID NO:16;
vi	SEQ ID NO:6	and	SEQ ID NO:17;
vj	SEQ ID NO:8	and	SEQ ID NO:19.

* Antibody name, *i.e.*, va, vb, vc, etc.

Antibodies that bind to the same epitope on EGFR as any of the antibodies set forth in Table I are also contemplated for use in the antibody combinations disclosed herein.

Other particular antibodies herein include those that comprise the following heavy and light chain CDR3, CDR2, and CDR1, sequences (each two digit number in this Table II represents a SEQ ID NO: as set forth in Tables VI and VII below).

**	TABLE II Heavy chain (SEQ ID NO:)			Light Chain (SEQ ID NO:)		
	CDR3	CDR2	CDR1	CDR3	CDR2	CDR1
ca	34	30	29	49	45	48;
cb	37	36	35	51	45	50;
cc	38	36	35	52	45	48;
cd	31	30	29	55	54	53;
ce	32	30	29	55 or 56	54	53;
cf	33	30	29	56	54	53;
cg	25	24	23	41	40	39;
ch	26	24	23	42	40	39;
ci	27	24	23	43	40	39;
cj	28	24	23	46	45	44;
ck	28	24	23	47	45	44.

** Antibody name, *i.e.*, ca, cb, cc, etc.

Antibodies that bind to the same epitope on EGFR as any of the antibodies set forth in Table 2 are also contemplated for use in the antibody combinations disclosed herein.

Also encompassed by the present invention are monoclonal antibodies that bind to the same or overlapping epitopes bound by any of the particular antibodies described herein below.

Antibodies disclosed herein include all known forms of antibodies and other protein scaffolds with antibody-like properties. For example, the antibody can be a human antibody, a humanized antibody, a bispecific antibody, an immunoconjugate, a chimeric antibody or a protein scaffold with antibody-like properties, such as fibronectin or ankyrin repeats. The antibody also can be a Fab, Fab'2, ScFv, AFFIBODY, nanobody, or a domain antibody. The antibody also can have any isotype, including any of the following isotypes: IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, and IgE. IgG antibodies are preferred.

Exemplary combinations of two antibodies (hereinafter "pair" or "pairs") herein provided include:

TABLE III

Each named pair also encompasses pairs of one each of antibodies that compete for binding to EGFR with each of the named antibodies of the pair

***		***	
a	ca and cd;	x	cc and ck;
b	ca and ce;	y	va and vd;
c	ca and cf;	z	va and ve;
d	ca and cg	aa	va and vf;
e	ca and ch;	ab	va and vg
f	ca and ci;	ac	va and vh;
g	ca and cj;	ad	va and vi;
h	ca and ck;	ae	va and vj;
i	cb and cd;	ag	vb and vd;
j	cb and ce;	ah	vb and ve;
k	cb and cf;	ai	vb and vf;
l	cb and cg	aj	vb and vg
m	cb and ch;	ak	vb and vh;
n	cb and ci;	al	vb and vi;
o	cb and cj;	am	vb and vj;
p	cb and ck;	ao	vc and vd;

	TABLE III Each named pair also encompasses pairs of one each of antibodies that compete for binding to EGFR with each of the named antibodies of the pair		
q	cc and cd;	ap	vc and ve;
r	cc and ce;	aq	vc and vf;
s	cc and cf;	ar	vc and vg
t	cc and cg	as	vc and vh;
u	cc and ch;	at	vc and vi;
v	cc and ci;	au	vc and vj;
w	cc and cj;		

***Name of antibody pair

As set forth in the above tables, a named antibody is considered to be an antibody that will compete with itself. Also contemplated are pairs of antibodies that compete for binding to EGFR with, or bind to the same epitopes on EGFR as, the pairs of antibodies set forth in Table III.

Exemplary combinations of three of more antibodies include each pair designated a-x immediately above in combination with a third antibody not present in the particular pair and selected from cd, ce, cf, cg, ch, ci, cj, and ck and further include each binary combination designated above as y, z, aa, ab, ac, ad, ae, ag, ah, ai, aj, ak, al ,am , ao, ap, aq, ar, as, at, au ,or av in combination with a third antibody not present in the particular pair and selected from vd, ve, vf, vg, vh, vi, and vj.

Exemplary combinations of three antibodies (hereinafter "trio" or "trios") herein provided also include those named in TABLE IV and Table V below, as well as trios comprising, for each trio named in TABLE IV and Table V, below, one each of antibodies that compete for binding to EGFR with each of the antibodies of that named trio. The antibodies disclosed herein were categorized into groups, or "bins" based on competition binding experiments, and are described accordingly in the Examples and Figures. Bin1 antibodies all compete with each other for binding to the extracellular domain of EGFR, Bin2 antibodies all compete with each other for binding to the extracellular domain of EGFR, and Bin3 antibodies all compete with each other for binding to the extracellular domain of EGFR. However, Bin1 antibodies do not compete with Bin 2 antibodies or Bin 3 antibodies for binding to the extracellular domain of EGFR, Bin2 antibodies do not compete with Bin 1 antibodies or Bin 3 antibodies for binding to the extracellular domain of EGFR, and Bin3 antibodies do not compete with

Bin 1 antibodies or Bin 2 antibodies for binding to the extracellular domain of EGFR. Thus each Bin corresponds to a distinct epitope to which the antibodies in that Bin will bind. Bin1, Bin2, and Bin3 antibodies are referred to in Tables IV and V below as "1st Antibody," "3rd Antibody," and "2nd Antibody," respectively.

TABLE IV

Trio Name	1st antibody (Bin1)	2nd Antibody (Bin3)	3rd Antibody (Bin2)
1c	ca	cg	cd
2c	ca	cg	ce
3c	ca	cg	cf
4c	ca	ch	cd
5c	ca	ch	ce
6c	ca	ch	cf
7c	ca	ci	cd
8c	ca	ci	ce
9c	ca	ci	cf
10c	ca	cj	cd
11c	ca	cj	ce
12c	ca	cj	cf
13c	ca	ck	cd
14c	ca	ck	ce
15c	ca	ck	cf
16c	cb	cg	cd
17c	cb	cg	ce
18c	cb	cg	cf
19c	cb	ch	cd
20c	cb	ch	ce
21c	cb	ch	cf
22c	cb	ci	cd
23c	cb	ci	ce
24c	cb	ci	cf
25c	cb	cj	cd

TABLE IV			
Trio Name	**1st antibody (Bin1)**	**2nd Antibody (Bin3)**	**3rd Antibody (Bin2)**
26c	cb	cj	ce
27c	cb	cj	cf
28c	cb	ck	cd
29c	cb	ck	ce
30c	cb	ck	cf
31c	cc	cg	cd
32c	cc	cg	ce
33c	cc	cg	cf
34c	cc	ch	cd
35c	cc	ch	ce
36c	cc	ch	cf
37c	cc	ci	cd
38c	cc	ci	ce
39c	cc	ci	cf
40c	cc	cj	cd
41c	cc	cj	ce
42c	cc	cj	cf
43c	cc	ck	cd
44c	cc	ck	ce
45c	cc	ck	cf

TABLE V			
Trio Name	**1st antibody (Bin1)**	**2nd Antibody (Bin3)**	**3rd Antibody (Bin2)**
1v	va	vi	vd
2v	va	vi	ve
3v	va	vi	vf
4v	va	vi	vg
5v	va	vi	vh
6v	va	vj	vd

TABLE V			
Trio Name	**1st antibody (Bin1)**	**2nd Antibody (Bin3)**	**3rd Antibody (Bin2)**
7v	va	vj	ve
8v	va	vj	vf
9v	va	vj	vg
10v	va	vj	vh
11v	vb	vi	vd
12v	vb	vi	ve
13v	vb	vi	vf
14v	vb	vi	vg
15v	vb	vi	vh
16v	vb	vj	vd
17v	vb	vj	ve
18v	vb	vj	vf
19v	vb	vj	vg
20v	vb	vj	vh
21v	vc	vi	vd
22v	vc	vi	ve
23v	vc	vi	vf
24v	vc	vi	vg
25v	vc	vi	vh
26v	vc	vj	vd
27v	vc	vj	ve
28v	vc	vj	vf
29v	vc	vj	vg
30v	vc	vj	vh

As set forth in the above Tables I-V, a named antibody is considered to be an antibody that will compete with itself for binding to EGFR.

Also encompassed by the invention are trios of antibodies that compete for binding to EGFR with, or bind to the same epitope on EGFR as, any of the trios of antibodies set forth in Table IV above.

As set forth in Table IV, Bin1 antibodies include antibodies va, vb and vc (as defined in Table I) and antibodies ca, cb and cc (as defined in Table II). Bin2 antibodies

include antibodies vd, ve, vf, vg and vh (as defined in Table I) and antibodies cd, ce and cf (as defined in Table II). Bin3 antibodies include antibodies vi and vj (as defined in Table I) and antibodies cg, ch, ci, cj and ck (as defined in Table II).

Also provided are compositions of pairs of anti-EGFR antibodies (two antibodies, or duo of antibodies), in which one antibody of the pair competes for binding with, or binds to the same epitope as, a Bin1 antibody as defined herein, and the second antibody of the pair competes for binding with, or binds to the same epitope as, a Bin2 antibody as defined herein. Thus, in one embodiment, a composition of a pair of anti-EGFR antibodies is provided comprising a first antibody and a second antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of va, vb and vc or an antibody selected from the group consisting of ca, cb and cc and (ii) the second antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vd, ve, vf, vg and vh or an antibody selected from the group consisting of cd, ce and cf.

Compositions of pairs of anti-EGFR antibodies (two antibodies, or duo of antibodies), are also provided in which one antibody of the pair competes for binding with, or binds to the same epitope as, a Bin1 antibody as defined herein, and the second antibody of the pair competes for binding with, or binds to the same epitope as, a Bin3 antibody as defined herein. Thus, in one embodiment, a composition of a pair of anti-EGFR antibodies is provided comprising a first antibody and a second antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of va, vb and vc or an antibody selected from the group consisting of ca, cb and cc and (ii) the second antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vi and vj or an antibody selected from the group consisting of cg, ch, ci, cj and ck.

Also provided are compositions of pairs of anti-EGFR antibodies (two antibodies, or duo of antibodies), in which one antibody of the pair competes for binding with, or binds to the same epitope as, a Bin2 antibody as defined herein, and the second antibody of the pair competes for binding with, or binds to the same epitope as, a Bin3 antibody as defined herein. Thus, in one embodiment, a composition of a pair of anti-EGFR antibodies is provided comprising a first antibody and a second antibody, wherein

(i) the first antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vd, ve, vf, vg and vh or an antibody selected from the group consisting of cd, ce and cf and (ii) the second antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vi and vj or an antibody selected from the group consisting of cg, ch, ci, cj and ck.

Also provided are compositions of trios of anti-EGFR antibodies (three antibodies, or triplets of antibodies), in which one antibody of the trio competes for binding with, or binds to the same epitope as, a Bin1 antibody as defined herein, a second antibody of the trio competes for binding with, or binds to the same epitope as, a Bin2 antibody as defined herein, and a third antibody of the trio competes for binding with, or binds to the same epitope as, a Bin3 antibody as defined herein. Thus, in one embodiment, a composition of a trio of anti-EGFR antibodies is provided comprising a first antibody, a second antibody and a third antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of va, vb and vc or an antibody selected from the group consisting of ca, cb and cc; (ii) the second antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vd, ve, vf, vg and vh or an antibody selected from the group consisting of cd, ce and cf; and (iii) the third antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vi and vj or an antibody selected from the group consisting of cg, ch, ci, cj and ck.

For example, a composition is provided comprising a trio of anti-EGFR antibodies comprising a first antibody, a second antibody and a third antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, antibody ca (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 34, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 48, 45 and 49, respectively); (ii) the second antibody competes for binding with, or binds to the same epitope as, antibody cd (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 31, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 53, 54 and 55, respectively); and (iii) the third antibody competes for binding with, or binds to the same epitope as, antibody ch (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 23, 24 and 26, respectively, and light chain CDRs 1, 2

and 3 set forth in SEQ ID NOs: 39, 40 and 42, respectively). The trio combination of antibodies ca, cd and ch is also described herein as antibody trio 4c set forth in Table IV.

In the following Tables VI and VII, CDR amino acid sequences are set forth immediately above the corresponding SEQ ID NOs in each column, while corresponding full heavy or light chain variable sequences are indicated by SEQ ID NO in the preceding row in the left hand column.

TABLE VI

Heavy Chain	CDR 1	CDR 2	CDR 3
SEQ ID NO:1			
	SYG	ISAYNGNT	DSGGYGSGS
	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:25
SEQ ID NO:2			
	SYG	ISAYNGNT	DLGGYGSGS
	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:26
SEQ ID NO:3			
	SYG	ISAYNGNT	DGGPYGFGP
	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:27
SEQ ID NO:4			
	SYG	ISAYNGNT	DLGGYGSGGV
	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:28
SEQ ID NO:5			
	SYG	ISAYNGNT	DLGGYGSGGV
	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:28
SEQ ID NO:6			
	SYA	IIPIFGTA	MGRGKV
	SEQ ID NO:29	SEQ ID NO:30	SEQ ID NO:31
SEQ ID NO:7			
	SYA	IIPIFGTA	MARGKV
	SEQ ID NO:29	SEQ ID NO:30	SEQ ID NO:32
SEQ ID NO:8			
	SYA	IIPIFGTA	MVRGKV
	SEQ ID NO:29	SEQ ID NO:30	SEQ ID NO:33
SEQ ID NO:9			
	SYA	IIPIFGTA	DPSVDL
	SEQ ID NO:29	SEQ ID NO:30	SEQ ID NO:34
SEQ ID NO:10			
	SGSYY	IYYSGST	DSPYYG
	SEQ ID NO:35	SEQ ID NO:36	SEQ ID NO:37
SEQ ID NO:11			
	SGSYY	IYYSGST	EPLYD
	SEQ ID NO:35	SEQ ID NO:36	SEQ ID NO:38

TABLE VII

Light Chain	CDR 1	CDR 2	CDR 3
SEQ ID NO:12			
	QSVSSN	GAS	QDYRSWPR
	SEQ ID NO:39	SEQ ID NO:40	SEQ ID NO:41
SEQ ID NO:13			
	QSVSSN	GAS	QDYRTWPR
	SEQ ID NO:39	SEQ ID NO:40	SEQ ID NO:42
SEQ ID NO:14			
	QSVSSN	GAS	QQYNDWPR
	SEQ ID NO:39	SEQ ID NO:40	SEQ ID NO:43
SEQ ID NO:15			
	QSVSSY	DAS	QQRGSWPR
	SEQ ID NO:44	SEQ ID NO:45	SEQ ID NO:46
SEQ ID NO:16			
	QSVSSY	DAS	HQRGTWPS
	SEQ ID NO:44	SEQ ID NO:45	SEQ ID NO:47
SEQ ID NO:17			
	QSVLYSSNNKNY	WAS	QQYYGSP
	SEQ ID NO:53	SEQ ID NO:54	SEQ ID NO:55
SEQ ID NO:19			
	QSVLYSSNNKNY	WAS	GQFYGS
	SEQ ID NO:53	SEQ ID NO:54	SEQ ID NO:56
SEQ ID NO:20			
	QSISSW	DAS	QQFAAHA
	SEQ ID NO:48	SEQ ID NO:45	SEQ ID NO:49
SEQ ID NO:21			
	QDITNY	DAS	HQYNAFP
	SEQ ID NO:50	SEQ ID NO:45	SEQ ID NO:51
SEQ ID NO:22			
	QSISSW	DAS	QQYIEYA
	SEQ ID NO:48	SEQ ID NO:45	SEQ ID NO:52

Contemplated compositions may further include, or be prepared for use as a medicament in combination therapy with, an additional therapeutic agent, e.g., an additional anti-cancer agent. An "anti-cancer agent" is a drug used to treat tumors, cancers, malignancies, and the like. Drug therapy (e.g., with antibody compositions disclosed herein) may be administered without other treatment, or in combination with other treatments such as surgery, heat, or radiation therapy (e.g., with ionizing radiation). Several classes of anti-cancer agents may be used in cancer treatment, depending on the nature of the organ or tissue involved. For example, breast cancers are commonly stimulated by estrogens, and may be treated with drugs which inactive sex hormones. Similarly, prostate cancer may be treated with drugs that inactivate androgens. Anti-cancer agents for use in combination with antibody compositions disclosed herein include, among others, those listed in APPENDIX A, which should not

be construed as limiting. One or more anti-cancer agents may be administered either simultaneously or before or after administration of an antibody composition disclosed herein. Antibody compositions disclosed herein can be administered sequentially or together with the additional anti-cancer agent, e.g., an anti cancer agent disclosed in Appendix A, below.

Methods for selecting particular combinations of antibodies are also provided. In one embodiment, such methods include selecting anti-EGFR antibodies that have a particular IC50 and/or IC90 with respect to an EGFR activity or function (*e.g.*, an IC90 of better than 80nM - e.g., an IC90 of 70 nM or lower - for inhibiting EGFR-mediated signaling). Such antibodies can then be administered in combination (*e.g.*, together) or sequentially. In another embodiment, the method includes the step of further selecting combinations of antibodies that do not compete with each other for binding to EGFR. Additional selection criteria include at least one of the following properties:

(i) inhibition of the growth of cells expressing EGFR in a xenograft model *in vivo*;

(ii) inhibition of an EGFR ligand binding to EGFR *in vitro*;

(iii) inhibition of EGFR dimerization *in vitro*; or

(iv) downregulation of EGFR on cell surfaces *in vitro*.

Kits comprising one or more antibody or composition disclosed herein are also contemplated, optionally, contained within a single vial and/or with instructions for use in treating or diagnosing a disease associated with EGFR, such as cancers. Such kits may comprise the antibody composition in unit dosage form, such as in a single dose vial or a single dose pre-loaded syringe.

