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

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
23 April 2015 (23.04.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/057461 A2

(71) Applicant: **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; National Institutes of Health, Office of Technology Transfer, 6011 Executive Boulevard, Suite 325, MSC 7660, Bethesda, MD 20852-7660 (US).

(72) Inventors: **PFISTER, Thomas, D.**; SAIC-Frederick, Building 425, Room 218, Frederick, MD 21702 (US). **MARRERO, Allison, M.**; SAIC-Frederick, Building 425, Room 212, Frederick, MD 21702 (US). **PARCHMENT, Ralph, E.**; SAIC-Frederick, Building 425, Room 221, Frederick, MD 21702 (US). **DOROSHOW, James, H.**; NCI, Building 31, Room 3A44, 31 Center Drive, Bethesda, MD 20814 (US).

(74) Agent: **SIEGEL, Susan, Alpert**; Klarquist Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).

(54) Title: ANTIBODIES THAT SPECIFICALLY BIND ATAXIA TELANGIECTASIA-MUTATED AND RAD3-RELATED KINASE PHOSPHORYLATED AT POSITION 1989 AND THEIR USE

(57) Abstract: Monoclonal antibodies that specifically bind ataxia telangiectasia-mutated and RAD3- related kinase phosphorylated at position 1989 (pT1989 ATR), antigen binding fragments, conjugates thereof, and the use of these antibodies, antigen binding fragments and conjugates are disclosed herein. Also disclosed are nucleic acids encoding these antibodies, vectors including these antibodies, and isolated host cells transformed with these nucleic acids and vectors. Methods are also disclosed for using these antibodies, such as to detect pT1989 ATR, or to determine the dose of an agent of use to treat a subject. The antibodies are also of use for identifying ATR inhibitors.

- 1 -

ANTIBODIES THAT SPECIFICALLY BIND ATAXIA TELANGIECTASIA-MUTATED AND RAD3-RELATED KINASE PHOSPHORYLATED AT POSITION 1989 AND THEIR USE

5   **CROSS REFERNCE TO RELATED APPLICATIONS**

This claims the benefit of U.S. Provisional Application No. 61/893,070, filed October 18, 2013, which is incorporated herein by reference.

FIELD OF THE DISCLOSURE

10   This is related to the field of kinases, particularly to antibodies that specifically bind ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989 (pT1989 ATR) and their use.

BACKGROUND

15   The DNA damage response coordinates cell cycle transitions, DNA replication, DNA repair, and apoptosis to guard against genomic instability. Three related kinases belonging to the PI3K-related protein kinase (PIKKs) family, ataxia telangiectasia-mutated (ATM), ATM and RAD3-related (ATR), and DNA-dependent protein kinase (DNA-PK), are involved in the DNA damage response. ATR is essential for viability in replicating human cells, and disruption of

20   ATR in mice results in embryonic lethality (see, for example, Cortez et al., *(2001) Science 294, 1713–1716*). ATR regulates replication fork stability, restart of collapsed forks, and late-origin firing during S-phase. This kinase also activates the G_2 checkpoint to prevent entry into mitosis in the presence of damaged DNA (see, for example, Sancar et al., *(2004) Annu. Rev. Biochem. 73, 39–85*).

25   Hypomorphic mutations in ATR cause Seckel syndrome and ATR mutations have been associated with poor prognosis of tumors with microsatellite instability (O'Driscoll, *(2003) Nat. Genet.* 33, 497–501; Zighelboim et al., *(2009) J. Clin. Oncol.* 27, 3091–3096: Lewis et al., Genes Chromosomes Cancer 46, 1061–1068). A key function of ATR is activation of checkpoint kinase 1 (Chk1), leading to cell cycle arrest. Many anti-cancer therapeutics induce

30   DNA damage and ATR is itself a drug target.

- 2 -

ATM, ATR and DNA-PK undergo autophosphorylation. Phosphorylation sites on ATM and DNA-PK are functionally significant and have been used as direct markers of activation (see, for example, Kozlov et al., *(2006) EMBO J. 25, 3504–3514*). Phosphorylation at the threonine at position 1989 (T1989) is important for ATR activation (Nam et al., *(2011), J. Biol.*

5   *Chem.* 286: 28707-28714). There is a need for reagents that can be used to detect ATR phosphorylation at this position.

SUMMARY OF THE DISCLOSURE

Monoclonal antibodies that specifically bind ataxia telangiectasia-mutated and RAD3-

10   related kinase phosphorylated at position 1989 (pT1989 ATR), antigen binding fragments, conjugates thereof, and the use of these antibodies, antigen binding fragments and conjugates are disclosed herein. In some embodiments, the isolated monoclonal antibody, or antigen binding fragment thereof, includes a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region includes a heavy chain complementarity determining

15   region (H-CDR)1, a H-CDR2, and a H-CDR3 of the heavy chain variable region set forth as SEQ ID NO: 6, and wherein the light chain variable domain includes a light chain complementarity determining region (L-CDR)1, a L-CDR2, and a L-CDR3 of the light chain variable region set forth as SEQ ID NO: 7. In additional embodiments, nucleic acids encoding are disclosed that encode these antibodies, as well as vectors expressing these nucleic acids, and

20   isolated host cells transformed with these nucleic acids and vectors.

In further embodiments, methods are also disclosed for using these antibodies, such as to detect pT1989 ATR, or to determine if an agent is of use to treat a subject. The antibodies disclosed herein are also of use for identifying ATR inhibitors. The antibodies have enhanced affinity for pT1989 ATR.

25

The foregoing and other features and advantages of the invention will become more apparent from the following detailed description of a several embodiments which proceeds with reference to the accompanying figures.

30

- 3 -

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Protein sequence (SEQ ID NO: 1) of human serine/threonine-protein kinase ATR (2644 amino acids, NP_001175.2).

FIG. 2. Peptide sequences, ap (SEQ ID NO: 2), bp (SEQ ID NO: 3), anp (SEQ ID NO: 4) and bnp (SEQ ID NO: 5) used for immunization and/or screening.

FIGS. 3A-3D. Rabbit bleed screening ELISA with phospho-peptides on 96 well plates. Fluorescence signal at 680nm from Licor ODYSSEY® infrared scanner for (3A) ATR phospho-peptide A conjugated to bovine serum albumin (BSA), and (3B) ATR phospho-peptide B conjugated to bovine serum albumin (BSA). Dilution curve for (3C) ATR phospho-peptide A conjugated to bovine serum albumin (BSA) and (3D) ATR phospho-peptide B conjugated to bovine serum albumin (BSA).

FIGS. 4A-4D. Rabbit bleed screen with phospho-peptides on 96 well plates. Fluorescence signal at 680nm from Licor Odyssey infrared scanner for (4A) ATR phospho-peptide A conjugated to bovine serum albumin (BSA), and (4B) ATR phospho-peptide B conjugated to bovine serum albumin (BSA). Dilution curve for (4C) ATR phospho-peptide A conjugated to bovine serum albumin (BSA) and (4D) ATR phospho-peptide B conjugated to bovine serum albumin (BSA).

FIGS. 5A-5C. Direct ELISA for dilution of hybridomas against (A) phospho-peptide A (ap). (B) non-phospho-peptide A (anp). Arrows indicate hybridomas that detected pT1989-ATR peptide but did not cross react with unphosphorylated peptide. (C) ATR pT1989 clone 7A7 titers for BSA conjugated phosphorylated and non-phosphorylated peptides. The antibody is specific for the phosphorylated polypeptides.

FIG. 6. Western blotting of hybridomas (7, 19, 27 and 35). Lane1, Molecular weight markers; lane 2, DLD-1 untreated; lane 3 DLD-1 treated with 100nM gemcitabine for 4h; lane 4 Seckel cell untreated; lane 5 Seckel cell treated with 100nM gemcitabine for 4h. Arrow indicates ATR pT1989 band observed at 250 kDa, corresponding to predicted molecular weight of ATR.

FIG. 7. Relative quantification of ATR band in Western blot of hybridoma # 7.

FIG. 8. Western blot of subclone 7A7. Lane1, molecular weight marker; lane 2, DLD-1 untreated; lane 3 DLD1 treated with 100nm gemcitabine for 4h; lane 4 Seckel cell untreated;

- 4 -

lane 5 Seckel cell treated with 100nM gemcitabine for 4h.

FIG. 9. ATR-pT1989 immunofluoresence (IFA) assay for formalin fixed paraffin embedded (FFPE) HT-29 cells treated with (A) water and (B) 150 nM gemcitabine, constant exposure, 20X. 4',6-diamidino-2-phenylindole (DAPI) signal in blue, ATR-pT1989 in green.

FIG. 10. ATR-pT1989 IFA assay for FFPE (10A) DLD-1 cells treated with water and (10B) DLD-1 Seckel cells treated with water, constant exposure, 20X. DAPI signal in blue, ATR-pT1989 in green.

FIG. 11. Western blot of Hybridoma 7, ATR expressing colon cancer cell lines (LoVo, HT29). Lane1, molecular weight marker; lane 2, LoVo vehicle; lane 3, LoVo VE-821, 1uM 4 hours (hr); lane 4, LoVo gemcitabine, 150 nM 4h; lane 5, LoVo VE-821 plus gemcitabine, lane 6, MW marker; lane 7, HT29 vehicle; lane 8, HT29 VE-821, 1uM 4 hr; lane 9, HT29 gemcitabine, 150 nM 4h; lane 10, HT29 VE-821 plus gemcitabine 34 ug protein per well.

FIG. 12. ATR-pT1989 IFA assay using purified recombinant 7A7, 4 ug/ml. HT29 cells treated with vehicle (DMSO), ATR inhibitor VE-821, gemcitabine or the combination for 4 hrs, gemcitabine as a single agent for 24 hrs. Constant exposure, 40X. DAPI signal in blue, ATR-pT1989 in green. Nuclear foci were counted using DEFINIENS® software.

FIG. 13A-13D shows the location of the CDRs, as identified using Kabat and Clothia. The CDR using two other identification systems (Enhanced Clothia (Clothia+) and Contact) are also shown. The Table presents the amino acid sequence of the heavy and light chains (SEQ ID NO: 6 and SEQ ID NO: 7), and provides the position numbers for each numbering schemes. In the bar graphs below the figure, the framework regions are represented with light grey bars (or no bar) and CDRs are represented by dark grey bars. The framework regions continue beyond the indicated bars, so that they abut each CDR and continue to the end of the sequence.

FIGS. 14A-C show that VE-821 inhibits the ATR-Chk1 response. (14A) Experimental protocol. Cells were treated with Top1 inhibitors without or with VE-821 as indicated. Samples were harvested at 0, 4, 8 and 18 h after Top1 inhibitor removal. (14B) Kinetics of ATR activation (phosphorylation on threonine 1989), Chk1 activation (phosphorylation on serine 345), Chk2 activation (phosphorylation on threonine 68) and γH2AX analyzed by immunoblotting. GAPDH was used as loading control. (14C) Same as panel B for LMP-400 instead of CPT.

- 5 -

FIGS. 15A-15D show results from A2780 xenografts. Representative images of the pATR T1989 immunofluorescence assay in A2780 xenografts from mice treated for 24 hours with either (A) vehicle, (B) 1.2 mg/kg cisplatin or (C) 6.0 mg/kg cisplatin. (D) Quantitation of the percent of cells with ≥7 pATR T1989 foci per nucleus, results are mean ± S.D. of an average of 10 representative fields per xenograft quadrant, 5 mice per group. Each point on the graph represents one xenograft.]

FIG. 16 shows detection of ATR foci in a human clinical sample. A human tumor biopsy sample from NCI Cancer Therapy Evaluation Program (CTEP) trial 9350 was prepared into an FFPE block and slides were sectioned to 5 um. A Backup-1 slide was used to stain with DAPI as a nuclear marker (left) and pATR T1989 (center). A merge of the two channels is shown on the right. pATR is visualized as discreet foci specifically localized to the nuclear region of the cells.

FIGS. 17A-17C are digital images of a Western Blots of colon cell lysates probed with anti-ATR-pT1989 antibodies. (17A) Rabbit Hybridoma clone 7-7 (7A7)1:50, (17B) Kerafast pT1989 1:25 and (17C) Kerafast pT1989 1:100. GAR-IR-680 was used at 0.1ug/ml. In each blot, Lane 1: MW markers, lane 2: DLD1 parental untreated, lane 3: DLD1 parental 100nM Gemcitabine 4 hour, lane 4: DLD-1 seckel untreated, lane: DLD-1 seckel 100nM Gemcitabine 4 hours. Clone 7A7 was specific to pATR, and detected pATR specifically at a dilution of 1:50. However, the commercially available antibody (Kerafast) provided weak detection of pATR in gemcitabine treated DLD-1 cells at a dilution of 1:25, but not at a dilution of 1:100. Thus, the commercially available antibody had poor specificity and sensitivity, and would be unsuitable for immunoassay applications such as pharmacodynamic immunofluorescence assay. The detection of cross-reactive proteins also indicated that the commercially available (Kerafast) antibody cannot be used for quantitation in unpurified samples.

SEQUENCES

The nucleic and amino acid sequences listed are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file [91933-02_Sequence.txt, October 8, 2014, 32.7 KB], which is

- 6 -

incorporated by reference herein.

SEQ ID NO: 1 is the amino acid sequence of human ATR, see Fig. 1.

SEQ ID NOs: 2-5 are the amino acid sequences of human ATR peptides, see Fig. 2.

SEQ ID NO: 6 is the amino acid sequence of a heavy chain of an antibody that

5   specifically binds pT1989 ATR.

SEQ ID NO: 7 is the amino acid sequence of a light chain of an antibody that

specifically binds pT1989 ATR.

SEQ ID NO: 8 is the nucleic acid sequence of an exemplary nucleic acid molecule that

encodes the heavy chain amino acid sequence set forth as SEQ ID NO: 6

10   SEQ ID NO: 9 is the nucleic acid sequence of an exemplary nucleic acid molecule that

encodes the light chain amino acid sequence set forth as SEQ ID NO: 7.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Monoclonal antibodies that specifically bind pT1989 ATR, antigen binding fragments of

15   these antibodies, conjugates of these antibodies and antigen binding fragments, and the use of

these antibodies, antigen binding fragments and conjugates are disclosed herein.

I.   Summary of Terms

Unless otherwise noted, technical terms are used according to conventional usage.

20   Definitions of common terms in molecular biology can be found in Benjamin Lewin, *Genes VII*,

published by Oxford University Press, 1999; Kendrew *et al.* (eds.), *The Encyclopedia of*

Molecular Biology, published by Blackwell Science Ltd., 1994; and Robert A. Meyers (ed.),

Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH

Publishers, Inc., 1995; and other similar references.

25   As used herein, the singular forms "a," "an," and "the," refer to both the singular as well

as plural, unless the context clearly indicates otherwise. For example, the term "an antigen"

includes single or plural antigens and can be considered equivalent to the phrase "at least one

antigen." As used herein, the term "comprises" means "includes." Thus, for example,

"comprising an antibody" means "including an antibody" without excluding other elements.

30   The phrase "and/or" means "and" or "or." It is further to be understood that any and all base

- 7 -

sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for descriptive purposes, unless otherwise indicated. Although many methods and materials similar or equivalent to those described herein can be used, particular suitable methods and materials are described below. In

5   case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. To facilitate review of the various embodiments, the following explanations of terms are provided:

Administration: To provide or give to a subject an agent, for example, a

10   chemotherapeutic agent or an antibody, by any effective route. Exemplary routes of administration include, but are not limited to, oral, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), sublingual, rectal, transdermal (for example, topical), intranasal, vaginal, and inhalation routes.

Agent: Any substance or any combination of substances that is useful for achieving an

15   end or result; for example, a substance or combination of substances useful for decreasing or reducing tumor growth in a subject. Agents include effector molecules, detectable markers, antibodies, antigen binding fragments of antibodies, and chemical compounds such as, but not limited to, chemotherapeutic compounds.

Antibody: A polypeptide substantially encoded by an immunoglobulin gene or

20   immunoglobulin genes, or antigen binding fragments thereof, which specifically binds and recognizes an analyte (antigen) such as pT1989 ATR or an antigenic fragment of pT1989 ATR. Immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable domain genes. The term "antibody," as used herein, also includes antigen binding fragments either produced by the

25   modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies.

Non-limiting examples of antibodies include, for example, intact immunoglobulins and variants and fragments thereof known in the art that retain specific binding affinity for the antigen. Antibodies also include genetically engineered forms such as chimeric antibodies (such

30   as humanized murine antibodies) and heteroconjugate antibodies (such as bispecific antibodies).

- 8 -

See also, *Pierce Catalog and Handbook*, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., *Immunology*, 3rd Ed., W.H. Freeman & Co., New York, 1997.

Examples of antigen binding fragments include: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')$_2$, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; (4) F(ab')$_2$, a dimer of two Fab' fragments held together by two disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable domain of the light chain and the variable domain of the heavy chain expressed as two chains; and (6) single chain antibody ("SCA"), a genetically engineered molecule containing the variable domain of the light chain, the variable domain of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

A single-chain antibody (scFv) is a genetically engineered molecule containing the V_H and V_L domains of one or more antibody(ies) linked by a suitable polypeptide linker as a genetically fused single chain molecule (see, for example, Bird *et al.*, *Science*, 242:423-426, 1988; Huston *et al.*, *Proc. Natl. Acad. Sci.*, 85:5879-5883, 1988). In a dsFv the heavy and light chain variable chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. Diabodies also are included, which are bivalent, bispecific antibodies in which V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see, for example, Holliger *et al.*, *Proc. Natl. Acad. Sci.*, 90:6444-6448, 1993; Poljak *et al.*, *Structure*, 2:1121-1123, 1994).

An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally-occurring immunoglobulin has two identical binding sites, a single-chain antibody or Fab fragment has one binding site, while a bispecific or bifunctional antibody has two different

binding sites.

Typically, a naturally occurring antibody has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each heavy and light chain contains a constant region and a variable region (the regions are also known as "domains"). In several embodiments, the heavy and the light chain variable regions combine to specifically bind the antigen. In additional embodiments, only the heavy chain variable region is required. For example, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain (see, *e.g.*, Hamers-Casterman *et al.*, *Nature*, 363:446-448, 1993; Sheriff *et al.*, *Nat. Struct. Biol.*, 3:733-736, 1996). Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs" (see, e.g., Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, 1991). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space.

The CDRs are primarily responsible for binding to an epitope of an antigen. The amino acid sequence boundaries of a given CDR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. ("Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991; "Kabat" numbering scheme), Al-Lazikani et al., (JMB 273,927-948, 1997; "Chothia" numbering scheme), and Lefranc et al. ("IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains," Dev. Comp. Immunol., 27:55-77, 2003; "IMGT" numbering scheme). The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3 (from the N-terminus to C-terminus), and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is the CDR3 from the variable region of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable region of the light chain of the antibody in which it is

- 10 -

found. Light chain CDRs are sometimes referred to as LCDR1, LCDR2, and LCDR3. Heavy chain CDRs are sometimes referred to as LCDR1, LCDR2, and LCDR3.

References to "V_H" or "VH" refer to the variable domain of an immunoglobulin heavy chain, including that of an antigen binding fragment, such as Fv, scFv, dsFv or Fab. References to "V_L" or "VL" refer to the variable domain of an immunoglobulin light chain, including that of an Fv, scFv, dsFv or Fab.

A "monoclonal antibody" is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected, or a progeny thereof. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. These fused cells and their progeny are termed "hybridomas." Monoclonal antibodies include chimeric, humanized and fully human monoclonal antibodies. In some examples monoclonal antibodies are isolated from a subject. The amino acid sequences of such isolated monoclonal antibodies can be determined. Monoclonal antibodies can have conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. (See, for example, Harlow & Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988).)

A "chimeric" antibody is an antibody including a framework region from one antibody and one or more CDRs from a heterologous antibody. The framework regions and the CDRs can be from antibodies from the same or different species.

A "humanized" antibody is an antibody including a human framework region and one or more CDRs from a non-human (such as a mouse, rat, or synthetic) immunoglobulin. The non-human antibody providing the CDRs is termed a "donor," and the human antibody providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the donor antibody in a humanized antibody. Constant regions need not be present, but if they are, they must be substantially identical to human antibody constant regions, such as at least about 85-90%, such as about 95% or more identical. Hence, all parts of a humanized antibody, except possibly the CDRs, are substantially identical to corresponding parts of natural human antibody sequences. A "humanized antibody" is an antibody including a humanized light chain and a humanized heavy chain. A humanized antibody binds to the same antigen as the donor antibody

- 11 -

that provides the CDRs. The acceptor framework of a humanized immunoglobulin or antibody may have a limited number of substitutions by amino acids taken from the donor framework.

Humanized, chimeric or other monoclonal antibodies can have additional conservative amino acid substitutions, such as in the framework region, which have substantially no effect on

5   antigen binding or other immunoglobulin functions. Humanized an chimeric immunoglobulins can be constructed by means of genetic engineering (for example, see U.S. Patent No. 5,585,089).

Ataxia telangiectasia-mutated and Rad3-related (ATR) kinase: A member of the phosphoinositide 3-kinase related kinase (PIKK) family. ATR includes a kinase domain, a FAT

10   domain and a FATC (FAT domain at the carboxy terminus) domain. The FAT domain is part of a long N-terminal region predicted to fold into alpha-helical HEAT repeats, each 37-47 amino acids long. ATR is a large kinase of 2644 amino acids in length, with a molecular weight of approximately 300 kD. ATR-interacting protein (ATRIP), the 85 kDa binding partner of ATR, binds to the N-terminus of ATR. ATR is an atypical kinase for checkpoint kinase 1 (Chk1),

15   which has a role in cell cycle arrest, DNA repair, and apoptosis. ATR is essential for viability in replicating human cells, and can be activated by a variety of types of DNA damage. ATR is phosphorylated at the threonine at position 1989 (T1989) upon activation. An exemplary amino acid sequence for ATR is provided as GENBANK® Accession No. NP_001175.2, September 29, 2013, incorporated by reference herein, and is shown in Fig. 1.

20   **Binding affinity:** Affinity of an antibody or antigen binding fragment thereof for an antigen. In one embodiment, affinity is calculated by a modification of the Scatchard method described by Frankel et al., *Mol. Immunol.*, 16:101-106, 1979. In another embodiment, binding affinity is measured by an antigen/antibody dissociation rate. In yet another embodiment, a high binding affinity is measured by a competition radioimmunoassay. In several examples, a high

25   binding affinity is at least about 1×10^{-8} M. In other embodiments, a high binding affinity is at least about 1.0×10^{-9}, at least about 5.0×10^{-9}, at least about 1.0×10^{-10}, at least about 5.0×10^{-10}, or at least about 1.0×10^{-11}.

