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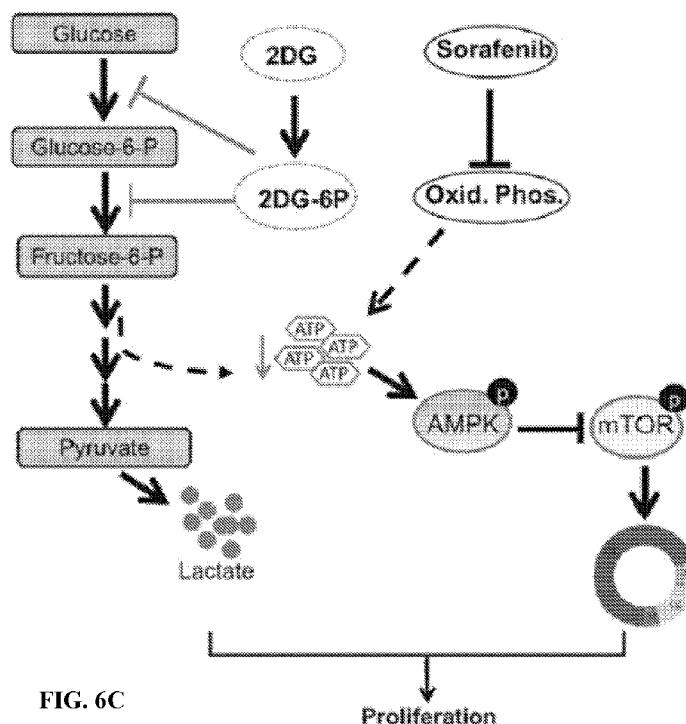


FIG. 6C

(57) Abstract: The present disclosure relates to methods and compositions for treatment of cancer in a subject in need thereof. Methods and compositions for treatment of cancer in a subject in need thereof are provided according to specific aspects which include administering both sorafenib and 2-deoxyglucose, as a combination formulation or as separate formulations. Methods for treatment of cancer (for example, hepatocellular carcinoma) in a subject in need thereof are provided according to specific aspects which include administering of fostamatinib or dasatinib alone or in combination with sorafenib.



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METHODS AND COMPOSITIONS FOR THE TREATMENT OF CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/354,454 filed June 24, 2016, which is expressly incorporated herein by reference.

5

FIELD

The present disclosure relates to methods and compositions for the treatment of cancer.

BACKGROUND

10 Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths globally. Sorafenib is the only first-line systemic drug for advanced HCC, but it has very limited survival benefits because patients treated with sorafenib either suffer from side effects or show disease progression after initial response. Thus, there is an urgent need to develop novel combination therapies and strategies for first-line and second-line therapy.

15 In addition, hepatocellular carcinoma (HCC) is frequently diagnosed in patients with late-stage disease who are ineligible for curative surgical therapies. Furthermore, the majority of patients become resistant to the only approved therapy, sorafenib. Recently, computational methods for drug repurposing have shown great promise to accelerate the discovery of new uses for existing drugs. Thus, there is a need to repurpose existing drugs for use in methods for treating
20 hepatocellular carcinoma (HCC), in particular, sorafenib resistant (SR)- hepatocellular carcinoma (HCC).

The compounds, compositions, and methods disclosed herein address these and other needs.

25 SUMMARY

Disclosed herein are compounds, compositions, and methods useful for treating cancer, for example, hepatocellular carcinoma. The inventors have unexpectedly found that the combination of glycolytic inhibitor 2-deoxyglucose (2DG) and sorafenib drastically inhibits viability of sorafenib sensitive and resistant cells. In contrast, the combination of other anti-
30 glycolytic drugs like lonidamine, gossypol, 3-bromopyruvate and imatinib with sorafenib does not show synergistic effect.

In addition, a transcriptomics-based drug repurposing method termed connectivity mapping was used to identify additional therapeutics for use against hepatocellular carcinoma (HCC). The use of two non-receptor tyrosine kinase inhibitors, dasatinib and fostamatinib, were determined to reduce viability of sorafenib-resistant HCC cells and up-regulated activity of Src family kinases, the targets of dasatinib, was observed in SR-HCC models.

In one aspect, disclosed herein is a pharmaceutical composition comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.

In some embodiments, the composition further comprises an additional chemotherapeutic agent.