Antibodies and compositions disclosed herein can be used in a broad variety of therapeutic and diagnostic applications, particularly oncological applications. Accordingly, in another aspect, provided herein are methods for inhibiting EGFR activity in a subject by administering one or more antibodies or compositions described herein in an amount sufficient to inhibit EGFR-mediated activity. Particular therapeutic indications which can be treated include, for example, cancers of organs or tissues such as skin, brain and central nervous system, head and neck, esophagus, stomach, colon, rectum, anus, liver, pancreas, bile duct, gallbladder, lung or bronchus, breast, ovary, uterus, cervix, vagina, testis, germ cells, prostate, kidney, ureter, urinary bladder, adrenal, pituitary, thyroid, bone, muscle or other connective tissues, leukemia, multiple myeloma, Hodgkin's lymphoma and non-Hodgkin's lymphoma.

Antibodies of disclosed herein also can be used to diagnose or prognose diseases (*e.g.*, cancers) associated with EGFR, for example, by contacting one or more antibodies, antibody pairs or antibody trios disclosed herein (e.g., *ex vivo* or *in vivo*) with cells from the subject, and measuring the level of binding to EGFR on the cells, wherein abnormally high levels of binding to EGFR indicate that the subject has a cancer associated with EGFR.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Figures

Figure 1: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5. Sequences are demarcated to show CDR positions as per the Vbase database. The sequence spanning the end of FR3 to the beginning of the JH region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 2: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5. Sequences are demarcated to show CDR positions as per the IMGT database. The sequence spanning the end of FR3 to the beginning of the JH region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 3: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9. Sequences are demarcated to show CDR positions as per the Vbase database. The sequence spanning the end of FR3 to the beginning of the JH region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 4: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9. Sequences are demarcated to show CDR positions as per the IMGT database. (The sequence spanning the end of FR3 to the beginning of the JH region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 5: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:10, and SEQ ID NO:11. Sequences are demarcated to show CDR positions as per the Vbase database. The sequence spanning the end of FR3 to the beginning of the JH

region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 6: Amino acid sequences for variable heavy regions comprised by SEQ ID NO:10, and SEQ ID NO:11. Sequences are demarcated to show CDR positions as per the IMGT database The sequence spanning the end of FR3 to the beginning of the JH region comprises CDR3. The amino acid sequence for the constant heavy region is also set forth.

Figure 7: Amino acid sequences for variable light regions (V kappa) comprised by SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14. Sequences are demarcated to show CDR positions as per the IMGT database. The amino acid sequence for the constant light region is also set forth.

Figure 8: Amino acid sequences for variable light regions comprised by SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14. Sequences are demarcated to show CDR positions as per the Vbase database. The amino acid sequence for the constant light region is also set forth.

Figure 9: Amino acid sequences for variable light regions comprised by SEQ ID NO:20 and SEQ ID NO:22. Sequences are demarcated to show CDR positions as per the IMGT database. The amino acid sequence for the constant light region is also set forth.

Figure 10: Amino acid sequences for variable light regions comprised by SEQ ID NO:20 and SEQ ID NO:22. Sequences are demarcated to show CDR positions as per the Vbase database. The amino acid sequence for the constant light region is also set forth.

Figure 11: Amino acid sequences for the variable light regions comprised by SEQ ID NO:21. Sequences are demarcated to show CDR positions as per the IMGT database. The amino acid sequence for the constant light region is also set forth.

Figure 12: Amino acid sequences for the variable light regions comprised by SEQ ID NO:21. Sequences are demarcated to show CDR positions as per the Vbase database. The amino acid sequence for the constant light region is also set forth.

Figure 13: Amino acid sequences for the variable light comprised by SEQ ID NO:15 and SEQ ID NO:16. Sequences are demarcated to show CDR positions as per the IMGT database. The amino acid sequence for the constant light region is also set forth.

Figure 14: Amino acid sequences for the variable light regions comprised by SEQ ID NO:15 and SEQ ID NO:16. Sequences are demarcated to show CDR positions as per the Vbase database. The amino acid sequence for the constant light region is also set forth.

Figure 15: Amino acid sequences for the variable light regions comprised by SEQ ID NO:17 and SEQ ID NO:19. Sequences are demarcated to show CDR positions as per the IMGT database. The amino acid sequence for the constant light region is also set forth.

Figure 16: Amino acid sequences for the variable light regions comprised by SEQ ID NO:17 and SEQ ID NO:19. Sequences are demarcated to show CDR positions as per the Vbase database. The amino acid sequence for the constant light region is also set forth.

Figures 17A-D: Epitope mapping of anti-EGFR antibodies by direct ELISA and Surface Plasmon Resonance. Figures 17A-17C are graphs showing the results of ELISA assays with wild-type (Figure 17A), Bin1 epitope mutant (Figure 17B) or Bin3 epitope mutant (Figure 17C) extracellular domain of EGFR (EGFR-ECD) as capture reagents. The x-axis denotes concentration of antibody and the y-axis denotes relative luminescence units (RLU). Figure 17D is the result of a Surface Plasmon Resonance assay demonstrating that the binding of antibodies from Bins 1, 2, and 3 bind to the extracellular domain of EGFR with distinct, non-overlapping epitopes. Antibody ch (Bin3) was conjugated to the surface of a BIACORE chip. Wild-type EGFR-ECD (0.5 μM) was injected followed by sequential injections of antibodies cd (Bin2), cb (Bin1), and ch (Bin3), the same antibody conjugated on the chip as a control. The result is depicted as a sensorgram in which the x-axis denotes time (seconds) and the y-axis denotes BIACORE Response Units (RU).

Figures 18A-C: Phospho-ERK signaling inhibition by combinations of two anti-EGFR antibodies with non-overlapping epitopes. A431 cells were incubated for 2 hr with indicated equimolar combinations of Bin 1 + Bin 3 (Figures 18A and 18B) or Bin 1 + Bin 2 antibodies (Figure 18C) and cell lysates analyzed by phospho-ERK ELISA. The x-axis denotes concentration of antibody and the y-axis denotes the amplitude of phospho-ERK activity, normalized to the amplitude observed in the +EGF ligand control. Solid lines depict a five parameter logistic fit to the data from each

combination. The table below each graph contains the potency (fraction phospho-ERK activity at saturating concentrations of antibody), IC50 value, and IC90 value for each combination, as calculated from the logistic function used to fit the data.

Figures 19A-B: Phospho-ERK signaling inhibition by combinations of two or three anti-EGFR antibodies with non-overlapping epitopes. A431 cells were treated with equimolar triple combination of Bin1, Bin 2 and Bin 3 antibodies (Figure 19A). The duo combination of antibodies (Bin 1) and cd (Bin 2) was then compared to the known anti-EGFR antibody cetuximab (Figure 19B) and shown to be more effective at lowering phospho-ERK than this antibody known in the art. Experiments were performed and data represented graphically as in Figure 18.

Figures 20A-D: Phospho-ERK signaling inhibition by equimolar and non-equimolar combinations of two anti-EGFR antibodies with non-overlapping epitopes. A431 cells were treated with varying ratios (in μg/ml) of antibody pairs cb and cd (Figure 20A), cb and ch (Figure 20B), cb and ci (Figure 20C) and ca and cd (Figure 20D). Experiments were performed and data represented graphically as in Figure 18.

Figure 21: Phospho-ERK signaling inhibition data from Figures 20A-D are represented in a heat map in which the x-axis (columns) denotes concentration of antibody, y-axis (rows) denotes pairs of antibodies combined at indicated ratios, and z-axis (into the plane) denotes fraction phospho-ERK activity in grayscale. The z-axis color map represents high phospho-ERK activity as white, low activity as black, and intermediary activities in shades of gray. Sample values are independently normalized to the mean of the +EGF ligand control on the ELISA plate on which they were analyzed and the mean values of EGF ligand controls (+/-) across plates are indicated at top left.

Figures 22A-C: Phospho-EGF receptor and phospho-ERK signaling inhibition by single and pairwise combinations of Bin 1 + Bin 2 or Bin 1 + Bin 3 antibodies and comparisons with other known anti-EGFR antibodies such as cetuximab, nimotuzumab, and zalutumumab. Figure 22A shows inhibition of ERK activation by the Bin1/2 antibodies cb and cd. Figure 22B shows the inhibition of EGFR activation by the Bin1/2 antibodies and cd. Figure 22C shows the inhibition of ERK activation by the Bin1/3 antibodies cb and ch. Experiments were performed and data represented graphically as in Figure 18.

Figures 23A-D are four graphs showing results of CTG Luminescent Cell Viability assays for cells treated with Bin1/Bin2/Bin3 mAb pairs or trios with and without EGF. Figure 23A shows CTG assay results for H1975 cells treated with the Bin1/3 antibodies ca and ch. Figure 23B shows CTG assay results for H1975 cells treated with the Bin1/2 antibodies ca and cd. Figures 23C (H1975 cells) and 23D (A431 cells) show CTG assay results for cells treated with the Bin1/2/3 trio of antibodies ca + cd + ch. Controls include cells treated with media containing 1% fetal calf serum (1% FCS) and cells treated with serum free media (SFM).

Figures 24A-B are two graphs showing inhibition of tumor growth by the pairs of antibodies cb with antibody ca +cd or cb +ch or ca +ch in the mouse xenograft tumor model using the epidermoid tumor cell line A431 (Figure 24A) and the NSCLC (non-small cell lung cancer) tumor cell line H1975 (Figure 24B). The x-axes represent days after implantation and the y-axes represent tumor size in mm^3.

Figure 25 is a graph of flow cytometry (FACS) analysis results showing inhibition of biotinylated EGF ligand binding to A431 cells by antibodies ca, cf, ch or cb alone (at a concentration of 2 μM), as compared to cetuximab or EGF ligand alone. The Y axis denotes the Mean Fluorescence Index (MFI) and the x-axis denotes varying concentrations of biotinylated human EGF ligand (in nM). Antibody ch showed some inhibition of EGF binding, antibody cf showed essentially complete inhibition of EGF binding up to a 20 nM concentration of EGF, and ca and cb showed essentially complete inhibition of EGF binding up to a 200 nM concentration of EGF.

Detailed Description

I. Definitions

The terms "EGFR," "ErbB1," and "EGF receptor" are used interchangeably herein to refer to human EGFR protein; see UniProtKB/Swiss-Prot entry P00533 (SEQ ID NO:57).

The term "inhibition" as used herein, refers to any statistically significant decrease in biological activity, including full blocking of the activity. For example, "inhibition" can refer to a statistically significant decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or about 100% in biological activity.

Inhibition of phosphorylation, as used herein, refers to the ability of an antibody to statistically significantly decrease the phosphorylation of a substrate protein relative to the signaling in the absence of the antibody (control). As is known in the art, intracellular signaling pathways include, for example, phosphoinositide 3′-kinase/Akt (PI3K/Akt/*PTEN* or "AKT") and/or mitogen-activated protein kinase (MAPK/ERK or "ERK") pathways. As is also known in the art, EGFR mediated signaling can be measured by assaying for the level phosphorylation of the substrate (*e.g.*, phosphorylation or no phosphorylation of AKT and/or ERK). Accordingly, in one embodiment, anti-EGFR antibody combinations and compositions provide statistically significant inhibition of the level of phosphorylation of either or both of AKT and ERK by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% relative to the level of phosphorylation of AKT and/or ERK in the absence of such antibody (control). Such EGFR mediated signaling can be measured using art recognized techniques which measure a protein in a cellular cascade involving EGFR, *e.g.*, ELISA, Western, or multiplex methods, such as Luminex®.

The phrase "inhibition of the growth of cells expressing EGFR," as used herein, refers to the ability of an antibody to statistically significantly decrease the growth of a cell expressing EGFR relative to the growth of the cell in the absence of the antibody (control) either *in vivo* or *in vitro*. In one embodiment, the growth of a cell expressing EGFR (*e.g.*, a cancer cell) may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% when the cells are contacted with an antibody composition of combination disclosed herein, relative to the growth measured in the absence of the antibody composition of combination (control). Cellular growth can be assayed using art recognized techniques which measure the rate of cell division, the fraction of cells within a cell population undergoing cell division, and/or the rate of cell loss from a cell population due to terminal differentiation or cell death (*e.g.*, using a cell titer glow assay or thymidine incorporation).

The phrase "inhibition of an EGFR ligand binding to EGFR," as used herein, refers to the ability of an antibody to statistically significantly decrease the binding of an EGFR ligand to its receptor, EGFR, relative to the EGFR ligand binding in the absence of the antibody (control). This means that, in the presence of the antibody, the amount

of the EGFR ligand that binds to EGFR relative to a control (no antibody), is statistically significantly decreased. The amount of an EGFR ligand which binds EGFR may be decreased in the presence of an antibody composition or combination disclosed herein by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% relative to the amount in the absence of the antibody (control). A decrease in EGFR ligand binding can be measured using art-recognized techniques that measure the level of binding of labeled EGFR ligand (*e.g.*, radiolabelled EGF or radiolabeled betacellulin) to cells expressing EGFR in the presence or absence (control) of the antibody.

The phrase "inhibition of EGFR dimerization," as used herein, refers to the ability of an antibody to statistically significantly decrease EGFR dimerization (pairing with another ErbB receptor to form homodimers, *e.g.*, ErbB1 / ErbB1 pairings, or heterodimers, *e.g.*, ErbB1 / ErbB3 pairings) relative to EGFR dimerization in the absence of the antibody (control). In one embodiment, dimerization of EGFR may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% when cells expressing EGFR are contacted with an antibody composition or combination disclosed herein, relative to dimerization of EGFR measured in the absence of the antibody (control). A decrease in EGFR dimerization can be measured using art-recognized techniques that measure the level of EGFR dimerization in the presence or absence (control) of the antibody.

The phrase "downregulation of EGFR expression," as used herein, refers to the ability of an antibody to statistically significantly decrease the expression of EGFR on a cell surface, for example, by increasing internalization of EGFR relative to EGFR expression in the absence of the antibody (control). In one embodiment, expression of EGFR may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% when cells expressing EGFR are contacted with an antibody composition of combination provided herein, relative to expression of EGFR on the cell surface measured in the absence of the antibody (control). Downregulation of EGFR expression on a cell surface includes, for example, an increase in internalization / recycling of the receptor, and/or an increase in internalization / degradation of the receptor. An increase

in EGFR internalization can be measured using art-recognized techniques that measure the level of EGFR internalization in the presence or absence (control) of the antibody.

With respect to combinations of EGFR antibodies (described herein), the words "additive" or "additivity," as used herein, refer to the activity of two or more antibodies wherein their combined activity (relative to a particular function, *e.g.*, inhibition of cell growth) is equal to the sum of their individual activities. That is, the sum of the activities of two or more antibodies provided herein, when acting individually on a cell expressing EGFR, is approximately equivalent to the combined effect of the same antibodies acting together on the same cell. In one embodiment, the additive effect is measured with respect to any of the properties discussed above (*e.g.*, inhibition of AKT or ERK phosphorylation, inhibition of the growth of cells expressing EGFR, *etc.*).

The words "synergy" or "synergistic," as used herein, refer to the activity of two or more antibodies wherein their combined activity (relative to a particular function, *e.g.*, inhibition of cell growth) is greater than the expected additive effect of their individual activities. For example, the expected additive effect can be defined according to Bliss independence criteria. In accordance with the Bliss criteria, the effect of two or more drugs (*e.g.*, antibodies) is equal to the sum of the effects of the individual drugs minus the multiplication of the effects of the individual drugs:

$$E12 = E1 + E2 - E1*E2$$

where E1 is the % inhibition by drug 1, E2 is the % inhibition by drug 2, and E12 is the expected % inhibition by the combination.

The synergistic effect can apply to any of the properties discussed herein (*e.g.*, inhibition of EGFR-dependant AKT or ERK phosphorylation, inhibition of the growth of cells expressing EGFR, *etc.*). In a particular embodiment, at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or greater increase in activity of the combined antibodies relative to the additive effect of their individual activities is achieved.

The term "antibody" or "immunoglobulin," as used interchangeably herein, includes whole antibodies and any antigen binding fragment (antigen-binding portion) or single chain cognates thereof. An "antibody" comprises at least one heavy (H) chain and one light (L) chain. In naturally occurring IgGs, for example, these heavy and light chains are inter-connected by disulfide bonds and there are two paired heavy and light

chains, these two also inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR) or Joining (J) regions (JH or JL in heavy and light chains respectively). Each V_H and V_L is composed of three CDRs three FRs and a J domain, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, J. The variable regions of the heavy and light chains bind with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) or humoral factors such as the first component (Clq) of the classical complement system. Thus one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, EGFR) may be used in the combinations disclosed herein. It has been shown that fragments of a full-length antibody can perform the antigen-binding function of an antibody. Examples of binding fragments denoted as an antigen-binding portion or fragment of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, CL and CH1 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 CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb including VH and VL domains; (vi) a dAb fragment (Ward *et al.* (1989) *Nature* 341, 544-546), which consists of a V_H domain; (vii) a dAb which consists of a VH or a VL domain; and (viii) an isolated complementarity determining region (CDR) or (ix) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. 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 protein chain in which the V_L and V_H regions are paired to form monovalent molecules (such a single chain cognate of an immunoglobulin fragment is known as a single chain Fv (scFv). Such single chain antibodies are also

intended to be encompassed within the term "antibody". 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 general manner as are intact antibodies. Antigen-binding portions can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Antigen binding fragments (including scFvs) of such immunoglobulins are also encompassed by the term "monoclonal antibody" as used herein. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations, which typically include different antibodies, directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies can be prepared using any art recognized technique and those described herein such as, for example, a hybridoma method, a transgenic animal, recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), or using phage antibody libraries using the techniques described in, for example, US Patent No. 7,388,088 and US patent application Ser. No. 09/856,907 (PCT Int. Pub. No. WO 00/31246). Monoclonal antibodies include chimeric antibodies, human antibodies and humanized antibodies and may occur naturally or be produced recombinantly.