Biological sample: A sample obtained from a subject. Biological samples include all clinical samples useful for detection of disease n subjects, including, but not limited to, cells,

30   tissues, and bodily fluids, such as blood, derivatives and fractions of blood such as serum,

- 12 -

cerebrospinal fluid; as well as biopsied or surgically removed tissue, for example tissues that are unfixed, frozen, or fixed in formalin or paraffin. In a particular example, a biological sample is obtained from a subject having or suspected of having a tumor; for example, a subject having or suspected of having breast, colorectal, lung, or skin cancer. In some examples, the subject has or is suspected of having a carcinoma.

Bispecific antibody: A recombinant molecule composed of two different antigen binding domains that consequently bind to two different antigenic epitopes. Bispecific antibodies include chemically or genetically linked molecules of two antigen-binding domains. The antigen binding domains can be linked using a linker. The antigen binding domains can be monoclonal antibodies, antigen-binding fragments (*e.g.*, Fab, scFv), eAds, bispecific single chain antibodies or combinations thereof. A bispecific antibody can include one or more constant domains, but does not necessarily include a constant domain. An example of a bispecific antibody is a bispecific single chain antibody including a scFv that specifically binds to pT1989 ATR joined (via a peptide linker) to a scFv that specifically binds to an antigen other than pT1989 ATR.

Cell cycle: The series of events that takes place in a cell leading to its division and duplication (replication). The cell cycle can be divided into two brief periods: interphase-during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA and t mitosis (M) phase, during which the cell splits itself into two distinct cells, often called "daughter cells." The cell cycle includes four distinct phases: G_1 phase (interphase or growth phase), S phase (DNA synthesis, chromosome replication), G_2 phase (growth phase) and M phase (mitosis, including karyokinesis and cytokines). In karyokinesis the cell's chromosomes are divided between the two daughter cells, and during cytokinesis the cell's cytoplasm divides in half forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase. Cells in a population that are "synchronized" are at the same phase of the cell cycle, such as G_1, S, G_2 or M.

Conditions sufficient to form an immune complex: Conditions which allow an antibody or antigen binding fragment thereof to bind to its cognate epitope to a detectably greater degree than, and/or to the substantial exclusion of, binding to substantially all other epitopes.

- 13 -

Conditions sufficient to form an immune complex are dependent upon the format of the binding reaction and typically are those utilized in immunoassay protocols or those conditions encountered *in vivo*. See Harlow & Lane, *supra*, for a description of immunoassay formats and conditions. The conditions employed in the methods are "physiological conditions" which

5   include reference to conditions (e.g., temperature, osmolarity, pH) that are typical inside a living mammal or a mammalian cell. While it is recognized that some organs are subject to extreme conditions, the intra-organismal and intracellular environment normally lies around pH 7 (e.g., from pH 6.0 to pH 8.0, more typically pH 6.5 to 7.5), contains water as the predominant solvent, and exists at a temperature above 0°C and below 50°C. Osmolarity is within the range that is

10   supportive of cell viability and proliferation.

        Conjugate: A complex of two molecules linked together, for example, linked together by a covalent bond. In one embodiment, an antibody is linked to an effector molecule; for example, an antibody that specifically binds to pT1989 ATR covalently linked to an effector molecule such as a detectable label. The linkage can be by chemical or recombinant means. In

15   one embodiment, the linkage is chemical, wherein a reaction between the antibody moiety and the effector molecule has produced a covalent bond formed between the two molecules to form one molecule. A peptide linker (short peptide sequence) can optionally be included between the antibody and the effector molecule. Because conjugates can be prepared from two molecules with separate functionalities, such as an antibody and an effector molecule, they are also

20   sometimes referred to as "chimeric molecules."

        Conservative variants: "Conservative" amino acid substitutions are those substitutions that do not substantially decrease the binding affinity of an antibody for an antigen (for example, the binding affinity of an antibody for pT1989 ATR). For example, an antibody that specifically binds pT1989 ATR can include at most about 1, at most about 2, at most about 5, at most about

25   10, or at most about 15 conservative substitutions and specifically bind the pT1989 ATR polypeptide. The term conservative variation also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid, provided that antibody retains binding affinity for pT1989 ATR. Non-conservative substitutions are those that reduce an activity or binding to pT1989 ATR.

30   Conservative amino acid substitution tables providing functionally similar amino acids

- 14 -

are well known to one of ordinary skill in the art. The following six groups are examples of amino acids that are considered to be conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Contacting: Placement in direct physical association, for example solid, liquid or gaseous forms. Contacting includes, for example, direct physical association of fully- and partially-solvated molecules.

Control: A reference standard. In some embodiments, the control is a negative control, such as tissue sample obtained from a patient that does not have cancer, or a tissue sample from a tissue that is non-cancerous. In other embodiments, the control is a positive control, such as a tissue sample obtained from a patient diagnosed with cancer, or a tissue sample from a cancerous tissue. In still other embodiments, the control is a historical control or standard reference value or range of values (such as a previously tested control sample, such as a group of cancer patients with known prognosis or outcome, or group of samples that represent baseline or normal values).

A difference between a test sample and a control can be an increase or conversely a decrease. The difference can be a qualitative difference or a quantitative difference, for example a statistically significant difference. In some examples, a difference is an increase or decrease, relative to a control, of at least about 5%, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, or at least about 500%.

Decrease or **Reduce:** To reduce the quality, amount, or strength of something; for example phosphorylation at position 1989 of ATR. In one example, an agent reduces phosphorylation at position 1989 of ATR, for example as compared to phosphorylation in the absence of the agent. In a particular example, a therapy decreases the phosphorylation at

- 15 -

position 1989 of ATR at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 100%. Such decreases can be measured using the methods disclosed herein.

Degenerate variant: In the context of the present disclosure, a "degenerate variant" refers to a polynucleotide encoding a protein (for example, an antibody that specifically binds pT1989 ATR) that includes a sequence that is degenerate as a result of the genetic code. There are twenty natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included as long as the amino acid sequence of the antibody that binds pT1989 ATR encoded by the nucleotide sequence is unchanged.

Detectable marker: A detectable molecule (also known as a label) that is conjugated directly or indirectly to a second molecule, such as an antibody, to facilitate detection of the second molecule. For example, the detectable marker can be capable of detection by ELISA, spectrophotometry, flow cytometry, microscopy or diagnostic imaging techniques (such as CT scans, MRIs, ultrasound, fiberoptic examination, and laparoscopic examination). Specific, non-limiting examples of detectable markers include fluorophores, chemiluminescent agents, enzymatic linkages, radioactive isotopes and heavy metals or compounds (for example super paramagnetic iron oxide nanocrystals for detection by MRI). In one example, a "labeled antibody" refers to incorporation of another molecule in the antibody. For example, the label is a detectable marker, such as the incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (for example, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (such as ^{35}S or ^{131}I), fluorescent labels (such as fluorescein isothiocyanate (FITC), rhodamine, lanthanide phosphors), enzymatic labels (such as horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (such as a leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), or magnetic agents, such as gadolinium chelates. In some embodiments,

- 16 -

labels are attached by spacer arms of various lengths to reduce potential steric hindrance. Methods for using detectable markers and guidance in the choice of detectable markers appropriate for various purposes are discussed for example in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 2012) and Ausubel *et al.* (In

5   Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1998).

Detecting: To identify the existence, presence, or fact of something. General methods of detecting are known to the skilled artisan and may be supplemented with the protocols and reagents disclosed herein. For example, included herein are methods of detecting a cell that expresses pT1989 ATR in a subject.

10   **Effective amount**: The amount of an agent (such as a pT1989 ATR specific antibody or a conjugate including a pT1989 ATR specific antibody) that alone, or together with one or more additional agents, induces the desired response, such as, for example formation of a detectable immune complex with pT1989 ATR.

Effector molecule: A molecule intended to have or produce a desired effect; for

15   example, a desired effect on a cell to which the effector molecule is targeted. Effector molecules include such molecules as polypeptides, radioisotopes and small molecules. Non-limiting examples of effector molecules include detectable labels, toxins, chemotherapeutic agents and anti-angiogenic agents. The skilled artisan will understand that some effector molecules may have or produce more than one desired effect. In one example, an effector molecule is the

20   portion of a chimeric molecule, for example a chimeric molecule that includes a disclosed antibody or fragment thereof, that is intended to have a desired effect on a cell to which the chimeric molecule is targeted.

Epitope: An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, *i.e.* that elicit a specific immune response. An

25   antibody specifically binds a particular antigenic epitope on a polypeptide. In some examples a disclosed antibody specifically binds to an epitope present on ATR phosphorylated at position 1989, but not to ATR that in not phosphorylated at position 1989.

Framework Region: Amino acid sequences interposed between CDRs in a heavy or light variable region of an antibody. Includes variable light and variable heavy framework

30   regions. The framework regions serve to hold the CDRs in an appropriate orientation.

- 17 -

IgA: A polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin alpha gene. In humans, this class or isotype includes IgA_1 and IgA_2. IgA antibodies can exist as monomers, polymers (referred to as pIgA) of predominantly dimeric form, and secretory IgA. The constant chain of wild-type IgA contains an 18-amino-acid extension at its C-terminus called the tail piece (tp). Polymeric IgA is secreted by plasma cells with a 15-kDa peptide called the J chain linking two monomers of IgA through the conserved cysteine residue in the tail piece.

IgG: A polypeptide belonging to the class or isotype of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans, this class includes IgG_1, IgG_2, IgG_3, and IgG_4. In mice, this class includes IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3.

Immune complex: The binding of antibody to a soluble antigen forms an immune complex. The formation of an immune complex can be detected through conventional methods known to the skilled artisan, for instance immunohistochemistry, immunoprecipitation, flow cytometry, immunofluorescence microscopy, ELISA, immunoblotting (for example, Western blot), magnetic resonance imaging, CT scans, X-ray and affinity chromatography. Immunological binding properties of selected antibodies may be quantified using methods well known in the art.

Increase: To increase the quality, amount, or strength of something; for example phosphorylation at position 1989 of ATR. In one example, an agent increase phosphorylation at position 1989 of ATR, for example as compared to phosphorylation in the absence of the agent. In a particular example, a therapy increase the phosphorylation at position 1989 of ATR at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 100%. Such increase can be measured using the methods disclosed herein.

Inhibiting or Treating a Disease: A therapeutic intervention (for example, a pharmaceutical compound) that reduces a sign or symptom of a disease or pathological condition related to a disease (such as a tumor). Treatment can also induce remission or cure of a condition, such as a tumor. In particular examples, treatment includes preventing a tumor, for example by inhibiting the full development of a tumor, such as preventing development of a metastasis or the development of a primary tumor. Prevention does not require a total absence

- 18 -

of a tumor.

Reducing a sign or symptom of a disease or pathological condition related to a disease, refers to any observable beneficial effect of the treatment. Reducing a sign or symptom associated with a tumor (such as pathological angiogenesis) can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject (such as a subject having a tumor which has not yet metastasized), a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease (for example by prolonging the life of a subject having tumor), a reduction in the number of relapses of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular tumor. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology.

Isolated and Purified: A biological component (such as a nucleic acid, peptide, protein or protein complex, for example an antibody) that has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, that is, other chromosomal and extra-chromosomal DNA and RNA, and proteins. Thus, isolated nucleic acids, peptides and proteins include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell, as well as, chemically synthesized nucleic acids. A isolated or purified nucleic acid, peptide or protein, for example an antibody or ATR, can be at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% pure.

Linker: A bi-functional molecule that can be used to link two molecules into one contiguous molecule, for example, to link an effector molecule or a detectable marker to an antibody. In some embodiments, the provided conjugates include a linker between the effector molecule or detectable marker and an antibody. In some embodiments, the linker is cleavable under intracellular conditions, such that cleavage of the linker releases the effector molecule or detectable marker from the antibody in the intracellular environment. In yet other embodiments, the linker is not cleavable and the effector molecule or detectable marker can be released, for example, by antibody degradation. In some cases, a linker is a peptide within an antibody

binding fragment (such as an Fv fragment) which serves to indirectly bond the variable heavy chain to the variable light chain.

The terms "conjugating," "joining," "bonding," or "linking" refer to making two molecules into one contiguous molecule; for example, linking two polypeptides into one

5   contiguous polypeptide, or covalently attaching an effector molecule or detectable marker radionuclide or other molecule to a polypeptide, such as an scFv. In the specific context, the terms include reference to joining a ligand, such as an antibody moiety, to an effector molecule. The linkage can be either by chemical or recombinant means. "Chemical means" refers to a reaction between the antibody moiety and the effector molecule such that there is a covalent

10   bond formed between the two molecules to form one molecule.

Nucleic acid: A polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Thus, the term includes

15   nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs, such as, for example and without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "oligonucleotide"

20   typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T. "

Conventional notation is used herein to describe nucleotide sequences: the left-hand end of a single-stranded nucleotide sequence is the 5'-end; the left-hand direction of a double-

25   stranded nucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand;" sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences;"

30   sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3'

- 20 -

end of the coding RNA transcript are referred to as "downstream sequences."

"cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Recombinant nucleic acid" refers to a nucleic acid having nucleotide sequences that are not naturally joined together. This includes nucleic acid vectors including an amplified or assembled nucleic acid which can be used to transform a suitable host cell. A host cell that includes the recombinant nucleic acid is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant polypeptide." A recombinant nucleic acid may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

A polynucleotide or nucleic acid sequence refers to a polymeric form of nucleotide at least 10 bases in length. A recombinant polynucleotide includes a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g.,

- 21 -

a cDNA) independent of other sequences. The nucleotides can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single- and double- stranded forms of DNA.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter, such as the CMV promoter, is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers provided herein are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Polypeptide: A polymer in which the monomers are amino acid residues that are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. A polypeptide includes both naturally occurring proteins, as well as those that are recombinantly or synthetically produced. A polypeptide has an amino terminal (N-terminal) end and a carboxy-terminal end. In some

- 22 -

embodiments, the polypeptide is a disclosed antibody or a fragment thereof.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein (such as an antibody) is more enriched than the peptide or protein is in its natural

5   environment within a cell. In one embodiment, a preparation is purified such that the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

Sequence identity: The similarity between amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the

10   higher the percentage, the more similar the two sequences are. Homologs or variants of a polypeptide will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.*

15   2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet et al., *Nucleic Acids Research* 16:10881, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul et al., *Nature Genet.* 6:119, 1994, presents a detailed consideration of sequence alignment methods and homology

20   calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of

25   how to determine sequence identity using this program is available on the NCBI website on the internet.

Homologs and variants of a V_L or a V_H of an antibody that specifically binds a polypeptide are typically characterized by possession of at least about 75%, for example at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity

30   counted over the full length alignment with the amino acid sequence of interest. Proteins with

- 23 -

even greater similarity to the reference sequences will show increasing percentage identities

when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 95%, at

least 98%, or at least 99% sequence identity. When less than the entire sequence is being

compared for sequence identity, homologs and variants will typically possess at least 80%

5   sequence identity over short windows of 10-20 amino acids, and may possess sequence identities

of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence.

Methods for determining sequence identity over such short windows are available at the NCBI

website on the internet. One of skill in the art will appreciate that these sequence identity ranges

are provided for guidance only; it is entirely possible that strongly significant homologs could be

10   obtained that fall outside of the ranges provided.

         Terms used to describe sequence relationships between two or more nucleotide

sequences or amino acid sequences include "reference sequence," "selected from," "comparison

window," "identical," "percentage of sequence identity," "substantially identical,"

"complementary," and "substantially complementary."

15   For sequence comparison of nucleic acid sequences, typically one sequence acts as a

reference sequence, to which test sequences are compared. When using a sequence comparison

algorithm, test and reference sequences are entered into a computer, subsequence coordinates are

designated, if necessary, and sequence algorithm program parameters are designated. Default

program parameters are used. Methods of alignment of sequences for comparison are well

20   known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the

local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981, by the

homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970, by the

search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444, 1988,

by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA

25   in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr.,

Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in*

Molecular Biology (Ausubel et al., eds 1995 supplement)).

         One example of a useful algorithm is PILEUP. PILEUP uses a simplification of the

progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360, 1987. The method

30   used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153, 1989. Using

- 24 -

PILEUP, a reference sequence is compared to other test sequences to determine the percent

sequence identity relationship using the following parameters: default gap weight (3.00), default

gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG

sequence analysis software package, e.g., version 7.0 (Devereaux et al., *Nuc. Acids Res.* 12:387-

5   395, 1984.

Another example of algorithms that are suitable for determining percent sequence

identity and sequence similarity are the BLAST and the BLAST 2.0 algorithm, which are

described in Altschul et al., *J. Mol. Biol.* 215:403-410, 1990 and Altschul et al., *Nucleic Acids

Res.* 25:3389-3402, 1977. Software for performing BLAST analyses is publicly available

10   through the National Center for Biotechnology Information (ncbi.nlm.nih.gov). The BLASTN

program (for nucleotide sequences) uses as defaults a word length (W) of 11, alignments (B) of

50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLASTP program

(for amino acid sequences) uses as defaults a word length (W) of 3, and expectation (E) of 10,

and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA*

15   89:10915, 1989). An oligonucleotide is a linear polynucleotide sequence of up to about 100

nucleotide bases in length.

Specifically bind: When referring to an antibody, refers to a binding reaction which

determines the presence of a target protein, peptide, or polysaccharide in the presence of a

heterogeneous population of proteins and other biologics. Thus, under designated conditions, an

20   antibody binds preferentially to a particular target protein or peptide (such as an epitope of ATR

that includes T1989 and is phosphorylated at position 1989) and does not bind in a significant

amount to other proteins or peptides (such as an epitope of ATR that includes T1989 but that is

not phosphorylated at position 1989). Specific binding can be determined by methods known in

the art. With reference to an antibody antigen complex, specific binding of the antigen and

25   antibody has a K_d of less than about 10^{-7} Molar (M), such as less than about 10^{-8} M, 10^{-9} M, 10^{-10}

M, or even less than about 10^{-11} M.

The antibodies disclosed herein specifically bind only to a defined target (or multiple

targets, in the case of a bispecific antibody). Thus, an antibody that specifically binds to pT1989

ATR is an antibody that binds substantially to only pT1989 ATR, including cells or tissue

30   expressing pT1989 ATR, a substrate to which the pT1989 ATR is attached, or pT1989 ATR in a

- 25 -

biological specimen. It is, of course, recognized that a very small degree of non-specific interaction may occur between an antibody or conjugate including an antibody (such as an antibody that specifically binds pT1989 ATR or conjugate including such antibody) and a non-target (such as a cell that does not express pT1989 ATR or ATR not phosphorylated at T1989).

5   Typically, specific binding results in a much stronger association between the antibody and protein or cells bearing the antigen than between the antibody and protein or cells lacking the antigen. Specific binding typically results in greater than 2-fold, such as greater than 5-fold, greater than 10-fold, or greater than 100-fold increase in amount of bound antibody (per unit time) to a protein including the epitope or cell or tissue expressing the target epitope as compared to a protein or cell

10   or tissue lacking this epitope. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats are appropriate for selecting antibodies or other ligands specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow & Lane,

15   *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

        Subject: Any mammal, such as humans, non-human primates, pigs, sheep, cows, rodents, and the like. In two non-limiting examples, a subject is a human subject or a murine

20   subject. Thus, the term "subject" includes both human and veterinary subjects.

        Therapeutically effective amount or Effective Amount: The amount of an agent (such as a pharmaceutical compound, antibody, antigen binding fragment or conjugate) that alone, or together with one or more additional agents, induces the desired response, such as, for example treatment of a tumor or identification of a specific protein. Ideally, a therapeutically effective

25   amount provides a therapeutic effect without causing a substantial cytotoxic effect in the subject. In one example, a desired response is to decrease the size, volume, or number (such as metastases) of a tumor in a subject.

        Several preparations disclosed herein are administered in therapeutically effective amounts. A therapeutically effective amount of an agent, such as, but not limited to, a

30   chemotherapeutic agent, that is administered to a human or veterinary subject will vary

- 26 -

depending upon a number of factors associated with that subject, for example the overall health of the subject. A therapeutically effective amount can be determined by varying the dosage and measuring the resulting therapeutic response, such as the regression of a tumor. Therapeutically effective amounts also can be determined through various *in vitro*, *in vivo* or *in situ*

5   immunoassays. The disclosed agents can be administered in a single dose, or in several doses, as needed to obtain the desired response. However, the therapeutically effective amount can be dependent on the source applied, the subject being treated, the severity and type of the condition being treated, and the manner of administration.

Tumor: An abnormal growth of cells, which can be benign or malignant. Cancer is a

10   malignant tumor, which is characterized by abnormal or uncontrolled cell growth. Other features often associated with malignancy include metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels and suppression or aggravation of inflammatory or immunological response, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc. "Metastatic disease" refers to

15   cancer cells that have left the original tumor site and migrate to other parts of the body for example *via* the bloodstream or lymph system.

The amount of a tumor in an individual is the "tumor burden" which can be measured as the number, volume, or weight of the tumor. A tumor that does not metastasize is referred to as "benign." A tumor that invades the surrounding tissue and/or can metastasize is referred to as

20   "malignant." Examples of hematological tumors include leukemias, including acute leukemias (such as 11q23-positive acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia

25   vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, and other sarcomas,

30   synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon

- 27 -

carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer (including basal breast carcinoma, ductal carcinoma and lobular breast carcinoma), lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, and CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyrgioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma and retinoblastoma).

Under conditions sufficient for: A phrase that is used to describe any environment that permits a desired activity. In one example the desired activity is formation of an immune complex. In particular examples the desired activity is treatment of a tumor.

II.   Description of Several Embodiments

A.   *Monoclonal Antibodies*

The threonine at position 1989 of ATR (T1989) is a DNA damage-regulated phosphorylation site; activation of ATR in vivo results in phosphorylation of this threonine (pT1989). Selective inhibition of ATR prevents phosphorylation at T1989.

Isolated monoclonal antibodies that specifically bind to pT1989 ATR, antigen binding fragments of such antibodies, conjugates thereof, and methods of using these molecules, are provided herein. The antibodies can be chimeric or humanized. Also disclosed herein are compositions including these monoclonal antibodies and a pharmaceutically acceptable carrier. Nucleic acids encoding these antibodies, expression vectors comprising these nucleic acids, and isolated host cells that express the nucleic acids are also provided. These antibodies have a high sensitivity and specificity, and can be used to detect pT1989 in biological samples, .

