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- (71) **Applicant:** OREGON HEALTH & SCIENCE UNIVERSITY [US/US]; 0690 SW Bancroft St, Portland, OR 97239 (US).
- (72) **Inventors:** DAVARE, Monika; 7180 SW 84th Ave, Portland, OR 97223 (US). DRUKER, Brian; 4100 SW Dosch Rd, Portland, OR 97239 (US).
- (74) **Agent:** JACKSON, Jeffrey; 0690 SW Bancroft St, Portland, OR 97239 (US).
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(54) **Title:** METHODS OF TREATING CANCERS CHARACTERIZED BY ABERRANT ROS1 ACTIVITY

(57) **Abstract:** Disclosed herein are methods of identifying a cancer characterized by aberrant ROS1 activity as being crizotinib resistant and foretinib (N1'-[3-fluoro-4-[[6-methoxy-7-(3-morpholinopropoxy)-4-quinolyl]oxy]phenyl]-N1-(4-fluorophenyl)cyclopropane-1,1-dicarboxamide) sensitive through the identification of mutations in the kinase domain of ROS1. Also disclosed kits used in facilitating the method as well as pharmaceutical compositions comprising foretinib for use in treating cancers characterized by aberrant ROS1 activity.



TITLE

METHODS OF TREATING CANCERS CHARACTERIZED BY ABERRANT ROS1 ACTIVITY

PRIORITY CLAIM

5 This application claims priority to US Provisional Application 61/769,936, filed on 27 February 2013, and US Provisional Application 61/901,148, filed 07 November 2013.

BACKGROUND

Chromosomal rearrangements of the receptor tyrosine kinase (RTK) ROS1 that
10 create oncogenic fusion kinases are reported in glioma, lung adenocarcinoma and
cholangiocarcinoma patients (Bergethon K *et al*, *Off J Am Soc Clin Oncol* **30**, 863-870
(2012); Charest A *et al*, *Genes, Chrom, Cancer* **37**, 58-71 (2003); and Gu TL *et al*, *PLoS*
One **6**, e15640 (2011)). With growing recognition of ROS1 as a therapeutic target, it is
necessary to identify ROS1 inhibitors with potential for clinical development Chin LP *et*
15 *al*, *Off Publ International Assoc for the Study of Lung Cancer* **7**, 1625-1630 (2012); El-
Deeb IM *et al*, *Med Res Rev* (2010); Ou SH *et al*, *Exp Rev Anticancer Therapy* **12**, 447-456
(2012)).

SUMMARY

A kinase inhibitor screen revealed that foretinib (GSK1363089) is a highly potent
20 ROS1 inhibitor, with selective efficacy for ROS1 compared to the related anaplastic
lymphoma kinase (ALK). *In vivo*, foretinib-treated FIG-ROS-driven tumors exhibited a
significant reduction in tumor volume in contrast to treated Pten-null tumors or upon
crizotinib treatment of either cohort. Hence foretinib is a more effective ROS1 inhibitor
than the FDA-approved inhibitor crizotinib that is now in clinical trial for lung
25 adenocarcinoma patients with ROS1-rearrangements. Patients treated with tyrosine
kinase inhibitors regularly develop clinical resistance due in part to emergence of kinase
domain (KD) mutations (Shah NP *et al*, *Cancer Cell* **2**, 117-125 (2002); incorporated by
reference herein.) Using an accelerated mutagenesis screen, ROS1 kinase domain

mutations were identified that confer resistance to crizotinib. Crizotinib-resistant ROS1 kinase domain mutants retain foretinib sensitivity at concentrations below a level already demonstrated in clinical trials to be safe for human subjects.

Disclosed herein are methods useful in identifying a sample from a subject as comprising a cancer cell that is sensitive to foretinib. In some aspects of the invention, the cell is also identified as resistant to crizotinib. The methods involve isolating a nucleic acid from the sample that includes a nucleic acid that encodes the ROS1 kinase domain (SEQ ID NO: 1) and identifying one or more mutations in the ROS1 kinase domain that results in one or more of the following amino acid substitutions: G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G. The presence of one or more of the mutations identifies the cell as sensitive to foretinib and resistant to crizotinib. The method may further comprise treating the subject with foretinib.

Disclosed herein are kits used to facilitate the performance of the method. The kits include a set of oligonucleotides used to amplify SEQ ID NO: 1 and a set of oligonucleotides used to identify a mutation in SEQ ID NO: 1 that results in one or more of the following amino acid substitutions: G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G.

Disclosed herein are pharmaceutical compositions for use in treating cancers characterized by aberrant activity of the ROS1 kinase domain. The pharmaceutical compositions include foretinib and a pharmaceutically acceptable carrier. The ROS1 kinase domain includes a mutation that results in one or more of the following amino acid substitutions: G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G.

BRIEF DESCRIPTION OF THE DRAWINGS

Some of the drawings in this disclosure are images that may not reproduce properly in a patent application publication. Additionally, some of the graphs, plots, and photographic images may be better understood using color, which is not available in a

patent application publication. Applicants consider all originally disclosed images and graphs (whether in color or not) part of the original disclosure and reserve the right to present high quality and/or color images of the herein described figures in later proceedings.

5 Figure 1A is a line graph depicting the viability of Ba/F3 FIG-ROS normalized to a vehicle treated control (control = 1.0) cells after 72 hours of exposure to the indicated concentrations of crizotinib, foretinib, Gö6976, TAE684 and GSK1838705A. The symbols represent the mean and the error bars represent the standard error of the mean (SEM) from three independent experiments with triplicate wells.

10 Figure 1B is a plot depicting the IC₅₀ values for the Ba/F3 FIG-ROS, Ba/F3 SLC-ROS, Ba/F3 ALK F1174L, Ba/F3 BCR-Abl, Ba/F3 JAK3 A572V and Ba/F3 parental cells treated with the indicated inhibitors. IC₅₀ was determined from non-linear regression curve fit analysis of the dose response curves.

 Figure 1C is an image of an immunoblot of lysates of Ba/F3 FIG-ROS and Ba/F3 SLC-ROS and the indicated downstream effectors after treatment with the indicated compounds. Images shown are representative of three independent experiments.

15 Figure 1D is a line graph depicting the viability of HCC78 cells after 72 hour exposure to the indicated concentrations of crizotinib and foretinib. The values are normalized to that of a vehicle treated control (control = 1.0). Symbols represent the mean and the error bars represent the standard error of the mean (SEM) from three independent experiments with triplicate wells.

 Figure 1E is an image of an immunoblot of lysates of HCC78 cells probing for phosphorylated and total SLC-ROS1 and ERK1/2, and phosphorylated protein S6 after treatment with the indicated concentration of the indicated inhibitor. The images are representative of two independent experiments.

25 Figure 1F top panel is an image of colonies formed by HCC78 cells plated after treatment with the indicated concentration of crizotinib or foretinib after 10 days.

Figure 1F bottom panel is a bar graph depicting the number of colonies observed on the

plates from Figure 1F top panel. Where indicated, ** = $p < 0.01$ and *** = $p < 0.001$ by Student's t-test.

Figure 2A is a plot of the IC_{50} values for shPten (lines 1 and 2) and FIG-ROS (lines 3 and 4) driven murine cholangiocarcinoma cell lines treated with the indicated inhibitors as determined from non-linear regression curve fit analysis of the dose response curves.

Figure 2B is a bar graph depicting the anchorage-independent colony formation of FIG-ROS (Line 4) and shPten (Line 2) cholangiocarcinoma cell lines treated with crizotinib and foretinib at the indicated concentrations. The numbers of colonies formed in the presence of crizotinib and foretinib are normalized to a vehicle treated control (control = 1.0). The values indicate the mean and the error bars indicate the standard error of the mean of 3 independent wells.

Figure 2C is an image of an immunoblot of lysates of lines 3 and 4 probed for phosphorylated and total ROS1, phosphorylated Shp2, phosphorylated Erk1/2, total Erk2, and tubulin as a loading control after treatment with the indicated doses of crizotinib and foretinib. Images shown are representative of three independent experiments.

Figure 3A is a set of 12 images of explanted FIG-ROS and shPten-driven subcutaneous tumors after 9 days of treatment with foretinib (25 mg/kg) or crizotinib (25 mg/kg) by oral gavage.

Figure 3B is a set of waterfall plots indicating the change in FIG-ROS and shPten tumor volume from treatment initiation to 24 hours after the administration of the last dose. Relative tumor growth was calculated as described in Example 1.

Figure 3C is a set of images of H&E stained tumor sections from Line 1 (shPten) and Line 3 (FIG-ROS) after 9 days of treatment with vehicle, crizotinib, or foretinib. Scale bar is 500 μ m.

Figure 3D is an image of an immunoblot of tumor lysates probed for phosphorylated and total ROS1, phosphorylated and total Shp2, phosphorylated and

total Stat3, phosphorylated and total Src as well as tubulin as a loading control. Tumors were harvested from vehicle or foretinib (25mg/kg) treated mice the indicated times after oral gavage.

Figure 4A is a bar graph depicting the percent frequency of the indicated ROS1 kinase domain mutations found in the ENU-treated Ba/F3 FIG-ROS clones isolated after growth and clonal expansion in the presence of 1000 nM crizotinib

Figure 4B is a structure of a homology model of the ROS1 kinase domain bound to crizotinib (magenta) and foretinib (orange). Foretinib and crizotinib are shown in colored stick representation, while the protein is shown in grey ribbon. Differences in the activation loop conformations are highlighted by coloring them the same as their respective ligands. Residues, which were found to confer resistance to crizotinib when mutated, are highlighted in red and shown in stick representation.

Figure 4C is a plot of the IC_{50} values for Ba/F3 FIG-ROS unmutated kinase domain and FIG-ROS kinase domain mutant cells treated with foretinib and crizotinib. IC_{50} was determined from non-linear regression curve fit analysis of the dose response curves. The dotted lines indicate steady state C_{max} for crizotinib and foretinib as reported in the literature.

Figure 4D is a plot of the growth of Ba/F3 CD74-ROS wild-type and G2032R mutant cells after a 72 hour exposure to graded concentrations of crizotinib or foretinib. Data are shown as normalized to vehicle-treated cells. The values are means $\pm$ SEM from three independent experiments performed in triplicate.

Figure 4E is a plot of the IC_{50} values for Ba/F3 CD74-ROS wild-type and G2032R mutant cells treated with foretinib and crizotinib as determined from non-linear regression curve fit analysis of the dose response curves. Dotted lines indicate the previously reported steady state C_{max} for crizotinib and foretinib as indicated.

Figure 4F is an image of an Immunoblot analysis of CD74-ROS (wild-type versus G2032R) phosphorylation and downstream effector modulation after treatment of cells

with varying doses of crizotinib and foretinib as shown. Cropped images representative of three independent experiments are shown.

Figure 5A is a set of three bar graphs depicting the induction of apoptosis in Ba/F3 FIG-ROS, Ba/F3 SLC-ROS and Ba/F3 ALK F1174L cells after 48 hours of treatment with the indicated doses of the indicated compounds. The number of cells that were Annexin V and 7-AAD positive after inhibitor treatment was normalized to number of cells staining positive with these markers with vehicle treatment (basal). A minimum of 2000 cells were counted per condition. Rate of apoptosis is shown as fold over basal. Basal apoptosis in Ba/F3 FIG-ROS, Ba/F3 SLC-ROS and Ba/F3 ALK F1174 cells was 3.6%, 2.8% and 1.2% respectively.

Figure 5B is a bar graph indicating the apoptosis induction in HCC78 cells after treatment with foretinib and crizotinib at the indicated concentrations for 72 hours. Basal (vehicle treated) apoptosis in HCC78 cells was 11% of total cell population. * - $p < 0.05$, ** - $p < 0.01$ and *** - $p < 0.0001$ by t-test.

Figure 6A is a set of six images on the left and a bar graph on the right. The images at left are of a gap at 10 minutes and at 18 hours after 'scratch' of cells on a surface. Cells were treated with vehicle, 50 nM crizotinib or 50 nM foretinib as indicated. The right panel is a bar graph depicting the percent of gap closure determined by measuring the length of a straight line drawn across the gap from imaging the same field of view at initiation of treatment and at 18 hours. Scale bar = 200 μm

Figure 6B is a plot depicting the IC_{50} values for HCC78, PC9 and HCC4011 cells treated with crizotinib, foretinib and erlotinib as indicated. IC_{50} was determined from non-linear regression curve fit analysis of the dose response curves.

Figure 6C is a plot depicting the IC_{50} values for HCC78 cells treated with crizotinib, foretinib, MGCD-265, SGX-523 and JNJ-38877605 as indicated. IC_{50} was determined from non-linear regression curve fit analysis of the dose response curves.

Figure 7A are images of immunoblots of lysates from NIH3T3 FIG-ROS and SLC-ROS cells treated with varying concentration of crizotinib and foretinib for 1 hour and probed for phosphorylated and total ROS1.

Figure 7B top panels are images of plates depicting NIH3T3 FIG-ROS and SLC-ROS colony formation in soft agar after treatment with crizotinib and foretinib as indicated. The bottom panels are bar graphs indicating the number of colonies formed from the plates depicted in the top panels. The values indicate average colony number $\pm$ SEM from 4 independent wells.

Figure 8 is a scatter plot of the individual weight distribution of treated tumors from Lines 1 and 2 (shPten tumors) and Lines 3 and 4 (FIG-ROS expressing tumors). Statistical significance was determined by a paired t-test.

Figure 9A is a bar graph depicting the viability of Ba/F3 FIG-ROS kinase domain mutant cell lines derived from an ENU mutagenesis screen after transfection with siRNA that silences either the ROS1 kinase domain (siROS1-KD) or nontargeting siRNA (siNT). Viability was normalized to that of vehicle (sterile water) transfected cells.

Figure 9B is a line graph depicting the results of an IL-3 withdrawal assay for Ba/F3 cells transfected with either the unmutated FIG-ROS or the indicated FIG-ROS kinase domain mutant. Total viable cell number was determined by counting cells on the indicated number of days after IL-3 withdrawal.

Figure 10A is a dose response curve showing the viability of the indicated Ba/F3 FIG-ROS kinase domain mutant cells after 72 hours of exposure to crizotinib. Viability is normalized to vehicle treated cells of the same genotype. Values are the means of three independent experiments with triplicate wells. Error bars indicate the standard error of the mean.

Figure 10B is a dose response curve showing the viability of the indicated Ba/F3 FIG-ROS kinase domain mutant cells after 72 hours of exposure to foretinib. Viability is normalized to vehicle treated cells of the same genotype. Values are the means of three

independent experiments with triplicate wells. Error bars indicate the standard error of the mean.

Figure 10C is an image of an immunoblot of lysates from wild type and the indicated kinase domain mutants probed for phosphorylated and total ROS1. Tubulin was probed as a loading control.

SEQUENCE LISTING

SEQ ID NO: 1 is the protein sequence of the kinase domain of *Homo sapiens* ROS1 (amino acids 1945 to 2222 in the whole molecule).

- 10 SEQ ID NO: 2 is a sense primer for SLC34A2.
SEQ ID NO: 3 is an antisense primer for ROS1.
SEQ ID NO: 4 is the FIG-ROS M13Kin3F primer.
SEQ ID NO: 5 is the ROS M13Kin1 Rev primer.
SEQ ID NO: 6 is the M13F primer.
15 SEQ ID NO: 7 is the M13REV primer.

DETAILED DESCRIPTION

Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin
20 Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9);
Kendrew et al. (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell
Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology
and Biotechnology: a Comprehensive Desk Reference*, published by VCR Publishers, Inc.,
25 1995 (ISBN 1-56081-569-8).

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents

unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base 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 description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes."

In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Aberrant activity of a tyrosine kinase: Inappropriate or uncontrolled activation of a tyrosine kinase, such as ROS1, for example by over-expression, upstream activation (for example, by upstream activation of a protein that affect a tyrosine kinase), and/or mutation (for example a truncation, deletion, insertion and/translocation which increases the activity, such as but not limited to, kinase activity of a tyrosine kinase), which can lead to uncontrolled cell growth, for example in cancer, non-small cell lung cancer and/or cholangiocarcinoma. In some examples, aberrant activity of a tyrosine kinase is a higher rate of kinase activity than the unmutated tyrosine kinase. In some examples, aberrant activity of a tyrosine kinase is a lower rate of kinase activity than the unmutated tyrosine kinase. Other examples of aberrant activity of a tyrosine kinase include, but are not limited to, mislocalization of the tyrosine kinase, for example mislocalization in an organelle of a cell or mislocalization at the cell membrane relative to the unmutated tyrosine kinase.

Administration: To provide or give a subject an agent, such as a composition that targets/inhibits a ROS1 kinase (such as foretinib) by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Amplifying a nucleic acid molecule: To increase the number of copies of a nucleic acid molecule, such as a gene or fragment of a gene, for example a region of a gene that encodes a tumor biomarker, such as a mutant in the ROS1 kinase domain. The resulting products are called amplification products. An example of in vitro amplification is the polymerase chain reaction (PCR). Other examples of in vitro amplification techniques include quantitative real-time PCR, strand displacement amplification (see USPN 5,744,311); transcription-free isothermal amplification (see USPN 6,033,881); repair chain reaction amplification (see WO90/01069); ligase chain reaction amplification; gap filling ligase chain reaction amplification (see USPN 5,427,930); coupled ligase detection and PCR (see USPN 6,027,889); and NASBA™ RNA transcription-free amplification (see USPN 6,025,134).