The term "recombinant antibody," refers to antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for immunoglobulin genes (*e.g.*, human immunoglobulin genes) or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library (*e.g.*, containing human antibody sequences) using phage display, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences (*e.g.*, human immunoglobulin genes) to other DNA sequences. Such recombinant antibodies may have variable and constant regions derived from human germline immunoglobulin sequences. In certain

embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term "chimeric immunoglobulin" or antibody refers to an immunoglobulin or antibody whose variable regions derive from a first species and whose constant regions derive from a second species. Chimeric immunoglobulins or antibodies can be constructed, for example by genetic engineering, from immunoglobulin gene segments belonging to different species.

The term "human antibody," as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat et al. (See Kabat, *et al.* (1991) *Sequences of proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The human antibody can have at least one or more amino acids replaced with an amino acid residue, *e.g.*, an activity enhancing amino acid residue that is not encoded by the human germline immunoglobulin sequence. Typically, the human antibody can have up to twenty positions replaced with amino acid residues that are not part of the human germline immunoglobulin sequence. In a particular embodiment, these replacements are within the CDR regions as described in detail below.

The term "humanized antibody" refers to an antibody that includes at least one humanized antibody chain (*i.e.*, at least one humanized light or heavy chain). The term "humanized antibody chain" (*i.e.*, a "humanized immunoglobulin light chain") refers to an antibody chain (*i.e.*, a light or heavy chain, respectively) having a variable region that

includes a variable framework region substantially from a human antibody and complementarity determining regions (CDRs) (*e.g.*, at least one CDR, two CDRs, or three CDRs) substantially from a non-human antibody, and further includes constant regions (*e.g.*, one constant region or portion thereof, in the case of a light chain, and preferably three constant regions in the case of a heavy chain).

"Isolated," as used herein, is intended to refer to an antibody or combination of two, three or four antibodies that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated composition of antibodies ca, cf, and ch, each of which specifically bind to EGFR, is substantially free of antibodies that specifically bind antigens other than EGFR). In addition, an isolated antibody is typically substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different EGFR binding specificities are combined in a well-defined composition.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by heavy chain constant region genes. In some embodiments, a monoclonal antibody composition provided herein comprises only antibodies of the IgG1 isotype. In other embodiments, a monoclonal antibody composition provided herein comprises only antibodies of the IgG2 isotype. In other embodiments, a monoclonal antibody composition provided herein comprises antibodies of two or three different isotypes.

An "antigen" is an entity (*e.g.*, a proteinaceous entity or peptide) to which an antibody binds. In various embodiments of the present invention, an antigen is EGF. In a particular embodiment according to the invention, an antigen is human EGFR.

Accordingly, also encompassed by the present disclosure are combinations of antibodies that bind to epitopes on EGFR which comprise all or a portion of the epitopes recognized by the particular antibodies of the combinations described herein. In another embodiment, the antibodies are provided that compete for binding to EGFR with the antibodies described herein. Competing antibodies and antibodies that recognize the same or an overlapping epitope can be identified using routine techniques such as an immunoassay, for example, by showing the ability of one antibody to block the binding of another antibody to a target antigen, *i.e.*, a competitive binding assay. Competitive binding may be determined using an assay such as described in the Examples below.

The terms "specific binding," "specifically binds," "selective binding," and "selectively binds," mean that an antibody exhibits appreciable affinity for a particular

antigen or epitope and, generally, does not exhibit significant cross-reactivity with other antigens and epitopes. "Appreciable" or preferred binding includes binding with a K_D of 10^6, 10^7, 10^8, 10^9, or 10^{10} M^{-1} or better. The K_D of an antibody antigen interaction (the affinity constant) indicates the concentration of antibody at which 50% of antibody and antigen molecules are bound together. Thus, at a suitable fixed antigen concentration, 50% of a higher (*i.e.*, stronger) affinity antibody will bind antigen molecules at a lower antibody concentration than would be required to achieve the same percent binding with a lower affinity antibody. Thus a lower K_D value indicates a higher (stronger) affinity. As used herein, "better" affinities are stronger affinities, and are of lower numeric value than their comparators, with a K_D of 10^7M^{-1} being of lower numeric value and therefore representing a better affinity than a K_D of 10^6M^{-1}. Affinities better (i.e., with a lower K_D value and therefore stronger) than 10^7M^{-1}, preferably better than 10^8M^{-1}, are generally preferred. Values intermediate to those set forth herein are also contemplated, and a preferred binding affinity can be indicated as a range of affinities, for example preferred binding affinities for anti-EGFR antibodies disclosed herein are, 10^6 to 10^{12} M^{-1}, preferably 10^7 to 10^{12} M^{-1}, more preferably 10^8 to 10^{12} M^{-1}. An antibody that "does not exhibit significant cross-reactivity" is one that will not appreciably bind to an off target antigen (*e.g.*, a non-EGFR protein). For example, in one embodiment, an antibody that specifically binds to EGFR will exhibit at least a two, and preferably three, or four or more orders of magnitude better binding affinity (i.e., binding exhibiting a two, three, or four or more orders of magnitude lower K_D value) for EGFR than for ErbB molecules other than ErbB1 (EGFR) or for non-ErbB proteins or peptides. Specific or selective binding can be determined according to any art-recognized means for determining such binding, including, for example, according to Scatchard analysis and/or competitive (competition) binding assays as described herein.

The term "K_D," as used herein, is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction or the affinity of an antibody for an antigen. In one embodiment, the antibody according to the present invention binds an antigen (*e.g.*, EGFR) with an affinity (K_D) of 100 nM or better (*i.e.*, or less) (*e.g.*, 90 nM, 80 nM, 70 nM, 60nM, 50nM, 40nM, 30nM, 20nM, or 10 nM or less), as measured using a surface plasmon resonance assay, a cell binding assay, or an equilibrium dialysis assay. In a particular embodiment, an antibody binds EGFR with an affinity (as represented by dissociation constant K_D) of 8 nM or better (*e.g.*, 7 nM, 6 nM, 5 nM, 4

nM, 2 nM, 1.5 nM, 1.4 nM, 1.3 nM, 1.2nM, 1.1nM, 1nM or lower), as measured by a surface plasmon resonance assay or a cell binding assay. In other embodiments, an antibody binds an antigen (*e.g.*, EGFR) with an affinity (K_D) of approximately less than 10^{-7} M, such as approximately less than 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by surface plasmon resonance (SPR) technology in a BIACORE 3000 instrument using recombinant EGFR as the analyte and the antibody as the ligand, and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. Other methods for determining K_D include equilibrium binding to live cells expressing EGFR via flow cytometry (FACS) or in solution using KinExA® technology.

The term "K_{off}," as used herein, is intended to refer to the off rate constant for the dissociation of an antibody from the antibody/antigen complex.

The terms "IC50" and "IC90," as used herein, refer to the measure of the effectiveness of a compound (*e.g.*, an anti-EGFR antibody) in inhibiting a biological or biochemical function (*e.g.*, the function or activity of EGFR) by 50% and 90%, respectively. For example, IC50 indicates how much of an anti-EGFR antibody is needed to inhibit the activity of EGFR (*e.g.*, the growth of a cell expressing EGFR) by half. That is, it is the half maximal (50%) inhibitory concentration (IC) of an anti-EGFR antibody (50% IC, or IC_{50}). According to the FDA, IC50 represents the concentration of a drug that is required for 50% inhibition *in vitro*. The IC50 and IC90 can be determined by techniques known in the art, for example, by constructing a dose-response curve and examining the effect of different concentrations of the antagonist (*i.e.*, the anti-EGFR antibody) on reversing EGFR activity.

As used herein, "glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin protein.

The term "nucleic acid molecule," as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding antibodies or antibody fragments (*e.g.*, V_H, V_L, CDR3), is intended to refer to a nucleic acid molecule in which the nucleotide sequences are essentially free of

other genomic nucleotide sequences, e.g., those encoding antibodies that bind antigens other than EGFR, which other sequences may naturally flank the nucleic acid in human genomic DNA.

The term "modifying," or "modification," as used herein, is intended to refer to changing one or more amino acids in the antibodies or antigen-binding portions thereof. The change can be produced by adding, substituting or deleting an amino acid at one or more positions. The change can be produced using known techniques, such as PCR mutagenesis. For example, in some embodiments, an antibody or an antigen-binding portion thereof identified using the methods of the invention can be modified, to thereby modify the binding affinity of the antibody or antigen-binding portion thereof to EGFR. "Conservative amino acid substitutions" in the sequences of the antibodies are provided, *i.e.*, nucleotide and amino acid sequence modifications which do not abrogate the binding of the antibody encoded by the nucleotide sequence or containing the amino acid sequence, to the antigen, *i.e.*, EGFR. Conservative amino acid substitutions include the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix or BLOSUM matrix. Six general classes of amino acid side chains have been categorized and include: Class I (Cys); Class II (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Trp). For example, substitution of an Asp for another class III residue such as Asn, Gln, or Glu, is a conservative substitution. Thus, a predicted nonessential amino acid residue in an anti-EGFR antibody is preferably replaced with another amino acid residue from the same class. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen binding are well-known in the art.

The term "non-conservative amino acid substitution" refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution of an Ala, a class II residue, with a class III residue such as Asp, Asn, Glu, or Gln.

Alternatively, in another embodiment, mutations (conservative or non-conservative) can be introduced randomly along all or part of an anti-EGFR antibody

coding sequence, such as by saturation mutagenesis, and the resulting modified anti-EGFR antibodies can be screened for binding activity.

A "consensus sequence" is a sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related sequences. In a family of proteins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence. A "consensus framework" of an immunoglobulin refers to a framework region in the consensus immunoglobulin sequence. Similarly, the consensus sequence for the CDRs of can be derived by optimal alignment of the CDR amino acid sequences of EGFR antibodies of the present invention.

For nucleic acids, the term "substantial homology" indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98% to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art.

The nucleic acid compositions, while often comprising a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures thereof may alternately be mutated, in accordance with standard techniques to provide altered gene sequences. For coding sequences, these mutations, may modify the encoded amino acid sequence as desired. In particular, DNA sequences substantially homologous to native V, D, J, constant, switches and other such sequences described herein are contemplated.

The term "operably linked" refers to a nucleic acid sequence placed into a functional relationship with another nucleic acid sequence. For example, DNA for a

presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms, "plasmid" and "vector" may be used interchangeably. However, other forms of expression vectors, such as viral vectors (*e.g.*, replication

defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions are also contemplated.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The terms "treat," "treating," and "treatment," as used herein, refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, an antibody or antibody pair or trio disclosed herein, for example, a subject having a disease or disorder associated with EGFR dependent signaling or predisposed to having such a disease or disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disease or disorder or recurring disease or disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

The term "disease associated with EGFR dependent signaling," or "disorder associated with EGFR dependent signaling," as used herein, includes disease states and/or symptoms associated with a disease state, where increased levels of EGFR and/or activation of cellular cascades involving EGFR are found. The term "disease associated with EGFR dependent signaling," also includes disease states and/or symptoms associated with the activation of alternative EGFR signaling pathways. In general, the term "disease associated with EGFR dependent signaling," refers to any disorder, the onset, progression or the persistence of the symptoms of which requires the participation of EGFR. Exemplary EGFR-mediated disorders include, but are not limited to, for example, cancer.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, glial cell tumors such as glioblastoma and neurofibromatosis, cervical cancer,

ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, melanoma, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. In a particular embodiment, a cancer treated or diagnosed using the methods disclosed herein is selected from melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal/colon cancer, lung cancer, and prostate cancer.

The term "effective amount," as used herein, refers to that amount of an antibody or an antigen binding portion thereof that binds EGFR, which is sufficient to effect treatment, prognosis or diagnosis of a disease associated with EGFR dependent signaling, as described herein, when administered to a subject. Therapeutically effective amounts of antibodies provided herein, when used alone or in combination, will vary depending upon the relative activity of the antibodies and combinations (*e.g.*, in inhibiting cell growth) and depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The dosages for administration can range from, for example, about 1 ng to about 10,000 mg, about 5 ng to about 9,500 mg, about 10 ng to about 9,000 mg, about 20 ng to about 8,500 mg, about 30 ng to about 7,500 mg, about 40 ng to about 7,000 mg, about 50 ng to about 6,500 mg, about 100 ng to about 6,000 mg, about 200 ng to about 5,500 mg, about 300 ng to about 5,000 mg, about 400 ng to about 4,500 mg, about 500 ng to about 4,000 mg, about 1 µg to about 3,500 mg, about 5 µg to about 3,000 mg, about 10 µg to about 2,600 mg, about 20 µg to about 2,575 mg, about 30 µg to about 2,550 mg, about 40 µg to about 2,500 mg, about 50 µg to about 2,475 mg, about 100 µg to about 2,450 mg, about 200 µg to about 2,425 mg, about 300 µg to about 2,000, about 400 µg to about 1,175 mg, about 500 µg to about 1,150 mg, about 0.5 mg to about 1,125 mg, about 1 mg to about 1,100 mg, about 1.25 mg to about 1,075 mg, about 1.5 mg to about 1,050 mg, about 2.0 mg to about 1,025 mg, about 2.5 mg to about 1,000 mg, about 3.0 mg to about 975 mg, about 3.5 mg to about 950 mg, about 4.0 mg to about 925 mg, about 4.5 mg to about 900 mg, about 5 mg to about 875 mg, about 10 mg to about 850 mg, about 20 mg to about 825 mg, about 30 mg to about 800 mg, about 40 mg to about 775 mg, about 50 mg to about 750 mg, about 100 mg to about 725 mg, about 200 mg to about 700 mg, about 300 mg to about 675 mg, about 400 mg to about

650 mg, about 500 mg, or about 525 mg to about 625 mg, of an antibody. Dosage regiments may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*i.e.*, side effects) of an antibody are minimized and/or outweighed by the beneficial effects.

The term "therapeutic agent" in intended to encompass any and all compounds that have an ability to decrease or inhibit the severity of the symptoms of a disease or disorder, or increase the frequency and/or duration of symptom-free or symptom-reduced periods in a disease or disorder, or inhibit or prevent impairment or disability due to a disease or disorder affliction, or inhibit or delay progression of a disease or disorder, or inhibit or delay onset of a disease or disorder, or inhibit or prevent infection in an infectious disease or disorder. Non-limiting examples of therapeutic agents include small organic molecules, monoclonal antibodies, bispecific antibodies, recombinantly engineered biologics, RNAi compounds, tyrosine kinase inhibitors, and commercial antibodies. In certain embodiments, tyrosine kinase inhibitors include, *e.g.*, one or more of erlotinib, gefitinib, and lapatinib, which are currently marketed pharmaceuticals. Commercially available pharmaceutical anti-EGFR antibodies include cetuximab and panitumumab. Other pharmaceutical anti-EGFR antibodies include zalutumumab, nimotuzumab, and matuzumab, which are in development.

The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

The term "subject" includes any mammal, e.g, a primate. For example, the methods and compositions herein disclosed can be used to treat a subject having cancer. In a particular embodiment, the subject is a human.

The term "sample" refers to tissue, body fluid, or a cell (or a fraction of any of the foregoing) taken from a patient or a subject. Normally, the tissue or cell will be removed from the patient, but *in vivo* diagnosis is also contemplated. In the case of a solid tumor, a tissue sample can be taken from a surgically removed tumor and prepared for testing by conventional techniques. In the case of lymphomas and leukemias, lymphocytes, leukemic cells, or lymph tissues can be obtained (e.g., leukemic cells from blood) and appropriately prepared. Other samples, including urine, tears, serum, plasma, cerebrospinal fluid, feces, sputum, cell extracts *etc.* can also be useful for particular cancers.

Various aspects of the disclosure are described in further detail in the following subsections.

II. Methods for Producing Antibodies

(i) Monoclonal Antibodies

Monoclonal antibodies can be produced using a variety of known techniques, such as the standard somatic cell hybridization technique, viral or oncogenic transformation of B lymphocytes, or yeast or phage display techniques using libraries of human antibody genes. In particular embodiments, the antibodies are fully human monoclonal antibodies.

Accordingly, in one embodiment, a hybridoma method is used for producing an antibody that binds EGFR. In this method, a mouse or other appropriate host animal can be immunized with a suitable antigen in order to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes can then be fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal. The monoclonal antibodies secreted by the subclones can be separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In another embodiment, antibodies that bind EGFR can be isolated from antibody libraries generated using well know techniques such as those described in, for example, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*. Additionally, production of high affinity (nM range) human antibodies by chain shuffling, as well as combinatorial

infection and *in vivo* recombination as a strategy for constructing very large phage libraries may also be used. See, e.g., US patent application Ser. No. 09/856,907 (PCT Int. Pub. No. WO 00/31246)

In a particular embodiment, the monoclonal antibody that binds EGFR is produced using phage display. This technique involves the generation of a human Fab library having a unique combination of immunoglobulin sequences isolated from human donors and having synthetic diversity in the heavy-chain CDRs is generated. The library is then screened for Fabs that bind to EGFR.

In yet another embodiment, human monoclonal antibodies directed against EGFR can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system (see *e.g.*, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*).

In another embodiment, human antibodies can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome (see *e.g.*, PCT Publication WO 02/43478 to Ishida *et al.*).

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-EGFR antibodies. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-EGFR antibodies. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome can be used. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art and can be used to raise anti-EGFR antibodies.

In yet another embodiment, antibodies can be prepared using a transgenic plant and/or cultured plant cells (such as, for example, tobacco, maize and duckweed) that

produce such antibodies. For example, transgenic tobacco leaves expressing antibodies can be used to produce such antibodies by, for example, using an inducible promoter. Also, transgenic maize can be used to express such antibodies and antigen binding portions thereof. Antibodies can also be produced in large amounts from transgenic plant seeds including antibody portions, such as single chain antibodies (scFv's), for example, using tobacco seeds and potato tubers.