In one aspect, disclosed herein is a kit comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof. In some embodiments, sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation. In some embodiments, sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.

In another aspect, disclosed herein is a method of treating or preventing a cancer in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.

In some embodiments, the cancer is selected from hepatocellular carcinoma, breast cancer, or chronic lymphocytic leukemia (CLL). In some embodiments, the cancer is hepatocellular carcinoma (HCC). In some embodiments, the cancer is resistant to sorafenib.

In some embodiments, the administration of sorafenib and 2-deoxyglucose provides a synergistic effect. In some embodiments, the administration of sorafenib and 2-deoxyglucose inhibits ATP production.

In some embodiments, sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation. In some embodiments, sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.

In some embodiments, the method further comprises the administration of an additional chemotherapeutic agent.

In a further aspect, disclosed herein is a method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of fostamatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

In one aspect, disclosed herein is a method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of dasatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

5 In some embodiments, the method further comprises the administration of an additional chemotherapeutic agent. In some embodiments, the additional chemotherapeutic is sorafenib.

BRIEF DESCRIPTION OF THE DRAWINGS

10 The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

FIGS. 1A-1C. Establishment and characterization of sorafenib resistant HCC cell lines. (FIG. 1A) Viability assay of Huh7-S, Huh7-R-Pool and Huh7-R-A7 cells. Cells were treated with various concentrations of sorafenib for 48 hours. Error bars represent the standard deviation of two biological replicates. (FIG. 1B) Glucose consumption and (FIG. 1C) lactate production of 15 Huh7-S, Huh7-R-Pool and Huh7-R-A7 cells. The error bars represent the standard deviation of two biological replicates. * $p < 0.05$ (t -test) compared to 0 μ M treated cells of the corresponding cell line.

FIGS. 2A-2H. The combination of sorafenib and 2-deoxyglucose synergistically inhibited HCC cell proliferation. (FIGS. 2A-2E) Huh7-R-Pool cells were treated with various 20 concentrations of sorafenib and (FIG. 2A) 2DG, (FIG. 2B) 3-bromopyruvate (3-BP), (FIG. 2C) gossypol, (FIG. 2D) imatinib and (FIG. 2E) lonidamine. Sorafenib and 2-DG combinations were also tested in the Huh7-S (FIG. 2F), Huh7-R-A7 (FIG. 2G) and Hep3B (FIG. 2H) cells. The x-axis of each plot is represented in units of “effective dose”. Each effective dose corresponds to a specific concentration of sorafenib and anti-glycolytic drug. Combination index 25 (CI) values were calculated using CompuSyn software.

FIGS. 3A-3C. Colony formation is inhibited by the combination of sorafenib and 2-deoxyglucose. (FIG. 3A) Huh7-S, (FIG. 3B) Huh7-R-Pool and (FIG. 3C) Huh7-R-A7 cells were subject to a colony formation assay following treatment with sorafenib, 2DG or a combination of both. Plates were imaged macroscopically (top) and microscopically (bottom) 30 after staining and fixing. Microscopic images are representative of average colony size.

FIGS. 4A-4B. The combination of sorafenib and 2DG induces cell cycle arrest in HCC cells. (FIGS. 4A and 4B) Huh7-R-Pool cells were subject to cell cycle analysis. Cells were seeded and allowed to attach overnight. Serum was then withdrawn from the media for 24 hours

to allow cell synchronization. Cells were then treated with no drug (1% DMSO), sorafenib (8 μ M), 2DG (5 mM) or a combination of both for 48 hours. Cells were then harvested, fixed, stained with propidium iodide and analyzed by flow cytometry. Cell cycle data shown is representative of three biological replicates.

5 FIGS. 5A-5C. Cellular energy is depleted upon combination therapy with sorafenib and 2-deoxyglucose. (FIG. 5A) Cellular ATP, (FIG. 5B) Glucose consumption, and (FIG. 5C) Lactate production was measured in Huh7-S, Huh7-R-Pool and Huh7-R-A7 cells. Cells were treated with no drug (1% DMSO), sorafenib (8 μ M), 2DG (5 mM) or a combination of both for 48 hours prior to the measurements. Error bars represent the standard deviation of two biological
10 replicates. * $p < 0.05$ (t -test) DMSO treated cells of the corresponding cell line.