A commonly used method for real-time quantitative polymerase chain reaction involves the use of a double stranded DNA dye (such as SYBR Green I dye). For example, as the amount of PCR product increases, more SYBR Green I dye binds to DNA, resulting in a steady increase in fluorescence. SYBR green binds to double stranded DNA, but not to single stranded DNA. In addition, SYBR green fluoresces strongly at a wavelength of 497 nm when it is bound to double stranded DNA, but does not fluoresce when it is not bound to double stranded DNA. As a result, the intensity of fluorescence at 497 nm may be correlated with the amount of amplification product present at any time during the reaction. The rate of amplification may in turn be correlated with the amount of template sequence present in the initial sample. Generally, Ct values are calculated similarly to those calculated using the TaqMan® system. Because the probe is absent, amplification of the proper sequence may be checked by any of a number of techniques. One such technique involves running the amplification products on an agarose or other gel appropriate for resolving nucleic acid fragments and comparing the amplification products from the quantitative real time PCR reaction with control DNA fragments of known size.

Another commonly used method is real-time quantitative TaqMan® PCR (Applied Biosystems). This type of PCR has reduced the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products to produce sensitive, accurate, and reproducible measurements of levels of gene expression. The PCR step can use any of a number of thermostable DNA-dependent DNA polymerases, it typically employs a Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. Thus, TaqMan® PCR typically utilizes the 5'-nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used.

Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is nonextendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

Examples of fluorescent labels that may be used in quantitative PCR include but need not be limited to: HEX, TET, 6-FAM, JOE, Cy3, Cy5, ROX TAMRA, and Texas Red.

Examples of quenchers that may be used in quantitative PCR include, but need not be limited to TAMRA (which may be used as a quencher with HEX, TET, or 6-FAM), BHQ1, BHQ2, or DABCYL. TAQMAN® RT-PCR can be performed using commercially available

equipment, such as, for example, ABI PRISM 7700® Sequence Detection System (Perkin-Elmer- Applied Biosystems), or Lightcycler (Roche Molecular Biochemicals).

In one embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700® Sequence Detection System. The system includes a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real time through fiber optic cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

In some examples, 5'-nuclease assay data are initially expressed as Ct, or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (Ct).

To minimize errors and the effect of sample-to-sample variation, RT-PCR can be performed using an internal standard. The ideal internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are the mRNA products of housekeeping genes.

Amplification of a nucleic acid sequence may be used for any of a number of purposes, including increasing the amount of a rare sequence to be analyzed by other methods. It may also be used to identify a sequence directly (for example, through an amplification refractory mutation system) or as part of a DNA sequencing method.

Antibody: A polypeptide including at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen (such as a mutant ROS1 kinase domain) or a fragment thereof. Antibodies are composed of a heavy and a light chain, each of which has a variable region, termed the

variable heavy (VH) region and the variable light (VL) region. Together, the VH region and the VL region are responsible for binding the antigen recognized by the antibody.

The term “antibody” encompasses intact immunoglobulins, as well the variants and portions thereof, such as Fab fragments, Fab' fragments, F(ab)'2 fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker. In dsFvs the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies, heteroconjugate antibodies (such as, bispecific antibodies). 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.

Anti-proliferative activity: An activity of a molecule, for example a small molecule, an inhibitory RNA, and the like, which reduces proliferation of at least one cell type, but which may reduce the proliferation (either in absolute terms or in rate terms) of multiple different cell types (e.g., different cell lines, different species, etc.). In specific embodiments, the anti-proliferative activity of a small molecule, such as an inhibitor of ROS1 kinase will be apparent against cancer cells obtained from a subject that has aberrant ROS1 tyrosine kinase activity, including cells that have aberrant ROS1 activity and one or more mutations that render the cancer susceptible to foretinib and not other tyrosine kinase inhibitors.

Array: An arrangement of molecules, such as biological macromolecules (such as peptides or nucleic acid molecules) or biological samples (such as tissue sections), in addressable locations on or in a substrate. A “microarray” is an array that is miniaturized so as to require or be aided by microscopic examination for evaluation or analysis. In certain example arrays, one or more molecules (such as an antibody or peptide) will occur on the array a plurality of times (such as twice), for instance to provide internal controls. The number of addressable locations on the array can vary, for example from

at least one, to at least 2, to at least 3, at least 4, at least 5, at least 6, at least 10, at least 20, at least 30, at least 50, at least 75, at least 100, at least 150, at least 200, at least 300, at least 500, least 550, at least 600, at least 800, at least 1000, at least 10,000, or more. In some examples, arrays include positive and/or negative controls, such as probes that bind housekeeping genes. In particular examples, an array includes nucleic acid molecules, such as oligonucleotide sequences that are at least 15 nucleotides in length, such as about 15-75 or 15-60 nucleotides in length. In particular examples, an array includes oligonucleotide probes or primers which can be used to detect nucleotides that encode tumor biomarker sequences (including RCC biomarkers). In an example, the array is a commercially available array such as Human Genome GeneChip® arrays from Affymetrix (Santa Clara, CA).

Within an array, each arrayed sample is addressable, in that its location can be reliably and consistently determined within at least two dimensions of the array. The feature application location on an array can assume different shapes. For example, the array can be regular (such as arranged in uniform rows and columns) or irregular. Thus, in ordered arrays the location of each sample is assigned to the sample at the time when it is applied to the array, and a key may be provided in order to correlate each location with the appropriate target or feature position. Often, ordered arrays are arranged in a symmetrical grid pattern, but samples could be arranged in other patterns (such as in radially distributed lines, spiral lines, or ordered clusters). Addressable arrays may be computer readable, in that a computer can be programmed to correlate a particular address on the array with information about the sample at that position (such as hybridization or binding data, including for instance signal intensity). In some examples of computer readable formats, the individual features in the array are arranged regularly, for instance in a Cartesian grid pattern, which can be correlated to address information by a computer.

Binding or stable binding: An association between two substances or molecules, such as the association of an antibody with a peptide, nucleic acid to another nucleic

acid, or the association of a protein with another protein or nucleic acid molecule, or the association of a small molecule drug with a protein (such as a tyrosine kinase) or other biological macromolecule. Binding can be detected by any procedure known to one skilled in the art, such as by physical or functional properties. For example, binding can
5 be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation, protein activity (including tyrosine kinase activity) and the like.

Biological signaling pathway: A system of proteins, such as tyrosine kinases, and other molecules that act in an orchestrated fashion to mediate the response of a cell
10 toward internal and external signals. In some examples, biological signaling pathways include tyrosine kinase proteins, such as ROS1, which can propagate signals in the pathway by selectively phosphorylating downstream substrates. In some examples a biological signaling pathway is dysregulated and functions improperly, which can lead to aberrant signaling and in some instances hyper-proliferation of the cells with the
15 aberrant signaling. In some examples, dysregulation of a biological signaling pathway can result in a malignancy, such as cancer, for example the aberrant activation of a ROS1 kinase such as the formation of a fusion protein comprising ROS1 (including FIG-ROS and SLC-ROS). A ROS1 biological signaling pathway is a signaling pathway, in which ROS1 plays a role, for example by phosphorylation of downstream targets.

Biomarker: Molecular, biological or physical attributes that characterize a physiological or cellular state and that can be objectively measured to detect or define disease progression or predict or quantify therapeutic responses. A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic
20 intervention. A biomarker may be any molecular structure produced by a cell or organism. A biomarker may be expressed inside any cell or tissue; accessible on the surface of a tissue or cell; structurally inherent to a cell or tissue such as a structural component, secreted by a cell or tissue, produced by the breakdown of a cell or tissue
25

through processes such as necrosis, apoptosis or the like; or any combination of these. A biomarker may be any protein, carbohydrate, fat, nucleic acid, catalytic site, or any combination of these such as an enzyme, glycoprotein, cell membrane, virus, cell, organ, organelle, or any uni- or multimolecular structure or any other such structure now
5 known or yet to be disclosed whether alone or in combination.

A biomarker may be represented by the sequence of a nucleic acid from which it can be derived or any other chemical structure. Examples of such nucleic acids include miRNA, tRNA, siRNA, mRNA, cDNA, or genomic DNA sequences including any complimentary sequences thereof. One example of a biomarker is a DNA coding
10 sequence for a protein comprising one or more mutations that cause amino acid substitutions in the protein sequence. Such a biomarker may be the coding sequence of a particular part of a protein such as the kinase domain of ROS1 comprising nucleic acid mutations that cause one or more of the following amino acid substitutions G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G.

Cancer: A disease or condition in which abnormal cells divide without control and are able to invade other tissues. Cancer cells spread to other body parts through the blood and lymphatic systems. Cancer is a term for many diseases. There are more than 100 different types of cancer in humans. Most cancers are named after the organ in which they originate. For instance, a cancer that begins in the colon may be called a
20 colon cancer. However, the characteristics of a cancer, especially with regard to the sensitivity of the cancer to therapeutic compounds, are not limited to the organ in which the cancer originates. A cancer cell is any cell derived from any cancer, whether in vitro or in vivo. Other features often associated with cancer include metastasis, interference with the normal functioning of neighboring cells, release of cytokines or
25 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” or “metastasis” refers to 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 “pathology” of cancer includes all phenomena that compromise the well-being of the subject. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

Chemotherapeutic agent or Chemotherapy: Any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms, and cancer as well as diseases characterized by hyperplastic growth. In one embodiment, a chemotherapeutic agent is an agent of use in treating cancer, such as cancers characterized by aberrant ROS1 activity, including cancers characterized by aberrant ROS1 activity comprising mutations in the kinase domain of ROS1. Such agents include ROS1 inhibitors such as foretinib and crizotinib. Combination chemotherapy is the administration of more than one agent to treat cancer.

Contacting: Placement in direct physical association, including contacting of a solid with a solid, a liquid with a liquid, a liquid with a solid, or either a liquid or a solid with a cell or tissue, whether in vitro or in vivo. Contacting can occur in vitro with isolated cells or tissue or in vivo by administering to a subject.

Diagnostic: Identifying the presence or nature of a pathologic condition, such as, but not limited to cancer, such as cancer caused by aberrant ROS1 activity, including cancer caused by aberrant ROS1 activity that is insensitive to crizotinib and/or sensitive to foretinib. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of true positives). The "specificity" of a diagnostic assay is 1 minus the

false positive rate, where the false positive rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

5 **Domain:** any part of polypeptide that can be demonstrated to mediate a particular protein function. For example, the kinase domain of human ROS1 is from amino acid 1945 to amino acid 2222. The kinase domain of mouse ROS1 is from amino acid 1938 to amino acid 2216. In both cases, the numbers are derived from the amino acid in the full-length wild type ROS1 protein.

10 **Effective amount:** An amount of agent, such as a tyrosine kinase inhibitor that is sufficient to generate a desired response, such as reduce or eliminate a sign or symptom of a condition or disease, such as cancer, for example cancers expressing an aberrant ROS1 kinase. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations that has been shown to achieve anti proliferative
15 activity in vitro. In some examples, an “effective amount” is one that treats (including prophylaxis) one or more symptoms and/or underlying causes of any of a disorder or disease, for example cancer, such as a cancer characterized by an aberrant ROS1 kinase. An effective amount can be a therapeutically effective amount, including an amount that prevents one or more signs or symptoms of a particular disease or condition from
20 developing, such as one or more signs or symptoms associated with cancer.

Inhibitor: Any chemical compound, specific for a protein or other gene product that can directly interfere with the activity of a protein, such as a kinase, particularly a ROS1 kinase and more particularly a ROS1 kinase with aberrant activity. An inhibitor can inhibit the activity of a protein either directly or indirectly. Direct inhibition can be
25 accomplished, for example, by binding to a protein and thereby preventing the protein from binding an intended target, such as a receptor. Indirect inhibition can be accomplished, for example, by binding to a protein's intended target, such as a receptor

or binding partner, thereby blocking or reducing activity of the protein. Examples of inhibitors of aberrant ROS1 kinase domains include crizotinib and foretinib.

Inhibit: To reduce to a measurable extent, for example, to reduce activity (including aberrant activity) of a protein such as a kinase. In some examples, the kinase activity of a protein is inhibited, for example using a small molecule inhibitor of ROS1 such as crizotinib or foretinib.

Inhibiting or treating a disease: Inhibiting the full development of a disease or condition, for example, in a subject who has or who is at risk for a disease such cancer, for example, a cancer characterized by a ROS1 kinase with aberrant activity.

“Treatment” refers to any therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition. The term “ameliorating,” with reference to a disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of metastases, an improvement in the overall health or well-being of the subject, or by other clinical or physiological parameters associated with a particular disease. 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. A “therapeutic” treatment is a treatment administered after the development of significant signs or symptoms of the disease and.

Kinase: An enzyme that catalyzes the transfer of a phosphate group from one molecule to another. Kinases play a role in the regulation of cell proliferation, differentiation, metabolism, migration, and survival. A “tyrosine kinase” transfers phosphate groups to a hydroxyl group of a tyrosine in a polypeptide. In some examples, a kinase is a ROS1 tyrosine kinase. Receptor protein tyrosine kinases (RTKs) contain a single polypeptide chain with a transmembrane segment. The extracellular end of this

segment contains a high affinity ligand-binding domain, while the cytoplasmic end comprises the catalytic core and the regulatory sequences.

Non-receptor tyrosine kinases, such as ROS1, can be located in the cytoplasm as well as in the nucleus. They exhibit distinct kinase regulation, substrate phosphorylation, and function. A “preferential” inhibition of a kinase refers to an inhibitor that has the characteristic of inhibiting the activity of one kinase, such as ROS1, as well as or more than it inhibits the activity of a second kinase, such as ALK or another tyrosine kinase.

Mass spectrometry: A method wherein, a sample is analyzed by generating gas phase ions from the sample, which are then separated according to their mass-to-charge ratio (m/z) and detected. Methods of generating gas phase ions from a sample include electrospray ionization (ESI), matrix-assisted laser desorption-ionization (MALDI), surface-enhanced laser desorption-ionization (SELDI), chemical ionization, and electron-impact ionization (EI). Separation of ions according to their m/z ratio can be accomplished with any type of mass analyzer, including quadrupole mass analyzers (Q), time-of-flight (TOF) mass analyzers, magnetic sector mass analyzers, 3D and linear ion traps (IT), Fourier-transform ion cyclotron resonance (FT-ICR) analyzers, and combinations thereof (for example, a quadrupole-time-of-flight analyzer, or Q-TOF analyzer). Prior to separation, the sample may be subjected to one or more dimensions of chromatographic separation, for example, one or more dimensions of liquid or size exclusion chromatography or gel-electrophoretic separation.

Mutation: A mutation is any difference in a nucleic acid or polypeptide sequence from a normal, consensus or “wild type” sequence. A mutant is any protein or nucleic acid sequence comprising a mutation. In addition a cell or an organism with a mutation may also be referred to as a mutant.

Some types of mutations include point mutations (differences in individual nucleotides or amino acids); silent mutations (differences in nucleotides that do not result in an amino acid changes); deletions (differences in which one or more nucleotides or amino acids are missing); frameshift mutations (differences in which

deletion of a number of nucleotides indivisible by 3 results in an alteration of the amino acid sequence beyond the mutation). A mutation that results in a difference in an amino acid may also be called an amino acid substitution mutation. Amino acid substitution mutations may be described by the amino acid change relative to wild type at a particular position in the amino acid sequence. For example, mutations in the kinase domain of ROS1 disclosed herein include G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R and E1935G. G2032R refers to a mutation from a glycine residue at position 2032 of the wild type protein to an arginine residue in the mutant; V2098I refers to a mutation from a valine residue at position 2098 to an isoleucine in the mutant; G1971E refers to a mutation from a glycine at position 1971 to glutamic acid; L1982F refers to a mutation from a leucine at position 1982 to a phenylalanine, etc.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. Remington's Pharmaceutical Sciences, by E.W. Martin, Mack Publishing Co., Easton, PA, 19th Edition, 1995, describes compositions and formulations suitable for pharmaceutical delivery of the compositions disclosed herein. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise 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 (such as 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.

Phospho-peptide or phospho-protein: A protein in which one or more phosphate moieties are covalently linked to one or more of the amino acids making up the peptide or protein. A peptide can be phosphorylated at multiple or single sites.

Sometimes it is desirable for the phospho-protein to be phosphorylated at one site regardless of the presence of multiple potential phosphorylation sites. In vivo the transfer of a phosphate to a peptide is accomplished by a kinase. For example a tyrosine kinase such as ROS1 transfers a phosphate to a tyrosine residue of a substrate peptide or protein.

Polypeptide: Any chain of amino acids, regardless of length or posttranslational modification (such as glycosylation, methylation, ubiquitination, phosphorylation, or the like). In one embodiment, a polypeptide is a ROS1 polypeptide. "Polypeptide" is used interchangeably with "protein," and is used to refer to a polymer of amino acid residues. A "residue" refers to an amino acid or amino acid mimetic incorporated in a polypeptide by an amide bond or amide bond mimetic.

Purified: The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nucleic acid preparation is one in which the protein referred to is more pure than the protein in its natural environment within a cell. For example, a preparation of a protein is purified such that the protein represents at least 50% of the total protein content of the preparation. Similarly, a purified nucleic acid preparation is one in which nucleic acid is more pure than in an environment including a complex mixture compounds, such as a cell or cell extract. The terms "isolated" or "isolating" are interchangeable with the term "purified" or "purifying."

Sample: A sample, such as a biological sample, is a sample obtained from a plant or animal subject. As used herein, biological samples include all clinical samples useful for detection of mutations in tumor DNA, particularly mutations in the kinase domain of ROS1. Samples include, but not limited to, cells, tissues, and bodily fluids, including tissues that are, for example, unfixed, frozen, fixed in formalin and/or embedded in paraffin. In one example, a sample includes a tissue biopsy obtained from a subject with a tumor.

Sequence identity/similarity: The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math. 2:482, 1981; Needleman & Wunsch, J. Mol. Biol. 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS 5:151-3, 1989; Corpet et al., Nuc. Acids Res. 16:10881-90, 1988; Huang et al. Computer Appls. in the Biosciences 8, 155-65, 1992; and Pearson et al., Meth. Mol. Bio. 24:307-31, 1994. Altschul et al., J. Mol. Biol. 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both

sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1154 nucleotides is 75.0 percent identical to the test sequence ($1166 \div 1554 * 100 = 75.0$). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is, $15 \div 20 * 100 = 75$).