The binding specificity of monoclonal antibodies (or portions thereof) that bind EGFR prepared using any technique including those disclosed here, can be determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of a monoclonal antibody or portion thereof also can be determined by Scatchard analysis.

In certain embodiments, an EGFR antibody produced using any of the methods discussed above may be further altered or optimized to achieve a desired binding specificity and/or affinity using art recognized techniques, such as those described herein.

In one embodiment, partial antibody sequences derived from an EGFR antibody may be used to produce structurally and functionally related antibodies. For example, antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties. Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences.

Thus, one or more structural features of an anti-EGFR antibody, such as the CDRs, can be used to create structurally related anti-EGFR antibodies that retain at least one desired functional property, *e.g.*, inhibiting growth of cells expressing EGFR.

In a particular embodiment, one or more CDR regions selected from SEQ ID NOs:23-56 is combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, anti-EGFR antibodies. The heavy

and light chain variable framework regions can be derived from the same or different antibody sequences.

It is well known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in certain embodiments, antibodies are generated that include the heavy and/or light chain CDR3s of the particular antibodies described herein. The antibodies can further include the heavy and/or light chain CDR1 and/or CDR2s of the antibodies disclosed herein.

The CDR 1, 2, and/or 3 regions of the engineered antibodies described above can comprise the exact amino acid sequence(s) as those disclosed herein. However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences may be possible, particularly for CDR1 and CDR2 sequences, which can tolerate more variation than CDR3 sequences without altering epitope specificity (such deviations are, e.g., conservative amino acid substitutions). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDR1s and CDR2s that are, for example, 90%, 95%, 98%, 99% or 99.5% identical to the corresponding CDRs of an antibody named herein.

In another embodiment, one or more residues of a CDR may be altered to modify binding to achieve a more favored on-rate of binding. Using this strategy, an antibody having ultra high binding affinity of, for example, 10^{10} M^{-1} or more, can be achieved. Affinity maturation techniques, well known in the art and those described herein, can be used to alter the CDR region(s) followed by screening of the resultant binding molecules for the desired change in binding. Accordingly, as CDR(s) are altered, changes in binding affinity as well as immunogenicity can be monitored and scored such that an antibody optimized for the best combined binding and low immunogenicity are achieved.

Modifications can also be made within one or more of the framework or joining regions of the heavy and/or the light chain variable regions of an antibody, so long as antigen binding affinity subsequent to these modifications is better than 10^{6} M^{-1}.

In another embodiment, the antibody is further modified with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody

thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers. Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities.

Also provided are bispecific antibodies and immunoconjugates, as discussed below.

(ii) Bispecific Antibodies

Bispecific antibodies herein include at least two binding specificities for EGFR which preferably bind non-overlapping or non-competing epitopes. Such bispecific antibodies can include additional binding specificities, *e.g.*, a third EGFR binding specificity and/or a binding specificity for another ErbB receptor (*e.g.*, ErbB3) or another antigen, such as the product of an oncogene. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are well known in the art (see, e.g., WO 05117973 and WO 06091209). For example, production of full length bispecific antibodies can be based on the coexpression of two paired immunoglobulin heavy chain-light chains, where the two chains have different specificities. Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported.

In a particular embodiment, the bispecific antibody comprises a first antibody (or binding portion thereof) which binds to EGFR derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. An antibody may be derivatized or linked to more than one other functional molecule to generate multispecific molecules that bind to more than two different binding sites and/or target molecules; such multispecific molecules are also intended to be encompassed by the term "bispecific molecule" as used herein. To create a bispecific molecule, an antibody disclosed herein can be functionally linked

(*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, peptide or binding mimetic, such that a bispecific molecule results.

Accordingly, bispecific molecules comprising at least one first binding specificity for EGFR and a second binding specificity for a second target epitope are contemplated. In a particular embodiment, the second target epitope is an Fc receptor, *e.g.*, human FcγRI (CD64) or a human Fcα receptor (CD89). Therefore, bispecific molecules capable of binding both to FcγR, FcαR or FcεR expressing effector cells (*e.g.*, monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing EGFR are also provided. These bispecific molecules target EGFR expressing cells to effector cell and trigger Fc receptor-mediated effector cell activities, such as phagocytosis of an EGFR expressing cells, antibody dependent cell-mediated cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

In one embodiment, the bispecific molecules comprise as a binding specificity at least one antibody, or an antibody fragment thereof, including, *e.g.*, an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner *et al.* U.S. Patent No. 4,946,778.

The bispecific molecules can be prepared by conjugating the constituent binding specificities, *e.g.*, the anti-FcR and anti-EGFR binding specificities, using methods known in the art. For example, each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohaxane-1-carboxylate (sulfo-SMCC). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A bispecific molecule can be a single chain molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific molecules may comprise at least two single chain molecules. Methods for preparing bispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the bispecific molecules to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using *e.g.*, an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a γ- counter or a scintillation counter or by autoradiography.

(iii) Immunoconjugates

Immunoconjugates provided herein can be formed by conjugating the antibodies described herein to another therapeutic agent. Suitable agents include, for example, a cytotoxic agent (*e.g.*, a chemotherapeutic agent), a toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), and/or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain,

modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-EGFR antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Immunoconjugates can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see, *e.g.*, WO94/11026).

III. Methods for Screening Antibodies

Subsequent to producing antibodies they can be screened for various properties, such as those described herein, using a variety of assays that are well known in the art.

In one embodiment, the antibodies are screened (*e.g.*, by flow cytometry or ELISA) for binding to EGFR using, for example, purified EGFR and/or EGFR-expressing cells, such as A431 cells. The epitopes bound by the anti-EGFR antibodies can further be identified and compared, for example, to identify non-competing antibodies (*e.g.*, antibodies that bind different epitopes), as well as antibodies which compete for binding and/or bind the same or overlapping epitopes.

Competitive antibodies (such as antibodies that compete for binding to EGFR with any of the antibodies identified in Tables I and II, above) and non-competitive antibodies can be identified using routine techniques. Such techniques include, for example, an immunoassay, which shows the ability of one antibody to block (or not block) the binding of another antibody to a target antigen, *i.e.*, a competitive binding assay. Competitive binding is determined in an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody to a common antigen, such as EGFR. Numerous types of competitive binding assays are known, for example: solid

phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay; solid phase direct biotin-avidin EIA; solid phase direct labeled assay, solid phase direct labeled sandwich assay; solid phase direct ^{125}I labeled RIA; solid phase direct biotin-avidin EIA; and direct labeled RIA..

The surface plasmon resonance technique set forth in the Materials and Methods of the Examples and in Example 2, below, can also be used advantageously for this purpose. Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabeled test immunoglobulin and a labeled reference immunoglobulin. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 50-55%, 55-60%, 60-65%, 65-70% 70-75% or more.

Other screening techniques for determining the epitope bound by antibodies disclosed herein include, for example, x-ray analysis of crystals of antigen:antibody complexes, which provides atomic resolution of the epitope. Other methods monitor the binding of the antibody to antigen fragments or mutated variations of the antigen where loss of binding due to a modification of an amino acid residue within the antigen sequence is often considered an indication of an epitope component. In addition, computational combinatorial methods for epitope mapping can also be used. These methods rely on the ability of the antibody of interest to affinity isolate specific short peptides from combinatorial phage display peptide libraries. The peptides are then regarded as leads for the definition of the epitope corresponding to the antibody used to screen the peptide library. For epitope mapping, computational algorithms have also been developed which have been shown to map conformational discontinuous epitopes.

In another embodiment, the antibodies (*e.g.*, non-competing antibodies anti-EGFR antibodies) are screened for the ability to bind to epitopes exposed upon binding to ligand, *e.g.*, EGF (*i.e.*, do not inhibit the binding of EGFR-binding ligands to EGFR). Such antibodies can be identified by, for example, contacting cells which express EGFR (*e.g.* A431 cells) with a labeled EGFR ligand (*e.g.*, radiolabeled or biotinylated EGF) in the absence (control) or presence of the anti-EGFR antibody. If the antibody does not inhibit EGF binding to EGFR, then no statistically significantly decrease in the amount of label recovered, relative to the amount in the absence of the antibody, will be

observed. Alternatively, if the antibody inhibits EGF binding to EGFR, then a statistically significantly decrease in the amount of label recovered, relative to the amount in the absence of the antibody, will be observed.

Antibodies also can be screened (tested) for their binding affinity. This can be done, for example, using a plasmon resonance assay, e.g., as described below.

Antibodies also can be screened for their ability to inhibit signaling through EGFR using signaling assays, such as, those described herein. For example, the ability of an antibody to inhibit EGFR ligand mediated phosphorylation of EGFRs can be assessed by treating cells expressing EGFR with an EGFR ligand (*e.g.*, EGF) in the presence and absence of the antibody. The cells can then be lysed, crude lysates centrifuged to remove insoluble material, and EGFR phosphorylation measured, for example, by Western blotting followed by probing with an anti-phosphotyrosine antibody.

Alternatively, the ability of an antibody to inhibit downstream signaling through EGFR can be measured by kinase assays for known substrates of EGFR such as, for example, AKT and/or ERK, following EGFR stimulation by EGF ligand. For example, cells expressing EGFR can be stimulated with EGF ligand and incubated with a candidate antibody. Cell lysates subsequently prepared from such cells can be immunoprecipitated with an antibody for a substrate of EGFR (or a protein in a cellular pathway involving EGFR) such as, an anti-AKT antibody, and assayed for kinase activity (*e.g.*, AKT kinase activity) using art-recognized techniques. A decrease in or complete disappearance in level or activity (*e.g.*, kinase activity) of a EGFR substrate or protein in a pathway involving EGFR in the presence of the antibody, relative to the level or activity in the absence of the antibody is indicative of an antibody which inhibits EGFR signaling.

Antibodies that decrease levels of EGFR on cell surfaces can be identified by their ability to downregulate or inhibit EGFR expression on tumor cells. In certain embodiments, the antibodies decrease EGFR on cell surfaces by inducing internalization (or increasing endocytosis) of EGFR (*e.g.*, by internalization and recycling of the receptor and/or internalization and degradation of the receptor). To test this, EGFR can be biotinylated and the number of EGFR molecules on the cell surface can be readily determined, for example, by measuring the amount of biotin on a monolayer of cells in culture in the presence or absence of an antibody, followed by immunoprecipitation of

EGFR and probing with streptavidin. A decrease in detection of biotinylated EGFR over time in the presence of an antibody is indicative of an antibody that decreases EGFR levels on cell surfaces.

Antibodies and antibody combinations can also be tested for their ability to inhibit growth of cells expressing EGFR (either *in vivo* or *in vitro*), such as tumor cells, using art recognized techniques, including the Cell Titer Glow Assay described in the Examples below and Tritium-labeled thymidine incorporation assay. Antibodies also can be screened for the ability to inhibit spheroid growth of cells expressing EGFR. This can be done by using an assay that approximates conditions of a developing tumor growth as described herein.

In another embodiment, combinations of anti-EGFR antibodies are screened for IC50 and/or IC90 values relative to inhibiting a particular EGFR activity or function, such as EGFR-mediated signaling (*e.g.*, as measured by ELISA, Western, or multiplex methods, such as Luminex®. Combinations of antibodies, each of which possesses a particularly desired IC50 and/or IC90 value (*e.g.*, an IC90 of about 80nM for inhibiting EGFR signaling) can then be selected. In one embodiment, the combination has a greater IC50 or IC90 value than a known reference antibody (*e.g.*, cetuximab). In another embodiment, the combination has an additive IC50 or IC90 (*i.e.*, the sum of the activities of the antibodies, when acting individually on a cell expressing EGFR, is approximately equivalent to the combined effect of the same antibodies acting together on the same cell) In another embodiment, the combination has a synergistic IC50 or IC90 (*i.e.*, the sum of the effects of the antibodies, when acting individually on a cell expressing EGFR, is less than the combined effect of the same antibodies acting together on the same cell).

IV. Pharmaceutical Compositions

In another aspect, herein provided is a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of monoclonal antibodies formulated together with a pharmaceutically acceptable carrier. In one embodiment, the compositions include a combination of multiple (*e.g.*, two or more) isolated antibodies that bind different epitopes on EGFR. Such antibodies preferably have an additive or synergistic effect relative to inhibiting a particular EGFR activity or function, such as EGFR-mediated signaling.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

Compositions can be administered alone or in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a composition provided herein with at least one or more additional therapeutic agents, such as the anti-cancer agents described herein. The compositions can also be administered in conjunction with radiation therapy and/or surgery. Particular combinations of anti-EGFR antibodies may also be administered separately or sequentially, with or without additional therapeutic agents.

Compositions can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The antibodies can be prepared with carriers that will protect the antibodies against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

To administer compositions by certain routes of administration, it may be necessary to coat the constituents, *e.g.*, antibodies, with, or co-administer the compositions with, a material to prevent its inactivation. For example, the compositions may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

Acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the

art. Except insofar as any conventional medium or agent is incompatible with the antibodies, use thereof in compositions provided herein is contemplated. Supplementary active constituents can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. 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), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Including in the composition an agent that delays absorption, for example, monostearate salts and gelatin can bring about prolonged absorption of the injectable compositions.

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

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. For example, human antibodies may be administered once or twice weekly by subcutaneous injection or once or twice monthly by subcutaneous injection.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be

treated; each unit contains a predetermined quantity of antibodies calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms provided herein are dictated by and directly dependent on (a) the unique characteristics of the antibodies and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such antibodies for the treatment of sensitivity in individuals. Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, and parenteral administration. Parenteral administration is the most common route of administration for therapeutic compositions comprising antibodies. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of antibodies that can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. This amount of antibodies will generally be an amount sufficient to produce a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.001 per cent to about ninety percent of antibody by mass, preferably from about 0.005 per cent to about 70 per cent, most preferably from about 0.01 per cent to about 30 per cent.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions provided herein include water, ethanol, polyols (such as

glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Particular examples of adjuvants which are well-known in the art include, for example, inorganic adjuvants (such as aluminum salts, e.g., aluminum phosphate and aluminum hydroxide), organic adjuvants (e.g., squalene), oil-based adjuvants, virosomes (e.g., virosomes which contain a membrane-bound heagglutinin and neuraminidase derived from the influenza virus).

Prevention of presence of microorganisms may be ensured both by sterilization procedures and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of one or more agents that delay absorption such as aluminum monostearate or gelatin.

When compositions are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.001 to 90% (more preferably, 0.005 to 70%, such as 0.01 to 30%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, compositions provided herein, may be used in a suitable hydrated form, and they may be formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the antibodies in the pharmaceutical compositions provided herein may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the

particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the composition required. For example, the physician or veterinarian could start doses of the antibodies at levels lower than that required to achieve the desired therapeutic effect and gradually increasing the dosage until the desired effect is achieved. In general, a suitable daily dose of compositions provided herein will be that amount of the antibodies which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for antibodies to be administered alone, it is preferable to administer antibodies as a formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art, such as, for example, those disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, 4,596,556, 4,487,603, 4.,486,194, 4,447,233, 4,447,224, 4,439,196, and 4,475,196.

In certain embodiments, the monoclonal antibodies can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that therapeutic antibodies cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Patents 4,522,811; 5,374,548; 5,399,331; 5,891,468; 6,056,973; 6,210,707, 6,224,903; 6,316,024; 7,122,202; 7,135,177; and 7,507,407 and US Patent Publication 20070116753. The liposomes may comprise one or more moieties that attach to and/or are selectively transported into specific cells or organs, thus enhance targeted drug delivery.

The invention provides pharmaceutical compositions that comprise trios of anti-EGFR antibodies, that is the composition comprises three different anti-EGFR

antibodies, preferably three different antibodies that do not cross-compete with each other for binding to EGFR and/or bind to different epitopes from each other on EGFR. In addition to the three antibodies, these pharmaceutical compositions can comprise a pharmaceutically acceptable carrier and/or other excipient(s) such as those described in detail above. The pharmaceutical composition can be supplied in a single container containing all three antibodies or, alternatively, the pharmaceutical composition can comprise a package comprising three distinct containers each containing one of the three different anti-EGFR antibodies (as well as a pharmaceutically acceptable carrier and/or other excipient(s) as described above).

For example, the invention provides pharmaceutical compositions of trios of anti-EGFR antibodies (three antibodies, or triplets of antibodies), in which one antibody of the trio competes for binding with, or binds to the same epitope as, a Bin1 antibody as defined herein, a second antibody of the trio competes for binding with, or binds to the same epitope as, a Bin2 antibody as defined herein, and a third antibody of the trio competes for binding with, or binds to the same epitope as, a Bin3 antibody as defined herein. Thus, in one embodiment, the invention provides a pharmaceutical composition of a trio of anti-EGFR antibodies comprising a first antibody, a second antibody and a third antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of va, vb and vc or an antibody selected from the group consisting of ca, cb and cc; (ii) the second antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vd, ve, vf, vg and vh or an antibody selected from the group consisting of cd, ce and cf; and (iii) the third antibody competes for binding with, or binds to the same epitope as, an antibody selected from the group consisting of vi and vj or an antibody selected from the group consisting of cg, ch, ci, cj and ck.

For example, the invention provides a pharmaceutical composition comprising a trio of anti-EGFR antibodies comprising a first antibody, a second antibody and a third antibody, wherein (i) the first antibody competes for binding with, or binds to the same epitope as, antibody ca (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 34, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 48, 45 and 49, respectively); (ii) the second antibody competes for binding with, or binds to the same epitope as, antibody cd (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 31, respectively, and light chain CDRs 1, 2 and 3 set

forth in SEQ ID NOs: 53, 54 and 55, respectively); and (iii) the third antibody competes for binding with, or binds to the same epitope as, antibody ch (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 23, 24 and 26, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 39, 40 and 42, respectively). The trio combination of antibodies ca, cf and ch is also described herein as antibody trio 4c set forth in Table IV.