Compositions comprising the monoclonal antibodies specific for pT1989 ATR can be used for drug screening and diagnostic purposes. These monoclonal antibodies can also be used to determine if a pharmacologic agent, such as a chemotherapeutic agent, is of use for treating a subject, and/or to determine the dose of the agent that is effective for treating a subject.

- 28 -

In several embodiments, the monoclonal antibodies include a heavy chain comprising a heavy chain complementarity determining region (HCDR)1, a HCDR2 and an HCDR3, and a light chain comprising a light chain complementarity determining region (LCDR) 1, LCDR2 and LCDR3. The disclosed antibodies specifically bind to pT1989 ATR. In some embodiments, the

5   pT1989 ATR specific antibodies include a variable heavy (V$_H$) and a variable light (V$_L$) chain and specifically bind pT1989 ATR. In several embodiments, the antibody or antigen binding fragment thereof includes heavy and light chain variable regions including the HCDR1, HCDR2, and HCDR3 of the amino acid sequence set forth as SEQ ID NO: 6 and LCDR1, LCDR2, and LCDR3 of the amino acid sequence set forth as SEQ ID NO: 7. These sequences are shown

10   below


```
    ETGLRWLLLVAVLKGVQCQSVEESGGRLVAPGTPLTLTCTVSGIDLMYNAMNWVRQAPGKGLEF
    IGMIGSGGNTVYASWAKGRFTISKTSTTVGLKMTSLTQEDTATYFCAREGSGGSMDFWGPGTLV
    TVSSGQPKAPSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGTLTNGVRTFPSVRQSS
15  GLYSLSSVVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCPPPELLGGPSVFIFPPKPK
    DTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHQD
    WLRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVYTMGPPREELSSRSVSLTCMINGFYPSD
    ISVEWEKNGKAEDNYKTTPAVLDSDGSYFLYSKLSVPTSEWQRGDVFTCSVMHEALHNHYTQKS
    ISRSPGK (SEQ ID NO: 6)
20

    DTRAPTQLLGLLLLWLPGATFALVMTQSPSSVSAAVGGTVTITCQASQSLYNNNYLAWFQQKPG
    QRPKLLIYKASTLASGVPSRFKGSGSGTQFTLTISDLECEDAATYYCLGEYKDNVDDGNAFGGG
    TEVVVEGDPVAPTVLIFPPAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQTTGIENSKTPQ
    NSADCTYNLSSTLTLTSTQYNSHKEYTCKVTQGTTSVVQSFNRGDC (SEQ ID NO: 7)
25
```

The discussion of monoclonal antibodies below refers to isolated monoclonal antibodies that include heavy and light chain variable domains including at least one complementarity determining region (CDR), such as a CDR1, CDR2 and CDR3. The person of ordinary skill in the art will understand that various CDR numbering schemes (such as the Kabat, Chothia or

30   IMGT numbering schemes) can be used to determine CDR positions. The person of skill in the art will readily understand use of various CDR numbering schemes when referencing particular amino acids of the antibodies disclosed herein.

The antibody, or an antigen binding fragment thereof, can include the HCDR1, HCDR2, and/or HCDR3 of SEQ ID NO: 6, and the LCDR1, LCDR2, and/or LCDR3 of SEQ ID NO: 7,

35   and specifically bind pT1989 ATR. In specific, non-liming examples, both the heavy and light chain CDRs are identified by Kabat, Clothia or IMGT numbering. Thus, the antibody or an

- 29 -

antigen binding fragment thereof can include the HCDR1, HCDR2, and HCDR3 of SEQ ID NO: 6, and the LCDR1, LCDR2, and LCDR3 of SEQ ID NO: 7, and specifically bind pT1989 ATR. In specific non-limiting examples, the heavy and light chain CDRs are identified by Kabat, Clothia or IMGT numbering. It should be noted that the CDRs can be identified using other

5   methods known to those of skill in the art, for example, Contact and Enhanced Clothia (also called Clothia+, see Fig. 13).

In some embodiments, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and/or HCDR3 including amino acids 48-52, 67-82, and 113-121 (H31-H35, H50-H65 and H95-H102 using Kabat numbering) of SEQ ID NO: 6,

10   respectively. In further embodiments, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and/or HCDR3 including amino acids 43-49, 69-73, and/or 113-121 (H26-H32, H52-H56 and H95-H102 using Chothia numbering) of SEQ ID NO: 6, respectively. In other embodiments, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and HCDR3 including

15   amino acids 48-52, 67-82, and 113-121 (H31-H35, H50-H65 and H95-H102 using Kabat numbering) of SEQ ID NO: 6, respectively. In additional embodiments, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and HCDR3 including amino acids 43-49, 69-73, and113-121 (H26-H32, H52-H56 and H95-H102 using Chothia numbering) of SEQ ID NO: 6, respectively.

20   In some embodiments, the antibody or antigen binding fragment includes a light chain variable region including a LCDR1, LCDR2, and/or LCDR3 including amino acids 45-57, 73-79 and/or 112-124 (L24-L34, L50-L56, and L89-L97 using Kabat numbering and L24-L34, L50-L56, and L89-L97 using Chothia numbering) of SEQ ID NO: 7, respectively. In other embodiments, the antibody or antigen binding fragment includes a light chain variable region

25   including a LCDR1, LCDR2, and LCDR3 including amino acids 45-57, 73-79 and/or 112-124 (L24-L34, L50-L56, and L89-L97 using Kabat numbering and L24-L34, L50-L56, and L89-L97 using Chothia numbering) of SEQ ID NO: 7, respectively.

In specific non-limiting examples, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and HCDR3 including amino acids

30   48-52, 67-82, and 113-121 (H31-H35, H50-H65 and H95-H102 using Kabat numbering) of SEQ

- 30 -

ID NO: 6. The antibody or antigen binding fragment also includes a light chain variable region including a LCDR1, LCDR2, and LCDR3 including amino acids 45-57, 73-79 and/or 112-124 (L24-L34, L50-L56, and L89-L97 using Kabat numbering and L24-L34, L50-L56, and L89-L97 using Chothia numbering) of SEQ ID NO: 7.

5   In additional non-limiting examples, the antibody or antigen binding fragment includes a heavy chain variable region including a HCDR1, HCDR2, and HCDR3 including amino acids 43-49, 69-73, and113-121 (H26-H32, H52-H56 and H95-H102 using Chothia numbering) of SEQ ID NO: 6. The antibody or antigen binding fragment also includes a light chain variable region including a LCDR1, LCDR2, and LCDR3 including amino acids 45-57, 73-79 and/or

10   112-124 (L24-L34, L50-L56, and L89-L97 using Kabat numbering and L24-L34, L50-L56, and L89-L97 using Chothia numbering) of SEQ ID NO: 7.

In some embodiments, the antibody includes a heavy chain variable region including an amino acid sequence at least 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence set forth as SEQ ID NO: 6. In more embodiments, the antibody includes a light chain variable

15   region including an amino acid sequence at least 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence set forth as SEQ ID NO: 7. In additional embodiments, the antibody includes a heavy chain variable region including an amino acid sequence at least 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence set forth as SEQ ID NO: 6, and a light chain variable region including an amino acid sequence at least 95%, 96%, 97%, 98%, or 99%

20   identical to the amino acid sequence set forth as SEQ ID NO: 7.

In additional embodiments, the antibody includes a heavy chain variable region including, or consisting of, the amino acid sequence set forth as SEQ ID NO: 6. In more embodiments, the antibody includes a light chain variable region including, or consisting of, the amino acid sequence set forth as SEQ ID NO: 7. In yet other embodiment, the antibody includes

25   a heavy chain variable domain including, or consisting of, the amino acid sequence set forth as SEQ ID NO: 6 and a light chain variable domain including, or consisting of, the amino acid sequence set forth as SEQ ID NO: 7.

In several embodiments, the disclosed antibodies can specifically bind pT1989 ATR with an affinity of at least about 1.0×10^{-8} M, at least about 5.0×10^{-8} M, at least about 1.0×10^{-9} M, at

30   least about 5.0×10^{-9} M, at least about 1.0×10^{-10} M, at least about 5.0×10^{-10} M, or at least about

- 31 -

1.0 x 10^{-11} M. In additional embodiments, the antibody does not significantly bind ATR that is not phosphorylated at position 1989 (T1989 ATR). In additional embodiments, the disclosed antibodies specifically bind the amino acid sequence set forth as SEQ ID NO: 2 and/or the amino acid sequence set forth as SEQ ID NO: 3 with an affinity of at least about 1.0 x 10^{-9} M, at least about 5.0 x 10^{-9} M, at least about 1.0 x 10^{-10} M, at least about 5.0 x 10^{-10} M, or at least about 1.0 x 10^{-11} M. In specific non-liming examples, the disclosed antibodies specifically bind the amino acid sequence set forth as SEQ ID NO: 2 and/or the amino acid sequence set forth as SEQ ID NO: 3 with an affinity of at least about at least about 1.0 x 10^{-10} M.

The monoclonal antibodies can be rabbit monoclonal antibodies. Chimeric antibodies are also provided. The antibodies can include any suitable framework region, such as (but not limited to) a human, mouse, rat, goat, sheep, or a rabbit framework region. Human framework regions, and mutations that can be made in a human antibody framework regions, are known in the art (see, for example, in U.S. Patent No. 5,585,089, which is incorporated herein by reference). Alternatively, a heterologous framework region, such as, but not limited to a mouse framework region, can be included in the heavy or light chain of the antibodies. (See, for example, Jones *et al.*, *Nature* 321:522, 1986; Riechmann *et al.*, *Nature* 332:323, 1988; Verhoeyen *et al.*, *Science* 239:1534, 1988; Carter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:4285, 1992; Sandhu, *Crit. Rev. Biotech.*12:437, 1992; and Singer *et al.*, *J. Immunol.*150:2844, 1993.)

In some embodiments, an antibody that specifically binds pT1989 ATR as disclosed herein includes up to 10 amino acid substitutions (such as up to 1, 2, 3, 4, 5, 6, 7, 8, or up to 9 amino acid substitutions) in the framework regions of the heavy chain of the antibody, or the light chain of the antibody, or the heavy and light chains of the antibody.

The antibodies or antigen binding fragments disclosed herein can be derivatized or linked to another molecule (such as another peptide or protein). In general, the antibodies or portion thereof is derivatized such that the binding to pT1989 ATR is not affected adversely by the derivatization or labeling. For example, the antibody can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (for example, a bispecific antibody or a diabody), a detectable marker, an effector molecule, or a protein or peptide that can mediate association of the antibody

- 32 -

or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, such as to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (such as m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (such as disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, Ill.

The monoclonal antibodies disclosed herein can be of any isotype. The monoclonal antibody can be, for example, an IgM or an IgG antibody, such as IgG_1, IgG_2, IgG_3 or an IgG_4. The class of an antibody that specifically binds pT1989 ATR can be switched with another (for example, IgG can be switched to IgM), according to well-known procedures. For example, a nucleic acid molecule encoding the V_L or V_H of a disclosed antibody can be operatively linked to a nucleic acid sequence encoding a C_L or C_H from a different class of immunoglobulin molecule. This can be achieved using a vector or nucleic acid molecule that comprises a C_L or C_H chain, as known in the art. For example, an antibody that specifically binds pT1989 ATR, that was originally IgG may be class switched to an IgM. Class switching can be used to convert one IgG subclass to another, such as from IgG_1 to IgG_2, IgG_3, or IgG_4.

In some examples, the disclosed antibodies are oligomers of antibodies, such as dimers, trimers, tetramers, pentamers, hexamers, septamers, octomers and so on. In some examples, the antibodies are pentamers.

B.   Conjugates

Human monoclonal antibodies specific for pT1989 ATR, or antigen binding fragments thereof, can be conjugated to an agent, such as an effector molecule or detectable marker, using any number of means known to those of skill in the art. Both covalent and non-covalent attachment means may be used. Conjugates include, but are not limited to, molecules in which there is a covalent linkage of an effector molecule or a detectable marker to an antibody or antigen binding fragment that specifically binds pT1989 ATR. One of skill in the art will appreciate that various effector molecules and detectable markers can be used, including (but not limited to) chemotherapeutic agents, toxins, radioactive agents such as ^{125}I, ^{32}P, ^{14}C, ^{3}H and [35]S

- 33 -

and other labels, target moieties and ligands, etc.

A monoclonal antibody that specifically binds pT1989 ATR (or antigen binding fragment thereof) can be conjugated with a detectable marker; for example, a detectable marker capable of detection by ELISA, spectrophotometry, flow cytometry, microscopy or diagnostic imaging

5   techniques. The detectable marker may be synthetic markers that are non-naturally occurring. Specific, non-limiting examples of detectable markers include fluorophores, chemiluminescent agents, enzymatic linkages, radioactive isotopes and heavy metals or compounds (for example super paramagnetic iron oxide nanocrystals for detection by MRI). For example, useful detectable markers include fluorescent compounds, including fluorescein, fluorescein

10   isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin, lanthanide phosphors and the like. Bioluminescent markers are also of use, such as luciferase, Green fluorescent protein (GFP), Yellow fluorescent protein (YFP). An antibody or antigen binding fragment can also be conjugated with enzymes that are useful for detection, such as horseradish peroxidase, β- galactosidase, luciferase, alkaline phosphatase, glucose oxidase and

15   the like. When an antibody or antigen binding fragment is conjugated with a detectable enzyme, it can be detected by adding additional reagents that the enzyme uses to produce a reaction product that can be discerned. For example, when the agent horseradish peroxidase is present the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is visually detectable. An antibody or antigen binding fragment may also be conjugated

20   with biotin, and detected through indirect measurement of avidin or streptavidin binding. It should be noted that the avidin itself can be conjugated with an enzyme or a fluorescent label.

An antibody or antigen binding fragment can be conjugated with a paramagnetic agent, such as gadolinium. Paramagnetic agents such as superparamagnetic iron oxide are also of use as labels. Antibodies can also be conjugated with lanthanides (such as europium and

25   dysprosium), and manganese. An antibody or antigen binding fragment may also be labeled with a predetermined polypeptide epitopes recognized by a secondary reporter (such as leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags).

An antibody or antigen binding fragment can be conjugated with a radiolabeled amino

30   acid. The radiolabel may be used for both diagnostic and therapeutic purposes. For instance, the

- 34 -

radiolabel may be used to detect pT1989 ATR and pT1989 ATR-expressing cells by x-ray, emission spectra, or other diagnostic techniques. Examples of labels for polypeptides include, but are not limited to, the following radioisotopes or radionucleotides: ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I.

5   Means of detecting such detectable markers are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric

10   labels are detected by simply visualizing the colored label.

The choice of a particular detectable marker (or effector molecule) depends, amongst other things, on the particular cell, the desired biological effect, and/or the detection method. Thus, for example, the effector molecule can be a cytotoxin that is used to bring about the death of a particular target cell, or a fluorescent molecule can be used if visualization will use

15   fluorescent light. Any effector molecule that can be detected, such as by using another antibody, can be utilized in the detection methods disclosed herein. This includes proteins, particles and other agents.

Effector molecules and detectable markers can be linked to an antibody or antigen binding fragment of interest using any number of means known to those of skill in the art. Both

20   covalent and noncovalent attachment means may be used. The procedure for attaching an effector molecule or detectable marker to an antibody or antigen binding fragment varies according to the chemical structure of the effector. Polypeptides typically contain a variety of functional groups; such as carboxylic acid (COOH), free amine (-NH$_2$) or sulfhydryl (-SH) groups, which are available for reaction with a suitable functional group on an antibody to result

25   in the binding of the effector molecule or detectable marker. Alternatively, the antibody or antigen binding fragment is derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of known linker molecules such as those available from Pierce Chemical Company, Rockford, IL. The linker can be any molecule used to join the antibody or antigen binding fragment to the effector molecule or

30   detectable marker. The linker is capable of forming covalent bonds to both the antibody, or

- 35 -

antigen binding fragment, and to the effector molecule or detectable lable. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the antibody or antigen binding fragment and the effector molecule or detectable marker are polypeptides, the

5   linkers may be joined to the constituent amino acids through their side groups (such as through a disulfide linkage to cysteine) or to the alpha carbon amino and carboxyl groups of the terminal amino acids.

Additionally, in several embodiments, the linker can include a spacer element, which, when present, increases the size of the linker such that the distance between the effector

10   molecule or the detectable marker and the antibody or antigen binding fragment is increased. Exemplary spacers are known to the person of ordinary skill, and include those listed in U.S. Pat. Nos. 7,964,5667, 498,298, 6,884,869, 6,323,315, 6,239,104, 6,034,065, 5,780,588, 5,665,860, 5,663,149, 5,635,483, 5,599,902, 5,554,725, 5,530,097, 5,521,284, 5,504,191, 5,410,024, 5,138,036, 5,076,973, 4,986,988, 4,978,744, 4,879,278, 4,816,444, and 4,486,414, as well as

15   U.S. Pat. Pub. Nos. 20110212088 and 20110070248, each of which is incorporated by reference in its entirety.

Thus, in several embodiments, the conjugate includes a linker that connects the effector molecule or detectable marker to the pT1989 ATR -specific antibody or antigen binding fragment thereof. In some embodiments, the peptide linker is at least two amino acids long or at

20   least three amino acids long. However, the linker can be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids long, such as 1-2, 1-3, 2-5, 3-10, 3-15, 1-5, 1-10, 1-15, amino acids long. In some embodiments, the linker is cleavable under intracellular conditions, such that cleavage of the linker releases the effector molecule or detectable marker from the antibody or antigen binding fragment in the intracellular environment. In yet other embodiments, the linker is not cleavable

25   and the effector molecule or detectable marker not released.

The antibodies or antigen binding fragments disclosed herein can be derivatized, for example, by cross-linking two or more antibodies (of the same type or of different types, such as to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (such as m-

- 36 -

maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (such as disuccinimidyl suberate). Such linkers are commercially available.

In view of the large number of methods that have been reported for attaching a variety of radiodiagnostic compounds, radiotherapeutic compounds, labels (such as enzymes or fluorescent 5   molecules), toxins, and other agents to antibodies one skilled in the art will be able to determine a suitable method for attaching a given agent to an antibody or antigen binding fragment or other polypeptide. For example, the antibody or antigen binding fragment can be conjugated with small molecular weight drugs such as Monomethyl Auristatin E (MMAE), Monomethyl Auristatin F (MMAF), maytansine, maytansine derivatives, including the derivative of 10   maytansine known as DM1 (also known as mertansine), or other agents.

The average number of effector molecule or detectable marker moieties per antibody or antigen binding fragment in a conjugate can range, for example, from 1 to 20 moieties per antibody or antigen binding fragment. For some conjugates, the average number of effector molecule or detectable marker moieties per antibody or antigen binding fragment may be limited 15   by the number of attachment sites on the antibody or antigen binding fragment. For example, where the attachment is a cysteine thiol, an antibody or antigen binding fragment may have only one or several cysteine thiol groups, or may have only one or several sufficiently reactive thiol groups through which a linker may be attached. In certain embodiments, the average number of effector molecule or detectable marker moieties per antibody or antigen binding fragment in a 20   conjugate range from 1 to about 8; from about 2 to about 6; from about 3 to about 5; from about 3 to about 4; from about 3.1 to about 3.9; from about 3.2 to about 3.8; from about 3.2 to about 3.7; from about 3.2 to about 3.6; from about 3.3 to about 3.8; or from about 3.3 to about 3.7. See, for example, U.S. Pat. No. 7,498,298, incorporated by reference herein in its entirety. The average number of effector molecule or detectable marker moieties per antibody or antigen 25   binding fragment in preparations of conjugates may be characterized by conventional means such as mass spectroscopy and, ELISA assay.

The loading (for example, effector molecule (such as a detectable marker)/antibody ratio) of an conjugate may be controlled in different ways, for example, by: (i) limiting the molar excess of effector molecule-linker intermediate or linker reagent relative to antibody, (ii) limiting 30   the conjugation reaction time or temperature, (iii) partial or limiting reductive conditions for

- 37 -

cysteine thiol modification, (iv) engineering by recombinant techniques the amino acid sequence of the antibody such that the number and position of cysteine residues is modified for control of the number or position of linker-effector molecule attachments (such as thioMab or thioFab prepared as disclosed in WO2006/03448, incorporated by reference herein in its entirety.

5

C. Nucleotides, Expression Vectors and Host Cells

Nucleic acids encoding the amino acid sequences of antibodies that specifically bind pT1989 ATR are provided. Nucleic acid molecules encoding these antibodies can readily be produced by one of skill in the art, using the amino acid sequences provided herein, and the genetic code. In addition, one of skill can readily construct a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids which differ in sequence but which encode the same effector molecule, detectable marker or antibody or antigen binding fragment sequence.

Nucleic acid sequences encoding the antibodies that specifically bind pT1989 ATR can be prepared by any suitable method including, for example, cloning of appropriate sequences or by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68:90-99, 1979; the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151, 1979; the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.* 22:1859-1862, 1981; the solid phase phosphoramidite triester method described by Beaucage & Caruthers, *Tetra. Letts.* 22(20):1859-1862, 1981, for example, using an automated synthesizer as described in, for example, Needham-VanDevanter *et al.*, *Nucl. Acids Res.* 12:6159-6168, 1984; and, the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This can be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template.

Exemplary nucleic acids including sequences encoding an antibody that specifically binds pT1989 ATR (or antigen binding fragment thereof) can be prepared by cloning techniques.

In some embodiments, the heavy chain is encoded by the nucleic acid sequence set forth as:

- 38 -

ATGGAGACTGGGCTGCGCTGGCTTCTCCTGGTCGCTGTGCTCAAAGGTGTCCAGTGT
CAGTCGGTGGAGGAGTCCGGGGGGTCGCCTGGTCGCCCCTGGGACACCCCTGACACT
CACCTGTACCGTCTCTGGAATCGACCTCATGTACAATGCAATGAACTGGGTCCGCCA
GGCTCCAGGGAAGGGCCTGGAATTCATCGGAATGATTGGTAGTGGTGGTAATACAG
5   TCTATGCGAGCTGGGCGAAAGGCCGATTCACCATCTCCAAAACCTCGACCACGGTG
GGTCTGAAGATGACCAGTCTGACACAGGAGGACACGGCCACCTATTCTGTGCCAG
AGAGGGTAGTGGTGGCAGTATGGACTTCTGGGGCCCAGGCACCCTGGTCACCGTCT
CCTCAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACA
CACCCAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGGTACCTCCCGGAGCCA
10   GTGACCGTGACCTGGAACTCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTC
CGTCCGGCAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAA
GCAGCCAGCCCGTCACCTGCAACGTGGCCCACCCAGCCACCAACACCAAAGTGGAC
AAGACCGTTGCGCCCTCGACATGCAGCAAGCCCACGTGCCCACCCCCTGAACTCCT
GGGGGGACCGTCTGTCTTCATCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTC
15   ACGCACCCCCGAGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGG
TGCAGTTCACATGGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCGCTA
CGGGAGCAGCAGTTCAACAGCACGATCCGCGTGGTCAGCACCCTCCCCATCGCGCA
CCAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCACAACAAGGCACTCC
CGGCCCCCATCGAGAAAACCATCTCCAAAGCCAGAGGGCAGCCCCTGGAGCCGAA
20   GGTCTACACCATGGGCCCTCCCCGGGAGGAGCTGAGCAGCAGGTCGGTCAGCCTGA
CCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAGAAC
GGGAAGGCAGAGGACAACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCT
CCTACTTCCTCTACAGCAAGCTCTCAGTGCCCACGAGTGAGTGGCAGCGGGGCGAC
GTCTTCACCTGCTCCGTGATGCACGAGGCCTTGCACAACCACTACACGCAGAAGTCC
25   ATCTCCCGCTCTCCGGGTAAATGA (SEQ ID NO: 8).