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost 5 of 1). Homologs are typically characterized by possession of at least 70% sequence identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database.

Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, Comput. Appl. Biosci. 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least about 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to a protein.

When aligning short peptides (fewer than around 30 amino acids), the alignment is performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when

assessed by this method, such as at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to a protein. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and can possess sequence identities of at least 85%, 90%, 95% or 98% depending on their identity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site.

One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions, as described above.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

One of skill in the art will appreciate that the particular sequence identity ranges are provided for guidance only; it is possible that strongly significant homologs could be obtained that fall outside the ranges provided.

Small molecule: A molecule, typically with a molecular weight less than about 1000 Daltons, or in some embodiments, less than about 500 Daltons, wherein the molecule is capable of modulating, to some measurable extent, an activity of a target molecule such as inhibiting the activity of a kinase, such as a ROS1 kinase with aberrant activity.

Subject: A living multicellular vertebrate organism, a category that includes, for example, mammals and birds. A "mammal" includes both human and non-human mammals, such as mice. In some examples, a subject is a patient, such as a patient

diagnosed with cancer, particularly cancer characterized by a ROS1 kinase with aberrant activity, more particularly a cancer characterized by a ROS1 kinase with aberrant activity comprising one or more mutations in its kinase domain.

Substrate: A molecule that is acted upon by an enzyme, such as ROS1. A

5 substrate binds with the enzyme's active site, and an enzyme-substrate complex is formed. In some examples, the enzyme catalyses the incorporation of an atom or other molecule into the substrate, for example a kinase can incorporate a phosphate into the substrate, such as a peptide, thus forming a phospho-substrate.

Tissue: A plurality of functionally related cells. A tissue can be a suspension, a
10 semi-solid, or solid. Tissue includes parts of organs collected from a subject such as the lung, the liver or a portion thereof.

Methods of Diagnosis

Disclosed herein are methods of predicting if a subject with a cancer characterized by aberrant activity of ROS1 would benefit from treatment with foretinib,
15 more particularly if the subject would benefit from treatment with foretinib as opposed to another tyrosine kinase inhibitor such as crizotinib.

In particular examples, the methods include identifying a nucleic acid mutation in the kinase domain of ROS1 (SEQ ID NO: 1) that results in an amino acid substitution selected from G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G,
20 either alone or in combination. Detection of one or more of the above-identified mutations in a tumor sample from a subject indicates that the subject should be treated with foretinib as opposed to another tyrosine kinase inhibitor, particularly crizotinib.

The disclosed mutations may be identified by any suitable method known in the art. For example, they may be detected by any method of nucleic acid sequencing,
25 through any method involving nucleic acid amplification, by any method of detecting a protein with one or more of the disclosed mutations, or any combination thereof. Examples of these methods are discussed in detail below.

In some embodiments, the expression of the disclosed biomarker is detected in a sample of a tumor obtained from a subject. Tumor samples can include cancer cells. Tumor samples can also include normal tissue adjacent to the tumor. This normal tissue may serve as an internal negative control, especially in the case of assays that detect expression of a biomarker in the context of tissue structure, including immunohistochemistry, in situ hybridization, in situ polymerase chain reaction, or microdissection followed by nucleic acid amplification. It will be appreciated by those of skill in the art that any method of obtaining tissue from a subject can be utilized, and that the selection of the method used will depend upon various factors such as the type of tissue, age of the subject, or procedures available to the practitioner. For example, the tissue sample can be obtained by a variety of procedures including, but not limited to, surgical excision, aspiration, or biopsy alone or in combination with each other or other methods.

Detecting Cancer Biomarkers

The disclosed ROS1 kinase domain mutations can be detected in a sample using any one of a number of methods well known in the art. Nucleic acids such as genomic DNA, particularly tumor genomic DNA can be isolated from a tumor sample from a subject. General methods of nucleic acid isolation are well known to those of skill in the art. Such methods are disclosed in standard textbooks and handbooks of molecular biology and embodied in commercially available kits.

The mutations can be detected through nucleic acid sequencing. Sequencing may be performed on genomic DNA from the tumor through any method known in the art including Sanger sequencing, pyrosequencing, SOLiD® sequencing, massively parallel sequencing, barcoded sequencing, or any other sequencing method now known or yet to be disclosed.

In Sanger Sequencing, a single-stranded DNA template, an oligonucleotide primer, a DNA polymerase, and nucleotides are used. A label, such as a radioactive label or a fluorescent label is conjugated to some of the nucleotides. One chain terminator

base comprising a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP, replaces the corresponding deoxynucleotide in each of four reactions. The products of the DNA polymerase reactions are electrophoresed and the sequence determined by comparing a gel with each of the four reactions. In another example of Sanger sequencing, each of the chain termination bases is labeled with a fluorescent label and each fluorescent label is of a different wavelength. This allows the polymerization reaction to be performed as a single reaction and enables greater automation of sequence reading.

In pyrosequencing, the addition of a base to a single stranded template to be sequenced by a polymerase results in the release of a pyrophosphate upon nucleotide incorporation. An ATP sulfurylase enzyme converts pyrophosphate into ATP which in turn catalyzes the conversion of luciferin to oxyluciferin which results in the generation of visible light that is then detected by a camera.

In SOLiD® sequencing, the molecule to be sequenced is fragmented and used to prepare a population of clonal magnetic beads (in which each bead is conjugated to a plurality of copies of a single fragment) with an adaptor sequence. The beads are bound to a glass surface. Sequencing is then performed through 2-base encoding.

In massively parallel sequencing, randomly fragmented targeted DNA is attached to a surface through the use of an oligonucleotide adaptor. The fragments are extended and bridge amplified to create a flow cell with clusters, each with a plurality of copies of a single fragment sequence. The templates are sequenced by synthesizing the fragments in parallel. Bases are indicated by the release of a fluorescent dye correlating to the addition of the particular base to the fragment.

In pyrosequencing, massively parallel sequencing or SOLiD® sequencing, an artificial sequence called a barcode may be added to primers used to clone fragmented sequences or to adaptor sequences. A barcode is a 4-10 nucleic acid sequence that uniquely identifies a sequence as being derived from a particular sample. Barcoding of samples allows sequencing of multiple samples in a single sequencing run. (See Craig DW et al, Nat Methods 5, 887-893 (2008) for descriptions and examples of barcodes.)

DNA sequencing methods can, but need not, rely on nucleic acid amplification of a nucleic acid encoding a protein such as ROS1 genomic DNA, ROS1 cDNA, or the ROS1 kinase domain.

Additional methods of detecting mutations in nucleic acids include detection through selective nucleic acid amplification of mutant sequences. An example of such a method is the amplification refractory mutation system (ARMS) Newton et al, Nucleic Acids Res 17, 2503-2515 (1989.) This method uses a primer that matches the nucleotide sequence immediately 5' of the mutation to be tested with the 3' end of the primer specific for the nucleotide sequence of the mutant. Such a primer will specifically amplify the mutant nucleic acid but not the wild type amino acid. Such reactions may be adapted to real-time PCR based systems such as TaqMan®.

The disclosed mutations may also be identified using a microarray technique. Sequences corresponding to one or more of the disclosed mutants may be plated or arrayed on a microchip substrate. The arrayed sequences are then hybridized to isolated tumor genomic DNA. An array may also be a multi well plate.

The disclosed mutations may also be identified in proteins by, for example, mass spectrometry or antibodies designed to detect proteins with the disclosed mutations.

Methods of treatment

Methods of treating a subject with a cancer characterized by an aberrant ROS1 kinase are provided herein. The methods include selecting a subject with a tumor or tumor clone that has an aberrant ROS1 kinase, such as a subject with a cancer likely to have a FIG-ROS fusion, such as a non-small cell lung cancer or cholangiocarcinoma. The subject is then treated with a ROS1 kinase inhibitor such as foretinib, particularly when the kinase domain of the aberrant ROS1 kinase comprises one or more of the following G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G, including the homologs thereof for non-human mammals.

The administration of foretinib can be for either a prophylactic or a therapeutic treatment. When provided prophylactically, foretinib is provided in advance of any

symptom. The prophylactic administration of the compounds serves to prevent or ameliorate any subsequent disease process. When provided therapeutically, the compounds are provided at (or shortly after) the onset of a symptom of disease. For prophylactic and therapeutic purposes, foretinib can be administered to the subject in a single bolus delivery, via continuous delivery (for example, continuous transdermal, mucosal or intravenous delivery) over an extended time period, or in a repeated administration protocol (for example, by an hourly, daily or weekly, repeated administration protocol). The therapeutically effective dosage of the compound can be provided as repeated doses within a prolonged prophylaxis or treatment regimen that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with a targeted disease or condition. One of skill in the art in light of this disclosure will be able to determine an effective dose of foretinib.

Kits

A kit is an assemblage of components that may be used in the performance of the method. Use of kits provides advantages to the end user of the method in that the components may have been standardized, the components may have been subject to quality assurance, the components may have been subject to sterilization, or the proportions and characteristics of the various components may have been optimized for maximal efficacy. In addition, a kit may provide the advantage that the components of the kit are obtained from a single source. This in turn makes preparations for the performance of the method as well as troubleshooting problems with the method more efficient. Components may be enclosed in one or more containers appropriate for their storage, such as vials, tubes, bottles, or any other appropriate container. The containers may be further packaged into secondary containers such as boxes, bags, or any other enclosure.

A diagnostic kit may contain reagents such as oligonucleotides configured to perform nucleic acid amplification (including TaqMan® amplification) that specifically recognize mutant nucleic acids that cause amino acid changes such as V2098I, G1971E,

L1982F, L1982R, C2060G, L1947R or E1935G in the ROS1 kinase domain. A diagnostic kit may also comprise an array that includes oligonucleotides that detect the disclosed mutations. A diagnostic kit may also contain a set of primers that amplify the kinase domain for sequencing or any other nucleic acid analysis. A diagnostic kit may also
5 comprise antibodies specific for mutant forms of the ROS1 kinase domain, including V2098I, G1971E, L1982F, L1982R, C2060G, L1947R or E1935G.

A kit may further comprise instructions describing how to perform the method. The instructions may be any description of the method that is provided with, referred to by, or otherwise indicated by a component of the kit. The instructions may be
10 communicated through any tangible medium of expression. The instructions may be printed on the package material, printed on a separate piece of paper or any other substrate and provided with or separately from the kit. They may be printed in any language and may be provided in picture form. The instructions may be posted on the internet, written into a software package, or provided verbally through a telephone or
15 by an email conversation or provided as a smart phone application. The instructions may be said to describe how to perform the method if the instructions provide a recipe of how to perform the method, if they refer a user to a publication wherein a description of the method may be found, or in any other way inform any end user of how to perform the method.

20 EXAMPLES

The following examples are illustrative of disclosed methods. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other examples of the disclosed method would be possible without undue experimentation.

25 ***Example 1 – Methods***

The methods described in this Example 1 were used in the generation of the results described in Examples 2-7 below.

Cell culture: The parental Ba/F3 cell line was cultured in RPMI 1640 medium with 10% FBS, L-glutamine, penicillin/streptomycin and 15% WEHI-conditioned media. Ba/F3 cell lines were maintained at densities of 0.5×10^6 to 1.5×10^6 per ml of media. FIG-ROS and SLC-ROS Ba/F3 lines were created by infecting the parental Ba/F3 with retrovirus expressing either human FIG-ROS-S (indicated FIG-ROS herein) or SLC-ROS-S (indicated SLC-ROS herein). Stable transfectant lines were sorted for GFP expression on a FACS Aria® flow cytometer. Cells were counted daily using Guava ViaCount® reagent and Guava Personal Cell Analysis® flow cytometer. NIH3T3 cells were cultured in DMEM-High glucose medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin. Early passage NIH3T3 cells were infected with retrovirus expressing either FIG-ROS or SLC-ROS and sorted for GFP expression as described above for Ba/F3 cells. HCC78 cells were cultured in RPMI 1640 medium with 10% FBS, L-glutamine, and penicillin/streptomycin. This media is referred to as RPMI-10%FBS herein.

Isolation of primary liver progenitor cells and murine cholangiocarcinoma tumor cell line generation: The isolation of liver progenitor cells was performed as previously described in Zender *et al*, *Cold Spring Harb Symp Quant Biol* **70**, 251-261 (2005) (incorporated by reference herein). Hepatoblasts of the genotype AlbCre^{+/-}; Isl Kras^{G12D+/-}; p53^{R172H/loxP} were isolated from ED14.5 mouse embryos and retrovirally transduced with either the FIG-ROS fusion, turbo RFP or a short hairpin RNA against Pten (shPten.1522). At 48 hours following transduction, 1×10^6 cells were resuspended in 25 μ l Matrigel and injected sub-capsular into the livers of immune-deficient mice. Upon tumor formation, tumors were explanted and subjected to collagenase digestion. Digests were plated onto gelatin-coated plates. Tumor cells were enriched by either differential trypsinization or cell sorting. Two cell lines derived by this method expressing FIG-ROS were designated lines 3, 4 and 6. Two cell lines derived by this method expressing shPten were designated lines 1 and 2. These four cell lines were

further cultured and maintained in DMEM-High glucose medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin.

Cell Viability Assay: Cell lines were distributed in 96-well plates and incubated with the indicated concentrations of inhibitor for 72 hours. Adherent cell lines (for example, HCC78, and the murine cholangiocarcinoma (mCC) cell lines described above) were plated 12 to 18 hours before adding inhibitor. Ba/F3 cell lines were plated at 4×10^3 cells/well. HCC78 and mCC cell lines were plated at 1.5×10^3 cells/well. All cell types were seeded in a final volume of 80 microliters. A 20 microliter volume of five-fold concentrated inhibitor media was added. Cell proliferation was measured using a methanethiosulfonate (MTS)-based viability assay (CellTiter96 Aqueous One® Solution; Promega). A BioTek Synergy 2® plate reader was used to read the absorbance at 490 nm at 30 minutes and 1 hour after addition of MTS. To facilitate comparison between experiments, the raw MTS absorbance of vehicle (0.1% DMSO) treated cells was set to 1 and absorbance from inhibitor treated wells was normalized to the vehicle treated value. These normalized values are used to plot the graphs shown in the Figures. Each experiment had a minimum of 3 wells per condition and the average and SEM was plotted for curve fit analysis. Data were normalized using Microsoft Excel® and further non-linear regression curve fit analysis of the normalized data for determination of IC₅₀ was performed using Graphpad Prism® software.

Plasmid Construction: The FIG-ROS-S fusion gene was synthesized using the GeneArt® service. FIG-ROS-S is denoted as FIG-ROS herein. FIG-ROS was further subcloned into the retroviral vector, pMSCV-IRES-GFP (pMIG) using the Gateway® Cloning system. SLC-ROS-S (SLC-ROS) was cloned from cDNA made from HCC78 cells. PCR amplification using a sense primer that was specific for the N-terminus of SLC34A2 (5' CAC CAT GGC TCC CTG GCC TGA ATT GG) (SEQ ID NO: 2) and an anti-sense primer specific for the C-terminus of ROS1 (5' TTA ATC AGA CCC ATC TCC ATA TCC ACT GTG AGT G) (SEQ ID NO: 3) amplified both SLC-ROS-L and SLC-ROS-S from HCC78 cDNA. The PCR products corresponding to SLC-ROS-L and SLC-ROS-S were gel purified and cloned into

the Gateway Cloning system compatible entry vector, pENTR-D/TOPO. They were then further subcloned into the pMIG retroviral vector as described above. The SLC-ROS-L fusion did not transform Ba/F3 after IL-3 withdrawal so all further inhibitor testing was performed with the SLC-ROS-S, indicated as SLC-ROS. A pMSCV-turboRFP-IRES-GFP construct was generated by inserting turboRFP into the pMIG backbone using BglII/XhoI restriction sites. A pMSCV-shPten.1522-SV40-GFP construct was also generated. FIG-ROS point mutations were created using the Quikchange® Site Directed mutagenesis kit according to the manufacturer's protocol.

IL-3 Withdrawal/Transformation assay: Parental Ba/F3, pMIG-alone, FIG-ROS (wildtype or ROS1 kinase domain mutant variants) or SLC-ROS expressing Ba/F3 cells (3×10^6 cells total) were washed three times with 50 ml of RPMI-10% FBS media and re-suspended in 6 mls of fresh RPMI-10% FBS media. The total number of viable cells was counted every other day using a Guava ViaCount® reagent and a Guava Personal Cell Analysis® flow cytometer. If cells grew to cell densities $> 1.5 \times 10^6$ /ml in withdrawal media, the cells were centrifuged and resuspended in fresh media to keep final culture density of 0.5×10^6 /ml.

In vivo treatment of tumor bearing mice with inhibitors: Foretinib was dissolved in DMSO (373 mg/ml), aliquoted and stored at -80°C . Prior to use, aliquots were thawed and further diluted in 1% Hydroxypropylmethylcellulose/0.2% SDS for a final foretinib concentration of 3.25 mg/ml. Crizotinib was reconstituted in 1% Hydroxypropylmethylcellulose and 0.2% SDS to 3.25 mg/ml, aliquoted and stored at 80°C until use. Mouse chorangiocarcinoma cell lines expressing either FIG-ROS or shPten were subcutaneously injected into 11 week old female immune-deficient mice. Upon reaching a tumor diameter of 4-6 mm, mice were treated with foretinib (25 mg/kg; Molecular Weight = 632.65), crizotinib (25 mg/kg; Molecular Weight = 450.34) or vehicle control by oral gavage once daily for 9 consecutive days. Caliper measurements were taken at the time of treatment initiation and 24 hrs after administration of the last dose. Data in waterfall plots is calculated as follows: $((\text{tumor volume } T_{\text{end}} / \text{tumor volume } T_0) -$

1). Caliper measurements of tumors were taken at T_0 and T_{end} . Tumor volume is calculated as: $0.5 \times L \times W^2$ ($L > W$).