The invention also provides use of the above-described trios of anti-EGFR antibodies in the manufacture of a medicament for the treatment of a disease associated with EGFR dependent signaling. The invention also provides use of the above-described trios of anti-EGFR antibodies in the manufacture of a medicament for the treatment of cancer, such as an EGFR-expressing cancer, such as a cancer including, but not limited to melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal cancer, colon cancer, lung cancer, pancreatic cancer, skin cancer, head and neck cancer glioblastoma, prostate cancer and other solid and/or metastatic tumors.

V. Methods of Using Antibodies

Also provided are methods of using antibodies that bind EGFR in a variety of *ex vivo* and *in vivo* diagnostic and therapeutic applications involving EGFR dependent signaling, including a variety of cancers.

Accordingly, in one embodiment, a method is provided for treating a disease associated with EGFR dependent signaling by administering to a subject an antibody or preferably a combination of antibodies provided herein in an amount effective to treat the disease. Suitable diseases include, for example, a variety of cancers including, but not limited to, melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal cancer, colon cancer, lung cancer, pancreatic cancer, skin cancer, head and neck cancer glioblastoma, prostate cancer and other solid and/or metastatic tumors.

The antibody can be administered alone or with another therapeutic agent that acts in conjunction with or synergistically with the antibody to treat the disease associated with EGFR mediated signaling. Such therapeutic agents include those described herein, for example, small organic molecules, monoclonal antibodies, bispecific antibodies, recombinantly engineered biologics, RNAi compounds, tyrosine kinase inhibitors, and commercial antibodies, as well as anticancer agents (*e.g.*, cytotoxins, chemotherapeutic agents, small molecules and radiation).

Also provided are kits comprising one or more anti-EGFR antibodies, optionally contained in a single vial, and include, *e.g.*, instructions for use in treating or diagnosing a disease associated with EGFR upregulation and/or EGFR dependent signaling. The kits may include a label indicating the intended use of the contents of the kit. The term label includes any writing, marketing materials or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

Other embodiments are described in the following non-limiting Examples.

Examples

Materials and Methods for Examples 1-5

Throughout examples 1-5, the following materials and methods are used unless otherwise stated.

In general, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, *e.g.*, antibody technology), and standard techniques of polypeptide preparation are used.

Cell Lines

All the cell lines to be used in the experiments described below are obtained from the National Cancer Institute or ATCC.

Cell Lines:

A431 – epidermoid carcinoma

OVCAR-3 – ovarian cancer

Du 145 - prostate carcinoma

ADRr - breast carcinoma

Pulverization of Tumor Cells

A cryopulverizer (COVARIS Inc.) is used for the pulverization of tumors. Tumors are stored in special bags (pre-weighed before the addition of the tumor) and placed in liquid nitrogen while handling them. For small tumors, 200 μL of Lysis buffer is first added to the bag containing the tumor, frozen in liquid nitrogen and then pulverized to improve the recovery of the tumor from the bag. Pulverized tumors are transferred to 2 mL EPPENDORF tubes and placed in liquid nitrogen until ready for further processing.

Lysis of Tumor Cells

Tumors are lysed in Lysis buffer supplemented with protease and phosphatase inhibitors. Lysis Buffer is added to the tumor aliquots in a final concentration of about 62.5 mg/mL. Tumor samples are homogenized by vortexing for 30 sec and incubating on ice for about 30 min. The lysates are spun for about 10 min in Qiagen QIASHREDDER columns for further homogenization of the samples. Cleared lysates are aliquoted into fresh tubes for further processing.

BCA Assay

BCA assay (Pierce) is performed following the manufacturer's protocol on all tumor samples. The total protein concentration (in mg/mL) of each tumor sample is later used in the normalization of the ELISA results.

ELISA Assay

All ELISA reagents for the total and phospho-EGFR ELISAs are purchased from R&D Systems as Duoset kits. 96-half well GREINER high binding plates (Cat. #675077; GREINER BIO-ONE, Monroe, NC) are coated with 50 µL of an antibody and incubated overnight at room temperature. Next morning, plates are washed 3 times with 1000 µl/well in a BIOTEK plate washer with PBST (0.05% Tween-20). Plates are subsequently blocked for about 1 hour at room temperature with 2 % BSA in PBS. The plates are washed 3 times with 1000 µl/well in the BIOTEK plate washer with PBST (0.05%Tween-20). 50 µL of cell lysates and standards diluted in 50% Lysis buffer and 1%BSA are used in duplicates for further processing. Samples are incubated for 2hrs at 4°C with shaking and washed 3 times with 1000 µl/well in the BIOTEK plate washer with PBST (PBS with 0.05%Tween-20). About 50 µl of a detection antibody diluted in 2% BSA, PBST is added and incubated for about 1 hour at room temperature. For phospho-EGFR, the detection antibody is directly conjugated to horse radish peroxidase (HRP) and incubated for 2 hrs at room temperature. The plate is washed 3 times with 1000 µl/well in the BIOTEK plate washer with PBST (0.05%Tween-20). About 50 µl of Streptavidin-HRP is added and incubate for 30 min at room temperature (except for pErbB3). The plates are washed 3 times with 1000 µl/well in the BIOTEK plate washer with PBST (0.05%Tween-20). About 50 µL of SUPERSIGNAL PICO ELISA substrate is added and the plate is read using a Fusion plate reader. The data is analyzed using EXCEL. Duplicate samples are averaged and the error bars are used to represent the standard deviation between the two replicates.

Inhibition of AKT and/or ERK Signaling

EGFR mediated signaling via AKT or ERK is measured using art recognized techniques using kinase assays for such proteins. See, for example, US patent publication No. 2010-0056761.

EGFR Dimerization Assay

To measure the extent of EGFR homodimerization in cells, co-precipitation assays using two kinds of epitope-tagged EGFR constructs are used, *e.g.*, EGFR-flag and EGFR-myc which contain Flag and Myc tags, respectively, in the C-terminal region of EGFR. EGFR-flag and EGFR-myc are co-transfected into cells, *e.g.*, COS-7 cells and incubated in serum-free media. The cell lysates are precipitated with anti-Flag antibody and the precipitated material is analyzed by Western blotting using the anti-Myc antibody, or vice versa. The amount of co-precipitation of EGFR-myc with the anti-Flag antibody, and EGFR-flag co-precipitated with anti-Myc antibody in the presence and absence (control) of antibody is measured and compared.

EGFR Internalization Assay

To measure the extent of EGFR internalization (*i.e.*, decrease in EGFR expression), cells expressing radiolabeled EGFR (*e.g.*, ^{125}I) are incubated in the presence and absence (control) of antibody. The ratio of internalized and surface radioactivity is determined and compared.

Binding Affinity

Dissociation constants of anti-EGFR antibodies may be measured using two independent techniques, *e.g.*, a Surface Plasmon Resonance Assay and a cell binding assay using A431 cells. Affinities and cross reactivity of antibodies are also measured in solution with recombinant EGF receptor using KinExA instrumentation (SAPIDYNE Instruments, Boise, ID). The procedure used 12 three-fold titrated dilutions of ErbB1.6his (1000 nM, 333 nM, 111 nM, 37 nM, 12.3nM, 4.1 nM, 1.37 nM, 450 pM, 150 pM, 50 pM, 17 pM) prepared in 5mL tubes keeping the concentration of antibodies in each tube constant at 250pM. 15mL of 2ug/mL Cy5 labeled Gα human IgG secondary antibody is prepared via a 1:1000 dilution of stock solution (2 mg/mL). ErbB1.6HIS conjugated PMMA beads are coupled according to SAPIDYNE's KinExA protocol. 100ug of ErbB1-6his is added to a pre-measured aliquot of 200mg PMMA beads. 1X PBS is then added to the solution to make the final volume 1 ml. This solution is then incubated at room temp for 1 hr. The beads are then transferred to a bead vial with 27 ml

1X PBS. The experiment is then setup by entering the parameters in the KinExA Pro software.

Surface Plasmon Resonance Assay

The Surface Plasmon Resonance Assay is performed as follows.

Either antibody or antigen (300 RU) is immobilized on a CM5 chip using amine coupling. Different concentrations of antibodies or antigens are then injected to study their association and dissociation with the immobilized protein. Between different injections, the chip is regenerated using suitable regeneration buffer (such as glycine, pH 2.5). The dissociation phase is fitted using Equation 1 to determine K_{off} (dissociate rate):

$$R = R_o * \exp(-K_{off} * t) \quad (1)$$

The association phase is fitted using this value of K_{off} and Equation 2 to determine K_{on} (association rate) and K_D (equilbrium constant).

$$R = \frac{R_{max} * C}{K_D + C}(1 - \exp(-(K_{on} * C + K_{off})t) \quad (2)$$

where C represents either the antigen or antibody concentration in solution, R_{max} represents the saturation signal and t represents the time.

Cell Binding Assay

Cell binding assays for determining the K_D values are performed as follows: A431 cells are detached with 3 mLs trypsin-EDTA at 37 °C for 5 minutes. Complete DMEM (10 mLs) is added immediately to the trypsinized cells, resuspended gently and spun down in a Beckman tabletop centrifuge at 1100 rpm for 5 minutes. Cells are resuspended in stain buffer (PBS + 0.2% BSA+ 0.1% sodium azide) at a concentration of 2 x 10^6 cells per ml and 50 µl (1 x 10^5 cells) aliquots are plated in a 96-well titer plate.

A 300 µl stock solution of 2000 nM anti-EGFR antibody is prepared in stain buffer and 100 µl of it is serially diluted into 200 µl of stain buffer. The concentrations of the diluted antibody range from 2000 nM to 0.1 nM. 150 µl aliquots of the different protein dilutions are then added directly to the 50 µl cell suspension giving final concentrations of 1500 nM, 500 nM, 166.7 nM, 55.6 nM, 18.5 nM, 6.17 nM, 2.05 nM, 0.68 nM, 0.23 nM and 0.076 nM of the antibody.

Aliquoted cells in the 96-well plate are incubated with the protein dilutions for 2 hr at room temperature with shaking and washed 3 times with 300 µl stain buffer. Cells are then incubated with 100 µl of a 1:750 dilution of Alexa 647-labeled goat anti-human IgG in BD stain buffer for 45 minutes with shaking at 4°C. Finally, cells are washed

twice, pelleted and resuspended in 250 µl stain buffer + 0.5 µg/ml propidium iodide. Analysis of 10,000 cells is done in a FACSCALIBUR flow cytometer using the FL4 channel. MFI values and the corresponding concentrations of the anti-EGFR-antibodies are plotted on the y-axis and x-axis, respectively. The K_D of the molecule is determined using GRAPHPAD PRISM software using the one-site binding model for a non-linear regression curve.

The K_D value is calculated based on the formula Y=Bmax* X/ K_D + X (Bmax = fluorescence at saturation. X= antibody concentration. Y = degree of binding).

Inhibition of Tumor Cell Proliferation

Inhibition of cellular proliferation of cells expressing EGFR (*e.g.*, cancer cells) is examined as follows: Du145, A431 or OVCAR-3 cells are seeded in 96 well tissue culture plates at 20,000 cells per well and grown in RPMI-1640 medium supplemented with antibiotics, 2mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37 degrees Celsius and 5% carbon dioxide. Medium is then switched to RPMI-1640 (with antibiotics, 2mM L-glutamine, 0.5% FBS) which is without or with antibody at 200nM, 20nM, 2nM, 0.2nM, 0.02nM and 0nM concentrations (a control IgG is used as an isotype control). Cells are grown for 36 hours at 37°C and 5% carbon dioxide. The cells then are pulsed with 25 µl of a 1:40 dilution of ^{3}H- Thymidine (20Ci/mmol, 1mCi/ml), incubated over night (18hrs), harvested to FILTERMATS and read on a beta counter

Inhibition of EGFR Phosphorylation in Tumor Cells

To assess inhibition of EGFR phosphorylation *in vivo*, samples are lysed and BCA and ELISA assays are performed as described above.

Inhibition of EGF induced EGFR phosphorylation

Inhibition of EGF induced EGFR phosphorylation is examined as follows: OVCAR-3 and ADRr cells are plated at a density of 35,000 cells/well in a 96 well plate . Cells are incubated in 10% serum for 24 hrs and then serum starved for 14 hrs. Cells are then preincubated with different concentrations of anti-EGFR antibodies for 40 minutes. Following pre incubation, the medium is removed and the cells are stimulated for 5 minutes at 37^{0}C, 5% CO_2 with 50nM human EGF. EGF controls (5 minutes, 5nM), 10% serum and 0% serum controls are also used. Cells are washed with 1X cold PBS and lysed in 50µl ice cold lysis buffer (R & D SYSTEMS ELISA Kit Buffer 12 with freshly added protease inhibitors) by incubating on ice for 30 minutes. Lysates are either analyzed immediately or frozen at -80°C until use.

Inhibition of EGF-Mediated Signaling in Tumor Cells

Inhibition of ligand-mediated tumor cell signaling is investigated as follows: OVCAR-3 or Du145 cells are seeded in 96 well tissue culture plates and grown in RPMI-1640 medium supplemented with antibiotics, 2mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37°C and 5% carbon dioxide. Cells are serum starved in RPMI-1640 medium with antibiotics and 2mM L-glutamine for 24 hours at 37°C and 5% carbon dioxide. Cells are pre-treated with and without the anti-EGFR antibody at 1μM, 250nM, 63nM, 16nM, 4.0nM, 1.0nM, 240pM and 61pM concentrations for 30 minutes then stimulated with EGF ligand for 10 minutes at 37°C and 5% carbon dioxide. Cells are washed with cold PBS then harvested with mammalian protein extract (MPER) lysis buffer (Pierce, 78505) containing 150mM NaCl 5mM sodium pyrophosphate, 10mM bpV (phen), 50mM phenalarsine, 1mM sodium orthovanadate, and protease inhibitor cocktail (Sigma, P714). Cell lysates are diluted two-fold with 4% bovine serum albumin in phosphate buffered saline with 0.1% Tween-20, then analyzed by ELISA for AKT (a downstream effector of EGFR) and EGFR phosphorylation.

To test for AKT phosphorylation, lysates are run on an ELISA plate with a capture antibody specific for AKT and biotinylated detection antibody specific to the phosphorylation site on serine 473 of AKT. Signal is generated with streptavidin conjugated to horseradish-peroxidase reacted with chemiluminescent substrate (Pierce, 37070).

Example 1: Production of Antibodies

In order to obtain human anti-EGFR antibodies, expression libraries are screened in accordance with methods disclosed in US Patent Publications 20100056386 and 20090181855.

Example 2: Binding Affinity / Epitope Binning

Surface Plasmon Resonance (SPR) is used to analyze binding affinity. Specifically, one of the proteins (antibody or target) is immobilized on the surface of the chip (as described herein) and the other protein is added. The association / dissociation interaction of the two proteins is measured. As the protein in solution associates with the immobilized protein, an increase in refractive index results which is captured by the resonance signal. As the protein dissociates, a decrease in signal results.

Epitope binning (EGF blocking) is analyzed using BIACORE analysis as described above. One of the antibodies is immobilized on the surface of the chip. As EGFR associates with the antibody, the resonance signal increases. The chip is then regenerated and a mixture of EGFR and another antibody (*e.g.*, an antibody previously determined to bind to EGFR) is injected. If the antibody binds overlapping epitopes with the injected antibody, then the signal will be less compared to EGFR injected alone. The chip is then regenerated again and a mixture of EGF ligand and EGFR is injected. Resonance signal is measured. A decrease in signal indicates overlapping epitopes with EGF ligand. The chip is regenerated for a final time and injected with EGFR to confirm the activity of the antibody.

Example 3: Inhibition of EGFR phosphorylation in tumor cells

Inhibition of EGFR phosphorylation in ADRr, Ovcar3, and A431 cells by particular anti-EGFR antibodies is analyzed as described above.

Example 4: Inhibition of AKT phosphorylation in tumor cells

Inhibition of AKT phosphorylation in Du145 and Ovcar3 cells for antibody combinations is analyzed as described above. Two cell lines are tested based on two parameters: (1) high ($>10^5$ receptors/cell) expression of ErbB1 and (2) dynamic range of induction of pAKT is ≥5-fold.

All anti-EGFR antibodies are combined with another anti-EGFR antibody without regard to epitope mapping. Six point inhibition curves are created (2μM peak concentration with serial 10X dilutions for many pairs and trios, while some are screened with 4 point dilution curves.

All antibodies that either additively or synergistically inhibit pAKT will map to distinct epitopes. Some pairs will be more potent inhibitors than cetuximab. Results in two cell lines mirror each other suggesting that, in the absence of mutated receptors or signaling pathways (*e.g.*, kRAS), high expression of ErbB1 (and perhaps ErbB1> ErbB2/3) is a beginning criteria for synergy.

Short and long-term inhibition studies are conducted using anti-EGFR antibody combinations with 40 minute pre-incubation of antibodies. Assays are repeated with 40 minute, compared to 24 hours, pre-incubation of antibodies. Data show that effects of internalization and degradation of EGFR change readouts.

Example 5: Inhibition of tumor cell proliferation

Inhibition of tumor cell proliferation is analyzed as described above. The inhibitory action of the pairs and trios provided herein on DU145 cells is preferably robust. Also, preferred pairs and trios of antibodies present a greater inhibition of Du145 cell proliferation than an equimolar concentration of cetuximab. Pairs and trios are evaluated in pERK assay as well. Preferred pairs and trios are potent antagonists of pERK signaling.

Materials and Methods for Examples 6-12

Throughout Examples 6-13, the following materials and methods are used unless otherwise stated.