 FIGS. 6A-6C. mTOR and its downstream signaling are inhibited by sorafenib and 2D combination therapy in sorafenib sensitive and resistant Huh7 cells. (FIG. 6A) Huh7-S and Huh7-R-Pool cells were treated for 6 hours with vehicle (1% DMSO), sorafenib (8 μ M), 2DG (5 mM) or a combination of both and cellular extracts were subjected to Western blot analysis for
15 p-mTOR (S2448), total mTOR, p-p70S6K (Thr389), p-4E-Bp (Thr37/46), pAMPK (Thr172), total AMPK and β -actin. (FIG. 6B) Quantification of the western data. (FIG. 6C) Mechanism of synergistic growth inhibition of HCC cells by 2-DG and sorafenib. (Left panel) Glycolysis is inhibited by glucose analogue, 2-deoxy-D-glucose (2DG). Both glucose and 2DG are phosphorylated by hexokinase to glucose-6-phosphate and 2DG 6-phosphate, respectively.
20 However, in contrast to glucose-6-phosphate, 2DG 6-phosphate cannot be further metabolized to fructose-6-phosphate by phosphogluco isomerase, thus blocking glycolysis, lactate and ATP production. (Right panel) Sorafenib reduces oxidative phosphorylation leading to reduced ATP levels. This reduction in ATP production increases AMP:ATP ratio, activates energy sensor AMPK, leading to inhibition of mTOR activation. Inhibition of mTOR also inhibits
25 phosphorylation of its downstream targets 4EBP1 and p70S6K that control protein translation, thereby blocking cell cycle progression. Therefore, the inhibition of cellular ATP levels leads to reduced HCC cell proliferation.

 FIG. 7. Overview of computational drug repurposing workflow and drug target network. Gene expression signatures of experimental models of HCC sorafenib resistance were i) assessed
30 for prognostic significance, and ii) queried against gene expression signatures characterizing drug perturbations in the HepG2 cell line contained in the Library of Integrated Network-based Cellular Signatures (LINCS) database. Connectivity scores were calculated by the rank-based, non-parametric weighted Kolmogorov-Smirnov (KS) statistic. Drugs with negative connectivity scores (i.e. anti-correlated) represent those that are hypothesized to reverse HCC sorafenib

resistance gene signature. Drug candidates were further prioritized based on FDA approval/clinical investigation status, known anti-neoplastic activity and literature evidence for drug target genes associated with HCC. Two drugs were subsequently selected for *in vitro* validation.

5 FIGS. 8A-8D. Sorafenib resistance gene signatures - prognostic significance and drug repurposing candidates. (FIG. 8A) Percentage of primary tumor samples in The Cancer Genome Atlas (TCGA) liver hepatocellular carcinoma (LIHC) dataset containing the four sorafenib resistance gene signatures (SR+). (FIG. 8B) Kaplan-Meier plot of overall survival of TCGA LIHC patients with primary tumors harboring the Huh7-R-A7 SR gene signature (n=181 SR+) and those with primary tumors containing the inverse SR gene signature (n=190 SR-). Heatmap visualization of hierarchical clustering analysis of connectivity scores for LINCS drugs derived from gene expression profiles of (FIG. 8C) HCC cell lines (n=18) from the CellMiner database and (FIG. 8D) HCC sorafenib resistance (SR) experimental models.

15 FIGS. 9A-9F. Validation of predicted drug candidates for action against sorafenib resistant HCC. Increased sensitivity of sorafenib-resistant Huh7 cells to dasatinib (FIG. 9A) and fostamatinib (FIG. 9B), as measured 48 hrs post-treatment using CellTiter-Glo viability assay. Growth of sorafenib-resistant Huh7 cells is inhibited by dasatinib (FIG. 9C) and fostamatinib (FIG. 9D), alone and in combination with sorafenib, as measured by colony formation assay 2 weeks post-treatment. (FIG. 9E) Enhanced phosphorylation of the Src family of kinases in sorafenib-resistant cells relative to sensitive cells, as measured by Proteome Profiler Human Phospho-Kinase Array (* $p < 0.05$, ** $p < 0.010$, two-tailed t test) at 48 hrs. (FIG. 9F) Ingenuity pathway analysis of gene expression profile from fostamatinib-treated HepG2 cells reveals significantly altered pathways.