Immunoblotting: Ba/F3 FIG-ROS, Ba/F3 SLC-ROS, HCC78 and mCC cell lines were treated with the indicated concentration of inhibitors for 1-2 hours. For Ba/F3 derived cell lines: after the completion of treatment, 5×10^6 cells were pelleted, washed once in ice-cold PBS and lysed in 200 μ l of cell lysis buffer supplemented with 0.25% deoxycholate, 0.05% SDS, and protease and phosphatase inhibitors. The protein content of the cell lysates was determined and lysates were either used for immunoprecipitation where indicated or extracted with SDS Sample buffer for 15 minutes at 80°C. Proteins were transferred to Immobilon-FL membranes and immunoblotted with antibodies specific for phospho-ROS1 (Cell Signaling Technology (CST) #3078), total ROS1 (CST #3266), phospho-Shp2 (CST #3751), total Shp2 (CST #3752), phospho-Stat3 (CST #9145), total Stat3 (CST #4904), phospho-Erk1/2 (CST #9101), total Erk2 (Santa Cruz #SC-1647), phospho-S6 (CST #4858), total S6 (CST #2216), phospho-Src (CST #2105), total Src (CST #2110), α -tubulin (Sigma T6199), or β -actin (Sigma A1978) as indicated. Images of the blots were generated by the LI-COR Odyssey® imaging system, following the manufacturer's protocol for immunoblot detection with use of IR-dye conjugated secondary antibodies.

Accelerated cell-based mutagenesis screen: Ba/F3 cells expressing FIG-ROS were treated overnight with N-ethyl-N-nitrosourea (ENU; 50 μ g/ml), pelleted, resuspended in fresh media, and distributed into 96-well plates at a density of 1×10^5 cells/well in 200 μ l complete media supplemented with crizotinib ranging from 500 nM to 2000 nM. The wells were observed for cell growth under an inverted microscope and media color change every two days for one month. The contents of wells exhibiting cell outgrowth were transferred to a 24-well plate containing 2 mL complete media supplemented with crizotinib at the same concentration as in the initial 96-well plate. At confluency, cells in 24-well plates were collected by centrifugation. DNA was extracted from the cell pellets using a DNEasy® Tissue kit (QIAGEN). The FIG-ROS kinase domain was amplified using

primers FIG-ROS M13-Kin3F (5' GTAAAACGACGGCCAGTGCAGACTAGCTGCCAAGTAC 3') (SEQ ID NO: 4) and ROS M13-Kin1 REV (5' CAGGAAACAGCTATGACCGCCATCTTCACCTTCAAAGC 3') (SEQ ID NO: 5) and the PCR products were bi-directionally sequenced using M13F (5' GTAAAACGACGGCCAGTG 3') (SEQ ID NO: 6) and M13Rev (5' CAGGAAACAGCTATGACC 3') (SEQ ID NO: 7) primers. The chromatographs were analyzed for mutations using Mutation Surveyor software (SoftGenetics). We note here that we chose to expand clones only from 750 to 1500 nM crizotinib-treated plates since we observed no outgrowth of clones at 2000 and 2500 nM and a 40% frequency at 500 nM crizotinib.

Statistical Analysis: Where indicated the Student's t-test was used to determine statistical significance. Comparisons with a p value less than 0.05 when were deemed statistically significant. An asterisk in the figures and/or figure legends indicates statistical significance.

Example 2 – Screen for ROS1 inhibitors

The FIG-ROS-S (FIG-ROS) fusion protein has been reported in non-small cell lung cancers (NSCLC), glioblastomas, and cholangiocarcinomas. The expression of FIG-ROS in Ba/F3 or NIH3T3 cell lines is sufficient to transform those lines (Gu TL *et al*, PLoS One **6**, e15640 (2011); incorporated by reference herein.) Disclosed herein are the results of using a screen to identify kinase inhibitors that are selectively cytotoxic to FIG-ROS transformed Ba/F3 cells. The screen is described in detail in Tyner JW *et al*, *Cancer Res* **73**, 285-296 (2013).

Foretinib (GSK1363089, XL-880) (N1'-[3-fluoro-4-[[6-methoxy-7-(3-morpholinopropoxy)-4-quinolyl]oxy]phenyl]-N1-(4-fluorophenyl)cyclopropane-1,1-dicarboxamide) and Gö6976 (Go6976) (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole) were shown to be potent ROS1 inhibitors (Fig. 1A) with 50% growth inhibitory concentration (IC₅₀) of 2.2 nM and 1.4 nM respectively. The complete list of inhibitors tested is in Table 1.

Table 1: MTS values of compounds tested for ROS1 inhibition – all normalized to an internal “no drug” control. Foretinib is referred to as XL-880 in this table. Shaded entries are mentioned in text of Example 2.

DRUG ID	Concentration of Drug (μM)						
	0.014	0.041	0.123	0.370	1.111	3.333	10.000
Nilotinib	0.509	0.808	0.770	0.656	0.754	0.889	0.613
PKC-412	1.000	1.100	0.839	0.083	-0.020	0.090	-0.024
MLN-518	0.872	0.841	0.889	0.878	0.900	0.702	0.151
Compound 7	0.967	0.976	1.145	0.970	1.009	1.000	1.118
CHIR-258	0.969	0.825	0.812	0.654	0.135	0.074	0.076
pan-JAK	1.000	1.200	0.910	1.013	0.897	0.874	0.715
Sunitinib	0.665	0.980	1.030	1.150	1.000	0.907	0.837
Sorafenib	1.160	1.120	1.000	1.050	1.120	1.100	0.155
Dasatinib	0.435	1.081	0.508	0.973	0.502	0.710	0.251
Cytosia	1.150	0.900	0.980	1.000	1.100	0.380	0.157
SYK	1.247	1.311	1.232	1.209	1.079	0.470	0.256
AKT IV	1.200	1.000	0.666	-0.014	0.090	0.008	0.151
PRT063607	1.137	1.014	1.108	0.968	1.020	1.083	1.050
PRT062070	1.125	1.278	1.060	1.250	1.130	1.000	0.207
SU-14813	0.944	0.907	0.993	1.054	0.972	0.718	0.433
AST-487	1.000	0.873	1.007	0.920	0.886	1.060	1.085
Cal101	0.973	1.059	0.989	1.038	0.906	0.885	0.385
Compound 9	1.000	1.183	1.058	0.989	1.050	0.976	1.066
LY294002	1.009	0.958	0.834	0.379	0.421	0.149	0.031
AKTX	0.891	0.967	0.904	0.962	0.981	0.346	1.112
Compound 2	1.046	1.094	1.040	0.990	0.997	0.861	0.737
PD98059	0.957	1.128	0.987	0.984	0.997	0.963	1.120
MEK1/2	1.080	1.107	1.077	0.766	0.766	0.986	0.791
JNK II	1.243	1.323	1.127	1.237	1.094	1.080	0.779
p38	0.507	0.873	0.460	0.944	0.344	0.868	0.348
BIRB-796	1.083	0.982	1.016	1.065	0.877	1.099	1.251
SB-202190	1.100	1.145	1.223	0.572	0.522	0.447	0.547
SB-203580	1.120	1.210	1.190	1.260	1.250	1.165	1.100
PP2	1.144	0.902	1.014	1.035	0.807	0.660	0.695
AC-220	0.976	1.084	1.030	1.090	0.690	0.963	0.484
AP24534	1.106	1.183	1.150	0.987	1.286	1.021	0.043
Velcade	1.000	0.940	0.765	0.175	0.039	0.030	0.035
Flavopiridol	1.124	0.741	0.368	0.152	0.134	0.139	0.136
H-90	1.026	0.995	1.173	0.957	1.084	0.617	0.401
NF-kB	0.761	0.631	0.580	0.587	0.512	0.470	0.397
GW-2580	1.000	1.120	1.090	1.065	0.985	0.192	0.524
Compound 6	1.070	0.796	0.921	0.871	0.834	0.844	0.792

VX-745	1.250	1.150	1.200	1.090	1.000	0.606	0.898
STO609	1.028	0.863	0.998	0.899	0.948	0.854	0.913
AMPK	0.900	0.947	0.990	1.020	1.000	0.419	0.700
Go6976	0.068	0.014	0.007	0.074	0.020	0.072	0.024
KN92	1.145	1.126	1.215	1.180	1.000	0.788	1.024
KN93	1.065	0.582	1.121	0.553	0.580	0.289	0.459
VX-680	1.043	0.800	1.139	0.436	0.057	0.065	0.026
MLN-8237	0.805	0.641	0.443	0.658	0.800	0.055	0.037
JNJ-7706621	1.213	1.103	1.056	0.847	0.693	0.186	0.039
CYC-202	0.968	1.011	0.898	0.940	0.921	0.683	0.346
LENALDAKAR	1.000	0.727	0.764	0.844	0.764	0.276	0.250
Nilotinib_2	1.125	1.230	1.210	1.150	1.000	0.399	0.238
PD153035	1.028	0.939	0.953	1.100	0.992	1.036	1.226
Crenolanib	0.870	0.877	0.809	0.532	0.194	0.149	0.020
EKB-569	1.121	1.058	0.891	0.610	0.173	0.160	0.144
Erlotinib	1.000	0.875	0.749	0.985	1.000	0.980	0.971
Gefitinib	1.252	1.250	1.190	1.145	1.203	1.060	0.356
Lapatinib	0.674	1.086	0.723	1.148	0.777	1.090	0.082
CI-1033	1.150	0.617	1.039	1.100	1.000	1.036	0.349
AP24534_2	1.022	1.035	1.172	1.156	1.120	1.082	0.144
ZD-6474	0.955	1.037	0.869	0.972	1.099	0.278	0.040
Sunitinib_2	0.991	1.033	0.876	0.915	1.024	1.161	0.618
AC-220_2	0.621	0.724	0.658	0.806	0.111	0.116	0.059
Sorafenib_2	1.054	1.273	1.174	1.228	1.315	1.003	0.384
PTK-787	1.137	1.260	1.180	1.060	1.170	1.000	1.060
GW-786034	0.932	0.893	1.022	1.012	0.697	0.379	0.005
AMG-706	1.000	0.978	0.966	1.182	1.056	0.998	0.924
ABT-689	1.107	1.057	1.159	1.091	1.175	0.789	0.068
CHIR-265	1.140	0.909	0.964	0.676	0.580	0.453	0.259
BMS-387032	0.952	0.930	0.738	0.475	0.113	0.025	0.061
AZD-1152	0.977	0.871	0.229	0.226	0.162	0.144	0.180
PI-103	0.873	0.733	0.501	0.168	0.142	0.066	0.059
SB-431542	0.896	0.851	0.939	0.839	0.872	0.799	0.634
SJB-66	1.019	1.010	0.278	0.233	0.210	0.245	0.229
CP-690550	2.820	3.414	3.195	4.917	2.865	2.797	2.511
CX5461	0.325	0.107	0.091	0.033	0.013	-0.094	-0.084
X-082	1.020	1.000	1.000	1.100	1.050	1.060	1.090
Rapamycin	0.953	0.872	0.730	0.429	0.622	0.242	0.212
CPI-267703	1.000	0.785	0.230	0.042	0.174	0.260	0.155
WS158	1.126	1.126	0.988	1.099	1.181	0.944	0.877
INK-128	0.561	0.291	0.076	-0.008	0.013	0.177	-0.008
Imatinib	0.915	1.130	0.888	1.120	0.987	0.790	0.186

R406	1.000	1.200	1.150	1.090	0.417	0.486	0.232
CX4945	1.133	0.966	1.054	0.971	1.014	0.752	0.262
CX6258	1.120	1.090	1.150	0.938	0.314	0.163	0.066
PWT-33597	0.863	0.800	0.679	0.433	0.241	0.062	0.027
X-319	0.897	0.981	0.838	0.718	0.446	0.198	0.289
Celator	0.642	0.319	0.081	-0.002	-0.005	-0.020	-0.005
X-376	0.862	0.966	0.819	0.552	0.179	0.112	0.110
X-379	0.651	1.108	0.578	0.468	0.159	0.089	0.034
X-389	0.872	0.887	0.558	0.426	0.071	0.234	0.069
X-367	0.849	0.767	0.833	0.614	0.586	0.414	0.454
Cal120	0.780	0.951	0.782	0.821	0.648	0.363	0.427
Cal129	1.000	0.741	0.845	1.120	1.080	1.060	0.765
Dasatinib_2	0.795	0.952	0.897	1.358	1.076	0.897	0.742
LY-333531	1.153	1.137	0.902	1.063	0.721	0.426	0.005
MerckAKT	0.743	0.585	0.511	0.578	0.269	0.069	0.022
GDC-0941	1.013	0.899	0.848	0.543	0.404	0.136	0.164
YM-155	1.000	0.960	0.939	1.567	1.061	1.614	0.484
Staurosporin	0.291	0.291	0.321	0.318	0.226	0.211	0.135
BEZ235	1.046	0.920	0.713	0.223	0.114	0.114	0.158
GSK-1120212	0.648	0.557	0.357	0.197	0.297	0.177	0.285
PP242	0.733	0.638	0.597	0.480	0.233	0.157	0.129
TG-101348	1.088	1.171	0.964	1.330	0.288	0.411	0.081
AB-1010	0.827	0.942	0.946	1.216	1.177	1.022	0.950
AG-013736	0.951	0.766	0.854	0.851	0.538	0.371	0.153
R457	1.013	0.971	0.775	0.768	0.267	0.233	0.226
CEP-701	1.263	1.031	0.799	0.984	0.206	0.147	
GSK-1838705A	0.807	0.889	1.012	1.003	0.468	0.135	0.103
MLN-120B	0.652	0.927	1.000	1.070	1.063	1.010	1.494
JNJ-28312141	1.084	1.003	1.177	0.994	1.049	1.026	0.760
SKI-606	0.894	1.073	0.882	0.911	0.342	0.383	0.046
AT-7519	1.081	0.917	1.088	0.822	0.593	0.278	0.322
AZD-1480	1.059	1.033	0.912	0.747	0.376	0.225	0.185
AZD-2171	0.892	1.014	1.203	1.017	1.014	0.326	0.084
AZD-6244	0.956	0.959	0.831	0.871	0.708	0.763	0.419
BI-2536	0.819	0.703	0.231	0.204	0.180	0.130	0.092
BIBW-2992	1.012	0.985	0.856	0.889	0.284	0.305	0.044
Crenolanib_2	0.986	0.979	0.773	0.678	0.258	0.323	0.119
XL-880	0.122	0.124	0.113	0.186	0.169	0.116	0.099
GDC-0879	0.981	1.314	1.221	1.324	1.289	1.794	1.227
HKI-272	1.728	3.779	3.250	1.882	1.838	3.404	3.096
GSK-690693	1.033	0.900	0.811	1.078	0.963	0.808	0.612
KW-2449	0.964	0.835	0.564	0.887	0.544	0.395	

NVP-TAE-684	0.556	0.098	0.128	0.123	0.104	0.047	0.104
PF-2341066	0.865	0.868	0.106	0.237	0.098	0.040	-0.057
PHA-665752	3.185	3.586	4.000	4.065	4.879	2.759	2.267
PLX-4720	0.825	0.724	0.724	0.929	0.893	0.663	0.750
SGX-523	1.064	0.769	1.013	0.845	0.916	0.656	0.811
TG-100-115	0.673	0.561	0.347	0.679	0.654	0.739	0.358
KI-20227	0.966	0.761	0.858	0.966	0.830	0.738	0.782
PD-173955	1.206	1.074	0.762	0.883	0.544	0.405	0.152
A-674563	1.305	1.201	1.150	0.942	0.715	0.497	0.225
BMS-345541	1.529	1.167	1.192	0.978	0.938	0.394	0.109
PD-184352	1.066	0.968	0.796	0.884	0.809	0.880	0.665
AP24534_3	1.501	0.975	1.181	1.518	1.207	1.252	0.185
Dasatinib_3	1.012	0.901	1.034	0.952	0.797	0.774	0.716
Nilotinib_3	1.240	1.092	1.071	0.989	0.779	0.742	0.162
Sorafenib_3	0.939	0.866	0.873	0.827	0.853	0.737	0.568
Sunitinib_3	1.473	1.401	1.068	1.068	1.080	0.777	
PCI-62735	1.453	1.093	0.962	0.852	1.058	0.930	0.859
PIM123	0.606	0.528	0.380	0.375	0.297	0.211	0.138
PIM13	1.034	0.727	0.794	0.541	0.371	0.392	0.386
Vargetef	0.807	1.013	0.898	1.190	0.213	0.440	0.202
GDC-0941_2	1.004	0.520	0.891	0.742	0.566	0.386	0.388

Human ROS1 and anaplastic lymphoma kinase (ALK) share 42-48% amino acid homology in their respective kinase domains, suggesting that ALK tyrosine kinase inhibitors will inhibit ROS1. Recent publications show that the MET/ALK inhibitor, crizotinib (PF-02341066 in Table 1 above), and the ALK inhibitor, TAE684 (NVP-TAE684 in Table 1 above) inhibit the proliferation of Ba/F3 cells transformed by ROS1-fusions as well as the human NSCLC cell line, HCC78, which is characterized by an SLC-ROS fusion (Rikova K *et al*, *Cell* **131**, 1190-1203 (2007)).