In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, *e.g.*, antibody technology), and standard techniques in polypeptide preparation. See, *e.g.*, Sambrook, Fritsch and Maniatis, Molecular Cloning Cold Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow et al., C.S.H.L. Press, Pub. (1999); and Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons (1992). In vitro and in vivo model systems for assaying HCV biology are described, for example, in Cell culture models and animal models of viral hepatitis. Part II: hepatitis C, Lab. Anim. (NY).;34(2):39-47 (2005) and in The chimpanzee model of hepatitis C virus infections, ILAR J.; 42(2): 117-26 (2001).

Cell Lines

All the cell lines to be used in the experiments described below are obtained from the National Cancer Institute or ATCC.

Cell Lines:

A431 – epidermoid carcinoma

Du 145 - prostate carcinoma

H1975-non-small cell lung carcinoma

Protein Purification of EGFR Extracellular Domain (EGFR-ECD) Mutants

Mutants of the EGFR extracellular domain (EGFR-ECD) are generated for mAb epitope binning. Mutations were designed based upon both the cetuximab (Li S. *et al.*, Cancer Cell. **7:** 301-311, 2005) and H11 (Spangler J. *et al.* PNAS. **107:** 13252-13257, 2010) epitopes and upon structural analysis of the EGFR-ECD structure (Protein Data Bank ID: 1NQL; Ferguson K.M. *et al.* Mol Cell. **11:** 507-517, 2003). Residues are mutated to alanines as noted in the protein sequences included in this application. DNA synthesis of expression constructs is completed by DNA2.0 (www.dna20.com). Subsequent DNA subcloning, protein expression in 293F cells and protein purification are completed as previously described.

Inhibition of EGF-Mediated Signaling of EGFR or ERK in Tumor Cells

Inhibition of ligand-mediated tumor cell signaling is investigated as follows: A431 or Du145 cells are seeded at a density of 35,000 cells/well or 17,500 cells per half well in 96 well tissue culture plates and grown in DMEM or RPMI-1640 medium supplemented with antibiotics, 2 mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37°C and 5% carbon dioxide. Cells are serum starved in 1% FBS medium with antibiotics and 2mM L-glutamine for about 20 hours at 37°C and 5% carbon dioxide. Cells are then preincubated with varying concentrations of anti-EGFR antibodies for 2 hrs, and then stimulated with human EGF ligand (50 ng/ml) (Preprotech, cat # AF-100-15) for 10 minutes at 37°C and 5% carbon dioxide. Cells are washed with ice-cold PBS and lysed in 50µl ice-cold Lysis buffer (Mammalian Protein Extract Lysis buffer (MPER-Pierce, 78505) amended with 150mM NaCl and protease inhibitor cocktail (Sigma, P714)) by incubating on ice for 30 minutes. Lysates are either analyzed immediately by ELISA for ERK (a downstream effector of EGFR) and EGFR phosphorylation, or frozen at -80°C until use.

ELISA Assays

For the phospho-EGFR sandwich ELISA, 96-half well GREINER high binding plates (Cat. #675077; GREINER BIO-ONE, Monroe, NC) are coated with 50 µL of an EGFR antibody (EGFR Ab-11, Clone: 199.12, without BSA and azide, Fisher Scientific, cat# MS396P1ABX), and incubated overnight at room temperature. Next morning, plates are washed 3 times with 100 µl/well PBST (0.05% Tween-20) on a BIOTEK plate washer. Plates are subsequently blocked for about 1 hour at room temperature with 2 %

BSA in PBS. The plates are washed 3 times with 100 µl/well PBST (0.05%Tween-20) on the BIOTEK plate washer. Cell lysates (50µl) or standards (pEGFR pY1068 ELISA kit, R&D Systems, cat# DYC3570) diluted in 50% Lysis buffer and 1%BSA-PBS (per the manufacturer's recommendations) are added to the plates in duplicates and incubated for 2 hrs at room temperature or overnight at 4°C with shaking. Plates are then washed 3 times with 100 µl/well in the BIOTEK plate washer with PBST (PBS with 0.05%Tween-20). About 50 µl of a detection antibody conjugated to horse radish peroxidase (HRP) (pEGFR pY1068 ELISA kit, R&D Systems, cat# DYC3570) diluted in 2% BSA, PBS is added and incubated for about 2 hour at room temperature. The plate is washed 3 times with 100 µl/well in the BIOTEK plate washer with PBST (0.05%Tween-20). About 50 µL of SUPERSIGNAL PICO ELISA substrate is added and the plate is read using an Envision (Perkin Elmer) plate reader. The data is analyzed and duplicate samples are averaged and the error bars are used to represent the standard deviation between the two replicates.

The phospho-ERK ELISA is performed similarly to the phospho-EGFR ELISA with the following changes: Human pERK ELISA Duoset kit is purchased from R&D Systems (cat# DYC1018-5) and used as recommended by the manufacturer.

A direct ELISA is performed using EGFR-ECD wild-type (WT), a Bin1 epitope mutant, or a Bin3 epitope mutant as capture reagents (4µg/ml). 96-half well GREINER high binding plates (Cat. #675077; GREINER BIO-ONE, Monroe, NC) were coated with 50 µL of capture reagent and incubated overnight at room temperature. Next morning, plates are washed 3 times with 100 µl/well in a BIOTEK plate washer with PBST (0.05% Tween-20) and blocked for about 1 hour at room temperature with 1% BSA in PBS, pH7.2. Varying concentrations (1, 0.25, 0.06, and 0.02 µg/ml) of monoclonal antibodies (mAbs) diluted in 1 % BSA in PBS, pH7.2 were incubated with the capture reagents at room temperature for 2 hours, followed by detection with 1:50,000 dilution in 1 % BSA in PBS, pH7.2 of Peroxidase-Conjugated AffiniPure Goat Anti-Human IgG Fc Fragment (Jackson Immunoresearch Catalog #109-035-008) for 2 hours. About 50 µL of Supersignal PICO ELISA substrate is added and the plate is read using an Envision (Perkin Elmer) plate reader. The data is analyzed and duplicate samples are averaged and the error bars are used to represent the standard deviation between the two replicates. Cells are pre-treated with and without the anti-EGFR

antibody at 2 μM, 666.6 nM, 222.2 nM, 74 nM, 24.7 nM, 8.2 nM, 2.7 nM, 914 pM and 304 pM concentrations for 2 hours

Binding Affinity: Kinetic Exclusion Assay (KinExA)

Affinities and cross reactivity of antibodies are also measured in solution with recombinant EGF receptor using KinExA instrumentation (SAPIDYNE Instruments, Boise, ID). Materials used for this assay are a KinExA 3000 instrument and software (Sapidyne Instruments, Boise, ID), polymethylmethacrylate (PMMA) beads (Sapidyne Instruments), human anti-EGFR IgG, recombinant human EGFR, Cy5-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA), phosphate buffered saline (PBS), and bovine serum albumin in PBS (100 mg/ml).

To couple the recombinant EGF receptor to PMMA beads, 100 μg of recombinant EGFR is added to a pre-measured aliquot of 200 mg PMMA beads, and PBS is added to make the total volume 1 ml. The beads are incubated for 1 hr at room temperature on a rotating wheel. Then the beads are briefly centrifuged and the supernatant is removed. 100 μl of 100 mg/ml BSA in PBS is added to the beads, with further addition of PBS to make a total volume of 1 ml. The beads are incubated again for 1 hr at room temperature on a rotating wheel. The beads are then transferred to a glass bottle containing 27 mL of PBS.

To determine the monovalent antibody binding affinity, a twelve-step dilution series of recombinant EGFR (75 nM, 25 nM, 8.3 nM, 2.8 nM, 0.9 nM, 0.3 nM, 100 pM, 33 pM, 11 pM, 4 pM, 1.3 pM, 0 pM) is prepared in 5 ml PBS having a constant concentration of anti-EGFR antibody. For accurate affinity measurement, the total antibody binding site concentration ("ABC"; twice the molar concentration of antibody, due to valence) should be less than the monovalent affinity of the antibody for EGFR. The antibody-receptor mixtures are incubated for 2 hr at room temperature in order to achieve equilibrium. Depending upon the expected affinity of the antibody-receptor complex, this equilibration time may be adjusted accordingly. In a separate tube, 15 mL of 2 μg/mL Cy5-conjugated anti-human IgG secondary antibody is prepared, using a 1:1000 dilution of stock (2 mg/mL) antibody into PBS. Then, the KinExA instrument lines are attached to each of the 12 antibody-receptor solution tubes. Each solution is injected through a packed EGFR-bead column. (The KinExA instrument automatically packs a fresh bead column for each injection.) After a wash step, the labeled secondary

antibody is passed through the column. Finally, using the measured amount of uncomplexed receptor at different receptor concentrations, the equilibrium titration data is fit to a 1:1 binding model in the KinExA software to yield an affinity value Kd.

To determine the binding on-rate using the KinExA "direct method", the equilibrium monovalent binding affinity (K_D) is determined using the above approach and total antibody binding site concentration (ABC). Then, using the "Theoretical Binding Curve Demonstration" software (Sapidyne Instruments), the starting antigen concentration (L0) is determined for the kinetics experiment. To do this, the affinity and ABC values determined in the monovalent binding affinity experiment are entered, and a starting antigen concentration is selected as that concentration where roughly 20% of antibody will be unbound to antigen at equilibrium. This assures good signal-to-noise ratio in the experiment. 15 mL of 2 μg/mL Cy5-conjugated anti-human IgG secondary antibody is prepared, using a 1:1000 dilution of stock (2 mg/mL) antibody into PBS. In a separate tube, 8 mL of anti-EGFR antibody solution is prepared at a concentration of 2 x ABC. This concentration is double the running concentration, since it is mixed with 8 mL of antigen solution prior to the experiment. In a separate tube, 8 mL of recombinant EGFR solution is prepared at a concentration of 2 x L0. This concentration is also double the running concentration, since it is mixed with 8 mL of antibody solution prior to the experiment. Then, the EGFR coated beads and secondary antibody solution are placed in the appropriate container and line, respectively. The antibody and antigen solution are mixed thoroughly and immediately connected to the appropriate line, and the KinExA software is used to measure the amount of free antibody as a function of time in the resulting solution. To determine the association constant k_{on} (Kon), the KinExA software is used to fit the depletion of the amount of free antibody as a function of time to a reversible bimolecular rate equation. The dissociation constant K_{off} (Koff) is equal to the K_{on}*K_D (Kd).

Binding Affinity: Surface Plasmon Resonance Assay

The Surface Plasmon Resonance Assay is performed as follows.

Either antibody or antigen (300 RU) is immobilized on a CM5 chip using amine coupling. Different concentrations of antibodies or antigens are then injected to study their association and dissociation with the immobilized protein. Between different

injections, the chip is regenerated using suitable regeneration buffer (such as glycine, pH 2.5). The dissociation phase is fitted using Equation 1 to determine K_{off} (dissociate rate):

$$R = R_o * \exp(-K_{off} * t) \quad (1)$$

The association phase is fitted using this value of K_{off} and Equation 2 to determine K_{on} (association rate) and K_D (equilibrium constant).

$$R = \frac{R_{max} * C}{K_D + C}(1 - \exp(-(K_{on} * C + K_{off})t)) \quad (2)$$

where C represents either the antigen or antibody concentration in solution, R_{max} represents the saturation signal and t represents the time.

Cell Binding Assay

Cell binding assays for determining the K_D values are performed as follows: A431 cells are detached with 3 mLs trypsin-EDTA at 37 °C for 5 minutes. Complete DMEM (10 mLs) is added immediately to the trypsinized cells, resuspended gently and spun down in a Beckman tabletop centrifuge at 1100 rpm for 5 minutes. Cells are resuspended in stain buffer (PBS + 0.2% BSA+ 0.1% sodium azide) at a concentration of 2 x 10^6 cells per ml and 50 µl (1 x 10^5 cells) aliquots are plated in a 96-well titer plate.

A 300 µl stock solution of 2000 nM anti-EGFR antibody is prepared in stain buffer and 100 µl of it is serially diluted into 200 µl of stain buffer. The concentrations of the diluted antibody range from 2000 nM to 0.1 nM. 150 µl aliquots of the different protein dilutions are then added directly to the 50 µl cell suspension giving final concentrations of 1500 nM, 500 nM, 166.7 nM, 55.6 nM, 18.5 nM, 6.17 nM, 2.05 nM, 0.68 nM, 0.23 nM and 0.076 nM of the antibody.

Aliquoted cells in the 96-well plate are incubated with the protein dilutions for 2 hr at room temperature with shaking and washed 3 times with 300 µl stain buffer. Cells are then incubated with 100 µl of a 1:750 dilution of Alexa 647-labeled goat anti-human IgG in BD stain buffer for 45 minutes with shaking at 4°C. Finally, cells are washed twice, pelleted and resuspended in 250 µl stain buffer + 0.5 µg/ml propidium iodide. Analysis of 10,000 cells is done in a FACSCALIBUR flow cytometer using the FL4 channel. MFI values and the corresponding concentrations of the anti-EGFR-antibodies are plotted on the y-axis and x-axis, respectively. The K_D of the molecule is determined using GRAPHPAD PRISM software using the one-site binding model for a non-linear regression curve.

The K_D value is calculated based on the formula Y=Bmax* X/ K_D + X (Bmax = fluorescence at saturation. X= antibody concentration. Y = degree of binding).

Inhibition of Tumor Cell Proliferation *In Vitro*

Inhibition of cellular proliferation of cells expressing EGFR (*e.g.*, cancer cells) is examined *in vitro* as follows: H1975 or A431 cells are seeded in 96 well tissue culture plates at 250, 500, 1000 or 2,000 cells per well and grown in RPMI-1640 medium supplemented with antibiotics, 2mM L-glutamine and 10% fetal calf serum (FCS) for 24 hours at 37 degrees Celsius and 5% carbon dioxide. Medium is then switched to RPMI-1640 (with antibiotics, 2mM L-glutamine, 1% FBS) supplemented with 50ng/mL EGF in the presence or absence of 1 μM antibody (final concentration). The cells are grown for 6 days at 37°C and 5% carbon dioxide. Cell viability is measured using the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (Promega Corporation) according to manufacturer's instructions. The CTG assay measures the number of viable cells in culture based upon quantitation of ATP present, which is an indicator of metabolically active cells. Control treatments include cells treated with RPMI-1640 with antibiotics, 2mM L-glutamine, 1% FCS but not supplemented with 50ng/mL EGF (referred to as 1% FCS treatment) and cells treated with RPMI-1640 with antibiotics, 2mM L-glutamine but not supplemented with either FCS or EGF (referred to as serum free media, or SFM, treatment).

Mouse Xenograft *in vivo* Studies

Nu/nu mice (Charles River Labs) are injected subcutaneously with 3.5 x 10^6 A431 cells or 2.0 x 10^6 H1975 cells and the resultant tumors allowed to grow until they reached an average size of 200 mm. Dosing is then initiated as follows. Antibodies cross-reactive with mouse EGFR are given every 2 days (q2d) and non-cross-reactive antibodies are given every 4 days (q4d). Measurements are taken at 3 day intervals. Dosing is stopped after 5 and 7 doses for A431 and H1975, respectively. A recovery period is maintained for both studies. Antibody doses were normalized to represent a uniform serum half-life.

Example 6: Binding Affinity / Epitope Binning

Epitope binning is analyzed using BIACORE analysis as described above. One of the antibodies is immobilized on the surface of the chip. As EGFR associates with

the antibody, the resonance signal increases. Sequential injections of antibodies that belong to the three bins 1, 2 and 3 are performed. If the antibody binds overlapping epitopes with the injected antibody, then the signal will not change compared to the previous injection. If the antibody binds to a non-overlapping epitope, the signal on the chip will be higher than the previous injection. The antibody conjugated to the chip is finally injected as free ligand to confirm lack of binding with overlapping epitopes (Figure 17D).

The Bins were designed so that selected antibodies would span three distinct, non-overlapping epitopes on the extracellular domain (ECD) of EGFR. These are grouped into three bins: Bin1 is mapped to Domain III of EGFR and represents the c225 epitope (the site of cetuximab binding); Bin2 is mapped to Domain I and represents the ICR10 epitope (Abcam Ab231) (Cochran *et al.* (2004) Journal of Immunological Methods, 287:147-158); and Bin3 is mapped to Domain III and represents the clone H11 epitope (Spangler J. *et al.* PNAS. **107:** 13252-13257, 2010). Bin1 (B1-7MT-Ala) and Bin3 (B3-4MT) mutants were generated for epitope mapping at the amino acid positions shown below in bold in the EGFR extracellular domain.