25 FIGS. 10A-10F. Impact of clinical and demographic factors on drug repurposing candidates and sorafenib resistance gene signature. (FIG. 10A) Heatmap visualization of hierarchical clustering analysis of connectivity scores for LINCS drugs derived from gene expression profiles of HCC patient tumor datasets from the GEO database representing distinct etiologies. (FIG. 10B) Proportions of SR+ and SR- patient primary HCC tumors in the TCGA LIHC dataset across five HCC etiologies (Chi-square test). Proportions of HCC patients with primary SR+ and SR- tumors in the TCGA LIHC dataset across (FIG. 10C) stage based on TNM classification (Chi-square test p value reported), (FIG. 10D) Child Pugh class (Chi-square test p value reported), (FIG. 10E) patient race (Chi-square test p value reported) and (FIG. 10F) patient gender (Fisher exact test p value reported). Note the mean age at diagnosis for SR+ and SR- patients is 59.5 years and 59.4 years, respectively. Abbreviations: AI= alcohol-induced, HBV=

hepatitis B virus, HCV= hepatitis C virus, NAFLD= non-alcoholic fatty liver disease, Hem= hemochromatosis.

FIG. 11. Sorafenib dose response curve for sorafenib sensitive and resistant Huh7 cells. Fold-change in cell viability of Huh7 cells treated with increasing concentrations of sorafenib, as measured 48 hours post-treatment using an ATP assay (CellTiter-Glo). The blue horizontal line marks the IC₅₀ values. Huh7-S: parental Huh7 cells; Huh7-R: pool of sorafenib-resistant cells; A7, E11, F3, F2: individual sorafenib-resistant clones. Increased IC₅₀ values are demonstrated for the sorafenib-resistant pool and individual clones relative to parental cells.

FIGS. 12A-12B. Comparison of HCC sorafenib resistance gene signatures. Overlap of up-regulated (FIG. 12A) and down-regulated (FIG. 12B) genes among the four HCC sorafenib resistance gene signatures.

FIG. 13. Effect of SYK mRNA expression on HCC patient survival. Kaplan-Meier plot visualizing disease-free survival of SR+ TCGA LIHC patients with up-regulated (≥ 2 -fold) SYK gene expression (cases with alteration) vs. unchanged SYK (cases without alteration) using the cBioPortal survival analysis tool. Log-rank P-value= 0.00316.

DETAILED DESCRIPTION

Disclosed herein are compounds, compositions, and methods useful for treating cancer, for example, hepatocellular carcinoma. The inventors have unexpectedly found that the combination of glycolytic inhibitor 2-deoxy-D-glucose (2DG) and sorafenib drastically inhibits viability of sorafenib sensitive and resistant cells. In contrast, the combination of other anti-glycolytic drugs like lonidamine, gossypol, 3-bromopyruvate and imatinib with sorafenib does not show synergistic effect.

In addition, a transcriptomics-based drug repurposing method termed connectivity mapping was used to identify additional therapeutics for use against hepatocellular carcinoma (HCC). The use of two non-receptor tyrosine kinase inhibitors, dasatinib and fostamatinib, were determined to reduce viability of sorafenib-resistant HCC cells and up-regulated activity of Src family kinases, the targets of dasatinib, in SR-HCC models.

Reference will now be made in detail to the embodiments of the invention, examples of which are illustrated in the drawings and the examples. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention

belongs. The following definitions are provided for the full understanding of terms used in this specification.

Terminology

5 As used in the specification and claims, the singular form “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes a plurality of cells, including mixtures thereof.

 As used herein, the terms “may,” “optionally,” and “may optionally” are used interchangeably and are meant to include cases in which the condition occurs as well as cases in
10 which the condition does not occur. Thus, for example, the statement that a formulation “may include an excipient” is meant to include cases in which the formulation includes an excipient as well as cases in which the formulation does not include an excipient.

 As used here, the terms “beneficial agent” and “active agent” are used interchangeably herein to refer to a chemical compound or composition that has a beneficial biological effect.
15 Beneficial biological effects include both therapeutic effects, i.e., treatment of a disorder or other undesirable physiological condition, and prophylactic effects, i.e., prevention of a disorder or other undesirable physiological condition. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of beneficial agents specifically mentioned herein, including, but not limited to, salts, esters, amides, prodrugs, active metabolites, isomers,
20 fragments, analogs, and the like. When the terms “beneficial agent” or “active agent” are used, then, or when a particular agent is specifically identified, it is to be understood that the term includes the agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, conjugates, active metabolites, isomers, fragments, analogs, etc.