Those results were confirmed by the screen disclosed herein: crizotinib and TAE684 inhibited Ba/F3 FIG-ROS-S cell growth with an IC₅₀ of 38 nM and 2 nM respectively. In addition, Davis *et al* published the binding affinities (K_D) of a total of 72 kinase inhibitors for a total of 442 kinases including ROS1 (Davis MI *et al*, *Nature Biotechnology* **29**, 1046-1051 (2011) and Fabian MA *et al*, *Nature Biotechnol* **23**, 329-336 (2005). In the Davis *et al* (2011) study, the binding affinity of crizotinib (4 nM) for ROS1

is lower than that of foretinib (14 nM). However, in the screen disclosed herein, the IC₅₀ of foretinib (2 nM) for FIG-ROS in a FIG-ROS transformed Ba/F3 cell is 19-fold lower than crizotinib (38nM). Davis *et al* (2011) reported 19 inhibitors as having disassociation constants of less than 10 μ M for ROS1. Of these, crizotinib, TAE684, foretinib and GSK1838705A were reported as having disassociation constants of less than 20 nM.

Table 2: Binding affinity of selected inhibitors of the indicated kinases

Inhibitor	ROS1	ALK	MET	PDGFRA	IGF1R	EGFR WT	Primary reported target
NVP-TAE684*	0.49	1.1	170	840	2.7	180	ALK
Crizotinib*	4.1	3.3	2.1	> 10,000	780	> 10,000	MET, ALK
Foretinib (XL-880)*	14	69	1.4	4.5	430	440	MET, VEGFR2
GSK1838705A*	15	0.55	> 10,000	> 10,000	7	4100	IGF1R, ALK
Go6976 [§]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	PKC, JAK2, PLK4

* - Davis et al, 2011 *supra*

§ inhibitory activity for PKC described in Martiny-Baron *et al*, *J Biol Chem* **268**, 9194-9197 (1993); inhibitory data for JAK2 reported in Grandage *et al*, *Br J Haematol* **135**, 303-316 (2006); both of which are incorporated by reference herein

N.A. – No data available for binding activity

Example 3 – Further selection of potential ROS-1 inhibitors

The relative potency of crizotinib, foretinib, Gö6976, TAE684 and GSK1838705A for inhibiting ROS1 and ALK were tested in Ba/F3 expressing FIG-ROS. The results of this experiment are shown in Figure 1A. In addition, IC₅₀ for the above listed inhibitors were determined in Ba/F3 cells expressing each of the following proteins: cytoplasmic FIG-ROS, membrane associated SLC-ROS, Bcr-Abl, JAK3 comprising an A572V mutation, and ALK comprising a F1174L mutation. The Ba/F3 parental line was also tested. (O'Dwyer ME and Druker BJ, *Lancet Oncology* **1**, 207-211 (2000); Walters DK *et al*, *Cancer Cell* **10**, 65-75 (2006); and Heukamp LC *et al*, *Science Translational Medicine* **4**, 141ra191 (2012)). The results are shown in Figure 1B. ALK F1174L was relatively insensitive to foretinib (IC₅₀ = 1800 nM), but foretinib was particularly potent in the cells expressing FIG-ROS (2 nM) and SLC-ROS (10 nM). Both TAE684 and GSK1838705A were effective ALK F1174L inhibitors with IC₅₀ of 0.5 nM and 10 nM respectively. FIG-ROS and SLC-ROS were

sensitive to TAE684 with IC_{50} of 1.8 nM and 28 nM respectively. Both ROS-fusions were relatively insensitive to GSK1838705A with IC_{50} of 250 nM for FIG-ROS) and 1000 nM for SLC-ROS.

The reported disassociation constants of foretinib and GSK1838705A for ROS1 are 14 nM and 15 nM respectively, yet these compounds have strikingly different inhibitory efficacy for Ba/F3 FIG-ROS cell growth (Figure 1B). As previously reported in Sasaki T *et al*, *Cancer Res* **70**, 10038-10043 (2010) ALK F1174L is partially crizotinib-resistant (IC_{50} = 103 nM), particularly when compared to TAE684. Ba/F3 Bcr-Abl, JAK3 A572V and parental cells are all insensitive to the crizotinib, foretinib, Gö6976 and TAE684 up to 2000 nM and resistant to GSK1838705A up to 10,000 nM.

Immunoblots in Figure 1C show a dose dependent decrease in autophosphorylation of FIG-ROS and SLC-ROS as well as ROS1-driven signaling pathways such as phospho-Shp2 and phospho-ERK1/2 in Ba/F3 cells when treated with increasing amounts of crizotinib, foretinib, Gö6976, and TAE684. Foretinib and Gö6976 (15 nM) induced apoptosis in Ba/F3 FIG-ROS and SLC-ROS cells but not ALK F1174 cells (Figure 5A).

Example 4 – Comparison between crizotinib and foretinib

The relative effects of crizotinib and foretinib to suppress the viability of HCC78 cells was compared. The HCC78 line comprises an SLC-ROS mutation. Foretinib inhibited HCC78 cell growth with an IC_{50} of 425 nM compared to crizotinib (IC_{50} = 2200 nM) (Figure 1D). Furthermore, foretinib inhibited both SLC-ROS and ERK1/2 phosphorylation in HCC78 cells at a lower concentration than did crizotinib (Figure 1E). In addition, HCC78 colony formation (Figure 1F) and cell migration (Figure 6A) were also more potently inhibited by foretinib than crizotinib.

Foretinib and crizotinib activity were also tested using the human non-small cell lung cancer cell lines, PC9 and HCC4011. Cell growth in both lines is driven by activated EGFR and both lines are sensitive to erlotinib. However, both cell lines are relatively insensitive to foretinib and crizotinib (Figure 6B).

In addition to the SLC-ROS fusion protein described above, the HCC78 cell line also has an activated MET activation. However, the silencing of ROS1 in HCC78 cells using ROS1 specific siRNA results in a loss of cell viability. Therefore, HCC78 cells require SLC-ROS for oncogenic growth. Crizotinib is a dual specificity, MET/ALK inhibitor. As a result, it is possible that the crizotinib sensitivity of HCC78 cells may be due to inhibition of MET and therefore independent of ROS1. (Komiya T *et al*, *Off J Am Soc Clin Oncol* **30**, 3425-3426 (2012) incorporated by reference herein) Foretinib is also a MET inhibitor. Therefore, it was possible that other MET inhibitors (MGCD-265, SGX-523 and JNJ38877605) could decrease HCC78 cell growth. However, HCC78 cells are resistant to MGCD-265, SGX-523 and JNJ38877605, suggesting that the effect of foretinib and crizotinib in these cells is due to the inhibition of SLC-ROS and not MET (Figure 6C). Foretinib strongly suppressed FIG-ROS and SLC-ROS phosphorylation and anchorage-independent colony formation at lower doses than crizotinib in NIH3T3 cells transformed with both FIG-ROS and SLC-ROS (Figure 7A and Figure 7B).

Example 5 – Inhibition of FIG-ROS kinase fusions in mouse cholangiocarcinoma cell lines by foretinib

Cholangiocarcinoma (CC) or cancer of the bile duct is the second most common hepatic malignancy. It is refractory to treatment and has a median survival of less than two years. Recently, FIG-ROS kinase fusions have been reported in 8.7% of cholangiocarcinoma patients, (Gu TL *et al*, 2011 *supra*). Murine cholangiocarcinoma tumor cell lines were therefore generated as described in Example 1 above. Pten null murine cholangiocarcinoma tumor cell lines expressing a short hairpin RNA that silences Pten (shPten) were generated as a control.

Cell viability assays were used to determine the efficacy and selectivity of crizotinib and foretinib to block cell growth of FIG-ROS (Lines 3, 4, 6) and shPten-expressing (Lines 1, 2) murine cholangiocarcinoma tumor cells. Both foretinib (IC_{50} = 300 nM) and crizotinib (IC_{50} = 690 nM) inhibited the growth of FIG-ROS but not of shPten cholangiocarcinoma tumor cell lines (IC_{50} > 2000 nM) (Figure 2A). Crizotinib and

foretinib also inhibited the ability of FIG-ROS cholangiocarcinoma tumor cells but not shPten cholangiocarcinoma tumor cells to form anchorage-independent colonies in soft agar. The inhibition was dose dependent (Figure 2B). To directly assess inhibition of FIG-ROS phosphorylation, immunoblot analyses of lysates from vehicle or inhibitor-treated cells were performed. At 100 nM, foretinib almost completely inhibited auto-phosphorylation of FIG-ROS and of phosphorylation of Shp2 and ERK1/2. Crizotinib at the same concentration had a more moderate effect on the phosphorylation (Figure 2C).

Example 6 – Foretinib treatment of FIG-ROS kinase fusions in mouse

cholangiocarcinoma cell lines in vivo

Tumors were formed by subcutaneous injection of the murine cholangiocarcinoma cell lines described above in the flanks of immunodeficient mice. Upon reaching a measurable tumor diameter, the mice were treated with either vehicle, foretinib (25 mg/kg), crizotinib (25 mg/kg) once daily by oral gavage. Caliper measurements were taken at the time of treatment initiation (T_i) and 24 h after administration of the last dose (T_{end}). After 9 days of treatment, the tumors were explanted and measured (Figure 3A). The waterfall plots down in Figure 3B, show the effect of inhibitor treatment on relative tumor growth suppression and/or regression as calculated from T_i and T_{end} caliper measurements taken. Foretinib dramatically and significantly reduced FIG-ROS tumor volume ($p < 0.0001$ for Lines 4 and 6) compared to shPTEN tumors ($P = 0.068$ and 0.058 for Lines 1 and 2) (Figure 3A, Figure 3B, and Figure 8). Equal dosing with crizotinib had a moderate but statistically insignificant effect on FIG-ROS tumor volume.

To biochemically assess the efficacy of oral foretinib treatment on intra-tumoral suppression of FIG-ROS and ROS-1 effector pathway phosphorylation, FIG-ROS tumors were harvested from mice treated with vehicle or 25mg/kg foretinib at 1, 2, 4 and 8 hours after oral gavage and processed for immunoblotting. Rapid and robust inhibition of FIG-ROS phosphorylation was observed in foretinib-treated animals compared to

vehicle-treated animals (Figure 3D). Concomitant with diminished FIG-ROS activation, phosphorylation of Shp2 and Stat3, but not Src is inhibited in the treated tumors.

Example 7 –ROS1 kinase domain mutations that confer resistance to crizotinib

A subset of patients treated with tyrosine kinase inhibitors develop resistance to therapy due to de novo acquisition of kinase domain mutations or other compensatory cellular mechanisms that promote cancer cell growth in presence of inhibitor (Garraway LA and Janne PA *Cancer Discovery* **2**, 214-226 (2012) and Zhang J *et al*, *Nature Rev Cancer* **9**, 28-39 (2009)). Patients with resistant tumors no longer respond to therapy and face poor prognosis unless they can be treated with a secondary agent that suppresses cell growth. For example, second generation Abl kinase inhibitors like dasatinib are successfully used to treat imatinib-resistant CML patients harboring Bcr-Abl KD mutations.

Crizotinib is currently being evaluated in clinical trials as a therapeutic option of ROS1-rerrangments in lung cancer. Partial response of ROS1-fusion bearing patients to crizotinib therapy was recently reported (Shaw AT *et al*, *J Clin Oncol* **30**, 7508 (2012)). Due to its FDA-approved status, partial therapeutic response and clinical momentum, it is likely that crizotinib will be routinely used to treat lung cancer patients harboring ROS1-fusions in near future. A subset of these ROS1-fusion patients may become resistant to crizotinib therapy, as observed for ALK-fusion lung cancer patients treated with crizotinib (Katayama R *et al*, *Science Translational Med* **4**, 120ra117 (2012) and Doebele RE *et al Clin Cancer Res* **18**, 1472-1482 (2012)).

ROS1 kinase domain mutations that confer resistance to crizotinib may be as a predictive strategy for future patient diagnostic and therapy. An N-ethyl-N-nitrosourea (ENU)-assisted accelerated mutagenesis screen was performed using Ba/F3 FIG-ROS cells in the presence of graded concentrations of crizotinib. Multiple ROS1 single and compound kinase domain mutations were found (C2060G, V2098I, G1971E, L1982F, L1974R, E1935G, G1971E/L1982F, C2060G/V2098I) that substantially reduce the efficacy

of crizotinib. The frequency of occurrence of these mutations in crizotinib resistant clones is shown in Figure 4A.

In order to understand how the mutations might confer resistance to inhibition homology models of the kinase domain of ROS1 bound to crizotinib and foretinib were constructed based on crizotinib-bound structure of ALK and foretinib-bound structure of MET. The L1947R, L1982FR, and V2098I mutations map close to structural features that are implicated in inhibitor binding (nucleotide binding loop, helix α C, and the activation loop respectively.) Mutations of L1152 in ALK, a residue homologous to L1982 in ROS1, also conferred crizotinib-resistance (Zhang S *et al*, *Chem Biol Drug Design* **78**, 999-1005 (2011)) and an ALK L1952R mutation was recently identified in a NSCLC patient that had developed clinical resistance to crizotinib (Sasaki T *et al*, *Cancer Res* **71**, 6051-6060 (2011)).

Example 7 – ROS1 kinase domain mutations in FIG-ROS confer resistance to crizotinib

FIG-ROS constructs comprising six of the most frequently recovered crizotinib-resistant mutants (V2098I, G1971E, L1982F, C2060G, L1947R, and E1935G) were generated. All transformed Ba/F3 cells (Figure 9B). All FIG-ROS mutants exhibited increased resistance to crizotinib (IC₅₀ range 350 to 2450 nM, Figure 4C and Figures 10A, 10B, and Table 3). This is close to the established steady state maximum plasma concentration C_{max} for crizotinib (411 ng/ml; 913 nM), suggesting that some of the mutations may be inefficiently inhibited at physiologically relevant concentrations.

Notably, while all the tested FIG-ROS mutants also demonstrated decreased sensitivity to foretinib relative to FIG-ROS without a kinase domain mutation, the foretinib IC₅₀ of each mutant was less than 20 nM (Figure 4C, Figure 10B, Table 3), which is well below the steady state C_{max} for foretinib of 340 nM. The differential resistance to crizotinib and foretinib was further evidenced by the reduced phosphorylation of FIG-ROS mutants relative to FIG-ROS without a kinase domain mutation (Figure 10C)

Table 3 – IC₅₀ of crizotinib and foretinib for Ba/F3 FIG-ROS kinase domain mutants

Mutant	Crizotinib IC ₅₀	Foretinib IC ₅₀
--------	-----------------------------	----------------------------

None	37.5	2.03
C2060G	690	13.6
V2098I	901	9.9
G1971E	605	8.69
L1982F	2450	15.27
L1947R	1420	17.87
E1935G	350	6.6

Example 8 – A G2032R mutation in the ROS1 kinase domain confers resistance to crizotinib, but sensitivity to foretinib

Therapeutic resistance to crizotinib in a CD74-ROS-rearranged lung
 5 adenocarcinoma patient resulting from the acquisition of a G2032R kinase domain point mutation which precludes crizotinib binding due to steric hindrance was reported in Awad MM *et al*, *New Engl J Med* **368**, 2395-2401 (2013); which is incorporated by reference herein.

To assess whether the G2032R mutation retained sensitivity to foretinib similarly
 10 to the ROS1 mutations recovered in the accelerated mutagenesis screen, the efficiency of foretinib to suppress ROS1 phosphorylation and ROS1-dependent cell growth in stably transformed Ba/F3 CD74-ROS wild-type and G2032R mutant cell lines was evaluated. Ba/F3 CD74-ROS^{G2032R} cells were highly resistant to crizotinib (IC₅₀: ~2200 nM) as compared to wild-type CD74-ROS (IC₅₀: 14 nM) (Fig. 4D). Despite being less
 15 sensitive to foretinib than the wildtype CD74-ROS fusion, the CD74-ROS^{G2032R} mutant kinase remained sensitive to foretinib (IC₅₀: 50nM) at concentrations below plasma levels that can be safely achieved in patients with oral dosing (Figure 4E). Thus, foretinib may offer an efficient second-line therapy in patients who are treatment refractory due to the acquisition of ROS1 mutations that confer resistance to crizotinib.

CLAIMS

1. A method of identifying a sample from a subject as comprising a crizotinib resistant and foretinib sensitive cancer cell, the method comprising:
 - isolating a nucleic acid from the sample, wherein the nucleic acid includes a nucleic acid sequence that encodes SEQ ID NO: 1;
 - identifying a mutation in the nucleic acid encoding SEQ ID NO: 1 that results in a mutation in G2032, V2098, G1971, L1982, C2060, L1947, or E1935;
 - wherein the presence of one or more of the mutations identifies the sample as comprising a crizotinib resistant and foretinib sensitive cancer cell.
2. The method of claim 1 wherein the mutation results in an amino acid substitution selected from G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R, or E1935G;
3. The method of claims 1-2 wherein the cancer cell is a glioblastoma, non small cell lung cancer cell or a cholangiocarcinoma.
4. The method of claims 1-2 further comprising amplifying the nucleic acid sequence that encodes SEQ ID NO: 1 using the nucleic acid from the sample as a template;
5. The method of claims 1-2 wherein the mutation is identified by nucleic acid sequencing or polymerase chain reaction.
6. A kit used to facilitate the performance of the method of any of claims 1-5, the kit comprising:
 - a first set of oligonucleotides configured to amplify the nucleic acid sequence that encodes SEQ ID NO: 1;

a second set of oligonucleotides configured to identify a mutation in SEQ ID NO: 1 that results in an amino acid substitution selected from G2032R, V2098I, G1971E, L1982F, C2060G, L1947R, L1982R or E1935G.