EGFR ECD (SEQ ID NO: 58)

```
1   MRPSGTAGAA LLALLAALCP ASRALEEKKV CQGTSNKLTQ LGTFEDHFLS LQRMFNNCEV
61  VLGNLEITYV QRNYDLSFLK TIQEVAGYVL IALNTVERIP LENLQIIRGN MYYENSYALA
121 VLSNYDANKT GLKELPMRNL QEILHGAVRF SNNPALCNVE SIQWRDIVSS DFLSNMSMDF
181 QNHLGSCQKC DPSCPNGSCW GAGEENCQKL TKIICAQQCS GRCRGKSPSD CCHNQCAAGC
241 TGPRESDCLV CRKFRDEATC KDTCPPLMLY NPTTYQMDVN PEGKYSFGAT CVKKCPRNYV
301 VTDHGSCVRA CGADSYEMEE DGVRKCKKCE GPCRKVCNGI GIGEFKDSLS INATNIKHFK
361 NCTSISGDLH ILPVAFRGDS FTHTPPLDPQ ELDILKTVKE ITGFLLIQAW PENRTDLHAF
421 ENLEIIRGRT KQHGQFSLAV VSLNITSLGL RSLKEISDGD VIISGNKNLC YANTINWKKL
481 FGTSGQKTKI ISNRGENSCK ATGQVCHALC SPEGCWGPEP RDCVSCRNVS RGRECVDKCN
541 LLEGEPREFV ENSECIQCHP ECLPQAMNIT CTGRGPDNCI QCAHYIDGPH CVKTCPAGVM
601 GENNTLVWKY ADAGHVCHLC HPNCTYGCTG PGLEGCPTNG PKIPSHHHHH H
```

The following substitution mutants were made at the indicated amino acid positions using standard recombinant DNA technology to create the Bin1 (B1) and Bin3 (B3) epitope mutants with the following mutant residues:

Mutants
Bin1 (B1) Mutant Residues
B1-7MT-Ala: Q408A, Q432M, H433E, K467A, K489A, I491A, N497A

Bin3 (B3) Mutant Residues
B3-4MT: S380A, F381G, T382A, H383G

A direct ELISA was performed using EGFR-ECD wild-type (WT), a Bin1 epitope mutant (c225 epitope), or a Bin3 epitope mutant (H11 epitope) as capture

reagents. Varying concentrations (1, 0.25, 0.06, and 0.02 μg/ml) of monoclonal antibodies (mAbs) ca (Bin1), cd (Bin2), and ch (Bin3) were incubated with the capture reagents at room temperature for 2 hours, followed by detection with HRP-conjugated anti-human Fc polyclonal antibody for 1 hour. As shown in Figure 17A, all three antibodies bound to the WT extracellular domain of EGFR, whereas the Bin1 antibody ca did not bind to the Bin1 mutant epitope (Figure 17B) and the ch antibody did not bind to the Bin3 mutant epitope (Figure 17C).

In order to further characterize the anti-EGFR antibodies, mAb ch (Bin3) was conjugated to the surface of a BIACORE chip. 0.5 μM EGFR extraceullular domain (EGFR-ECD) was injected followed by sequential injections of mAb cd (Bin2), mAb cb (Bin1), and finally mAb ch(Bin3) as a negative control for epitopes blocked by the binding of EGFR ECD to the Bin3 antibody conjugated to the chip surface. As shown in Figure 17D, antibodies from the three bins sequentially bound the extracellular domain of EGFR, demonstrating that mAbs belonging to Bins 1, 2, and 3 have non-overlapping, distinct epitopes.

A summary of the epitope mapping by ELISA and BIACORE is found in Table VIII below and a summary of binding affinities as determined by KinExA® is found below in Table IX. All affinities were better than 0.8 nM.

Table VIII:

		ELISA		BIACORE								
Antibody Bin	Name	Bin3 MT	Bin1 MT	H11	ICR10	cetuximab	ci	ch	cf	cd	cb	ca
Bin 1	ca	Yes	No	n.d.	Yes	No	n.d.	Yes	n.d.	Yes	n.d.	
Bin 1	cb	Yes	No	n.d.	Yes	No	Yes	Yes	Yes	Yes		
Bin 2	cd	Yes	Yes	n.d.	No	Yes	n.d.	Yes	n.d.			
Bin 2	cf	n.d.	n.d.	n.d.	n.d.	n.d.	Yes	Yes				
Bin 3	ch	No	Yes	n.d.	Yes	Yes	No					
Bin 3	ci	n.d.	n.d.	n.d.	n.d.	n.d.						
	cetuximab	n.d.	No	n.d.	n.d.							
	ICR10	n.d.	n.d.	n.d.								
	H11	No	n.d.									

n.d. – not determined (experiment not performed)

Yes, denotes binding events, corresponding to non-overlaping epitopes

No, denotes lack of binding events, corresponding to overlapping epitopes

Table IX

Antibody	Bin	Kd (M) By KinExA
ca	1	1.45E-10
cb	1	2.40E-11
cc	1	1.53E-10
cd	2	5.40E-10
ch	3	7.57E-10
ci	3	1.80E-09

Example 7: Phospho-ERK signaling inhibition by combinations of two anti-EGFR antibodies with non-overlapping epitopes

Non-overlapping anti-EGFR antibody combinations were tested for their ability to inhibit EGFR-mediated signaling, as measured by pERK inhibition, in A431 tumor cells by selecting one antibody from each of two or three bins (Bin1, Bin2, and Bin3 described above). A431 cells were incubated for 2 hr with indicated equimolar combinations of Bin 1 + Bin 3 (Figures 18A and 18B) or Bin 1 + Bin 2 antibodies (Figure 18C) and cell lysates analyzed by phospho-ERK ELISA. Results demonstrate that all pairwise combinations cause complete inhibition of pERK signaling, but exhibit different efficacies at the IC90s of the inhibition curves.

Example 8: Inhibition of ERK phosphorylation by combinations of two or three anti-EGFR antibodies with non-overlapping epitopes.

Additional experiments on the ability of antibody combinations to inhibit EGFR signaling, as measured by pERK inhibition, were carried out as described in Example 7 and in the methodology section above, A431 cells were treated with equimolar amounts of double (pair-wise) combinations of indicated Bin1/Bin2 antibodies (Figure 19A), Bin1/Bin2 or Bin1/Bin3 antibodies (Figure 19B) or triple combination of Bin1, Bin 2 and Bin 3 antibodies (Figure 19A). Treatment with the combinations of Bin1 + Bin2 antibodies, Bin1 + Bin3 antibodies or Bin1 + Bin2 + Bin3 antibodies all showed a dose dependent decrease in pERK production. As shown in Figure 19A, the equimolar combination of the three antibodies with non-overlapping epitopes completely inhibited ERK activation. When compared to the anti-EGFR antibody cetuximab, the duo combination of antibodies cb (Bin 1) and cd (Bin 2) exhibited 100 and 1000-fold higher

efficacy at reducing pERK signaling at the IC50 and IC90 of the inhibition curve (Figure 19B). Moreover, the duo mixture completely inhibited pERK signaling, while cetuximab did not reach maximal inhibition at the highest dose (only 50%).

Example 9: Phospho-ERK signaling inhibition by equimolar and non-equimolar combinations of two anti-EGFR antibodies with non-overlapping epitopes

A431 cells were treated with varying ratios (in μg/ml) of antibody pairs cb and cd (Figure 20A), cb and ch (Figure 20B), cb and ci (Figure 20C) and ca and cd (Figure 20D) and the ability of the antibody pair combinations to inhibit EGFR signaling, as measured by pERK inhibition, was determined. When used individually (300 μg/ml alone without a second antibody), anti-EGFR antibodies from Bin2 (cd) and Bin3 (ch and ci) were inefficacious at reducing pERK signaling (Figures 20A, 20B and 20C), while antibodies from Bin1 (cb and ca) showed improved efficacy. In all cases, the duo combinations resulted in complete inhibition of pERK signaling. Shifting the ratios away from equimolar concentrations did not result in a equivalent shift in the potency of the mixtures except for the ca and cd duo (Figure 20D). These results demonstrate improved potency of the mixtures over the single antibody components, and a tolerance of at least 10% of each component to contribute to complete inhibition of *in vitro* signaling (*i.e.*, a ratio of 270+30 μg/ml of cb+ch is equal in potency to a 150+150 μg/ml combination). A summary of these data is represented by a heat map (Figure 21) in which the x-axis (columns) denotes concentration of antibody, y-axis (rows) denotes pairs of antibodies combined at indicated ratios, and z-axis (into the plane) denotes fraction phospho-ERK activity in grayscale. The z-axis color map represents high phospho-ERK activity as white, low activity as black, and intermediary activities in shades of gray. Sample values are independently normalized to the mean of the +EGF ligand control on the ELISA plate on which they were analyzed and the mean values of EGF ligand controls (+/-) across plates are indicated at top left.

Example 10: Phospho-EGF receptor and phospho-ERK signaling inhibition by single and pairwise combinations of Bin 1 + Bin 2 or Bin 1 + Bin 3 antibodies

and comparisons with other known anti-EGFR antibodies such as cetuximab, nimotuzumab, and zalutumumab.

A431 cells were treated with single antibodies or antibody pairs and their ability to inhibit EGFR-dependent signaling was compared to that of known anti-EGFR antibodies cetuximab, nimotuzumab, or zalutumumab. Experiments were performed as described above for pERK and pEGFR signaling and the results are shown in Figures 22A-C. Only mixtures of Bin1/Bin2 antibodies cb and cd (Figure 22A) and Bin1/Bin3 antibodies cb and ch (Figure 22C) were effective at completely inhibiting phospho-ERK signaling when compared to the antibodies known in the art as well as individual components of the mixtures, cb, cd, and ch. All antibodies including the mixtures were effective at complete inhibition of Phospho-EGF receptor signaling with the exception of nimotuzumab (Figure 22B).

Example 11: Inhibition of tumor cell proliferation *in vitro*

Inhibition of tumor cell proliferation *in vitro* is analyzed by the methods described above or minor variations thereof. A431 cells (epidermoid) or H1975 cells (NSCLC) were plated at 250, 500, 1000, or 200 cells/well and treated with antibody combinations at 1 μM. Figures 23A-D show inhibition of cell proliferation using CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (Promega Corporation) that measures the number of viable cells in culture based upon quantitation of ATP present, which is an indicator of metabolically active cells. Figure 23A shows inhibition of growth of H1975 cells treated with a combination of the Bin1 and 3 antibodies ca antibody ca or an antibody that competes for binding to EGFR extracellular domain with antibody ca and ch, but not by the Serum Free Medium (SFM) or assay medium alone (1% FCS) in the presence or absence of the EGF ligand. Figure 23B shows similar results to Figure 23A, but with a combination of the Bin1 and 2 antibodies ca and cd. Figures 23C (H1975 cells) and 23D (A431 cells) show inhibition of cell proliferation with cells treated with the Bin1/2/3 trio of antibodies ca + cd + ch. All combinations used in this example clearly demonstrate the ability of antibody mixtures to inhibit tumor cell proliferation *in vitro*.

Example 12: Inhibition of tumor growth in a xenograft model *in vivo*

In order to evaluate inhibition of tumor growth *in vivo,* antibody pairs are used in the mouse xenograft tumor model using the epidermoid tumor cell line A431 (Figure 24A) and the NSCLC tumor cell line H1975 (Figure 24B). 75 4-5 week old nu/nu mice are injected (implanted) subcutaneously with 3.5 x 10^6 cells per mouse of A431 cells or 2.0 x10^6 cells per mouse of H1975 cells. Mice are dosed with antibody pairs beginning at day 8 (A431 cells) or day 4 (H1975 cells) post-implantation. Using the preceding methods or minor variations thereof, the Bin1/2 (cb + cd) and Bin 1/3 (cb + ch; ca + ch) antibody pairs were at least as effective in inhibiting tumor growth as cetuximab in A431 tumors. All treatments resulted in tumor stasis that was maintained through the recovery period after discontinuing the dosing regimen. In H1975 cells, however, all treatments resulted in tumor stasis, but tumors regressed rapidly after dosing was discontinued in all mice except those treated with the ca+ch pair.

Example 13: Inhibition of EGF Binding to Cells by Anti-EGFR Antibodies

A431 cells are grown to confluency in DMEM complete medium (as described above) in a T-150 cm^2 tissue culture flask, washed with 10 mL ice-cold PBS and detached with 5 mL 0.25% trypsin while being carefully monitored for detachment in order to minimize trypsinization of cell surface proteins (~5-7 min). As soon as cells began to lift, 15 mL of complete media is added to dilute trypsin, and cells in suspension are transferred to 50 mL conical centrifuge tubes, and centrifuged at 1200 rpm for 7 min. Supernatant is discarded and cell pellet is re-suspended in flow cytometry buffer (2% Fetal Bovine Serum, 0.1% Sodium Azide in 1XPBS buffer pH 7.2). Cells are transferred to a V-bottom 96-well plate at a density of 30,000 cells per well in a 100µl volume. A total of 12 wells are seeded for each technical replicate (11 for ligand dilution curve, 1 for ligand-only control), to which 100µL of antibody reaction mixture at a concentration of 4uM is added to each well (final concentration in wells at 2µM), except for the ligand-only control wells to which only flow cytometry buffer is added. Cells are incubated with the antibodies at room temperature for 1 hr, followed by the addition of 100 µL of 3-fold dilutions in flow cytometry buffer of biotin-XX-EGF ligand (Invitrogen cat# E3477) starting at 600nM (final concentrations in wells raging from 200nM-0.003nM), and incubated for 10 min at room temperature. Cells are then washed with 100 µL of ice-cold flow cytometry buffer, centrifuged at 1800 rpm for 2 min, and

the supernatant discarded. Cells are then incubated in 100µL of 1:500 dilution of Streptavidin-Alexa Fluor 647 conjugate (Invitrogen cat# S21374)in flow cytometry buffer for 30 min at room temperature. This step is followed by washing cells in 100µL of ice-cold flow cytometry buffer, centrifugation at 1800 rpm for 2 min, after which the supernatant is discarded. Wash step is repeated and cells are re-suspended in 80 µL fixing buffer (2% Fetal Bovine Serum, 2% Paraformaldehyde in 1XPBS, pH7.2), and transferred to U-bottom 96-well plates. Cells with bound ligand are detected by flow cytometry, gating for live cells (FSC/SSC) and for FL4-channel (measuring Alexa-Fluor 647 signal) positive cells. Results obtained using the methods here disclosed, or minor variations thereof, are shown in Figure 25 and the outcomes are described in the description thereof.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims. Any combination of the embodiments disclosed in the any plurality of the dependent claims are contemplated to be within the scope of the disclosure.

Incorporation by Reference

All, patents, pending patent applications and patent publications referred to hereinabove are hereby incorporated by reference in their entireties.

APPENDIX A
ANTI-CANCER AGENTS

Anti-Cancer Agent	Comments	Examples
Antibodies	Antibodies which bind IGF-1R (insulin-like growth factor type 1 receptor), which is expressed on the cell surface of must human cancers	A12 (fully humanized mAb) 19D12 (fully humanized mAb) CP751-871 (fully humanized mAb) H7C10 (humanized mAb) alphaIR3 (mouse) scFV/FC (mouse/human chimera) EM/164 (mouse) AMG 479 (fully humanized mAb; Amgen) IMCA 12 (fully humanized mAb; Imclone) NSC-742460 (Dyax) MR-0646, F50035 (Pierre Fabre Medicament, Merck)
	Antibodies which bind EGFR; Mutations affecting EGFR expression or activity can result in cancer	matuzumab (EMD72000) Erbitux® / cetuximab (Imclone) Vectibix® / panitumumab (Amgen) mAb 806 nimotuzumab (TheraCIM®) INCB7839 (Incyte) panitumumab (Vectibix®; Amgen)
	Antibodies which bind cMET (mesenchymal epithelial transition factor); a member of the MET family of receptor tyrosine kinases)	AV299 (AVEO) AMG102 (Amgen) 5D5 (OA-5D5) (Genentech)
	Anti-ErbB3 antibodies	MM-121 (Merrimack Pharmaceuticals) Ab #14 described in WO 2008/100624 1B4C3; 2D1D12 (U3 Pharma AG) U3-1287/AMG888 (U3 Pharma/Amgen)

	Anti-ErbB2 (HER2) antibodies	Herceptin® (trastuzumab; Genentech/Roche); Omnitarg® (pertuzumab; 2C4,R1273; Genentech/Roche)
Small Molecules Targeting IGF1R	IGF-1R (insulin-like growth factor type 1 receptor), which is expressed on the cell surface of must human cancers	NVP-AEW541-A BMS-536,924 (1H-benzoimidazol-2-yl)-1H-pyridin-2-one) BMS-554,417 Cycloligan TAE226 PQ401
Small Molecules Targeting EGFR	EGFR; Mutations affecting EGFR expression or activity can result in cancer	Iressa® / gefitinib (AstraZeneca) CI-1033 (PD 183805) (Pfizer) TYVERB / lapatinib (GlaxoSmithKline) Tykerb® / lapatinib ditosylate (SmithKline Beecham) Tarceva®/ Erlotinib HCL (OSI Pharma) PKI-166 (Novartis) PD-158780 EKB-569 Tyrphostin AG 1478(4-(3-Chloroanillino)-6,7-dimethoxyquinazoline)
Small Molecules Targeting ErbB2	ErbB2, also known as HER2, a member of the ErbB family of receptors, which is expressed on certain cancer cells	HKI-272 (neratinib; Wyeth) KOS-953 (tanespimycin; Kosan Biosciences)
Small Molecules Targeting cMET	cMET (Mesenchymal epithelial transition factor); a member of the MET family of receptor tyrosine kinases)	PHA665752 ARQ 197 (ArQule) ARQ-650RP (ArQule)