In additional embodiments, the light chain is encoded by the nucleic acid sequence set
forth as:
ATGGACACGAGGGCCCCCACTCAGCTGCTGGGGCTCCTGCTGCTCTGGCTCCCAGGT
30   GCCACATTTGCCCTGGTGATGACCCAGTCTCCATCCTCCGTGTCTGCAGCTGTGGGA

- 39 -

GGCACAGTCACCATCACTTGCCAGGCCAGTCAGAGTCTTTATAATAACAACTACTTA

GCCTGGTTTCAGCAGAAACCAGGGCAGCGTCCCAAGCTCCTGATCTACAAGGCATC

CACTCTGGCAAGTGGGGTCCCATCGCGCTTCAAAGGCAGTGGATCTGGGACACAGT

TCACTCTCACCATCAGCGACCTGGAGTGTGAAGATGCTGCCACTTACTACTGCCTAG

5   GCGAATATAAGGATAATGTAGATGATGGTAATGCTTTCGGCGGAGGGACCGAGGTG

GTGGTCGAAGGTGATCCAGTTGCACCTACTGTCCTCATCTTCCCACCAGCTGCTGAT

CAGGTGGCAACTGGAACAGTCACCATCGTGTGTGTGGCGAATAAATACTTTCCCGA

TGTCACCGTCACCTGGGAGGTGGATGGCACCACCCAAACAACTGGCATCGAGAACA

GTAAAACACCGCAGAATTCTGCAGATTGTACCTACAACCTCAGCAGCACTCTGACA

10   CTGACCAGCACACAGTACAACAGCCACAAAGAGTACACCTGCAAGGTGACCCAGG

GCACGACCTCAGTCGTCCAGAGCTTCAATAGGGGTGACTGTTAG (SEQ ID NO: 9)

SEQ ID NO: 8 and SEQ ID NO: 9 include an ATG encoding the N terminal methionine.
In some embodiments, this ATG is deleted. Examples of appropriate cloning and sequencing
techniques, and instructions sufficient to direct persons of skill through cloning are found in
15   Sambrook *et al.*, *supra*, Berger and Kimmel (eds.), *supra*, and Ausubel, *supra*. Product
information from manufacturers of biological reagents and experimental equipment also provide
useful information. Such manufacturers include the SIGMA Chemical Company (Saint Louis,
MO), R&D Systems (Minneapolis, MN), Pharmacia Amersham (Piscataway, NJ), CLONTECH
Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company
20   (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg,
MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland),
Invitrogen (San Diego, CA), and Applied Biosystems (Foster City, CA), as well as many other
commercial sources known to one of skill.

Nucleic acids can also be prepared by amplification methods. Amplification methods
25   include polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-
based amplification system (TAS), the self-sustained sequence replication system (3SR). A
wide variety of cloning methods, host cells, and *in vitro* amplification methodologies are well
known to persons of skill.

In one example, an antibody or antigen binding fragment of use is prepared by inserting
30   the cDNA which encodes a variable region from an antibody into a vector which includes the

- 40 -

cDNA encoding an effector molecule or detectable marker, such as an enzyme or label. The insertion is made so that the variable region and the effector molecule or detectable marker are read in frame so that one continuous polypeptide is produced. Thus, the encoded polypeptide contains a functional Fv region and a functional effector molecule or detectable marker region.

5   In one embodiment, cDNA encoding an enzyme is ligated to a scFv so that the enzyme is located at the carboxyl terminus of the scFv. In several examples, cDNA encoding a horseradish peroxidase or alkaline phosphatase, or a polypeptide marker of interest is ligated to a scFv so that the enzyme (or polypeptide marker) is located at the amino terminus of the scFv. In another example, the label is located at the amino terminus of the scFv. In a further example, cDNA

10   encoding the protein or polypeptide marker is ligated to a heavy chain variable region of an antibody or antigen binding fragment, so that the enzyme or polypeptide marker is located at the carboxyl terminus of the heavy chain variable region. The heavy chain-variable region can subsequently be ligated to a light chain variable region of the antibody or antigen binding fragment using disulfide bonds. In a yet another example, cDNA encoding an enzyme or a

15   polypeptide marker is ligated to a light chain variable region of an antibody or antigen binding fragment, so that the enzyme or polypeptide marker is located at the carboxyl terminus of the light chain variable region. The light chain-variable region can subsequently be ligated to a heavy chain variable region of the antibody or antigen binding fragment using disulfide bonds.

        Once the nucleic acids encoding the conjugate, antibody, or antigen binding fragment

20   thereof, are isolated and cloned, the protein can be expressed in a recombinantly engineered cell such as bacteria, plant, yeast, insect and mammalian cells using a suitable expression vector. One or more DNA sequences encoding the antibody or fragment thereof can be expressed *in vitro* by DNA transfer into a suitable host cell. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not

25   be identical to the parental cell since there may be mutations that occur during replication. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

        Polynucleotide sequences encoding the antibody or antigen binding fragment or conjugate thereof, can be operatively linked to expression control sequences. An expression

30   control sequence operatively linked to a coding sequence is ligated such that expression of the

- 41 -

coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit

5   proper translation of mRNA, and stop codons.

The polynucleotide sequences encoding the antibody, or antigen binding fragment or conjugate thereof can be inserted into an expression vector including, but not limited to a plasmid, virus or other vehicle that can be manipulated to allow insertion or incorporation of sequences and can be expressed in either prokaryotes or eukaryotes. Hosts can include

10   microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art.

Transformation of a host cell with recombinant DNA may be carried out by conventional

15   techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired, or by electroporation.

20   When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with polynucleotide sequences encoding the antibody, labeled antibody, or antigen binding fragment thereof, and a second foreign DNA

25   molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982). One of skill in the art can readily use expression systems such as plasmids and vectors of use in

30   producing proteins in cells including higher eukaryotic cells such as the COS, CHO, HeLa and

- 42 -

myeloma cell lines.

Isolation and purification of recombinantly expressed polypeptide can be carried out by conventional means including preparative chromatography and immunological separations. Once expressed, the conjugate, antibody, or antigen binding fragment thereof, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y., 1982). Substantially pure compositions of at least about 90 to 95% homogeneity are disclosed herein, and 98 to 99% or more homogeneity can be used for pharmaceutical purposes. Once purified, partially or to homogeneity as desired, if to be used therapeutically, the polypeptides should be substantially free of endotoxin.

Methods for expression of single chain antibodies and refolding to an appropriate active form, including single chain antibodies, from bacteria such as *E. coli* have been described and are well-known and are applicable to the antibodies disclosed herein. See, Buchner *et al.*, *Anal. Biochem.* 205:263-270, 1992; Pluckthun, *Biotechnology* 9:545, 1991; Huse *et al.*, *Science* 246:1275, 1989 and Ward *et al.*, *Nature* 341:544, 1989, all incorporated by reference herein. Often, functional heterologous proteins from *E. coli* or other bacteria are isolated from inclusion bodies and require solubilization using strong denaturants, and subsequent refolding. During the solubilization step, as is well known in the art, a reducing agent must be present to separate disulfide bonds. An exemplary buffer with a reducing agent is: 0.1 M Tris pH 8, 6 M guanidine, 2 mM EDTA, 0.3 M DTE (dithioerythritol). Reoxidation of the disulfide bonds can occur in the presence of low molecular weight thiol reagents in reduced and oxidized form, as described in Saxena *et al.*, *Biochemistry,* 9: 5015-5021, 1970, incorporated by reference herein, and especially as described by Buchner *et al.*, *supra*. Renaturation is typically accomplished by dilution (for example, 100-fold) of the denatured and reduced protein into refolding buffer. An exemplary buffer is 0.1 M Tris, pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione (GSSG), and 2 mM EDTA.

As a modification to the two chain antibody purification protocol, the heavy and light chain regions are separately solubilized and reduced and then combined in the refolding solution. An exemplary yield is obtained when these two proteins are mixed in a molar ratio such that a 5 fold molar excess of one protein over the other is not exceeded. Excess oxidized glutathione or

- 43 -

other oxidizing low molecular weight compounds can be added to the refolding solution after the redox-shuffling is completed.

D.   Compositions

Compositions are provided that include one or more of the disclosed conjugates, antibodies, or antigen binding fragments, that specifically bind pT1989 ATR, in a carrier (such as a pharmaceutically acceptable carrier). The compositions can be prepared in unit dosage forms for detection methods.

The compositions for administration can include a solution of the conjugate, antibody or antigen binding fragment dissolved in a pharmaceutically acceptable carrier, such as an aqueous carrier. A variety of aqueous carriers can be used, for example, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antibody or antigen binding fragment or conjugate in these formulations can vary with the particular method of use selected and the subject's needs. Actual methods of preparing such forms are known, or will be apparent, to those skilled in the art.

Antibodies, antigen binding fragments, or conjugates may be provided in lyophilized form and rehydrated with sterile water before administration, although they are also provided in sterile solutions of known concentration. The antibody or antigen binding fragment or conjugate solution is then added to an infusion bag containing 0.9% sodium chloride, USP, and in some cases administered at a dosage of from 0.5 to 15 mg/kg of body weight. Considerable experience is available in the art in the reconstitution of antibody or antigen binding fragment and conjugate drugs. Additional agents, including preservatives can be added.

E.   Kits

Kits are also provided. For example, kits for detecting a cell that expresses pT1989 ATR in a subject, or determining the dose of a chemotherapeutic agent for administration to a subject. The kits will typically include an antibody or antigen binding fragment that specifically binds

- 44 -

pT1989 ATR and/or a conjugate thereof.

More than one of the conjugates or antibodies or antigen binding fragments that specifically bind pT1989 ATR can be included in the kit. Thus, in several non-limiting examples, the kit can include two or more antibodies that specifically bind pT1989 ATR, or an antibody or antigen binding fragment that specifically binds pT1989 ATR and a conjugate thereof, or a combination thereof. In some embodiments, an antigen binding fragment or conjugate including an antigen binding fragment, such as an Fv fragment, is included in the kit. In one example, such as for *in vivo* uses, the antibody can be a scFv fragment.

The kit can include a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container typically holds a composition including one or more of the disclosed pT1989 ATR specific antibodies, antigen binding fragments, or conjugates. In several embodiments the container may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle).

A label or package insert indicates that the composition is used for detecting pT1989 ATR. The label or package insert typically will further include instructions for use of a disclosed pT1989 ATR specific antibodies or fragments thereof, or conjugates thereof, for example. The package insert typically includes instructions customarily included in commercial packages of products that contain information about the indications, usage, contraindications and/or warnings concerning the use of such products. The instructional materials may be written, in an electronic form (such as a computer diskette or compact disk) or may be visual (such as video files). The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, the kit may additionally contain means of detecting a label (such as enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a secondary antibody, or the like). The kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

- 45 -

F.   Methods of Detection

The antibodies disclose herein specifically bind pT1989 and can be used to identify ATR phosphorylated at position 1989 (pT1989 ATR). Methods are provided for detecting the presence of a pT1989 ATR in a cell extract or biological sample, for example detecting a tumor that expresses pT1989 ATR. In some embodiments, the methods include contacting a biological sample or cell extract, such as samples that include tumor cells, or an extract thereof, with one or more of the antibodies that specifically bind pT1989 ATR to form an immune complex. The amount of pT1989 ATR can be quantitated.

A biological sample can be obtained from a mammalian subject of interest, such as human. The biological sample can be cells, bodily fluids, such as blood, derivatives and fractions of blood (such as purified B and/or T cell populations), cerebrospinal fluid; as well as biopsied or surgically removed tissue, for example tissues that are unfixed, frozen, or fixed in formalin or paraffin. The biological sample can be from any organ, including any solid tissue that can be biopsied and blood. The sample can be tissue autopsies and pathology specimens. In specific non-limiting examples, the biological sample is peripheral blood mononuclear cells, cerebral spinal fluid, sputum, or bronchial lavage fluid. In additional non-limiting examples, the biological sample includes circulating tumor cells or circulating cancer stem cells. In additional non-liming examples, the biological sample is ascites fluid, a bone marrow aspirate, a skin punch biopsy or a buccal swab. In further non-liming examples, the sample includes hair follicles. The sample can include circulating endothelial cells.

Thus, methods are provided for detecting a cell that expresses pT1989 ATR, for example, a tumor cell that expresses pT1989ATR, or the amount of pT1989 in a biological sample. In a specific non-limiting example, the cell is a tumor cell. In some embodiments, the tumor is a hematological tumor such as a leukemia, for example an acute leukemia (such as 11q23-positive acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), or a chronic leukemia (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic

- 46 -

syndrome, hairy cell leukemia and myelodysplasia. In other embodiments, the tumor is a solid

tumor, such as a sarcoma or carcinoma, such as a fibrosarcoma, myxosarcoma, liposarcoma,

chondrosarcoma, osteogenic sarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's

tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic

5   cancer, breast cancer (including basal breast carcinoma, ductal carcinoma and lobular breast

carcinoma), lung cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous

cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid

carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma,

papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma,

10   renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical

cancer, testicular tumor, seminoma, bladder carcinoma, or a tumor of the central nervous system

(such as a glioma, astrocytoma, medulloblastoma, craniopharyrgioma, ependymoma, pinealoma,

hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma,

neuroblastoma and retinoblastoma).

15   Without being bound by theory, DNA damage, such as, but not limited to, DNA damage

caused by ultraviolet light, chemotherapeutic agents, hydroxyurea and replication stress activate

ATR. These agents cause polymerases to stall during replication at damage sites on DNA, such

that helicases continue to unwind the DNA, leading to long stretches of single-stranded DNA

(ssDNA) that result from the continued activity of helicases after replication forks are stalled.

20   These effects activate the ATR pathway. In this pathway, ATR (together with the Rad9-Hus1-

Rad1 complex) activates Chk1, which phosphorylates Cdc25A and Cdc25C. Ultimately, the

cells arrest in S or G2 (see Wagner and Kaufmann, *(2010), Pharmaceuticals* 3: 1311-1334).

Thus, in some embodiments, Cdc25A, Cdc25C, and/or Chk1 activation can be assayed. In

additional embodiments, cells are assayed to determine if they are in S or G2 arrest. Assays for

25   Cdc25A, Cdc25C, and/or Chk1 activation are known in the art. Methods for determining cell

cycle phase are also known in the art.

       In one embodiment, a sample is obtained from a subject, and the presence of pT1989

ATR is assessed *in vitro*. In additional embodiments, the sample is cells, such as from a cell

line, that are cultured *in vitro*.

30   These methods can include contacting the sample of interest, or an extract thereof, with

- 47 -

an antibody, antigen binding fragment or conjugate provided herein that specifically bind pT1989 ATR to form an immune complex. The presence (or absence) of the immune complex is then detected. The presence of the immune complex indicates the presence of pT1989 ATR. In some non-limiting examples, the amount of pT1989 ATR is quantified. In additional non-

5   limiting examples, the amount of pT1989 ATR is compared to a control. An increase in the presence of this immune complex in the sample, compared to the presence of the immune complex in a control sample, such as a sample not contacted with the antibody or antigen binding fragment, a sample contacted with an unrelated antibody, or a reference standard, detects the presence of cells that express pT1989 ATR in the biological sample. For example, an

10   increase in the presence of the immune complex in the sample as compared to formation of the immune complex in a control sample can indicate the presence of pT1989 in the sample from the subject, or in the cell cultured *in vitro*.

In some embodiments, the antibody that specifically binds pT1989 ATR or antigen binding fragment is conjugated to a detectable marker. In additional embodiments, the methods

15   include contacting a second antibody that specifically binds the antibody that specifically binds pT1989 ATR, antigen binding fragment thereof, or a conjugate including these molecules, for a sufficient amount of time to form an immune complex, and then detecting the presence of this immune complex. An increase in the presence of this immune complex in a biological sample (as described above) compared to the presence of the immune complex in a control sample, such

20   as a sample not contacted with the antibody or antigen binding fragment, a sample contacted with an unrelated antibody, or a reference standard, detects the presence of cells that express pT1989 ATR in the biological sample. In some examples, the second antibody is conjugated to a detectable marker.

Suitable detectable markers for the antibody, antigen binding fragment, or secondary

25   antibody are described and known to the skilled artisan. For example, various enzymes, prosthetic groups, fluorescent materials, luminescent materials, magnetic agents and radioactive materials. Non-limiting examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase. Non-limiting examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin. Non-limiting

30   examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein

isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin. A non-limiting exemplary luminescent material is luminol; a non-limiting exemplary a magnetic agent is gadolinium, and non-limiting exemplary radioactive labels include ^{125}I, ^{131}I, ^{35}S or ^{3}H. Additional examples are disclosed above.

5   In some embodiments, the assay is an enzyme-linked immunosorbant assay (ELISA) or a radioimmunoassay (RIA). In other embodiments the assay is a Western blot. The antibodies that specifically bind pT1989 ATR and conjugates thereof can be used in immunohistochemical assays. These assays are well known to one of skill in the art (see Harlow & Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York (1988), for a description of

10   immunoassay formats.

G. Determining the Effectiveness of Treatment and/or Dose Determination

Methods are disclosed for determining if a tumor can be treated with an ATR inhibitor. As disclosed above, pT1989 ATR is detected in a sample from a subject using the methods

15   disclosed herein. The sample can be any biological sample that includes tumor cells. The presence of pT1989 in the sample indicates that the subject can be treated with an ATR inhibitor.

In some embodiments, methods are provided to determine if a tumor can be treated using an ataxia telangiectasia-mutated and RAD3-related kinase (ATR) inhibitor. The methods

20   include contacting a sample comprising cells from the tumor with an effective amount of the monoclonal antibody or antigen binding fragment that specifically binds ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989, under conditions sufficient to form an immune complex; and detecting the presence of the immune complex. In specific non-limiting examples, the presence of the immune complex indicates that the tumor can be

25   treated with the ATR inhibitor. In additional non-limiting examples, absence of the immune complex indicates that the tumor cannot be treated with the ATR inhibitor. In further non-limiting examples, a cut-off value is determined. The presence of an amount the immune complex greater than the cut-off value indicates that the tumor can be treated with the ATR inhibitor. In additional non-limiting examples, the presence of an amount of the immune

- 49 -

complex lower than the cut-off value indicates that the tumor cannot be treated with the ATR inhibitor.

Methods are also disclosed herein for determining the effectiveness of a dose, or the duration of a dose, of a therapeutic agent, such as a chemotherapeutic agent. The therapeutic agent can be an ATR inhibitor or a chemotherapeutic agent. The method can determine if a therapeutic agent of interest is of use for treating the subject, or if the therapeutic agent has been administered for a sufficient period of time to treat the subject. In some embodiments, the subject has cancer. The methods can be used to determine the lowest effective therapeutic dose of an agent for the treatment of a subject. These methods include detecting pT1989 ATR in a biological sample from the subject administered the therapeutic agent. In some embodiments, the methods include administering the therapeutic agent to the subject.

In some embodiments, the quantity and molar fraction of phosphorylated ATR is measured as a consequence of drug administration. This fraction may be either increased or decreased. The fraction may be represented as the level of phosphorylation per ATR molecule (such as the ratio of P:ATR ratio) or simply the quantity of pT1989ATR in the specimen.

In some embodiments, the methods can be used to determine the effectiveness of a treatment, for example a treatment for a tumor that expresses pT1989. As disclosed above, pT1989 ATR is detected in a sample from a subject using the methods disclosed herein. The sample can be any biological sample that includes tumor cells.

In some embodiments, the presence and/or amount of pT1989 ATR is determined in a sample from a subject administered a dose of a therapeutic agent of interest. The method includes contacting a biological sample from a subject administered the dose of the therapeutic agent, such as a sample that includes tumor cells, or an extract thereof, with an antibody, or antigen binding fragment thereof, that specifically binds pT1989 ATR, in order to form an immune complex. The presence (or absence) of the immune complex indicates the effectiveness of the treatment. The amount of the immune complex can be quantitated.

In additional embodiments, the amount of the immune complex can be compared to a control, such as the amount of pT1989 ATR in a sample taken prior to the treatment. In some embodiments, a decrease in the amount of pT1989 ATR in the sample, indicates that the first dose of the therapeutic agent is effective for the treatment, and/or that the therapeutic agent has

- 50 -

been administered for a sufficient duration of time to treat the subject. In other embodiments, an increase or no change in the amount of the immune complex, as compared to a control, indicates that the first dose of the therapeutic agent is not effective for the treatment and/or that the therapeutic agent has not been administered for a sufficient duration of time to treat the subject.

5   The biological sample can be any biological sample of interest, such as blood or a fraction thereof (for example, peripheral blood mononuclear cells), an extract from a biopsy or any tissue sample, such as platelets, white blood cell, kidney cells, muscle cells, heart cells, breast cells, brain cells, lung cells, and liver cells, hair follicle cells amongst others. The biological sample can be urine or cerebrospinal fluid. In specific non-limiting examples, the

10   biological sample is peripheral blood mononuclear cells, sputum, or bronchial lavage fluid. In additional non-limiting examples, the biological sample includes circulating tumor cells, circulating blast cells or circulating cancer stem cells. In additional non-liming examples, the biological sample is ascites fluid, a bone marrow aspirate, a skin punch biopsy or a buccal swab. In further non-liming examples, the sample includes hair follicles.