7. The kit of claim 6 wherein the second set of oligonucleotides is configured to form a microarray.
8. The kit of claim 6 further comprising reagents that facilitate nucleic acid sequencing and/or polymerase chain reaction.
9. The kit of claim 6 wherein the first set of oligonucleotides comprises an oligonucleotide that includes SEQ ID NO: 4 and an oligonucleotide that includes SEQ ID NO: 5.
10. A pharmaceutical composition for use in treating a cancer comprising a mutant form of the ROS1 kinase domain, the composition comprising:
 - foretinib and a pharmaceutically acceptable carrier;
 - wherein the ROS1 kinase domain (SEQ ID NO: 1) comprises a nucleic acid mutation that results in a point mutation in G2032, V2098, G1971, L1982, G1971, C2060, L1947, or E1935.
11. The pharmaceutical composition of claim 10 wherein the mutation results in an amino acid substitution selected from G2032R, V2098I, G1971E, L1982F, L1982R, C2060G, L1947R, or E1935G;
12. The pharmaceutical composition of claim 10 wherein the cancer comprises a ROS1 fusion protein.

13. The method of claim 8 wherein the ROS1 fusion protein is selected from FIG-ROS and SLC-ROS.

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Figure 1A

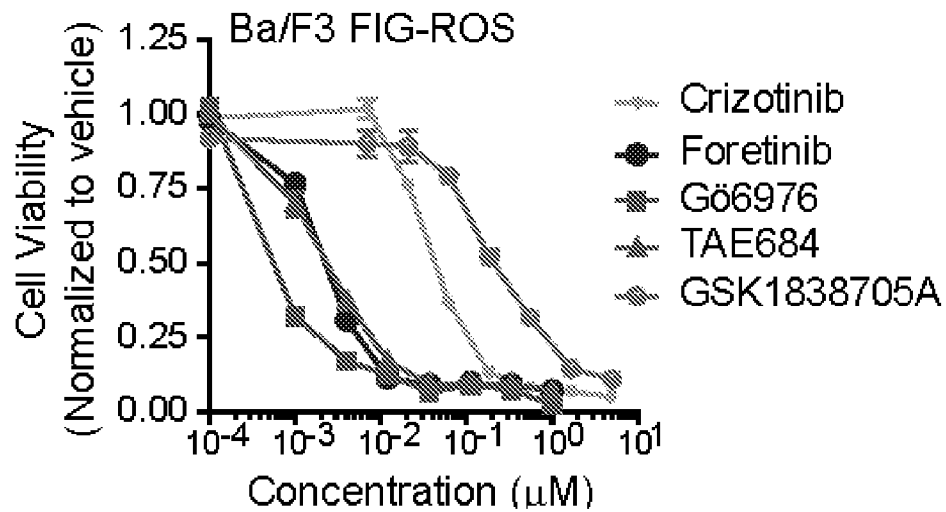
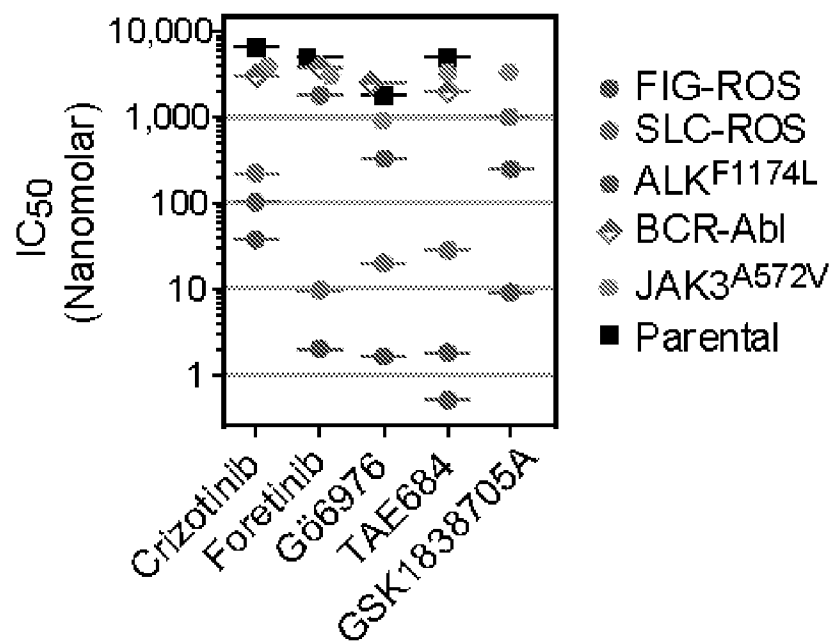


Figure 1B



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Figure 1C

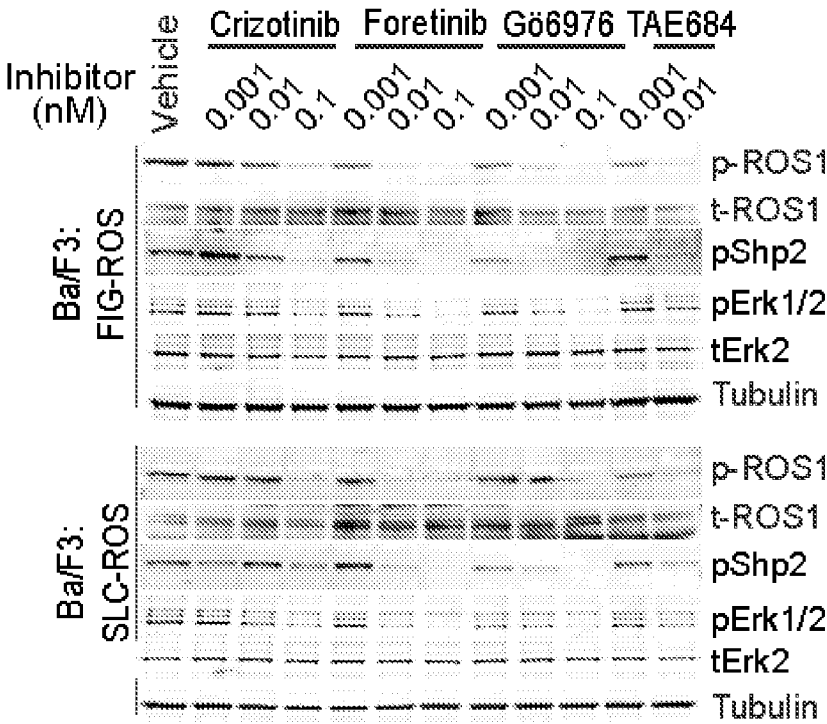


Figure 1D

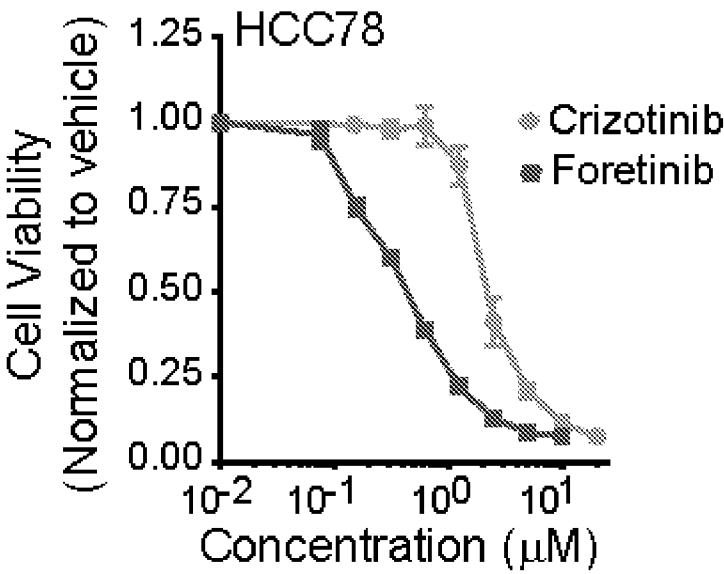


Figure 1E

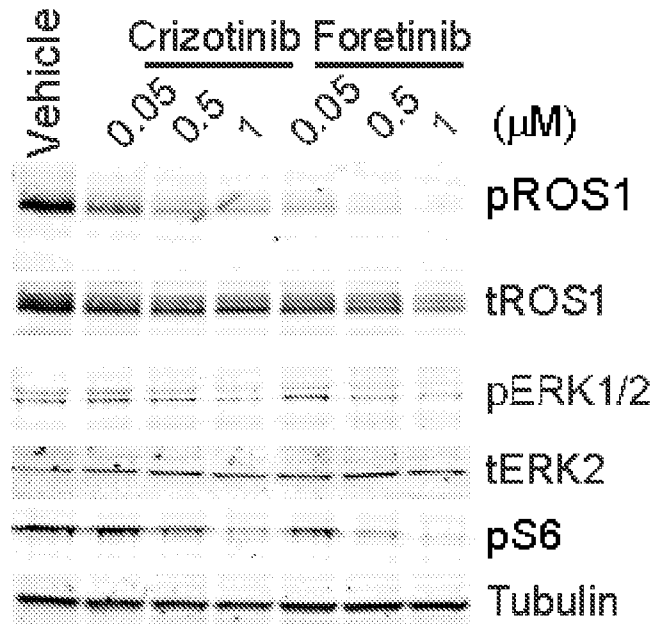
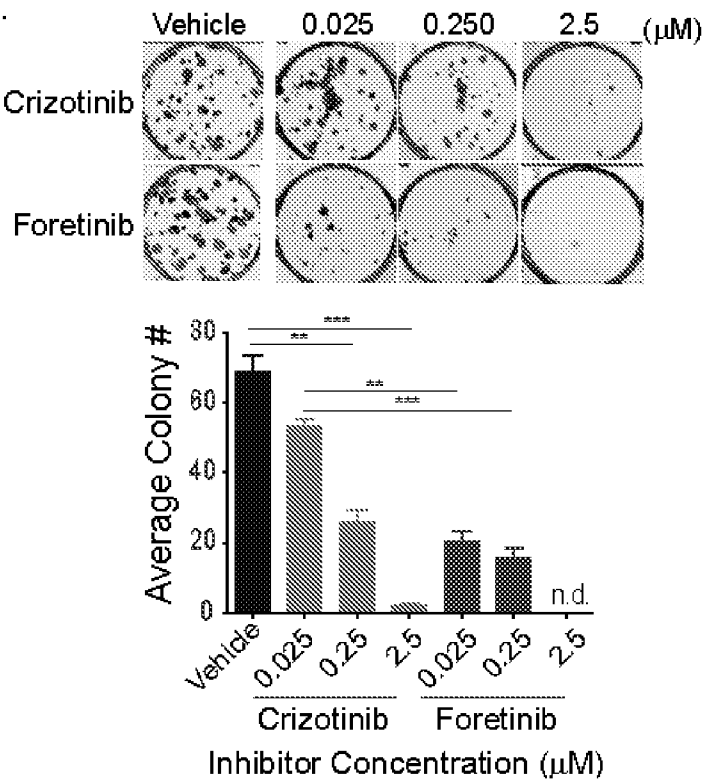


Figure 1F



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Figure 2A

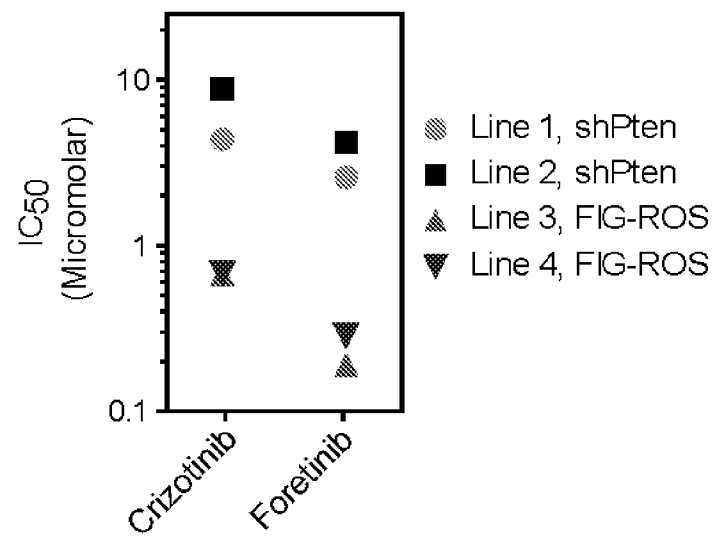
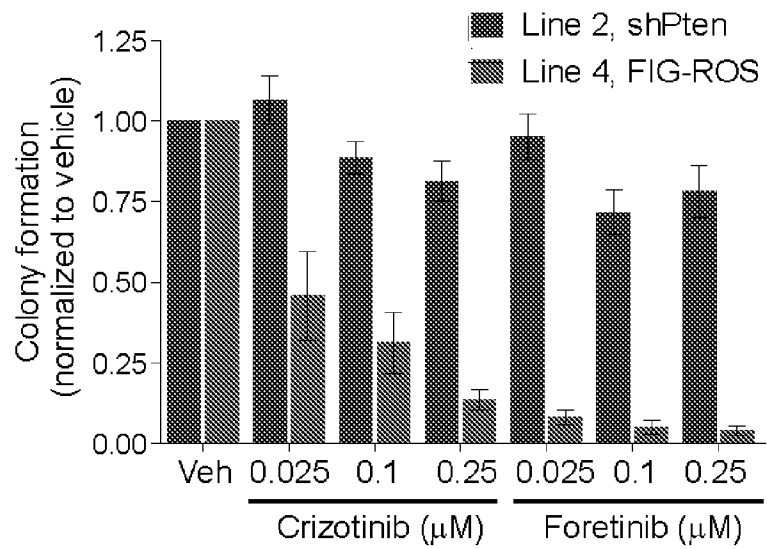


Figure 2B



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Figure 2C

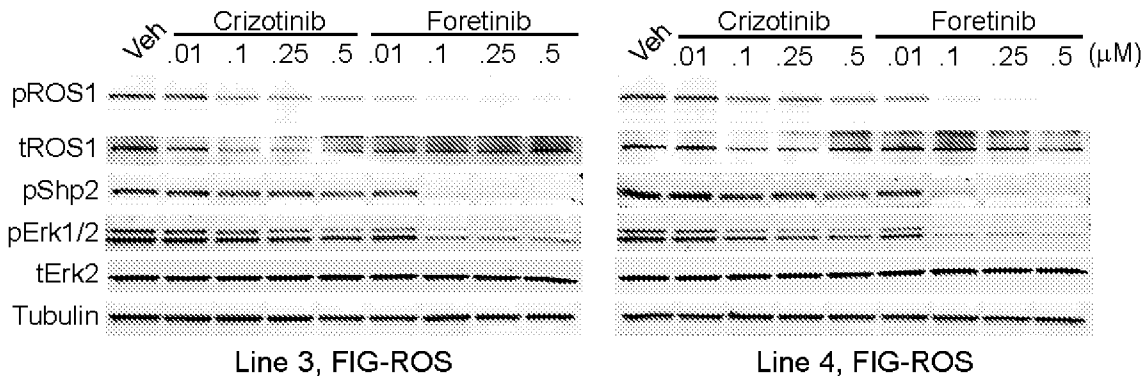


Figure 3A

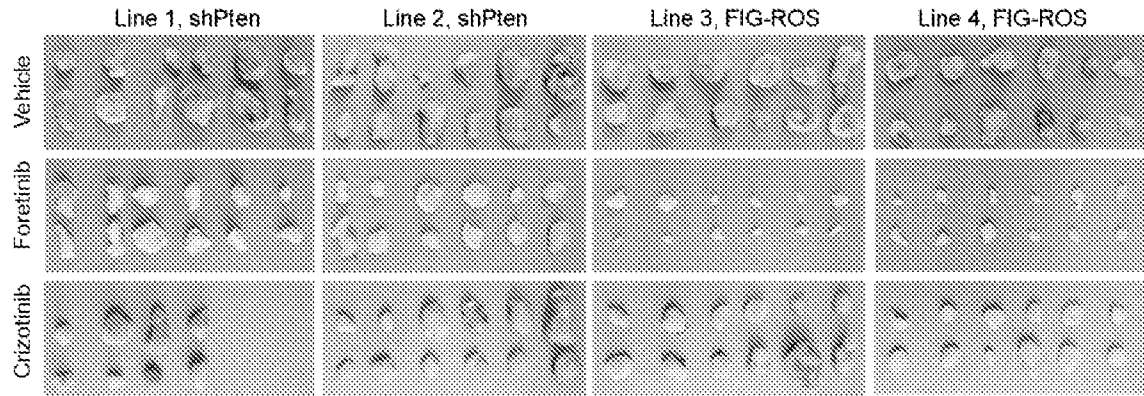
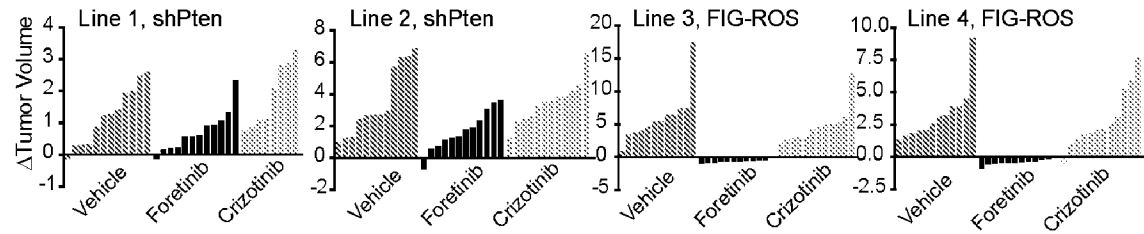