Antimetabolites	An antimetabolite is a chemical with a similar structure to a substance (a metabolite) required for normal biochemical reactions, yet different enough to interfere with the normal functions of cells, including cell division.	flourouracil (5-FU) capecitabine / XELODA® (HLR Roche) 5-trifluoromethyl-2'-deoxyuridine methotrexate sodium (Trexall) (Barr) raltitrexed / Tomudex® (AstraZaneca) pemetrexed / Alimta® (Lilly) tegafur cytosine arabinoside (Cytarabine, Ara-C) / tioguanine / Lanvis® (GlaxoSmithKline) 5-azacytidine 6-mercaptopurine (Mercaptopurine, 6-MP) azathioprine / Azasan® (AAIPHARMA LLC) 6-thioguanine (6-TG) / Purinethol® (TEVA) pentostatin / Nipent® (Hospira Inc.) fludarabine phosphate / Fludara® (Bayer Health Care) cladribine / Leustatin® (2-CdA, 2-chlorodeoxyadenosine) (Ortho Biotech) floxuridine (5-fluoro-2'-deoxyuridine) / FUDR® (Hospira, Inc,)
Alkylating agents	An alkylating antineoplastic agent is an alkylating agent that attaches an alkyl group to DNA. Since cancer cells generally proliferate unrestrictively more than do healthy cells they are more sensitive to DNA damage, and alkylating agents are used clinically to treat a variety of tumors.	Ribonucleotide Reductase Inhibitor (RNR) cyclophosphamide / Cytoxan® (BMS) / Neosar® (TEVA) ifosfamide /Mitoxana® (ASTA Medica) ThioTEPA (Bedford, Abraxis, Teva) BCNU→ 1,3-bis(2-chloroethyl)-1-nitosourea CCNU→ 1,-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (methyl CCNU) hexamethylmelamine (altretamine, HMM) / Hexalen® (MGI Pharma Inc.) busulfan / Myleran® (GlaxoSmithKline)

		procarbazine HCL / Matulane® (Sigma Tau) Dacarbazine (DTIC®) chlorambucil / Leukaran® (SmithKline Beecham) Melphalan / Alkeran® (GlaxoSmithKline) cisplatin (Cisplatinum, CDDP) / Platinol (Bristol Myers) carboplatin / Paraplatin (BMS) oxaliplatin / Eloxitan® (Sanofi-Aventis US) Bendamustine carboquone carmustine chloromethine dacarbazine (DTIC) fotemustine lomustine mannosulfan nedaplatin nimustine prednimustine ranimustine satraplatin semustine streptozocin temozolomide treosulfan triaziquone triethylene melamine triplatin tetranitrate trofosfamide uramustine
Topoisomerase inhibitors	Topoisomerase inhibitors are chemotherapy agents designed to interfere with the action of	doxorubicin HCL / Doxil® (Alza) daunorubicin citrate / Daunoxome® (Gilead) mitoxantrone HCL/Novantrone (EMD

	topoisomerase enzymes (topoisomerase I and II), which are enzymes that control the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle.	Serono) actinomycin D etoposide / Vepesid® (BMS)/ Etopophos® (Hospira, Bedford, Teva Parenteral, Etc.) topotecan HCL / Hycamtin® (GlaxoSmithKline) teniposide (VM-26) / Vumon® (BMS) irinotecan HCL(CPT-11) / camptosar® (Pharmacia & Upjohn) camptothecin (CPT) belotecan rubitecan
Microtubule targeting agents	Microtubules are one of the components of the cytoskeleton. They have diameter of apporximately 24 nm and length varying from several micrometers to possibly millimeters in axons of nerve cells. Microtubules serve as structural components within cells and are involved in many cellular processes including mitosis, cytokinesis, and vesicular transport.	vincristine / Oncovin® (Lilly) vinblastine sulfate/Velban®(discontinued) (Lilly) vinorelbine tartrate / Navelbine® (PierreFabre) vindesine sulphate / Eldisine® (Lilly) paclitaxel / Taxol® (BMS) docetaxel / Taxotere® (Sanofi Aventis US) Nanoparticle paclitaxel (ABI-007) / Abraxane® (Abraxis BioScience, Inc.) ixabepilone / IXEMPRA™ (BMS) larotaxel ortataxel tesetaxel vinflunine
Kinase inhibitors	Tyrosine kinases are enzymes within the cell that function to attach phosphate groups to the amino acid tyrosine. By blocking the ability of protein tyrosine kinases	imatinib mesylate / Gleevec (Novartis) sunitinib malate / Sutent® (Pfizer) sorafenib tosylate / Nexavar® (Bayer) nilotinib hydrochloride monohydrate / Tasigna® (Novartis) AMG 386 (Amgen) axitinib (AG-013736; Pfizer, Inc.)

	to function, these compounds provide a tool for controlling cancerous cell growth.	bosutinib (SKI-606; Wyeth) brivanib alalinate (BMS-582664; BMS) cediranib (AZD2171; Recentin, AstraZeneca) dasatinib (BMS-354825: Sprycel®; BMS) lestaurtinib (CEP-701; Cephalon) motesanib diphosphage (AMG-706; Amgen/Takeda) pazopanib HCL (GW786034; Armala, GSK) semaxanib (SU5416; Pharmacia) vandetanib (AZD647; Zactima; AstraZeneca) vatalanib (PTK-787; Novartis, Bayer Schering Pharma) XL184 (NSC718781; Exelixis, GSK)
Protein synthesis inhibitors	Induces cell apoptosis	L-asparaginase / Elspar® (Merck & Co.)
Immunotherapeutic agents	Induces cancer patients to exhibit immune responsiveness	Alpha interferon Angiogenesis Inhibitor / Avastin® (Genentech) IL-2→ Interleukin 2 (Aldesleukin) / Proleukin® (Chiron) IL-12→ Interleukin 12
Hormonal therapies	Hormonal therapies associated with menopause and aging seek to increase the amount of certain hormones in the body to compensate for age- or disease-related hormonal declines. Hormonal therapy as a cancer treatment	Ttoremifene citrate / Fareston® (GTX, Inc.) fulvestrant / Faslodex® (AstraZeneca) raloxifene HCL / Evista® (Lilly) anastrazole / Arimidex® (AstraZeneca) letrozole / Femara® (Novartis) fadrozole (CGS 16949A) exemestane / Aromasin® (Pharmacia & Upjohn) leuprolide acetate / Eligard® (QTL USA) Lupron® (TAP Pharm.)

generally either reduces the level of one or more specific hormones, blocks a hormone from interacting with its cellular receptor or otherwise alters the cancer's ability to be stimulated by hormones to grow and spread. Such hormonal therapies thus include hormone antagonists and hormone synthesis inhibitors. In some instances hormone agonists may also be used as anticancer hormonal therapies.	goserelin acetate / Zoladex® (AstraZeneca) triptorelin pamoate / Trelstar® (Watson Labs) buserelin / Suprefact® (Sanofi Aventis) nafarelin cetrorelix / Cetrotide® (EMD Serono) bicalutamide / Casodex® (AstraZeneca) nilutamide / Nilandron® (Aventis Pharm.) megestrol acetate / Megace® (BMS) somatostatin Analogs (e.g., Octreotide acetate / Sandostatin® (Novartis)) abarelix (Plenaxis™ ; Amgen) abiraterone acetate (CB7630; BTG plc) afimoxifene (TamoGel; Ascend Therapeutics, Inc.) aromatase inhibitor (Atamestane plus toremifene; Intarcia Therapeutics, Inc.) arzoxifene (Eli Lilly & Co) Asentar™; DN-101 (Novacea; Oregon Health Sciences U) flutamide (Eulexin®, Schering; Prostacur, Laboratorios Almirall, S.A) letrozole (CGS20267) (Femara®, Chugai; Estrochek®, (Jagsonpal Pharmaceuticals Ltd;) Delestrogen®, estradiol valerate (Jagsonpal) magestrol acetate / Megace® medroxyprogesteone acetate (Veraplex®; Combiphar) MT206 (Medisyn Technologies, Inc.) nandrolone decanoate (Zestabolin®; Mankind Pharma Ltd) tamoxifen (Taxifen®, Yung Shin Pharmaceutical; Tomifen®, Alkem Laboratories Ltd.) tamoxifen citrate (Nolvadex,

		AstraZeneca; soltamox, EUSA Pharma Inc; tamoxifen citrate SOPHARMA, Sopharma JSCo.)
Glucocorticoids	Anti-inflammatory drugs used to reduce swelling that causes cancer pain.	predinsolone dexamethasone / Decadron® (Wyeth) prednisone (Deltasone, Orasone, Liquid Pred, Sterapred®)
Aromatase inhibitors	Includes imidazoles	ketoconazole
mTOR inhibitors	The mTOR signaling pathway was originally discovered during studies of the immunosuppressive agent rapamycin. This highly conserved pathway regulates cell proliferation and metabolism in response to environmental factors, linking cell growth factor receptor signaling via phosphoinositide-3-kinase (PI-3K) to cell growth, proliferation, and angiogenesis.	sirolimus (Rapamycin) /Rapamune® (Wyeth) Temsirolimus (CCI-779) / Torisel® (Wyeth) Deforolimus (AP23573) (Ariad Pharm.) Everolimus (RAD001) /Certican® (Novartis)
Chemotherapeutic agents		adriamycin, 5-fluorouracil, cytoxin, bleomycin, mitomycin C, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins, clofarabine, mercaptopurine, pentostatin, thioguanine, cytarabine, decitabine, floxuridine, gemcitabine (Gemzar), enocitabine,

	sapacitabine
Protein Kinase B (PKB) Inhibitors	AKT Inhibitor Astex® (Astex Therapeutics) AKT Inhibitors NERVIANO (Nerviano Medical Sciences) AKT Kinase Inhibitor TELIK (Telik Inc) AKT DECIPHERA (Deciphera Pharmaceuticals, LLC) perifosine (KRX0401, D-21266; Keryx Biopharmaceuticals Inc, AEterna Zentaris Inc) perifosine with Docetaxel (Keryx Biopharmaceuticals Inc, AEterna Zentaris Inc) perifosine with Gemcitabine (AEterna Zentaris Inc) perifosine with paclitaxel (AEterna Zentaris Inc) protein kinase-B inhibitor DEVELOGEN (DeveloGen AG) PX316 (Oncothyreon, Inc.) RX0183 (Rexahn Pharmaceuticals Inc) RX0201 (Rexahn Pharmaceuticals Inc) VQD002 (VioQuest Pharmaceuticals Inc) XL418 (Exelixis Inc) ZEN027 (AEterna Zentaris Inc)
Phosphatidylinositol 3-Kinase (PI3K) Inhibitors	BEZ235 (Novartis AG) BGT226 (Novartis AG) CAL101 (Calistoga Pharmaceuticals, Inc.) CHR4432 (Chroma Therapeutics Ltd) Erk/PI3K Inhibitors ETERNA (AEterna Zentaris Inc) GDC0941 (Genentech Inc/Piramed Limited/Roche Holdings Ltd) enzastaurin HCL (LY317615; Enzastaurin; Eli Lilly)

		LY294002/Wortmannin PI3K Inhibitors SEMAFORE (Semafore Pharmaceuticals) PX866 (Oncothyreon, Inc.) SF1126 (Semafore Pharmaceuticals) VMD-8000 (VM Discovery, Inc.) XL147 (Exelixis Inc) XL147 with XL647 (Exelixis Inc) XL765 (Exelixis Inc) PI-103 (Roche/Piramed)
Cyclin Dependent Kinase Inhibitors		CYC200, R-roscovitine (Seliciclib; Cyclacel Pharma) NSC-649890, L86-8275, HMR-1275 (alvocidib; NCI)
TLr9, CD289		IMOxine (Merck KGaA) HYB2055 (Idera) IMO-2055 (Isis Pharma) 1018 ISS (Dynavax Technologies/UCSF) PF-3512676 (Pfizer)
Enzyme Inhibitor		lonafarnib(SCH66336; Sarasar; SuperGen, U Arizona)
Anti-TRAIL		AMG-655 (Aeterna Zentaris, Keryx Biopharma) Apo2L/TRAIL, AMG951 (Genentech, Amgen) APOMAB (fully humanized mAb; Genentech)
MEK Inhibitors	[Mitogen-Activated Protein Kinase Kinase 1 (MAP2K1); Mitogen-Activated Protein Kinase Kinase 2 (MAP2K2)]	ARRY162 (Array BioPharma Inc) ARRY704 (Array BioPharma Inc) ARRY886 (Array BioPharma Inc) AS703026 (Merck Serono S.A) AZD6244 (AstraZeneca Plc) AZD8330 (AstraZeneca Plc)

	RDEA119 (Ardea Biosciences, Inc.) RDEA436 (Ardea Biosciences, Inc.) XL518 (Exelixis Inc; Genentech Inc)
Miscellaneous Inhibitors	Imprime PGG (Biothera) CHR-2797 (AminopeptidaseM1 inhibitor; Chroma Therapeutics) E7820, NSC 719239 (Integrin-alpha2 inhibitor, Eisai) INCB007839 (ADAM 17, TACE Inhibitor; Incyte) CNF2024,BIIB021 (Hsp90 Inhibitor; Biogen Idec) MP470, HPK-56 (Kit/Mel/Ret Inhibitor; Schering-Plough) SNDX-275/MS-275 (HDAC Inhibitor; Syndax) Zarnestra™ ,Tipifarnib, R115777 (Ras Inhibitor; Janssen Pharma) volociximab; Eos 200-4,M200 (alpha581 integrin inhibitor; Biogen Idec; Eli Lilly/UCSF/PDL BioPharma) apricoxib (TP2001; COX-2 Inhibitor, Daiichi Sankyo; Tragara Pharma)

SEQ ID NO:57 (Human EGFR)

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        10          20          30          40          50          60
MRPSGTAGAA LLALLAALCP ASRALEEKKV CQGTSNKLTQ LGTFEDHFLS LQRMFNNCEV
        70          80          90         100         110         120
VLGNLEITYV QRNYDLSFLK TIQEVAGYVL IALNTVERIP LENLQIIRGN MYYENSYALA
       130         140         150         160         170         180
VLSNYDANKT GLKELPMRNL QEILHGAVRF SNNPALCNVE SIQWRDIVSS DFLSNMSMDF
       190         200         210         220         230         240
QNHLGSCQKC DPSCPNGSCW GAGEENCQKL TKIICAQQCS GRCRGKSPSD CCHNQCAAGC
       250         260         270         280         290         300

TGPRESDCLV CRKFRDEATC KDTCPPLMLY NPTTYQMDVN PEGKYSFGAT CVKKCPRNYV
       310         320         330         340         350         360
VTDHGSCVRA CGADSYEMEE DGVRKCKKCE GPCRKVCNGI GIGEFKDSLS INATNIKHFK
       370         380         390         400         410         420
NCTSISGDLH ILPVAFRGDS FTHTPPLDPQ ELDILKTVKE ITGFLLIQAW PENRTDLHAF
       430         440         450         460         470         480
ENLEIIRGRT KQHGQFSLAV VSLNITSLGL RSLKEISDGD VIISGNKNLC YANTINWKKL
       490         500         510         520         530         540
FGTSGQKTKI ISNRGENSCK ATGQVCHALC SPEGCWGPEP RDCVSCRNVS RGRECVDKCN
       550         560         570         580         590         600
LLEGEPREFV ENSECIQCHP ECLPQAMNIT CTGRGPDNCI QCAHYIDGPH CVKTCPAGVM
       610         620         630         640         650         660
GENNTLVWKY ADAGHVCHLC HPNCTYGCTG PGLEGCPTNG PKIPSIATGM VGALLLLLVV
       670         680         690         700         710         720
ALGIGLFMRR RHIVRKRTLR RLLQERELVE PLTPSGEAPN QALLRILKET EFKKIKVLGS
       730         740         750         760         770         780
GAFGTVYKGL WIPEGEKVKI PVAIKELREA TSPKANKEIL DEAYVMASVD NPHVCRLLGI
       790         800         810         820         830         840
CLTSTVQLIT QLMPFGCLLD YVREHKDNIG SQYLLNWCVQ IAKGMNYLED RRLVHRDLAA
       850         860         870         880         890         900
RNVLVKTPQH VKITDFGLAK LLFAEEKEYH AEFFKCPIKW MALESILHRI YTHQSDVWSY
       910         920         930         940         950         960
GVTVWELMTF GSKPYDGIPA SEISSILEKG ERLPQPPICT IDVYMIMVKC WMIDADSRPK
       970         980         990        1000        1010        1020
FRELIIEFSK MARDPQRYLV IQGDERMHLP SPTDSNFYRA LMDEEDMDDV VDADEYLIPQ
      1030        1040        1050        1060        1070        1080
QGFFSSPSTS RTPLLSSLSA TSNNSTVACI DRNGLQSCPI KEDSFLQRYS SDPTGALTED
      1090        1000        1110        1120        1130        1140
SIDDTFLPVP EYINQSVPKR PAGSVQNPVY HNQPLNPAPS RDPHYQDPHS TAVGNPELYN
      1150        1160        1170        1180        1190        1200
TVQPTCVNST FDSPAHWAQK GSHQISLDNP DYQQDFFPKE AKPNGIFKGS TAENAEYLRV
      1210
APQSSEFIGA
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Claims:

1. A composition comprising a trio of anti-EGFR antibodies comprising a first monoclonal antibody, a second monoclonal antibody and a third monoclonal antibody, wherein (i) the first antibody is or binds to the same epitope as, antibody ca (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 34, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 48, 45 and 49, respectively); (ii) the second antibody is or binds to the same epitope as, antibody cd (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 29, 30 and 31, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 53, 54 and 55, respectively); and (iii) the third antibody is or binds to the same epitope as, antibody ch (comprising heavy chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 23, 24 and 26, respectively, and light chain CDRs 1, 2 and 3 set forth in SEQ ID NOs: 39, 40 and 42, respectively).

2. A monoclonal antibody of claim 1, wherein the antibody is selected from the group consisting of a bispecific antibody, immunoconjugate, Fab, Fab'2, and ScFv.

3. A composition comprising a trio of antibodies of claim 1, wherein each antibody of the trio is an IgGl, IgG2, IgG3, IgG4, IgM, IgAl, IgA2, IgAsec, IgD, or IgE.

4. The composition of claim 1, wherein each of the antibodies comprised by the composition binds to EGFR with a K_D of 10^7 to 10^{12} M^{-1}.

5. The composition of claim 1, wherein the composition exhibits at least one of the following properties:

(a) inhibition of AKT phosphorylation or ERK phosphorylation, as measured in a cell-based *in vitro* assay;

(b) inhibition of the growth of tumor cells expressing EGFR *in vitro;*

(c) inhibition of the growth of tumor cells expressing EGFR in a xenograft model *in vivo;*

(d) inhibition of ligand binding to EGFR extracellular domain *in vitro;*

(e) inhibition of EGFR dimerization *in vitro;* or.

(f) downregulation of EGFR on cell surfaces *in vitro.*

6. The composition of claim 5, wherein the inhibition or downregulation is additive or synergistc as compared to a preparation comprising an individual antibody comprised by the composition in an amount (in moles) equivalent to the total amount (in moles) of combined antibodies in the composition.

7. A kit comprising the composition of claim 1 in a container.