15   In some embodiments, the sample includes tumor cell. In some embodiments, the tumor is a hematological tumor such as a leukemia, for example an acute leukemia (such as 11q23-positive acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), or a chronic leukemia (such as chronic myelocytic (granulocytic) leukemia,

20   chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia. In other embodiments, the tumor is a solid tumor, such as a sarcoma or carcinoma, such as a fibrosarcoma, myxosarcoma, liposarcoma,

25   chondrosarcoma, osteogenic sarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer (including basal breast carcinoma, ductal carcinoma and lobular breast carcinoma), lung cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid

30   carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma,

- 51 -

papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, or a tumor of the central nervous system (such as a glioma, astrocytoma, medulloblastoma, craniopharyrgioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma and retinoblastoma).

In certain aspects, these assays are performed at a diagnostic laboratory, and the information is then provided to the subject or a physician or other healthcare provider. The methods can further include administration of the therapeutic agent of interest, such as a chemotherapeutic agent, such as an ATR inhibitor.

In some embodiments, the dose of the therapeutic agent is decreased based on the results of the assay, and a second lower dose of the therapeutic agent is administered to the subject. In additional embodiments, these methods can be used to determine the lowest effective dose of the therapeutic agent of use to treat the subject. In yet other embodiments, the dose of the therapeutic is increased based on the results of the assay and administered to the subject. In other examples, and additional dose of the therapeutic agent is administered to the subject.

Thus, in additional embodiments, the method can include administering to the subject a second dose of the therapeutic agent, wherein the second dose is the same, greater, or less than the first dose of the therapeutic agent. The presence (or absence) of pT1989 ATR is then detected in a second biological sample obtained from the subject after administration of the second dose of the therapeutic agent. The biological sample is then contacted with an antibody or antigen binding fragment thereof, that specifically bind pT1989 ATR to form an immune complex. The amount of the immune complex can be quantitated. The presence (or absence) of the immune complex indicates the effectiveness of the treatment.

The amount of the immune complex can be compared to a control, such as the amount of pT1989 ATR in a sample taken from the subject after the first dose of the therapeutic agent, or a standard value. In some embodiments, a decrease or no change in the amount of pT1989 ATR in the sample, as compared to the control, indicates that the second dose of the therapeutic agent is effective for the treatment, and/or that the therapeutic agent has been administered for a sufficient duration of time to treat the subject. In additional embodiments, an increase in the

- 52 -

amount of the immune complex, as compared to the control, indicates that the second dose of the therapeutic agent is less effective for the treatment and/or that the therapeutic agent has not been administered for a sufficient duration of time to treat the subject. Thus, in some embodiments, the methods disclosed herein can be repeated to determine the lowest dose of an agent that is

5   effective for the treatment of the subject.

The methods can be repeated 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more times to determine the lowest dose of a therapeutic agent that is effective for treating the subject, and/or the shortest duration of administration that is effective for treating the subject. The methods can also be used over the course of a therapeutic regimen to monitor the efficacy of a therapeutic agent for the

10   treatment of the subject. In some embodiments, the lowest amount of a therapeutic agent is determined that inhibits activation of ATR in the subject.

The therapeutic agent can be any ATR kinase inhibitor of interest. In some embodiments, the ATR inhibitor is VE-821
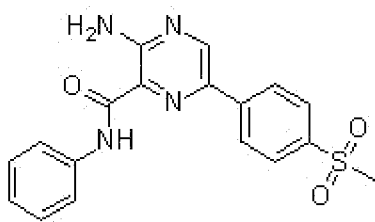
15   In other embodiments, the ATR kinase inhibitor isNU6027
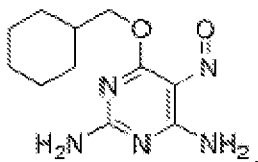
In another embodiment, the therapeutic agent is VX-970 (also referred to as VE-822, see Fokas et al., Cell Death & Disease 3: e441, 2012, see also Hall et al., Oncotarget 5: 5674-5685, 2014).
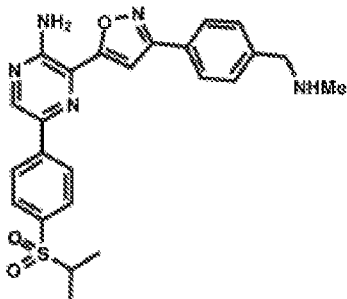
- 53 -

See also U.S. Published Patent Application No. 20130089626, which is incorporated herein by reference.

The methods disclosed herein can also be used to evaluate the effectiveness of a combination of therapeutic moieties, such as a combination of an ATR inhibitor and another chemotherapeutic compound, ionizing radiation, and DNA damaging agents. The method can also be used to evaluate the effectiveness of a chemotherapeutic agent, ionizing radiation, DNA damaging agent or any other agent of interest. . The chemotherapeutic agent can be, for example, an antimetabolite (for example, gemcitabine or cytarabine (cytosine arabinoside), or 5-fluorouracil), a Chk1 inhibitor (for example, XL9844, Exelixis, Inc.), a platinating agent (for example, cisplatin, carboplatin, oxaliplatin), an alkylating agent (for example, mitomycin C, temozolomide, and nitrogen mustards), or a topoisomerase inhibitor (for example camptothecin or etoposide). Suitable agents for combination with an ATR inhibitor can include, but are not limited to, those disclosed for example, in Wagner and Kaufman, 2010, *op. cit.*

As noted above, the subject can be administered an ATR inhibitor in conjunction with another moiety, such as a chemotherapeutic agent. As noted above, the subject can be administered an ATR inhibitor in conjunction with additional chemotherapeutic agent is a cytotoxic agent such as but not limited to an antimetabolite, a platinating agent, an alkylating agent, or DNA cross-linkers or a targeted agent such as a PARP inhibitor or a checkpoint kinase inhibitor, or ionizing radiation or any other therapeutic that damages DNA either by design or happenstance.

h. Assays for Modulators of ATR

Modulation of ATR can be assessed using a variety of *in vitro* and *in vivo* assays, including cell-based models. Such assays can be used to test for inhibitors and activators of ATR. In some embodiments, the assays use any cell that expresses ATR.

In one embodiment, the methods include contacting a biological sample, such as a cell that expresses ATR with the agent of interest. The phosphorylation of T1989 is then detected using the methods disclosed herein. The methods include contacting the cell, or an extract thereof with an antibody, antigen binding fragment or conjugate provided herein that specifically bind pT1989 ATR to form an immune complex. The presence (or absence) of the immune

- 54 -

complex is then detected and compared to a control. The control can be the binding of the antibody, antigen binding fragment or conjugate thereof to the cell, or extract thereof, wherein the cell is not contacted with the agent of interest. The control can be a standard value.

An increase in the presence of the immune complex in the sample as compared to formation of the immune complex in a control sample indicates that the agent increases phosphorylation of ATR, and activates ATR. A decrease in the presence of the immune complex in the sample as compared to formation of the immune complex in a control sample indicates that the agent decreases phosphorylation of ATR, and inhibits ATR. In some non-limiting examples, the amount of pT1989 ATR is quantified.

In some examples of the disclosed methods, the antibody that specifically binds pT1989 ATR or antigen binding fragment is conjugated to a detectable marker. In some examples, the methods include contacting a second antibody that specifically binds the antibody that specifically binds pT1989 ATR, antigen binding fragment thereof, or a conjugate including these molecules, for a sufficient amount of time to form an immune complex, and then detecting the presence of this immune complex. In some examples, the second antibody is conjugated to a detectable marker.

In one embodiment, high throughput screening methods involve providing a combinatorial small organic molecule or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

- 55 -

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al., Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al., Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al., J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al., J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al., I Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al., Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al., I Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Sambrook, all *supra)*, peptide nucleic acid libraries (see, e.g., U.S. Patent 5,539,083), antibody libraries (see, e.g., Vaughn *et al., Nature Biotechnology,* 14(3):309-314 (1996)), carbohydrate libraries (see, e.g., Liang *et al., Science,* 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

The compounds tested as modulators of ATR can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, a sugar, a nucleic acid, e.g., an antisense oligonucleotide or a ribozyme or siRNA, or a lipid. Typically, test compounds will be small organic molecules, peptides, circular peptides, siRNA, antisense molecules, ribozymes, and lipids.

Essentially any chemical compound can be used as a potential modulator, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel

- 56 -

(e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

5   Devices for the preparation of combinatorial libraries are commercially available *(see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available *(see, e.g.,* ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, 10   Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

Any of the assays disclosed herein can be adapted for high throughput screening. In high throughput assays, either soluble or solid state, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or 15   incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay many plates per day; assay screens for up to about 6,000, 20,000, 50,000, or more than 100,000 different compounds are possible using the integrated systems of the 20   invention.

EXAMPLES

The following examples are provided to illustrate particular features of certain embodiments, but the scope of the claims should not be limited to those features exemplified.

25

Example I
Development of monoclonal antibodies

A. Antigen

30   Design and synthesis of a phospho-peptide comprising of the sequence of amino acids surrounding and including (phospho) threonine 1989 of the ATR protein pT1989-ATR

- 57 -

(NP_001175.2) that will produce a suitable immunogen. The peptide included the sequence of amino acids surrounding and including phospho-threonine 1989 of the ATR protein (pT1989-ATR) and be conjugated to C-term and N-term via a terminal Cys. Sequences are shown in FIG. 2. The peptides were then conjugated to KLH and OVA for immunization, and to BSA for

5   ELISA screening. Corresponding non-phospho peptides were made for screening (conjugated to BSA).

B.   Immunization

Two three-month old New Zealand white rabbits (ID: E5571 and E5572) were

10   immunized using a protocol of five injections and two test bleeds per rabbit. At the time of each injection, aliquots of peptide mixture (SAIC-1011ap and bp) were thawed and combined with Complete Freund's Adjuvant (CFA) (for the first injection) or with incomplete Freund's Adjuvant (IFA) for the subsequent injections. The injection route was subcutaneous. Immunization details are summarized in Table 1.

15

Table 1. Immunization and Bleed Schedule

Rabbit ID	Date	Type	immunization / bleed #	amount	Immunogen
E5571	5/15/2012	Bleed	0	5ml	
	5/16/2012	Injection	1	0.5mg	mix ap, bp KLH peptides
	6/6/2012	Injection	2	0.25mg	mix ap, bp KLH peptides
	6/20/2012	Injection	3	0.25mg	mix ap, bp KLH peptides
	7/4/2012	Injection	4	0.25mg	mix ap, bp OVA peptides
	7/16/2012	Bleed	1	5ml	
	7/18/2012	Injection	5	0.25mg	mix ap, bp OVA peptides
	7/30/2012	Bleed	2	5ml	
E5572	5/15/2012	Bleed	0	5ml	
	5/16/2012	Injection	1	0.5mg	mix ap, bp KLH peptides
	6/6/2012	Injection	2	0.25mg	mix ap, bp KLH peptides
	6/20/2012	Injection	3	0.25mg	mix ap, bp KLH peptides
	7/4/2012	Injection	4	0.25mg	mix ap, bp OVA peptides
	7/16/2012	Bleed	1	5ml	
	7/18/2012	Injection	5	0.25mg	mix ap, bp OVA peptides
	7/30/2012	Bleed	2	5ml	

KLH: Keyhole limpet hemocyanin, carrier protein for conjugation to antigenic peptide to make them more immunogenic, OVA: ovalbumin, carrier protein, ap: peptide a, bp: peptide b

- 58 -

C.   Blood screening

        The titer results from the pair of rabbits for each peptide antigen were determined in a
colorimetric ELISA against BSA-conjugated peptides. Plates were coated with 50ng/well
peptide-BSA conjugate in bicarbonate buffer pH 9.6 and incubated at 4°C overnight, then and
blocked with 1% BSA in TBS. Diluted (1:250 -1:256,000) rabbit anti-sera (50ul/well) were
added and incubated at room temperature. Plates were washed with Tris-buffered saline with
0.05% Tween (TBST). Anti-rabbit secondary conjugated to alkaline phosphatase was used for
detection with p-nitrophenyl phosphate (PNPP) substrate. Plates were read at 405nm with a plate
reader. The results are shown in Table 2.

Table 2. Bleed Screening Data from Direct ELISA with ATR peptides								
	Peptide - BSA conjugates							
	ap-BSA		anp-BSA		bp-BSA		bnp-BSA	
Bleed #	B1	B2	B1	B2	B1	B2	B1	B2
Rabbit ID E5571								
1:0.25K	1.23	1.22	1.14	1.14	1	0.96	0.94	0.99
1:1K	1.3	1.26	1.25	1.21	1.09	1.05	1.07	1.05
1:4K	1.2	1.23	1.07	1.01	1.1	0.93	0.93	0.97
1:16K	0.91	0.87	0.68	0.66	0.77	0.63	0.56	0.53
1:64K	0.47	0.43	0.32	0.31	0.4	0.33	0.28	0.29
1:256K	0.21	0.2	0.17	0.15	0.17	0.16	0.13	0.13
0	0.08	0.08	0.08	0.1	0.07	0.07	0.06	0.07
Pre-Bleed	0.07	0.07	0.06	0.07	0.07	0.06	0.06	0.07
Rabbit ID E5572								
1:0.25K	1.17	1.13	1.19	1.18	1.03	0.98	0.94	1.07
1:1K	1.28	1.22	1.19	1.19	1.05	0.97	1.03	1
1:4K	1.16	1	0.96	0.96	1.04	0.87	0.89	0.9
1:16K	0.79	0.63	0.53	0.5	0.66	0.6	0.52	0.48
1:64K	0.39	0.28	0.25	0.26	0.34	0.26	0.26	0.24
1:256K	0.18	0.13	0.13	0.13	0.16	0.13	0.13	0.12
0	0.07	0.06	0.09	0.09	0.07	0.08	0.07	0.07
Pre-Bleed	0.07	0.06	0.07	0.07	0.06	0.06	0.06	0.07

Dilution of pre-bleed was 1:60K. Peptide sequences shown in FIG.2.

Independent screening of blood was conducted. Flat bottom, black, high binding 360 μL plates with clear bottom (VWR catalog # 29444-037) were coated with 100 ng/well peptide-BSA conjugate in bicarbonate buffer pH 9.6 (Sigma, catalog # C3041-100CAP) and incubated at 4°C overnight, then and blocked with ODYSSEY® blocking buffer (LiCor, catalog # 927-

5   40010). Diluted (1:250 -1:1,000,000) rabbit anti-sera (100ul/well) were added and incubated at 24C. Plates were washed with phosphate-buffered saline with 0.05% tween-20 (PBS-T). Anti-rabbit secondary conjugated to IR680 fluorescent dye (LiCor, catalog # 926-68021) was used for detection. Plates were read at 700nm with a Licor ODYSSEY® scanner. Images were quantified using Licor ODYSSEY® application software v3.0 (FIGS. 3 and 4).

10   Results from both sites indicated Rabbit E5571 had good titers against both phospho-peptides while E5572 has lower titers against the two phospho-peptides. Rabbit E5571 was selected for splenectomy and fusion.

D.   Fusion

15   A final i.v. boost was given and splenectomy was performed. Splenocytes were isolated. Four hundred million lymphocyte cells from each spleen were fused with 200 million fusion partner cells and plated on 40 96-well plates. The plates were kept in tissue culture incubators under standard conditions. Fusion data is shown in Table 3.

Table 3. Fusion data

Rabbit ID	Tissue Type	Weight (g)	Size (cm)	Cell Viability (%)	Total Cells (M)
E5571	spleen	4.06	7	92	2300

20

E.   Hybridoma screening

Cell growth was monitored 2-3 weeks after fusion. Hybridoma supernatants were screened by standard colorimetric ELISA against BSA-conjugated peptides as described in section C. The screening process consisted of a Primary screen on 40 plates (using bleed 2 of

25   E5571 at 1:100 dilution as a Positive control) clones (44) with O.D. greater than 0.5 were considered putatively positive and were further expanded to 24-well plate. Confirmatory screen

- 60 -

was performed and 6 clones were confirmed positive against phospho-specific peptides (Table 4). Additional screening was performed by ELISA using the same assay as for bleed screening (FIG.5). Western blotting was done on selected clones # 7, 27 and 35 for subcloning (FIG.6). DLD-1 cells and DLD-1 Seckel cells with and without gemcitabine treatment (100nM for 4 h)

5   were lysed with cell extraction buffer (Invitrogen, catalog # FNN0011). Lysates were run on NUPAGE® NOVEX® 3-8% gels (Invitrogen, catalog # EA0378BOX) and transferred to nitrocellulose membranes (Invitrogen catalog # IB3010-01) using the IBLOT® apparatus from Invitrogen. Blots were blocked with Licor ODYSSEY® blocking buffer (LiCor, catalog # 927-40010) and incubated overnight with hybridoma supernatants (1:30). Blots were then washed

10   with PBS-T (0.05% TWEEN®) and incubated with Licor goat anti-rabbit IR-680 antibody for 1h at room temperature. Blots were then washed with phosphate-buffered saline with 0.05% TWEEN®-20 (PBS-T) and scanned at 700nm on Licor ODYSSEY® scanner.

Table 4. Hybridoma Screening Data

Hybridoma	Peptide				ap/anp	bp/bnp	AVG p/np
	ap	bp	anp	bnp			
35	2.38	2.15	0.08	0.08	30.9	25.6	28.3
27	2.27	2.39	0.10	0.08	23.9	28.8	26.4
23	1.11	1.21	0.08	0.08	14.8	15.9	15.4
7	2.26	2.20	0.24	0.12	9.3	18.8	14.1
1	1.03	0.93	0.09	0.07	11.4	13.3	12.4
17	1.99	1.12	1.80	0.07	1.1	16.0	8.6
6	0.85	0.36	0.07	0.08	11.5	4.4	8.0
40	0.13	1.73	0.08	1.99	1.7	0.9	1.3
11	0.63	0.09	0.45	0.10	1.4	1.0	1.2
34	1.54	1.50	1.29	1.61	1.2	0.9	1.1
19	2.25	2.11	2.11	2.18	1.1	1.0	1.0
37	1.77	1.98	1.88	1.90	0.9	1.0	1.0
29	0.82	0.75	0.82	0.80	1.0	0.9	1.0
8	2.10	1.91	2.22	1.96	0.9	1.0	1.0
16	1.63	1.68	1.72	1.90	1.0	0.9	0.9
20	1.93	1.96	2.29	2.07	0.8	0.9	0.9

Hybridoma screening data from direct ELISA against peptide antigens. Table shows analysis of raw data. Hybridomas were ranked by average ratio of signal from ATR pT1989 phospho peptide:corresponding unphosphorylated peptide (p/np). Peptide sequences shown in FIG.2.

F.   Subcloning

Subcloning was done using limited cell dilution method using standard methods. Positive subclones were selected for screening.

- 62 -

G. Subclone screening

Subclones were screened by several assays. Screening was performed by enzyme-linked immunosorbant assay (ELISA) and Western blot for subclones of clones # 7, 27 and 35 for subcloning. Subclones of clone # 7 were then tested in an immunofluroescent (IFA) assay. Clone # 7 was then tested in IFA assay. Formalin fixed paraffin embedded (FFPE) blocks were prepared from DLD-1 cells and DLD-1 Seckel cells treated with and without gemcitabine treatment (100nM for 4 h). Slides were stained using neat hybridomas supernatants and imaged at 20X.

Subclones (7 of 7 tested) were positive for specific detection of pT1989ATR foci in cell nuclei in IFA assay demonstrating stability, specificity and clonality of the subclones.

H. Cloning of cDNA

The cDNA was cloned to generate a recombinant antibody. Briefly the cDNAs of IgG heavy and light chains were polymerase chain reaction (PCR) amplified and cloned into a mammalian expression vector. The recombinant monoclonal antibody was then transiently expressed using a HEK293 cell system. The expressed recombinant antibody was then tested and confirmed in standard ELISA against specific antigen. The cDNA was sequenced.

I. Purification of recombinant antibody

Recombinant antibodies were produced in serum-free medium and purified by protein-A. Briefly, protein A was pre-wash with dH2O until OD280 < 0.05, then re-equilibrated extensively with 1x PBS. Protein A was incubated with antibody containing supernatant and binding buffer (Pierce, Cat# 21007, pH8.0) (Vol. Ratio: 1: 1) with rotation, for overnight, at 4°C. The column was packed with the protein A gel, and flow-through collected. Gel was washed with 1 X PBS until OD280 < 0.05. Antibody was eluted with elution buffer (Pierce, Cat#21009, pH2.8) elute with 10ml, and collect the elute in 1 ml fractions into the glass tubes containing 100ul Neutralizing Buffer (1M Tris hydrochloride solution, pH8.0). Absorbance at 280nm of each eluted fraction was measured. Fraction with positive OD280 were pooled and dialyzed against 1 x PBS (at least 2X changes, for overnight at 4°C). The antibody was then concentrated

- 63 -

to desired concentration by filtration (Amicon, Millipore).

Example II

Specificity of monoclonal antibodies and use in immunoassays

5   *A.   Use and Specificity via ELISA*

The specificity of the pT1989 epitope was demonstrated by direct ELISA with

corresponding phospho and non phospho peptide conjugated at N- or C terminal to BSA as

described in Example I, section C (FIG.5). Clone 7A7 bound the phosphopeptide conjugated at

either C term or N-term and did not cross-react with the corresponding non-phospho peptide–

10   BSA conjugates.

B.   Use and Specificity for Western Blot

Additional specificity was demonstrated by Western blotting (shown in FIGS. 6-8) of

lysates from cells expressing ATR (HT-29, LoVo and DLD-1). Details for lysate preparation

15   and Western blotting are given in Example I, section E. The antibody detected a single band at

about 250kDa consistent with the predicted molecular weight (MW) for ATR. The antibody

also detected an increase in phospho-ATR (pT1989) upon gemcitabine (SIGMA) treatment

which is known to increase ATR activity (*Mol. Pharmacol.* (2005), *68*, 1636–1644). Finally, an

isogenic pair of DLD-1 cells was used. The antibody showed greatly decreased ATR level in the

20   ATR pathway deficient Seckel cells (Oncogene (2007) 26, 2535–2542) compared to the parental

DLD-1 cell lysate.