Figure 3B



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Figure 3C

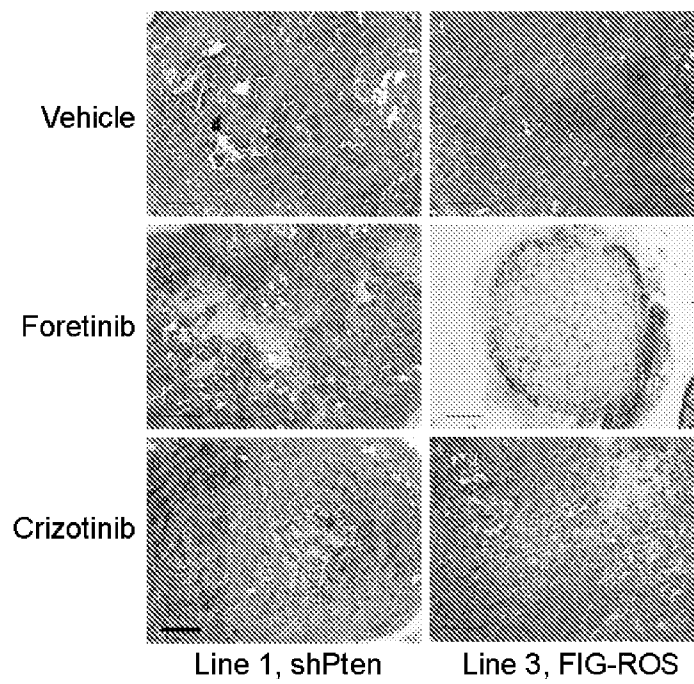
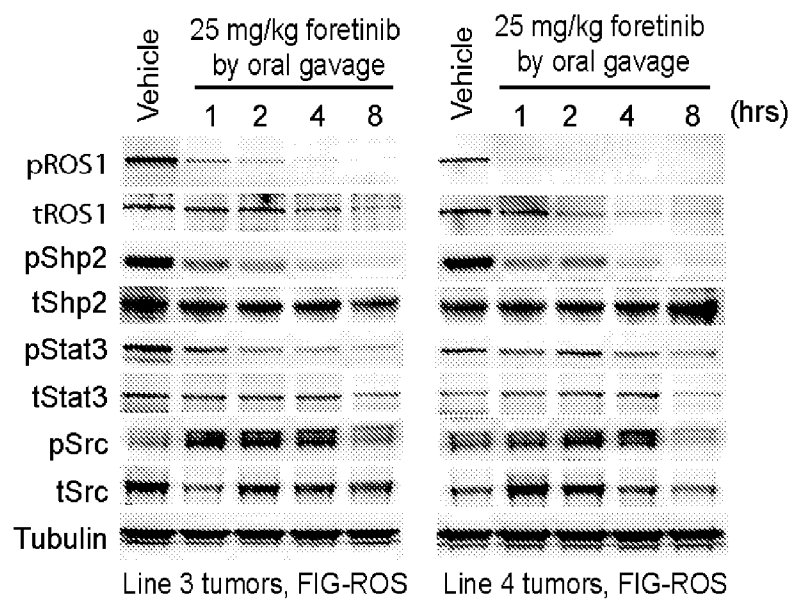


Figure 3D



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Figure 4A

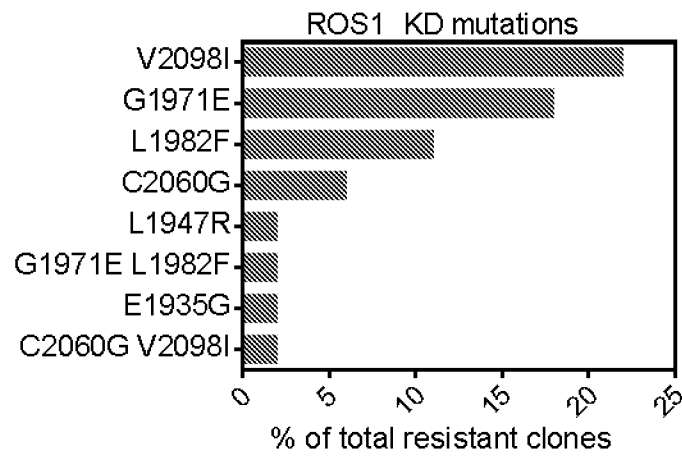
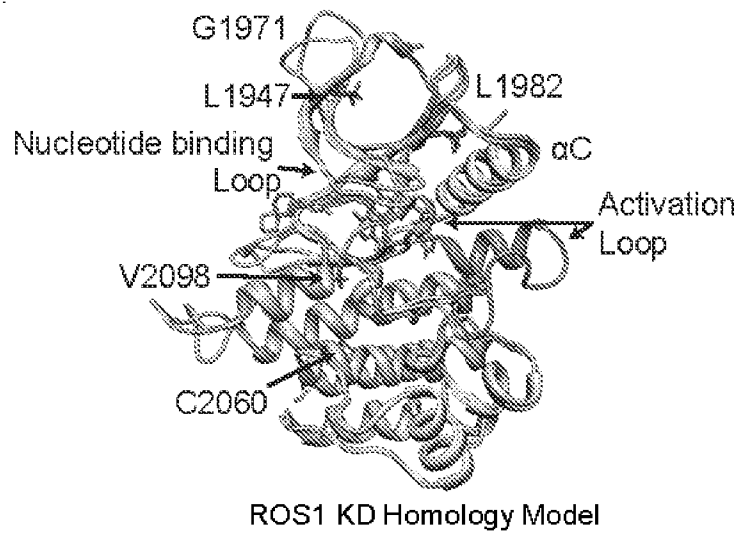


Figure 4B



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Figure 4C

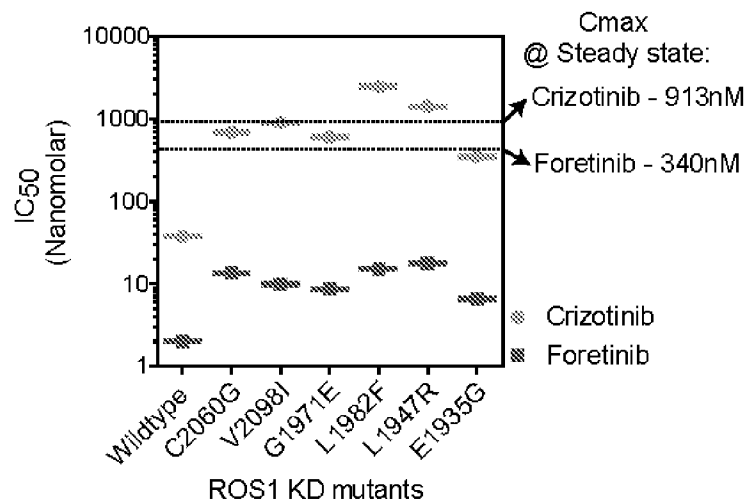
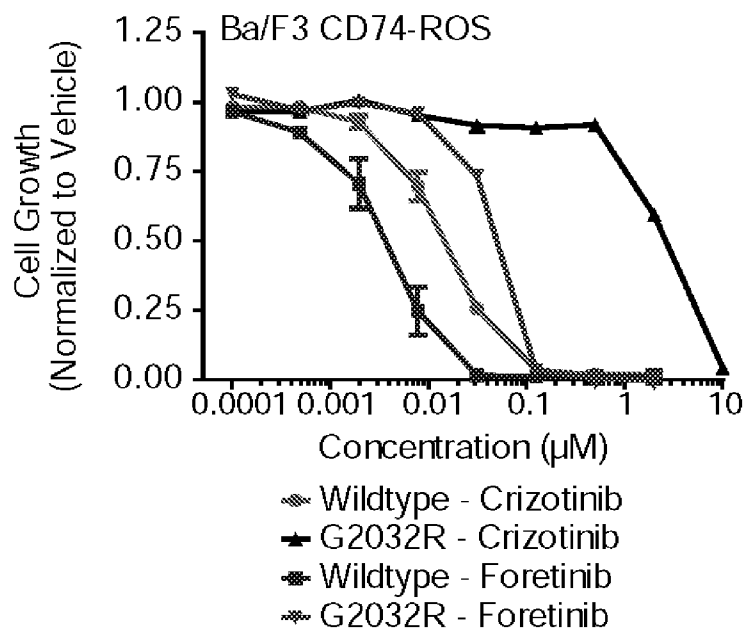


Figure 4D



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Figure 4E

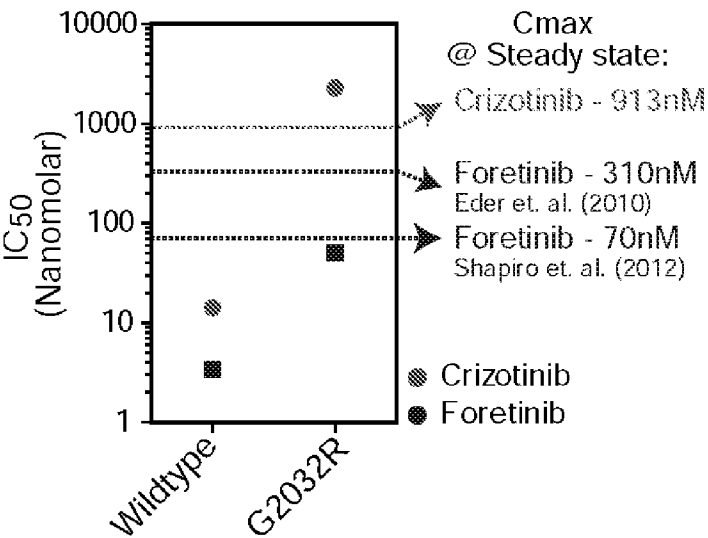
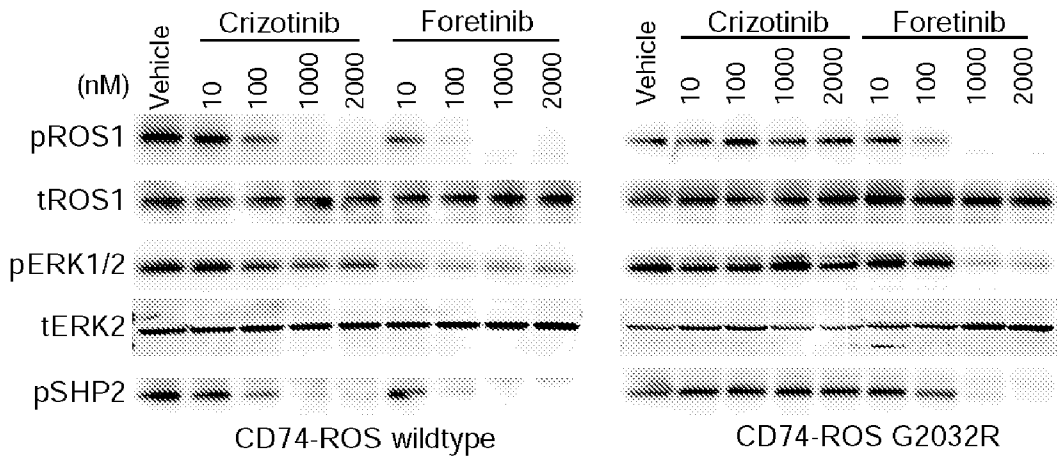


Figure 4F



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Figure 5A

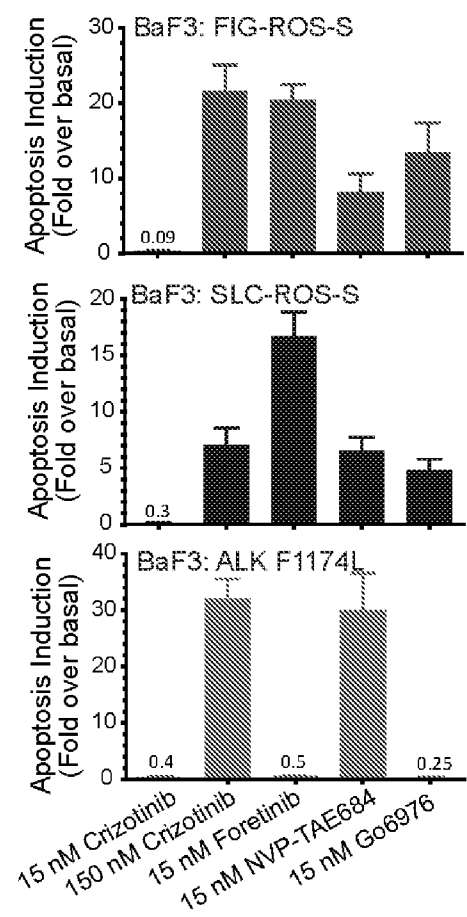


Figure 5B

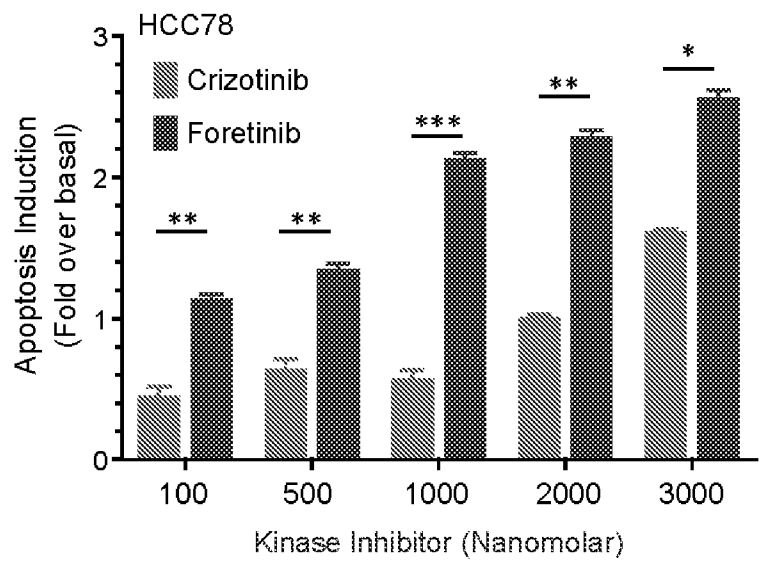
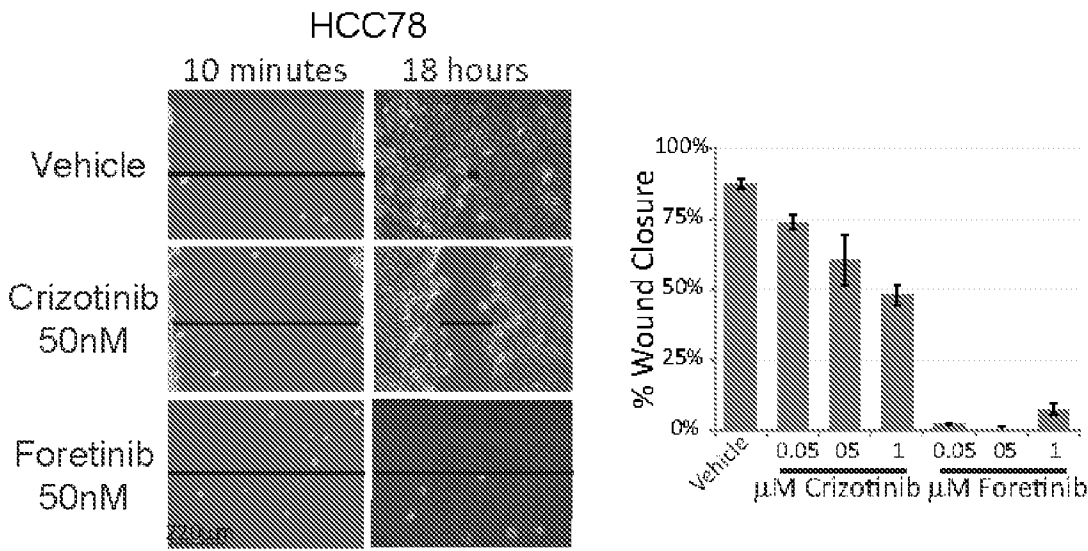