C.   Use and Specificity for IFA

Clone 7A7 was demonstrated to work in a slide based immunofluorescent assay

25   (IFA,shown in FIGS. 9 and 10). Formalin fixed paraffin embedded (FFPE) pellets were

generated using cells expressing WT ATR (HT29, DLD-1 Parental) or mutated ATR (DLD-1

Seckel (Horizon)) , with and without gemcitabine (SIGMA) treatment to induce ATR

phosphorylation at pT1989 (Mol. Pharmacol. 2005, 68, 1636–1644). Cells were harvested by

trypsinization, transferred into a sterile 50 ml conical centrifuge tubes with media containing

30   10% serum and pelleted at 1000x g for 5 minutes. Medium was replaced with fresh serum

- 64 -

containing medium and centrifuged again at 1000x g, 5 minutes. All but 1 ml (approximately) of medium was removed and the pellet was re-suspended by flicking the tube. Suspended cells were transferred using a 2 ml pipette into a clean 1.5 ml conical Eppendorf tube. Cells were pelleted for 30 seconds at 8,000 rpm in an Eppendorf micro centrifuge. The supernatant was

5   discarded leaving approximately 100-200 ul behind. The cell pellet was loosened by gently flicking the tube and Thrombin stock (8 ul of 1 unit/ul water, Sigma #T-7009) was added. The cells were mixed briefly by gently flicking the tube then held on ice for 2-5 minutes. Fibrinogen stock (5 ul of at 10 mg/ml water, Sigma #F-3879) was added, and the tube was incubated for 2-5 minutes at room temperature. Clotted cells were pelleted by a brief centrifuge for 20 seconds at

10   10K. The supernatant was discarded and 1.0 ml of room temperature 10% neutral buffered formalin (Sigma) was added. The cells were fixed at room temperature for 8-16 hours. Following fixation, cells were centrifuged 30 seconds at 8,000 rpm in an Eppendorf micro centrifuge. The supernatant (fixative) was removed, and 1 ml of 70% ethanol in nuclease free water was added. Paraffin processing was next performed within 3 days. Slides were generated

15   and stained with clone 7A7 and DAPI.

The gemcitabine treated cells had higher signal intensity for pT1989 staining compared to untreated cells and the Seckel cells had lower signal intensity, as compared to the parental line.

Example III

20   **Use of monoclonal antibodies for PD applications of ATR inhibitors//modulators**

    A.   *Use and Specificity for Western blot*

Additional specificity was demonstrated by Western blotting (FIG.11) of lysates from cells expressing ATR (HT-29 and LoVo), that were treated with an ATR inhibitor. Cells were treated for 4 hours with either vehicle (DMSO, 0.01%), 150nM Gemcitabine (Sigma), 1 uM

25   ATR inhibitor VE-821 or combination of drugs. The antibody detected a single band at about 250kDa consistent with the predicted MW for ATR. The antibody also detected an increase in phospho-ATR (pT1989) upon Gemcitabine treatment, which is known to increase ATR activity (Molecular Pharmacology (2005) 68, 1636-1644). Treatment with the ATR inhibitor VE-821alone did not affect phospho-ATR (pT1989) compared to vehicle, but combined treatment of

30   gemcitabine and VE-821 had reduced phospho-ATR (pT1989) compared to gemcitabine alone.

Lysates were prepared in buffer as follows: 0.94 ml RIPA buffer, 40 μl Protease Inhibitor

Cocktail (25X stock, Roche Cat# 11697498001), 10 μl Phosphatase Inhibitor (Roche Cat#

04906845001) and 10 μl PMSF (Sigma cat# 93482). Lysates were sonicated prior to use.

Lysates were run on 3-8% Tris-Acetate gels from NOVEX® (Invitrogen, Cat# EA0378BOX)

5   and transferred to nitrocellulose membranes using the IBLOT® transfer apparatus, program

setting P3, 20V, 13 min. Blots were blocked with Licor ODYSSEY® blocking buffer and

incubated overnight with Clone 7 hybridoma supernatant (1:30). B lots were then washed with

PBS-T (0.05% Tween) and incubated with Licor goat anti-rabbit IR-680 antibody for 1h at room

temperature. Mouse anti-β-actin was used as a loading control, probed with Licor goat anti-

10   mouse IR-800 antibody for 1h at room temperature. Blots were washed with PBS-T (0.05%

TWEEN®) and scanned at 700nm and 800 nm on Licor ODYSSEY® scanner.

B.   Use and Specificity for IFA

Clone 7A7 was demonstrated to work in a slide based IFA (FIG.12). Formalin fixed

15   paraffin embedded (FFPE) pellets were generated (see example II, part C).

Treatment protocol: HT29 cells were treated for 4 hrs with either vehicle, 150nM

Gemcitabine, 1 uM ATR inhibitor VE-821 or combination of drugs to monitor induction of ATR

phosphorylation at pT1989. A 24 hrs exposure to 150 nM Gemcitabine was also included.

Slides were then generated and stained with clone 7A7. The gemcitabine treated cells had

20   higher signal intensity for pT1989 staining compared to untreated cells.

Example IV
Effect of VE-281

VE-281 was chosen for molecular analysis because it targets ATR (Charrier et al.,

25   Journal Med. Chem. 2011;54(7):2320-30; Reaper et al., Nature Chem. Biol. 2011;7(7):428-30)

upstream from Chk1. The structure of VE-281 is shown below:

- 66 -
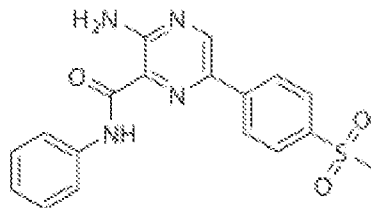
For these studies, auto-phosphorylation of ATR on threonine 1989, a DNA damage

activation site (Nam et al., J Biol Chem 2011;286(33):28707-14; Liu et al., Molecular cell

5   2011;43(2):192-202), was determined, as well as Chk1 phosphorylation on serine 345, which is

mediated by ATR. Histone H2AX phosphorylation was also determined on serine 139

(γH2AX), a validated pharmacodynamic biomarker for DNA damage and apoptosis (Bonner et

al., Nat Rev Cancer 2008;8(12):957-67; Kinders et al., Clin Cancer Res 2010;16(22):5447-57;

Solier et al., Mol Cell Biol 2009;29(1):68-82).

10   The protocol is shown in Fig. 14A. Briefly, cell lysates were electrophoresed on 4-20%

Tris-glycine or 3-8% Tris-acetate polyacrylamide gels, and transferred onto nitrocellulose

membranes. Antibodies against GAPDH, ATR and pS345-Chk1 were obtained from Cell

Signaling Technologies, γH2AX and pT68-Chk2 from Abcam and Top1 from BD Biosciences.

The antibody against pT1989-ATR is disclosed above. After incubation with secondary

15   antibody, signals were detected by enhanced chemiluminescence (Pierce).

CPT and LMP-400 quickly induced the rapid phosphorylation of Chk1, Chk2 and H2AX,

while phosphorylation of ATR was delayed and best detectable after drug removal. Chk1 and

ATR remained phosphorylated for 18 hours after drug removal, consistent with the persistent

checkpoint activation for hours after Top1 inhibitor removal (Seiler et al., Mol. Cell. Biol.

20   2007;27(16):5806-18; Shao et al., EMBO J 1999;18:1397-406). VE-821 inhibited the

phosphorylation of both ATR and Chk1 without reducing Chk2 phosphorylation, confirming the

specificity of VE-821 for ATR. Notably, VE-821 strongly induced γH2AX over time (Figs. 14B

and 14C). LMP-400 showed more pronounced effects than CPT, consistent with the enhanced

Top1 inhibitory potency of LMP-400 (Antony et al., Cancer Res 2007;67(21):10397-405). See

25   also Josse et al., "The ATR inhibitors VE-821 and VX-970 sensitize cancer cells to

topoisomerase I inhibitors by disabling DNA replication initiation and fork elongation

- 67 -

responses," Cancer Res., Pub. OnlineFirst September 30, 2014; doi: 10.1158/0008-5472.CAN-13-3369, which is incorporated herein by reference.

Example V

A2780 Xenografts

In vivo studies were performed. A2780 xenografts were evaluated in mice treated with 1.2 mg/kg cisplatin or 6.0 mg/kg cisplatin for 4, 7 and 24 hours. The immunofluoresecnce assay with the ATR-pT1989 antibody detected an increase in ATR foci in the treated samples compared to vehicle in both a dose and time dependent manner (FIG. 15A-15D). The following methods were used in these studies:

Xenograft Samples and In Vivo quantitation: Athymic nu/nu mice were implanted with A2780 cells as previously described (Kinders et al., Clin Cancer Res 2010;16(22):5447-57). Xenograft quadrants were collected and flash frozen from mice following exposure to vehicle (0.9% saline) or various doses (1.2, 6.0 mg/kg) of cisplatin administered intraperitoneally. The samples were fixed in neutral-buffered formalin and paraffin-embedded as previously described (Kinders et al., Clin Cancer Res 2010;16(22):5447-57). Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.), and all studies were conducted under an approved Animal Care and Use Committee protocol.

Immunofluorescence staining: The method used for staining tissue sections was carried out using a BOND-MAX™ Autostainer Staining protocol. The ATR 1989-P antibody was run at a 10 ug/ml dilution and ALEXA FLUOR® 546 goat anti-rabbit (Invitrogen) was used as the reporter. After completion of the staining, PROLONG® Gold Antifade Reagent (Invitrogen) was dropped onto the slides and coverslips; slides were cured overnight in the dark and imaged the following day. Image capture of the tissue sections was carried out using a Nikon 90i Microscope with an A1 confocal head with a 20X objective.

Quantitative Marker Analysis: The DEFINIENS TISSUE STUDIO™ (Version 2.0.3; Build 2057) software program was used for analysis of foci count/nucleus. For the analysis, fields were scored first for the number of nuclei and then individual counts of the number of foci count/per nucleus were performed. An average of 10 representative fields was taken per

- 68 -

xenograft quadrant, with a total of 5 mice per treatment group.

Example VI

Detection of ATR Foci in Human Clinical samples

5   ATR foci were also detected in human clinical samples (FIG. 16). Human clinical

biopsy samples (Cancer Therapy Evaluation Program (CTEP) trial 9350) were prepared as FFPE

blocks following the Tumor Frozen Needle Biopsy Preparation for Immunofluorescence Assay

(IFA) on Tumor Biopsies protocol (LHTP003.07.01). The biopsy samples were stained using

the BOND-MAX™ autostainer and a modified staining protocol, as outlined above in Example

10   V. All patients gave written informed consent for study inclusion and were enrolled on

institutional review board-approved protocols.

Example VII

Comparison of 7A7 and Commercially Available Antibodies

15   Clone 7A7 was compared with other clones and commercial antibodies including the

Kerafast monoclonal antibody on identical replicate Western blots of crude cell lysates. Clone 7

detected pT1989 ATR specific bands while the Kerafast monoclonal antibody did not detect

pT1989 ATR (see FIGs 17A-17C). The result shows that 7A7 is specific for pT1989 ATR, and

that it is far more effective than a commercially available antibody.

20   For these studies, DLD-1 parental and DLD-1 Seckel (Horizon) cells were treated with

gemcitabine for 4 hours. Clone 7A7 detected ATR in the parental DLD-1 cell lysate by Western

blotting and detects an increase in pT1989-ATR with gemcitabine treatment as expected. In

contrast, the level of pT-1989 detected by clone 7A7 was much lower in the DLD-1 Seckel cells

which have suppressed ATR expression.

25   The commercially available (Kerafast) pT1989 antibody was very weak and non-specific

(highly cross-reactive), since multiple bands were seen in a Western blot. The commercially

available (Kerafast) antibody was determined to be unsuitable for many immunoassay

applications such as pharmacodynamic immunofluorescence assays (IFAs) or other techniques

wherein ATR is not present in a purified form. In contrast, Clone 7A7 was successfully used in

30   pharmacodynamic IFA assay applications.

- 69 -

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that illustrated embodiments are only examples of the invention and should not be considered a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

- 70 -

We claim:

1.   An isolated monoclonal antibody, or antigen binding fragment thereof, comprising a heavy chain variable region and a light chain variable region,

wherein the heavy chain variable region comprises a heavy chain complementarity determining region (H-CDR)1, a H-CDR2, and a H-CDR3 of the heavy chain variable region set forth as SEQ ID NO: 6, and

wherein the light chain variable region comprises a light chain complementarity determining region (L-CDR)1, a L-CDR2, and a L-CDR3 of the light chain variable region set forth as SEQ ID NO: 7,

wherein the monoclonal antibody or antigen binding fragment specifically binds to ataxia telangiectasia-mutated and RAD3-related phosphorylated kinase at position 1989.

2. The isolated monoclonal antibody or antigen binding fragment of claim 1, wherein

(a) the H-CDR1, H-CDR2, and H-CDR3, comprise amino acids 48-52, 67-82, and 113-121 of SEQ ID NO: 6, respectively, and the L-CDR1, L-CDR2, and L-CDR3 comprise amino acids 45-57, 73-79 and 112-124 of SEQ ID NO: 7, respectively; or

(b) the H-CDR1, H-CDR2, and H-CDR3, comprise amino acids 43-49, 69-73, and 113-121 of SEQ ID NO: 6, respectively, and the L-CDR1, L-CDR2, and L-CDR3 comprise amino acids 45-57, 73-79 and 112-124 of SEQ ID NO: 7, respectively.

3. The isolated monoclonal antibody or antigen binding fragment of claim 1 or claim 2, wherein the heavy chain variable region comprises the amino acid sequence set forth as SEQ ID NO: 6.

4.   The isolated monoclonal antibody or antigen binding fragment of claim 1, wherein the light chain variable region comprising the amino acid sequence set forth as SEQ ID NO: 7.

5. The isolated monoclonal antibody or antigen binding fragment of claim 1 or claim 2, wherein the heavy and light chain variable regions comprise the amino acid sequences set forth

as SEQ ID NO: 6 and SEQ ID NO: 7, respectively.

6.   The isolated monoclonal antibody of any one of claims 1-5, wherein the antibody is an IgG.

7.   The antigen binding fragment of any one of claims 1-5.

8.   The antigen binding fragment of claim 7, wherein the antigen binding fragment is a Fv, Fab, F(ab')$_2$, scFV or a scFV$_2$ fragment.

9.   The isolated monoclonal antibody or antigen binding fragment of any one of claims 1-8, conjugated to a detectable label.

10.   The isolated monoclonal antibody or antigen binding fragment of claim 9, wherein the detectable marker is a fluorescent, enzymatic, heavy metal or radioactive marker.

11.   An isolated nucleic acid molecule encoding the monoclonal antibody or antigen binding fragment of any one of claims 1-9.

12.   The isolated nucleic acid molecule of claim 11, operably linked to a promoter.

13.   An expression vector comprising the nucleic acid molecule of claim 11 or claim 11.

14.   A host cell, comprising the nucleic acid molecule or expression vector of any one of claims 11-13.

15.   A composition, comprising an effective amount of the isolated monoclonal antibody or antigen binding fragment of any one of claims 1-10, the nucleic acid molecule of

- 72 -

claim 11 or claim 12, the expression vector of claim 13, or the host cell of claim 14, and a pharmaceutically acceptable carrier.

16. A kit for detecting the presence of ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989 in a cell, comprising

a container comprising the isolated monoclonal antibody or antigen binding fragment of any one of claims 1-10, the nucleic acid molecule of claim 11 or claim 12, the expression vector of claim 13, the host cell of claim 14 or the composition of claim 15, and

instructions for using the kit.

17. Use of the isolated monoclonal antibody or antigen binding fragment of any one of claims 1-10 to detect the presence of ataxia telangiectasia-mutated and RAD3-related phosphorylated kinase at position 1989 in a cell.

18. A method of detecting the presence of ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989, comprising:

contacting a sample comprising a cell or an extract thereof with an effective amount of the monoclonal antibody or antigen binding fragment of any one of claims 1-10 that specifically binds ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989, under conditions sufficient to form an immune complex; and

detecting the presence of the immune complex, wherein the presence of the immune complex indicates the presence of ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989 in the sample.

19. The method of claim 18, wherein the cell is cultured *in vitro*.

20. The method of claim 18, wherein the sample is a biological sample obtained from a subject of interest.

- 73 -

21.   The method of claim 20, wherein the subject of interest has cancer and wherein the cell is a cancer cell.

22.   The method of claim 20, wherein the presence of the immune complex indicates that the subject can be treated with an ataxia telangiectasia-mutated and RAD3-related kinase inhibitor.

23.   The method of claim 18, further comprising comparing the amount of the immune complex to a control.

24.   The method of any one of claims 18-23, further comprising
contacting the sample with a second antibody, wherein the second antibody specifically binds the monoclonal antibody or antigen binding fragment that specifically binds ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989.

25.   The method of claim 24, wherein the second antibody is labeled.

26.   A method of determining the effectiveness of a dose of a therapeutic agent for treatment of a subject, comprising
contacting a sample from the subject comprising a cell or an extract thereof with an effective amount of the monoclonal antibody or antigen binding fragment of any one of claims 1-10 that specifically binds ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989 (pT1989 ATR), under conditions sufficient to form an immune complex, wherein the sample is obtained following administration of the therapeutic agent to the subject; and
measuring the binding of the monoclonal antibody or antigen binding fragment to pT1989 ATR in the sample,
wherein a decrease in the amount of binding of the monoclonal antibody or antigen binding fragment thereof to pT1989 ATR in the sample as compared to the control indicates that the dose is effective for treating the subject.

- 74 -

27.   The method of claim 26, wherein the control is the binding of the monoclonal antibody to pT1989 in a sample from the subject prior to treatment with the therapeutic agent.

28.   The method of claim 26 or claim 27, further comprising

administering to the subject a additional dose of the therapeutic agent,

contacting an additional sample from the subject comprising a cell or an extract thereof with an effective amount of the monoclonal antibody or antigen binding fragment of any one of claims 1-10 that specifically binds pT1989 ATR, under conditions sufficient to form an immune complex; and

measuring the binding of the monoclonal antibody or antigen binding fragment to pT1989 ATR in the additional sample,

wherein a decrease or no change in the amount of binding of the monoclonal antibody or antigen binding fragment thereof to pT1989 ATR as compared to binding of the monoclonal antibody or antigen binding to pT1989 in an initial sample indicates that the additional dose is effective for treating the subject.

29.   The method of any one of claims 26-28, wherein the subject has cancer, and wherein cell is a cancer cell.

30.   The method of any one of claims 26-29, wherein the sample and the additional sample comprise circulating tumor cells or a bodily fluid.

31.   The method of any one of any one of claims 26-30, wherein the therapeutic agent comprises an ataxia telangiectasia-mutated and RAD3-related kinase modulator.

32.   The method of claim 31, wherein the therapeutic agent further comprises an additional chemotherapeutic agent.

33.   The method of claim 32, wherein the additional chemotherapeutic agent is a

- 75 -

cytotoxic agent such as but not limited to an antimetabolite, a platinating agent, an alkylating agent, or DNA cross-linkers or a targeted agent such as a poly (ADP-ribose) polymerase (PARP) inhibitor or a checkpoint kinase inhibitor, ionizing radiation or a DNA damaging agent.

5   34.   A method for determining if an agent inhibits ataxia telangiectasia-mutated and RAD3-related kinase activity, comprising

contacting a sample comprising a cell contacted with the agent, or an extract of the cell, with an effective amount of the monoclonal antibody or antigen binding fragment of any one of claims 1-10 under conditions sufficient to form an immune complex; and

10   detecting the presence of the immune complex, wherein the presence of the immune complex indicates that the agent affects ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989 in the sample.

        35.   The method of claim 34, wherein the cell is a cell from a cell line.

15

        36.   The method of claim 34, wherein the cell is a cancer cell.

        37.   The method of claim 36, wherein the cancer cell is in a tissue section.

20   38.   A method to determine if a tumor can be treated using an ataxia telangiectasia-mutated and RAD3-related kinase (ATR) inhibitor, comprising

contacting a sample comprising cells from the tumor with an effective amount of the monoclonal antibody or antigen binding fragment of any one of claims 1-10 that specifically binds ataxia telangiectasia-mutated and RAD3-related kinase phosphorylated at position 1989,

25   under conditions sufficient to form an immune complex; and

detecting the presence of the immune complex, wherein the presence of the immune complex indicates that the tumor can be treated with the ATR inhibitor.

        39.   The method of claim 38, wherein the absence of the immune complex indicates

30   that the tumor cannot be treated with the ATR inhibitor.

- 76 -

40.   The method of claim 18, 21, 26, 34 or 38, wherein the cell is in a biopsy, surgical specimen or fine needle aspirate.

41.   The method of claim 18, 21, 26, 34 or 38, wherein the cell is a breast cancer cell or a bone marrow cell.

42.   The method of claim 18, 26, 34 or 38 wherein the cell is a peripheral blood mononuclear cell or a hair follicle cell.

```
   1 MGEHGLELAS MIPALRELGS ATPEEYNTVV QKPRQILCQF IDRILTDVNV VAVELVKKTD
  61 SQPTSVMLLD FIQHIMKSSP LMFVNVSGSH EAKGSCIEFS NWIITRLLRI AATPSCHLLH
 121 KKICEVICSL LFLFKSKSPA IFGVLTKELL QLFEDLVYLH RRNVMGHAVE WPVVMSRFLS
 181 QLDEHMGYLQ SAPLQLMSMQ NLEFIEVTLL MVLTRIIAIV FFRRQELLLW QIGCVLLEYG
 241 SPKIKSLAIS FLTELFQLGG LPAQPASTFF SSFLELLKHL VEMDTDQLKL YEEPLSKLIK
 301 TLFPFEAEAY RNIEPVYLNM LLEKLCVMFE DGVLMRLKSD LLKAALCHLL QYFLKFVPAG
 361 YESALQVRKV YVRNICKALL DVLGIEVDAE YLLGPLYAAL KMESMEIIEE IQCQTQQENL
 421 SSNSDGISPK RRRLSSSLNP SKRAPKQTEE IKHVDMNQKS ILWSALKQKA ESLQISLEYS
 481 GLKNPVIEML EGIAVVLQLT ALCTVHCSHQ NMNCRTFKDC QHKSKKKPSV VITWMSLDFY
 541 TKVLKSCRSL LESVQKLDLE ATIDKVVKIY DALIYMQVNS SFEDHILEDL CGMLSLPWIY
 601 SHSDDGCLKL TTFAANLLTL SCRISDSYSP QAQSRCVFLL TLFPRRIFLE WRTAVYNWAL
 661 QSSHEVIRAS CVSGFFILLQ QQNSCNRVPK ILIDKVKDDS DIVKKEFASI LGQLVCTLHG
 721 MFYLTSSLTE PFSEHGHVDL FCRNLKATSQ HECSSSQLKA SVCKPFLFLL KKKIPSPVKL
 781 AFIDNLHHLC KHLDFREDET DVKAVLGTLL NLMEDPDKDV RVAFSGNIKH ILESLDSEDG
 841 FIKELFVLRM KEAYTHAQIS RNNELKDTLI LTTGDIGRAA KGDLVPFALL HLLHCLLSKS
 901 ASVSGAAYTE IRALVAAKSV KLQSFFSQYK KPICQFLVES LHSSQMTALP NTPCQNADVR
 961 KQDVAHQREM ALNTLSEIAN VFDFPDLNRF LTRTLQVLLP DLAAKASPAA SALIRTLGKQ
1021 LNVNRREILI NNFKYIFSHL VCSCSKDELE RALHYLKNET EIELGSLLRQ DFQGLHNELL
1081 LRIGEHYQQV FNGLSILASF ASSDDPYQGP RDIISPELMA DYLQPKLLGI LAFFNMQLLS
1141 SSVGIEDKKM ALNSLMSLMK LMGPKHVSSV RVKMMTTLRT GLRFKDDFPE LCCRAWDCFV
1201 RCLDHACLGS LLSHVIVALL PLIHIQPKET AAIFHYLIIE NRDAVQDFLH EIYFLPDHPE
1261 LKKIKAVLQE YRKETSESTD LQTTLQLSMK AIQHENVDVR IHALTSLKET LYKNQEKLIK
1321 YATDSETVEP IISQLVTVLL KGCQDANSQA RLLCGECLGE LGAIDPGRLD FSTTETQGKD
1381 FTFVTGVEDS SFAYGLLMEL TRAYLAYADN SRAQDSAAYA IQELLSIYDC REMETNGPGH
1441 QLWRRFPEHV REILEPHLNT RYKSSQKSTD WSGVKKPIYL SKLGSNFAEW SASWAGYLIT
1501 KVRHDLASKI FTCCSIMMKH DFKVTIYLLP HILVYVLLGC NQEDQQEVYA EIMAVLKHDD
1561 QHTINTQDIA SDLCQLSTQT VFSMLDHLTQ WARHKFQALK AEKCPHSKSN RNKVDSMVST
1621 VDYEDYQSVT RFLDLIPQDT LAVASFRSKA YTRAVMHFES FITEKKQNIQ EHLGFLQKLY
1681 AAMHEPDGVA GVSAIRKAEP SLKEQILEHE SLGLLRDATA CYDRAIQLEP DQIIHYHGVV
1741 KSMLGLGQLS TVITQVNGVH ANRSEWTDEL NTYRVEAAWK LSQWDLVENY LAADGKSTTW
1801 SVRLGQLLLS AKKRDITAFY DSLKLVRAEQ IVPLSAASFE RGSYQRGYEY IVRLHMLCEL
1861 EHSIKPLFQH SPGDSSQEDS LNWVARLEMT QNSYRAKEPI LALRRALLSL NKRPDYNEMV
1921 GECWLQSARV ARKAGHHQTA YNALLNAGES RLAELYVERA KWLWSKGDVH QALIVLQKGV
1981 ELCFPENETP PEGKNMLIHG RAMLLVGRFM EETANFESNA IMKKYKDVTA CLPEWEDGHF
2041 YLAKYYDKLM PMVTDNKMEK QGDLIRYIVL HFGRSLQYGN QFIYQSMPRM LTLWLDYGTK
2101 AYEWEKAGRS DRVQMRNDLG KINKVITEHT NYLAPYQFLT AFSQLISRIC HSHDEVFVVL
2161 MEIIAKVFLA YPQQAMWMMT AVSKSSYPMR VNRCKEILNK AIHMKKSLEK FVGDATRLTD
2221 KLLELCNKPV DGSSSTLSMS THFKMLKKLV EEATFSEILI PLQSVMIPTL PSILGTHANH
2281 ASHEPFPGHW AYIAGFDDMV EILASLQKPK KISLKGSDGK FYIMMCKPKD DLRKDCRLME
2341 FNSLINKCLR KDAESRRREL HIRTYAVIPL NDECGIIEWV NNTAGLRPIL TKLYKEKGVY
2401 MTGKELRQCM LPKSAALSEK LKVFREFLLP RHPPIFHEWF LRTFPDPTSW YSSRSAYCRS
2461 TAVMSMVGYI LGLGDRHGEN ILFDSLTGEC VHVDFNCLFN KGETFEVPEI VPFRLTHNMV
2521 NGMGPMGTEG LFRRACEVTM RLMRDQREPL MSVLKTFLHD PLVEWSKPVK GHSKAPLNET
2581 GEVVNEKAKT HVLDIEQRLQ GVIKTRNRVT GLPLSIEGHV HYLIQEATDE NLLCQMYLGW
2641 TPYM
```

FIG. 1

ap (aa1983-1997): CFPENE-pT-PPEGKNML
bp (aa1984-1997): FPENE-pT-PPEGKNML-C
anp (aa1983-1997): CFPENETPPEGKNML
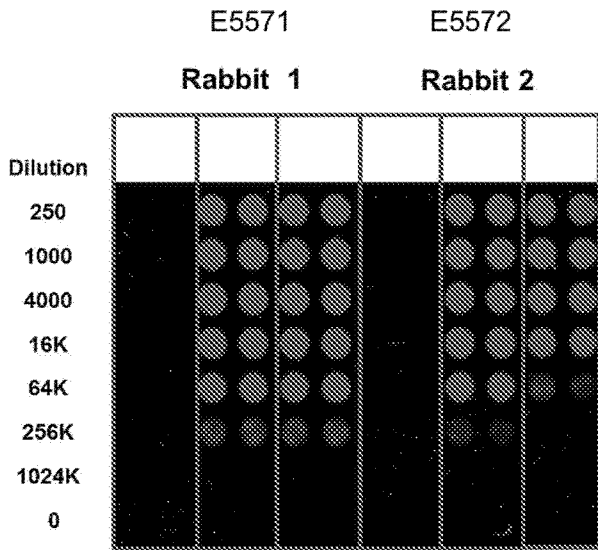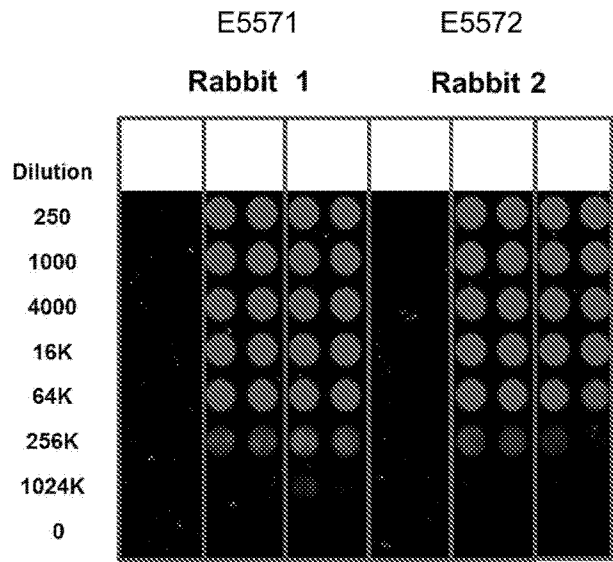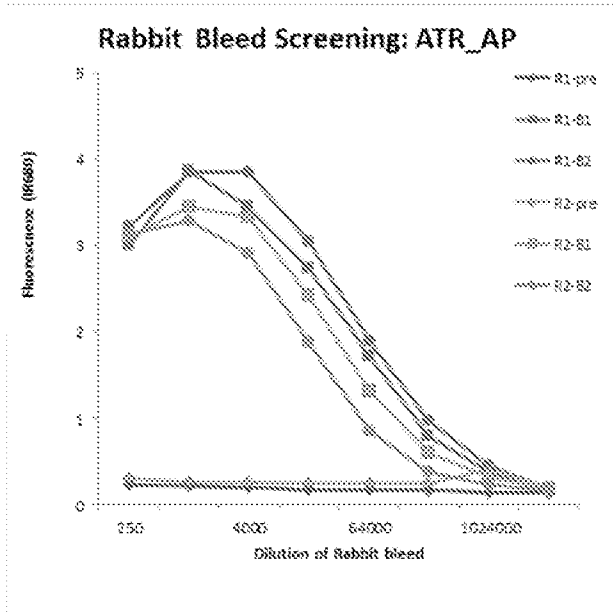bnp (aa1984-1997): FPENETPPEGKNML-C

FIG. 2

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D
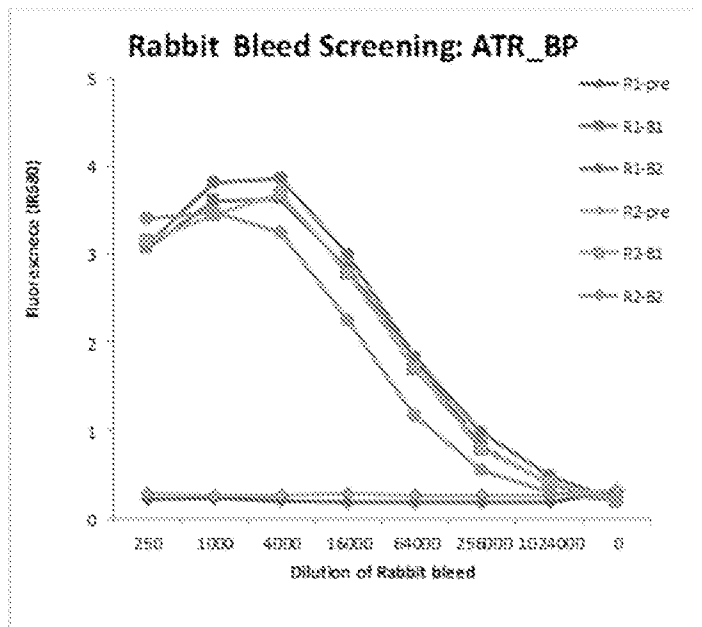
FIG. 4A
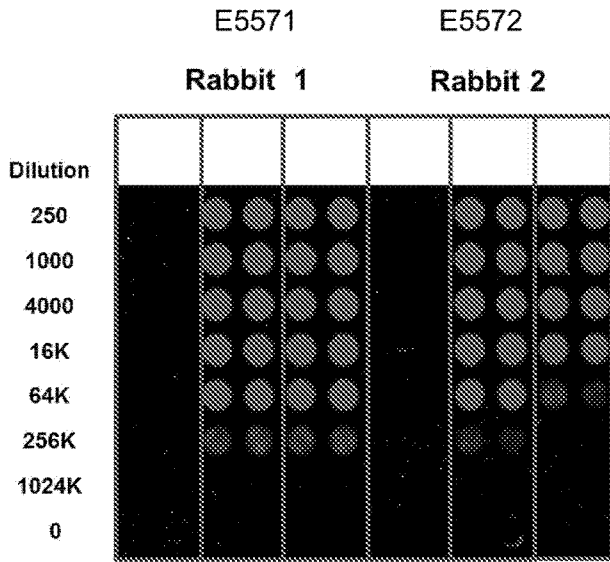
FIG. 4B
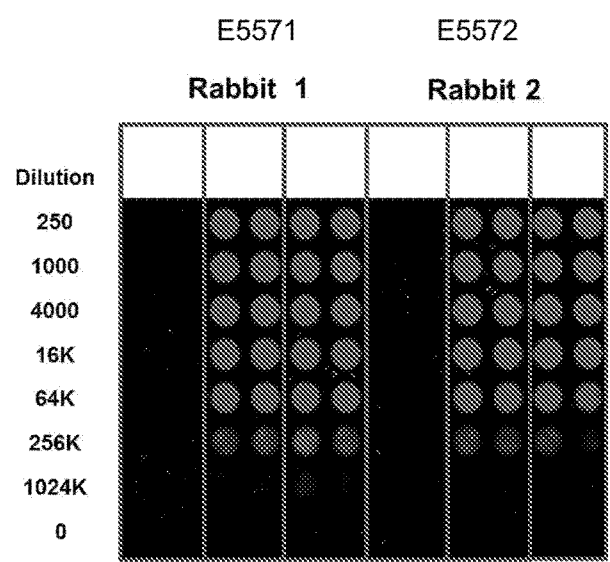
FIG. 4C