Figure 6A



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Figure 6B

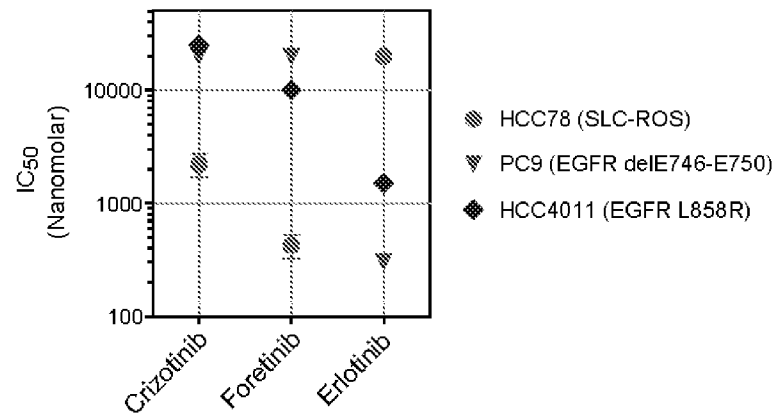


Figure 6C

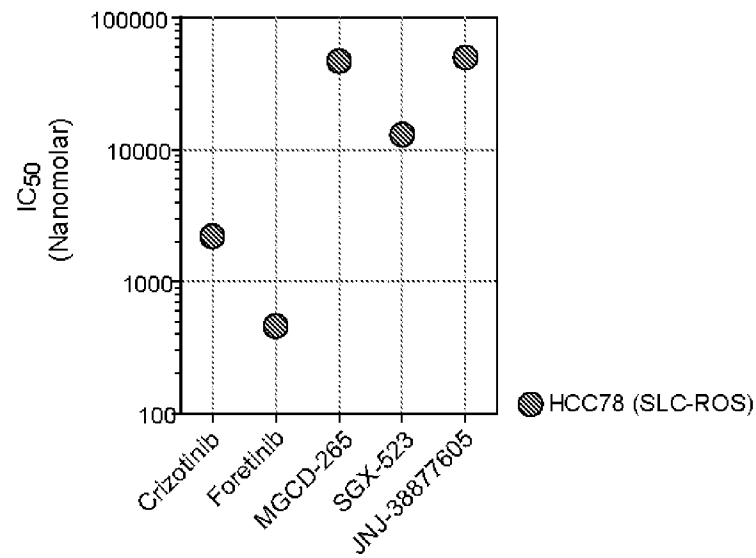
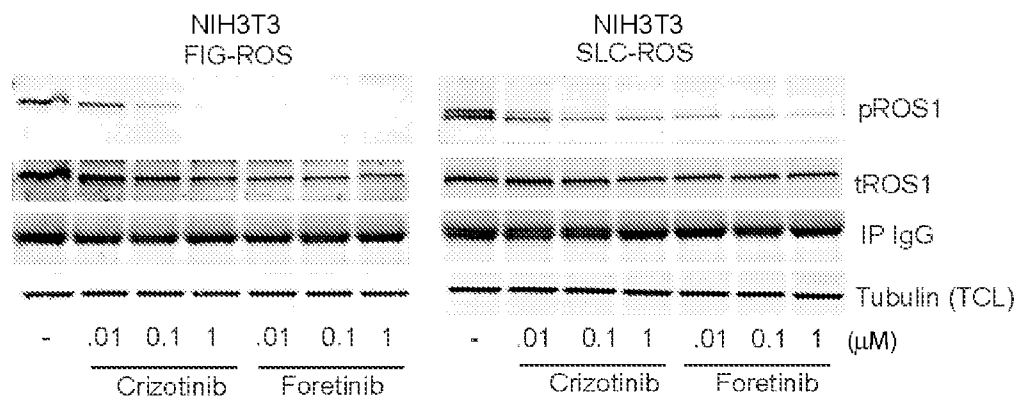
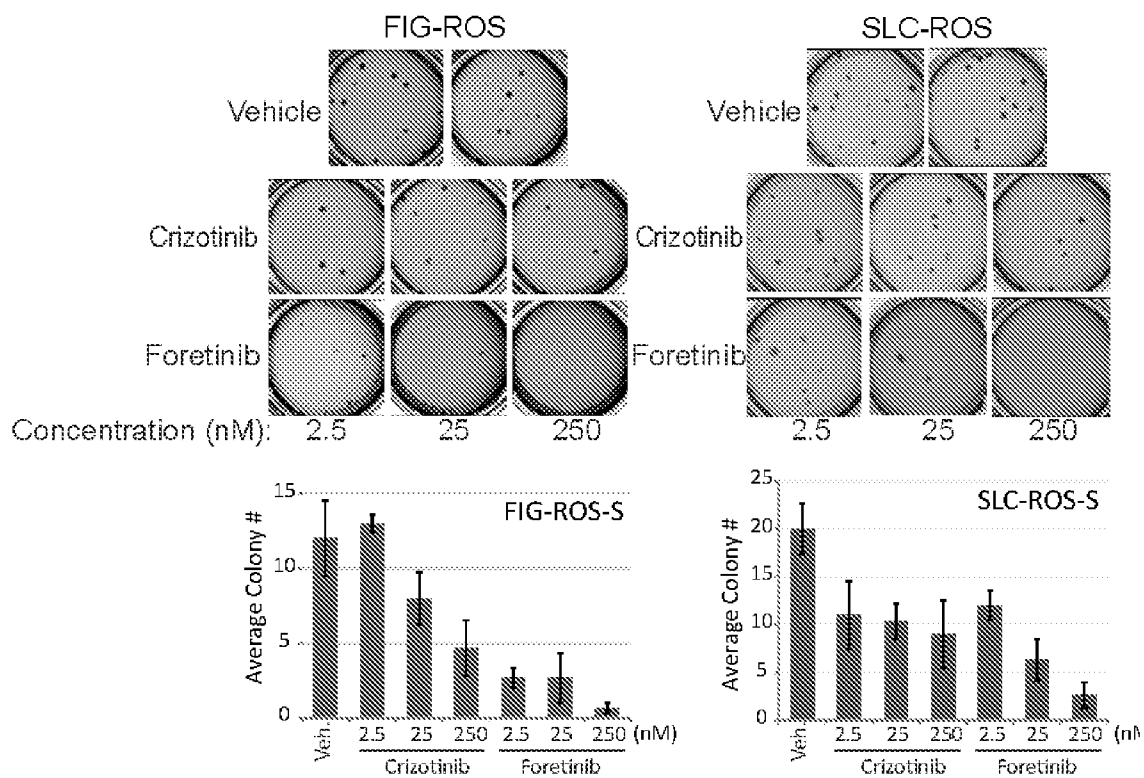


Figure 7A



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Figure 7B



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Figure 8

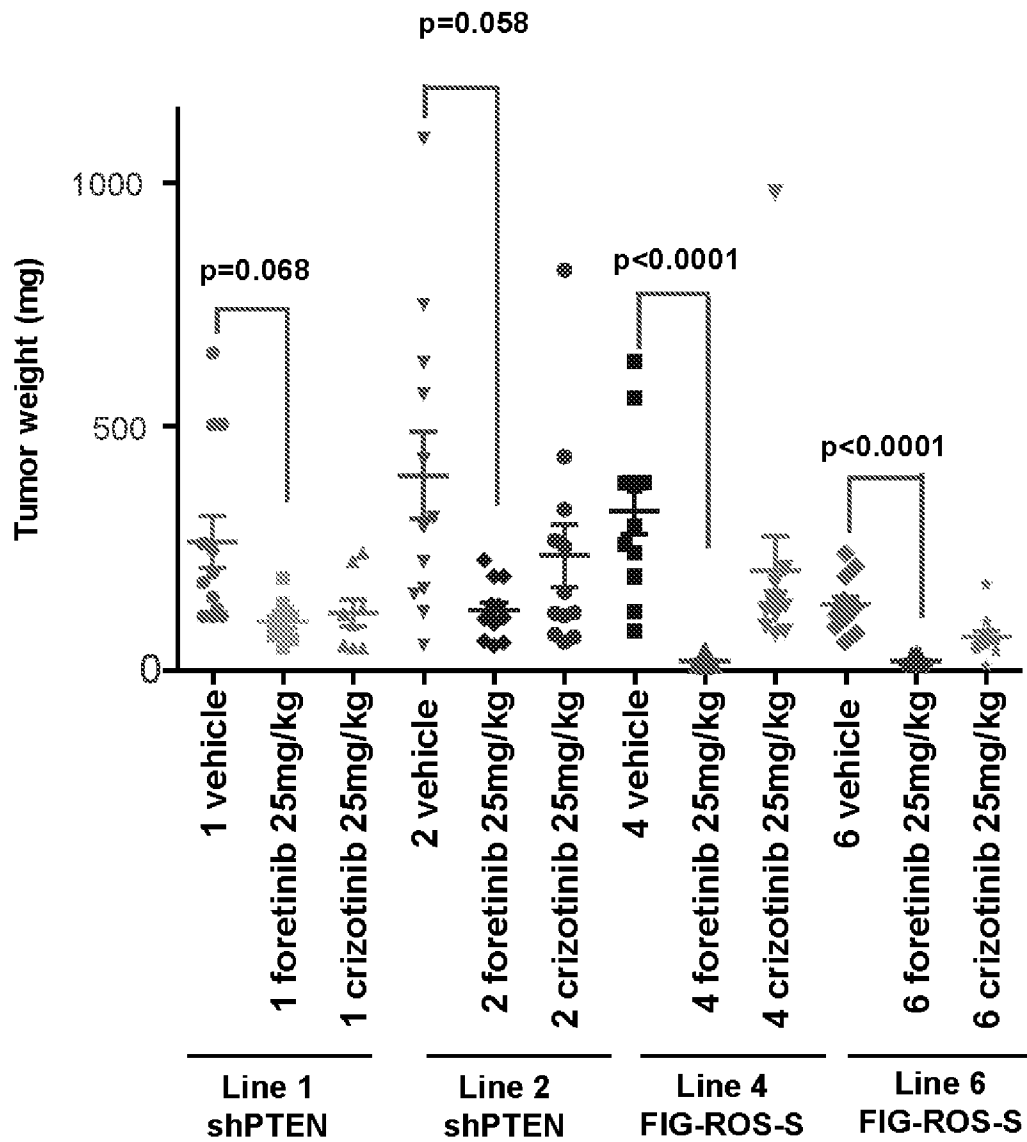


Figure 9A

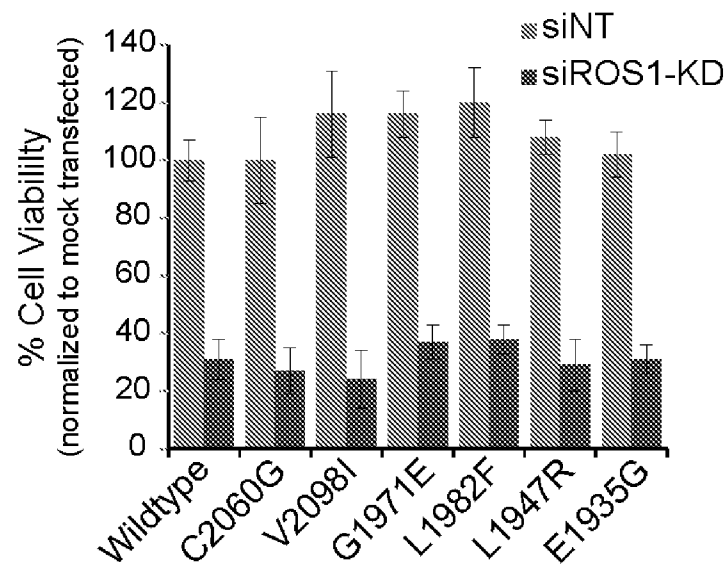
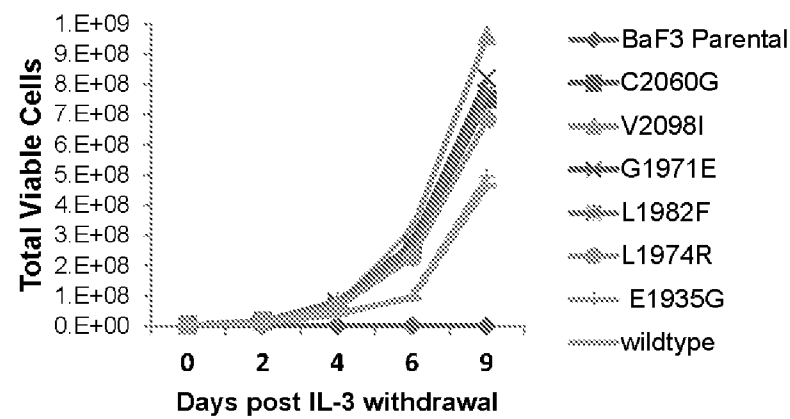


Figure 9B



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Figure 10A

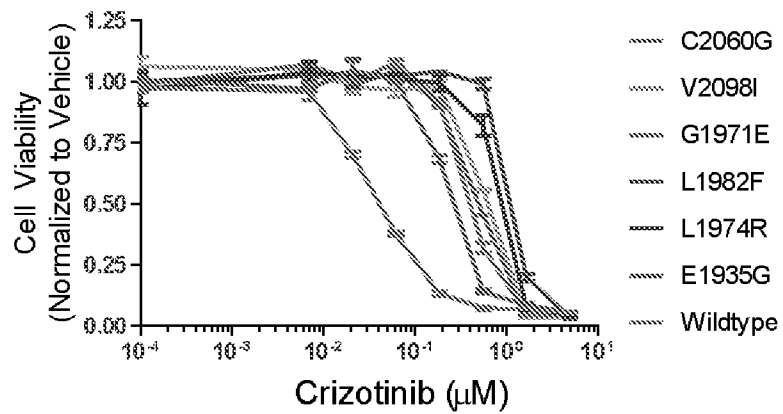
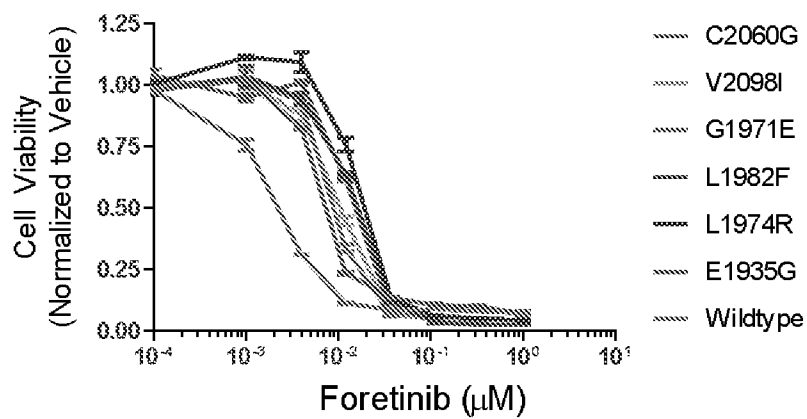
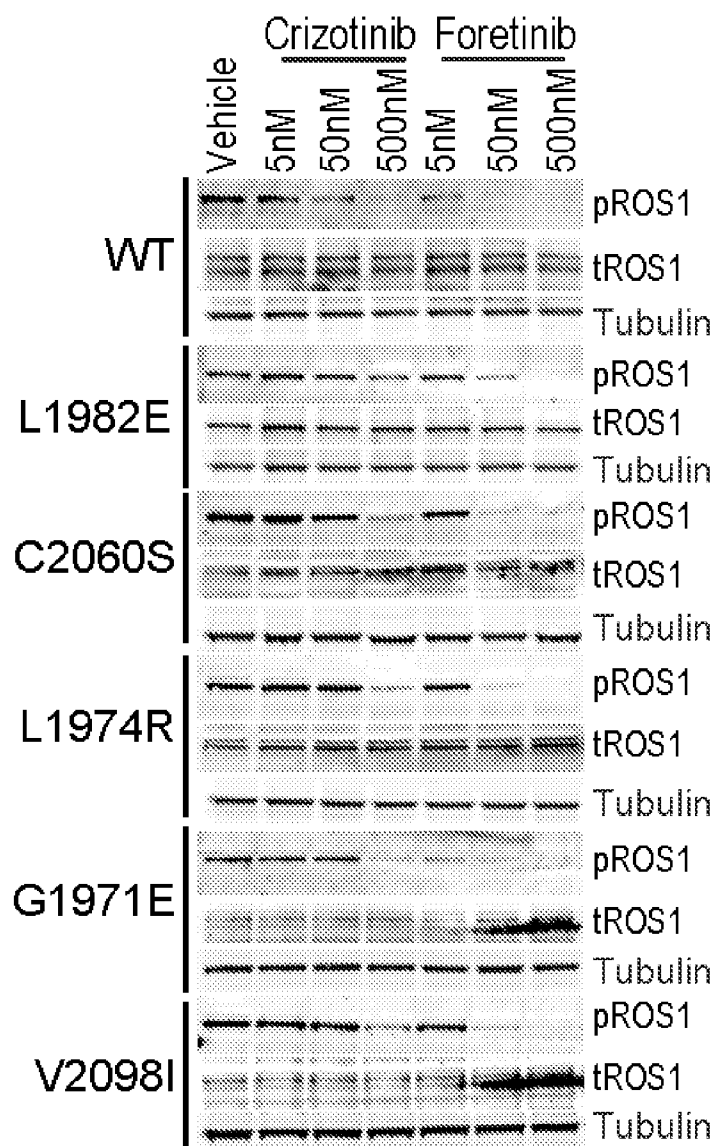


Figure 10B



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Figure 10C



A. CLASSIFICATION OF SUBJECT MATTER**C12Q 1/68(2006.01)i, C12N 15/12(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68; C12N 15/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: crizotinib resistant, foretinib sensitive, ROS1, cancer, mutation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHANG et al., 'Crizotinib-resistant mutants of EML4-ALK identified through an accelerated mutagenesis screen' Chemical Biology & Drug Design, Vol.78, No.6, pp.999-1005 (2011) See abstract; page 1004; and table 2.	1-2, 10-12
A	KATAYAMA et al., 'Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK' PNAS, Vol.108, No.18, pp.7535-7540 (2011) See abstract and pages 7536-7538.	1-2, 10-12
A	CECCON et al., 'Crizotinib-resistant NPM-ALK mutants confer differential sensitivity to unrelated ALK inhibitors' Molecular Cancer Research, Vol.11, No.2, pp.122-132 (13 December 2012) See the whole document.	1-2, 10-12
A	DOEBELE et al., 'Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer' Clinical Cancer Research, Vol.18, No.5, pp.1472-1482 (2012) See the whole document.	1-2, 10-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 July 2014 (04.07.2014)

Date of mailing of the international search report

07 July 2014 (07.07.2014)

Name and mailing address of the ISA/KR

International Application Division
Korean Intellectual Property Office
189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea

Facsimile No. +82-42-472-7140

Authorized officer

KIM, Seung Beom

Telephone No. +82-42-481-3371



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/018491

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NCBI, GenBank accession no. AAA60277.1 (09 January 1995) See the whole sequence.	1-2, 10-12
A	RIMKUNAS et al., 'Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: identification of a FIG-ROS1 fusion' Clinical Cancer Research, Vol.18, No.16, pp.4449-4457 (2012) See the whole document.	1-2, 10-12
PX	DAVARE et al., 'Foretinib is a potent inhibitor of oncogenic ROS1 fusion proteins' PNAS, Vol.110, No.48, pp.19519-19524 (26 November 2013) See the whole document.	1-2, 10-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/018491

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

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

a. a sequence listing filed or furnished

- ☐ on paper
☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
☐ filed together with the international application in electronic form
☐ furnished subsequently to this Authority for the purposes of search

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

3. Additional comments:

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This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7-9, 13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 7-9 and 13 are unclear since they are referring to unsearchable claims which do not comply with PCT Rule 6.4(a); Claim 13 is worded in reference to the ROS1 fusion protein of claim 8. However, said ROS1 fusion protein has not been worded in claim 8. Additionally, claim 8 relates to the kit, but claim 13 dependent on claim 8 relates to the method. Therefore, claim 13 is not clear and concise contrary to PCT Article 6.
3. ☒ Claims Nos.: 3-6
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/018491Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

None