FIG. 4D

FIG. 5A

FIG. 5B

FIG. 5C

ATR pT1989 clone 7A7 titers
for BSA conjugated phospho and non-phospho peptides

FIG. 6

FIG. 7

FIG. 8

water

150 nM Gemcitabine
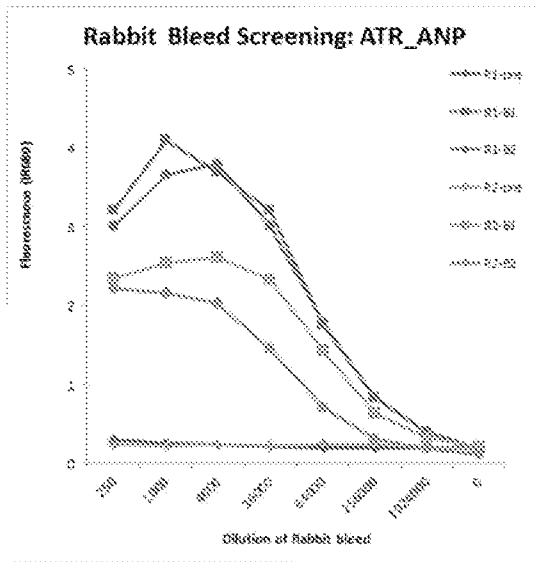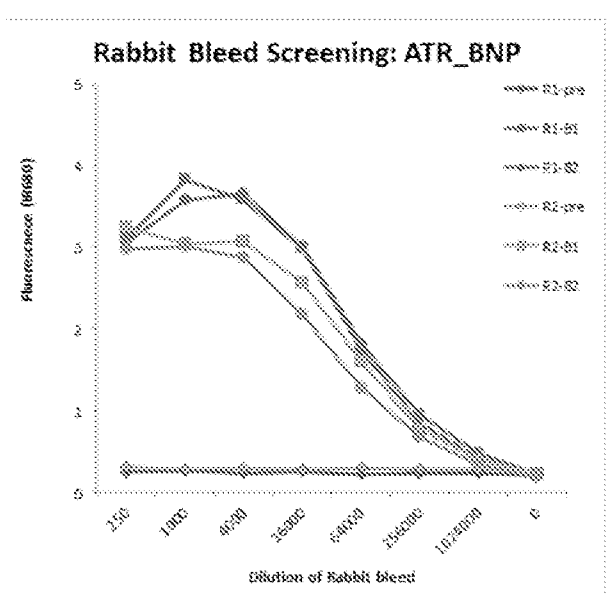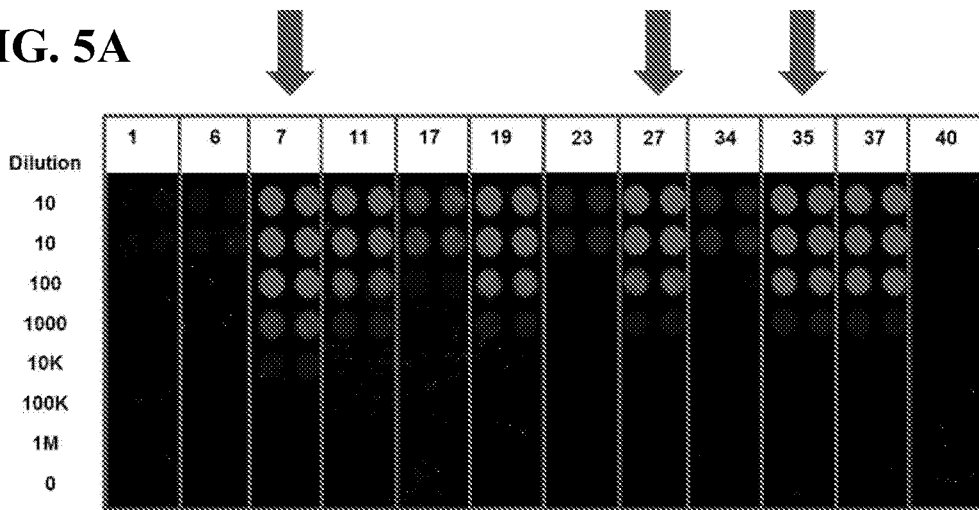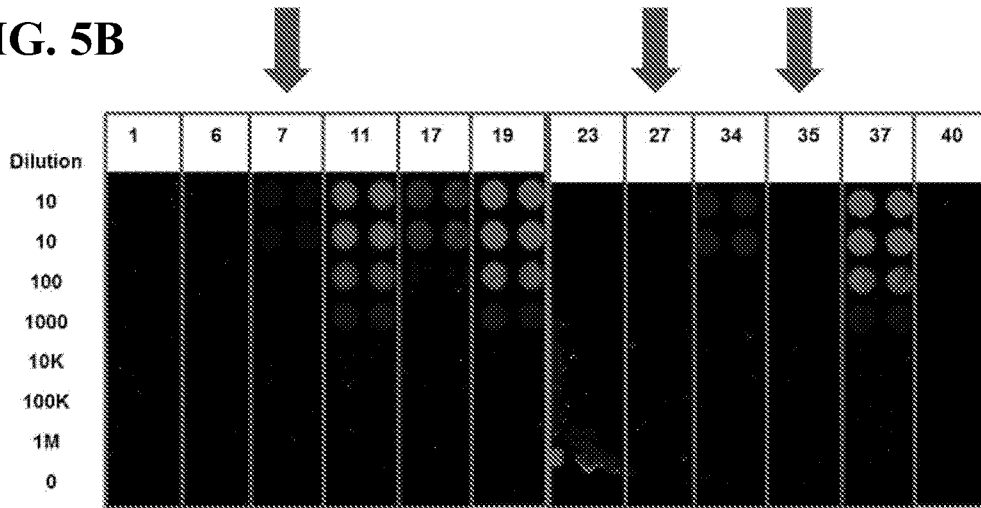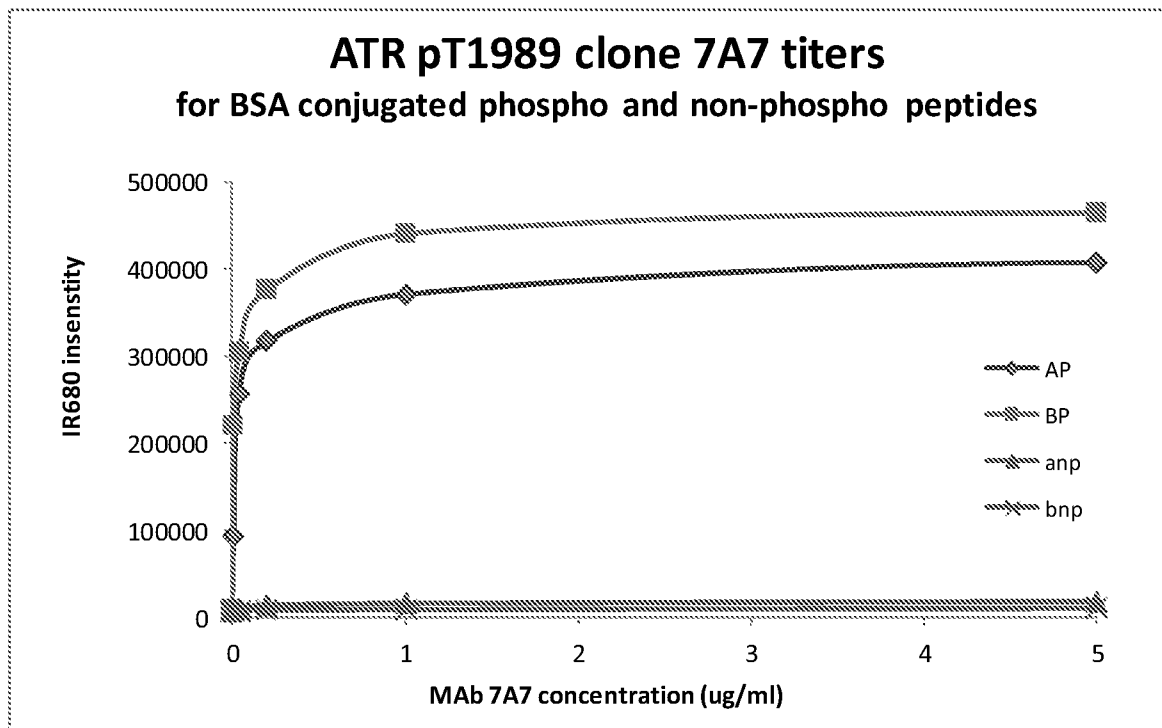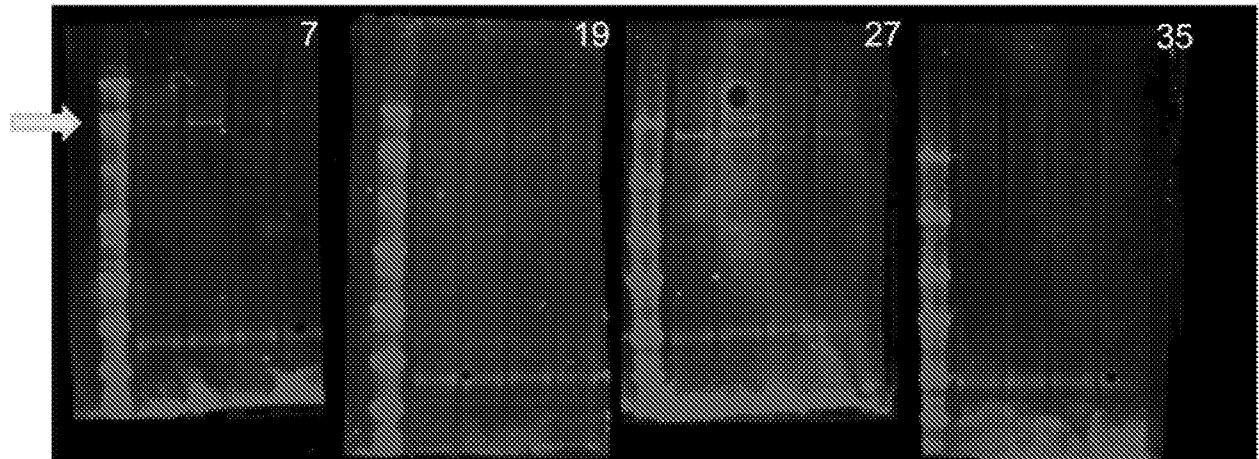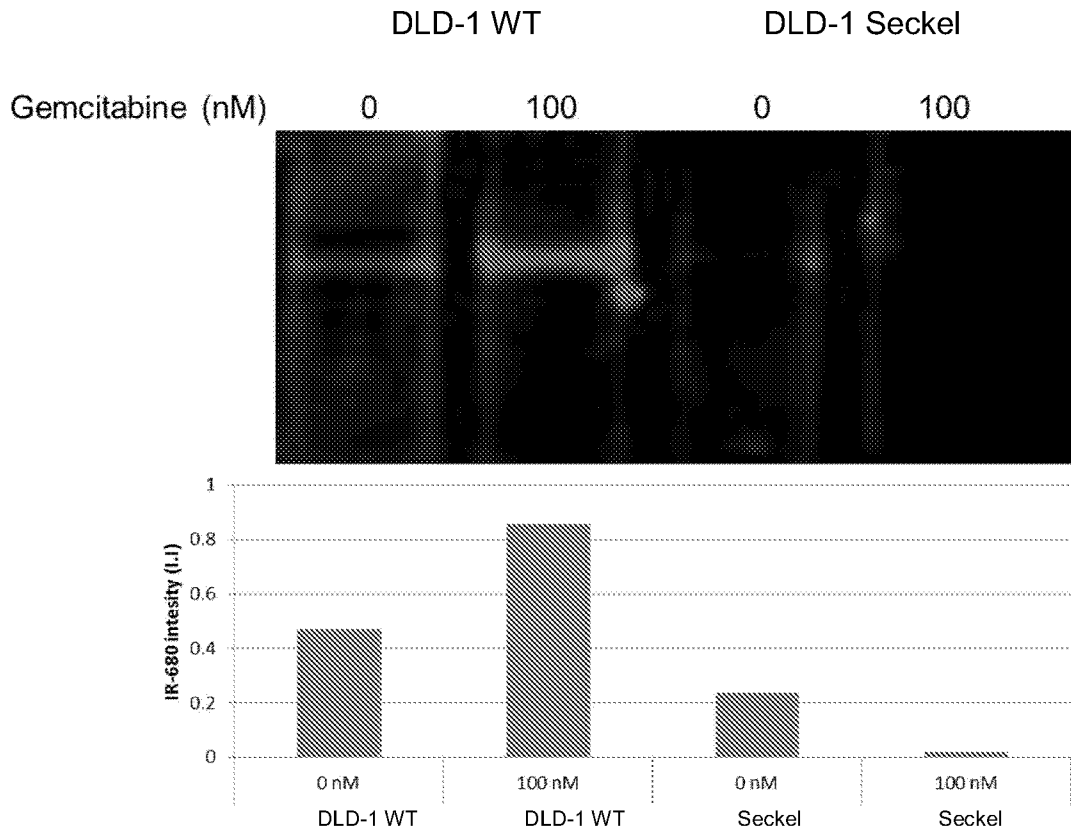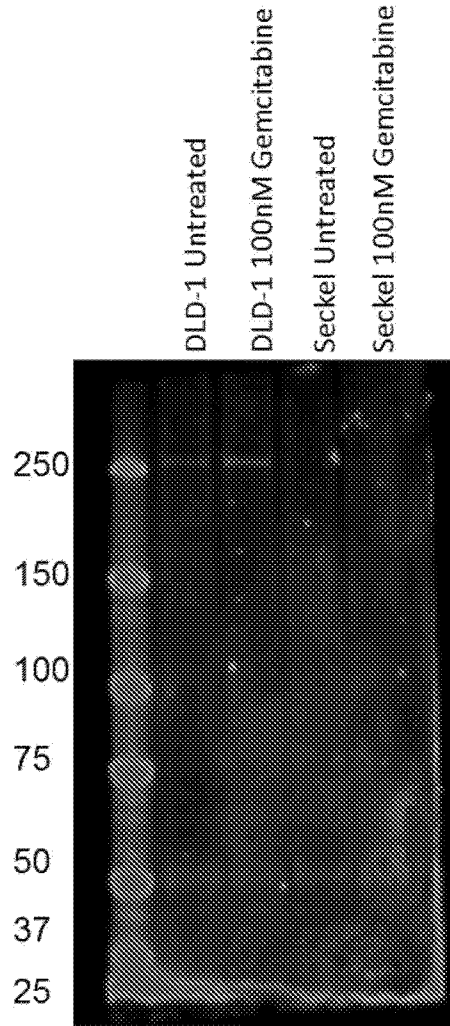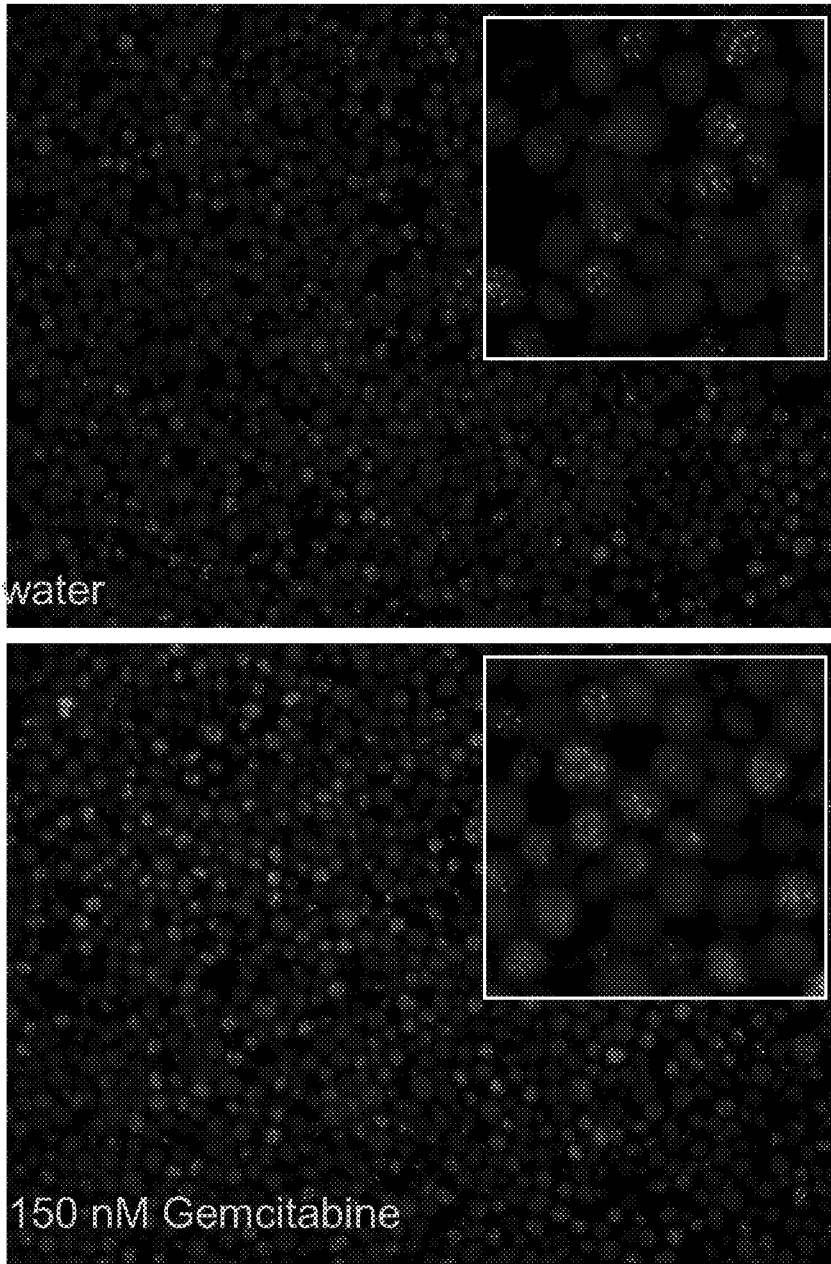
FIG. 9
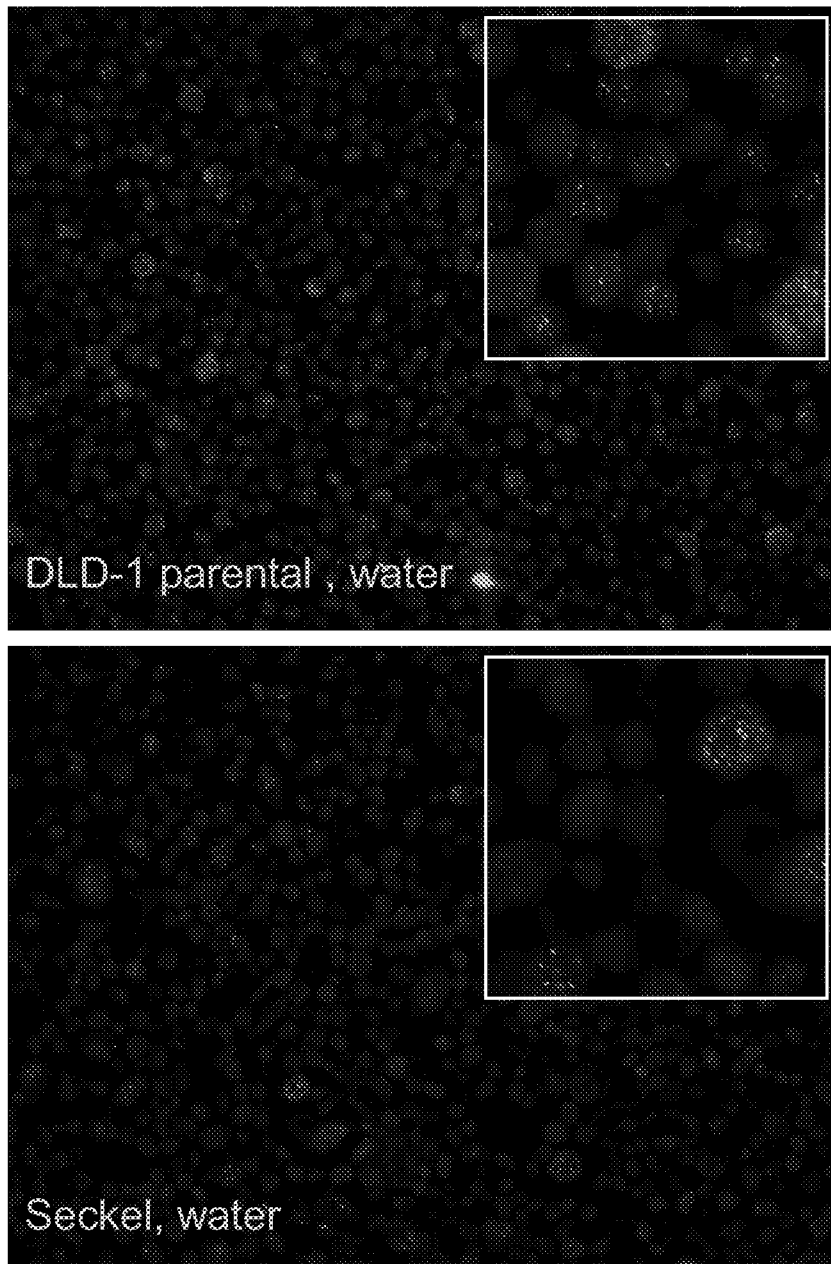
FIG. 10
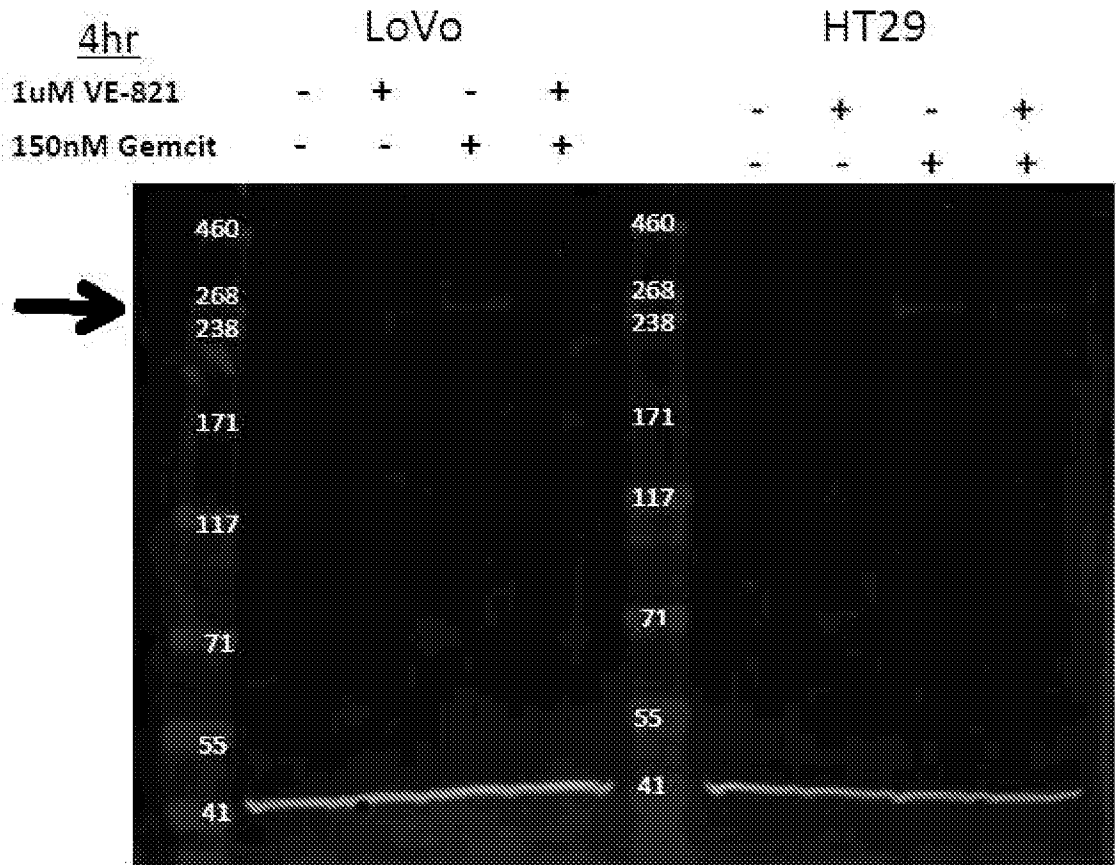
FIG. 11
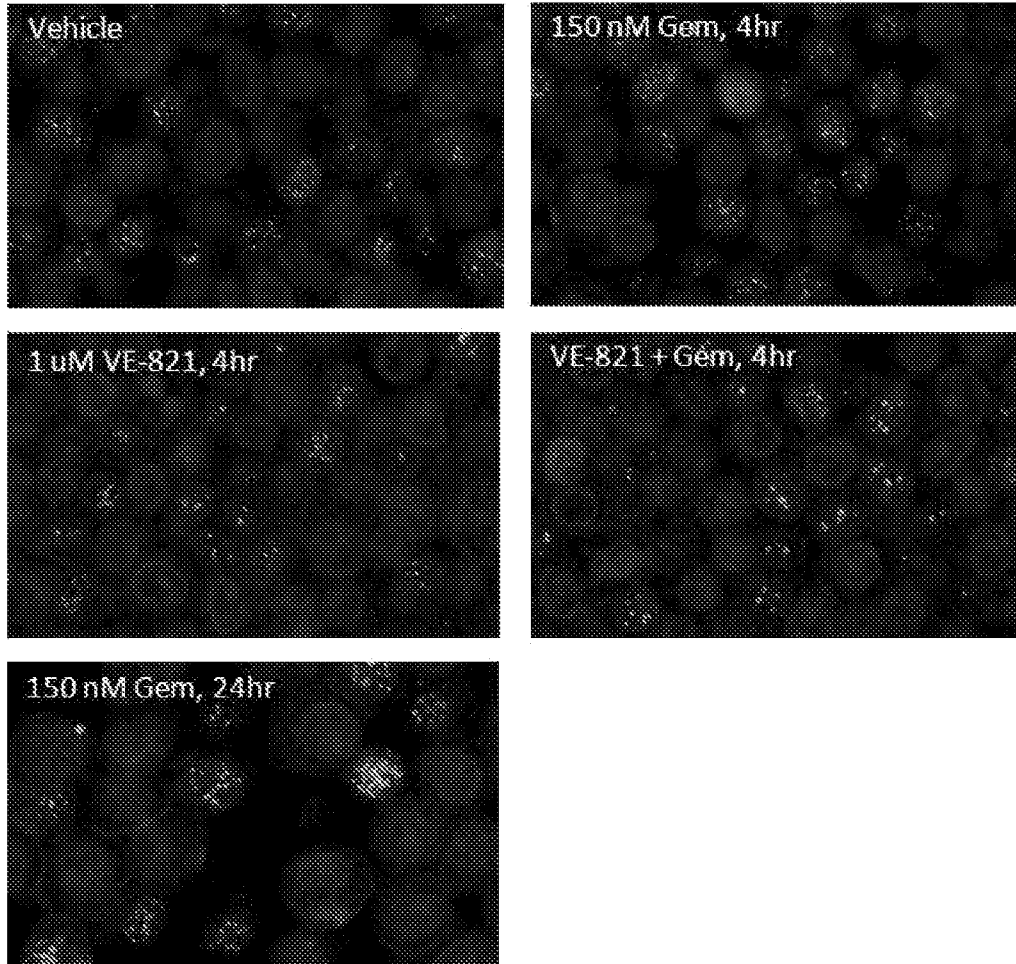
	% Cells >8 foci	fold	% Cells >5 foci	fold	total cell # per 5 fields
Vehicle	12.2	1.0	19.8	1.0	1089
150uM Gem, 4h	14.9	1.2	24.5	1.2	1126
1uM VE-821, 4h	2.3	0.2	8.3	0.4	1175
Gem+VE	3.1	0.3	7.3	0.4	1147
150uM Gem, 24h	26.3	2.2	31.9	1.6	631

FIG. 12

FIG. 13A

Displaying 23 - 138 of 239 residues:

Block 1 (region: LFR1)

Query protein sequence	A	L	V	M	T	Q	S	P	S	S	V	S	A	A	V	G	G	T	V	T	I
Chothia numbering	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
Chothia+ numbering	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
Kabat numbering	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
REGIONS: CHOTHIA	LFR1																				
ABM	LFR1																				
KABAT	LFR1																				
CONTACT	LFR1																				

Block 2

Query protein sequence	T	R	C	Q	A	S	Q	S	L	Y	N	N	N	Y	L	A	W	F	Q	Q	K	P	G
Chothia numbering	L22	L23	L24	L25	L26	L27	L28	L29	L30	L30A	L30B	L31	L32	L33	L34	L35	L36	L37	L38	L39	L40	L41	
Chothia+ numbering	L22	L23	L24	L25	L26	L27	L28	L29	L30	L30A	L30B	L31	L32	L33	L34	L35	L36	L37	L38	L39	L40	L41	
Kabat numbering	L22	L23	L24	L25	L26	L27	L27A	L27B	L28	L29	L30	L31	L32	L33	L34	L35	L36	L37	L38	L39	L40	L41	
REGIONS: CHOTHIA										CDR													
ABM										CDR													
KABAT										CDR								LFR2					
CONTACT										CDR							LFR2						

Block 3 (region: LFR2)

Query protein sequence	Q	R	P	K	L	L	I	Y	K	A	S	T	L	A	S	G	V	P	S	R	F	N	G
Chothia numbering	L42	L43	L44	L45	L46	L47	L48	L49	L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	L60	L61	L62	L63	L64
Chothia+ numbering	L42	L43	L44	L45	L46	L47	L48	L49	L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	L60	L61	L62	L63	L64
Kabat numbering	L42	L43	L44	L45	L46	L47	L48	L49	L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	L60	L61	L62	L63	L64
REGIONS: CHOTHIA	LFR2																						
KABAT	LFR2																						
CONTACT	LFR2																						

FIG. 13B

LFR3

S	G	S	G	T	Q	F	T	L	L	T	I	S	D	L	E	C	E	D	A	A	T	Y	Y
L65	L66	L67	L68	L69	L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	L80	L81	L82	L83	L84	L85	L86	L87
L65	L66	L67	L68	L69	L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	L80	L81	L82	L83	L84	L85	L86	L87
L65	L66	L67	L68	L69	L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	L80	L81	L82	L83	L84	L85	L86	L87

C	L	G	E	Y	K	D	N	V	D	D	V	N	A	F	G	G	G	T	E	V	V	V
L88	L89	L90	L91	L92	L93	L94	L95	L95A	L95B	L95C	L95D	L96	L97	L98	L99	L100	L101	L102	L103	L104	L105	L106
L88	L89	L90	L91	L92	L93	L94	L95	L95A	L95B	L95C	L95D	L96	L97	L98	L99	L100	L101	L102	L103	L104	L105	L106
L88	L89	L90	L91	L92	L93	L94	L95	L95A	L95B	L95C	L95D	L96	L97	L98	L99	L100	L101	L102	L103	L104	L105	L106

LFR4

E	G	D	P
L107	L108	L109	L110
L107	L108	L109	L110
L107	L108	L109	L110

Insertion

Unusual residue (<1% of sequences)

Displaying 19 - 133 of 456 residues:

Block 1

Query protein sequence	C	Q	S	V	E	E	S	N	G	G	R	L	V	A	P	G	T	P	L	T	L
Chothia numbering	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	
Chothia+ numbering	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	
Kabat numbering	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	

REGIONS: CHOTHIA — HFR1
ABM — HFR1
KABAT — HFR1
CONTACT — HFR1

Block 2

	T	C	T	V	S	G	I	D	L	M	Y	N	A	M	N	W	V	R	Q	A	P	G	K
	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43
	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43
	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43

HFR1 / HFR2

Block 3

	G	L	E	F	I	G	W	I	G	S	G	G	N	T	V	Y	A	S	W	A	K	G	R
	H44	H45	H46	H47	H48	H49	H50	H51	H52	H53	H54	H55	H56	H57	H58	H59	H60	H61	H62	H63	H64	H65	H66
	H44	H45	H46	H47	H48	H49	H50	H51	H52	H53	H54	H55	H56	H57	H58	H59	H60	H61	H62	H63	H64	H65	H66
	H44	H45	H46	H47	H48	H49	H50	H51	H52	H53	H54	H55	H56	H57	H58	H59	H60	H61	H62	H63	H64	H65	H66

HFR2 / HFR3

FIG. 13C

FIG. 13D

Insertion

Unusual residue (<1% of sequences)

FIG. 14A

± Top1 inhibitor, 1 μM
± VE-821, 1 μM

1   4   8   18 h

FIG. 14B

CPT

0 4 8 18   0 4 8 18 (h)

VE-821, 1 μM   - + - - - - + + + +

pT1989-ATR
ATR
pS345-Chk1
pT68-Chk2
γH2AX
Top1
GAPDH

FIG. 14C

LMP-400

0 4 8 18   0 4 8 18 (h)

VE-821, 1μM   - - - - - + + + +

pT1989-ATR
ATR
pS345-Chk1
pT68-Chk2
γH2AX
Top1
GAPDH

FIG. 15A   **FIG. 15B**   **FIG. 15C**

FIG. 15D

FIG. 16

DAPI   ATR-T1989-P   Merge

FIG. 17A   **FIG. 17B**   **FIG. 17C**