 As used herein, the terms “treating” or “treatment” of a subject includes the
25 administration of a drug to a subject with the purpose of preventing, curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving, stabilizing or affecting a disease or disorder, or a symptom of a disease or disorder. The terms “treating” and “treatment” can also refer to reduction in severity and/or frequency of symptoms, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms and/or their underlying cause, and
30 improvement or remediation of damage.

 As used herein, the term “preventing” a disorder or unwanted physiological event in a subject refers specifically to the prevention of the occurrence of symptoms and/or their

underlying cause, wherein the subject may or may not exhibit heightened susceptibility to the disorder or event.

By the term “effective amount” of a therapeutic agent is meant a nontoxic but sufficient amount of a beneficial agent to provide the desired effect. The amount of beneficial agent that is “effective” will vary from subject to subject, depending on the age and general condition of the subject, the particular beneficial agent or agents, and the like. Thus, it is not always possible to specify an exact “effective amount.” However, an appropriate “effective” amount in any subject case may be determined by one of ordinary skill in the art using routine experimentation. Also, as used herein, and unless specifically stated otherwise, an “effective amount” of a beneficial can also refer to an amount covering both therapeutically effective amounts and prophylactically effective amounts.

An “effective amount” of a drug necessary to achieve a therapeutic effect may vary according to factors such as the age, sex, and weight of the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

As used herein, a “therapeutically effective amount” of a therapeutic agent refers to an amount that is effective to achieve a desired therapeutic result, and a “prophylactically effective amount” of a therapeutic agent refers to an amount that is effective to prevent an unwanted physiological condition. Therapeutically effective and prophylactically effective amounts of a given therapeutic agent will typically vary with respect to factors such as the type and severity of the disorder or disease being treated and the age, gender, and weight of the subject.

The term “therapeutically effective amount” can also refer to an amount of a therapeutic agent, or a rate of delivery of a therapeutic agent (e.g., amount over time), effective to facilitate a desired therapeutic effect. The precise desired therapeutic effect will vary according to the condition to be treated, the tolerance of the subject, the drug and/or drug formulation to be administered (e.g., the potency of the therapeutic agent (drug), the concentration of drug in the formulation, and the like), and a variety of other factors that are appreciated by those of ordinary skill in the art.

As used herein, the term “pharmaceutically acceptable” component can refer to a component that is not biologically or otherwise undesirable, i.e., the component may be incorporated into a pharmaceutical formulation of the invention and administered to a subject as described herein without causing any significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the formulation in which it is contained.

When the term “pharmaceutically acceptable” is used to refer to an excipient, it is generally implied that the component has met the required standards of toxicological and manufacturing testing or that it is included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug Administration.

Also, as used herein, the term “pharmacologically active” (or simply “active”), as in a “pharmacologically active” derivative or analog, can refer to a derivative or analog (e.g., a salt, ester, amide, conjugate, metabolite, isomer, fragment, etc.) having the same type of pharmacological activity as the parent compound and approximately equivalent in degree.

As used herein, the term “mixture” can include solutions in which the components of the mixture are completely miscible, as well as suspensions and emulsions, in which the components of the mixture are not completely miscible.

As used herein, the term “subject” or “host” can refer to living organisms such as mammals, including, but not limited to humans, livestock, dogs, cats, and other mammals. Administration of the therapeutic agents can be carried out at dosages and for periods of time effective for treatment of a subject. In some embodiments, the subject is a human. In some embodiments, the pharmacokinetic profiles of the systems of the present invention are similar for male and female subjects.

As used herein, the term “controlled-release” or “controlled-release drug delivery” or “extended release” refers to release or administration of a drug from a given dosage form in a controlled fashion in order to achieve the desired pharmacokinetic profile in vivo. An aspect of “controlled” drug delivery is the ability to manipulate the formulation and/or dosage form in order to establish the desired kinetics of drug release.

The phrases "concurrent administration", "administration in combination", "simultaneous administration" or "administered simultaneously" as used herein, means that the compounds are administered at the same point in time or immediately following one another. In the latter case, the two compounds are administered at times sufficiently close that the results observed are indistinguishable from those achieved when the compounds are administered at the same point in time.

Compositions and Methods

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men worldwide. Due to late diagnosis and lack of effective drugs for treatment, HCC is the 2nd highest cause of male death from cancer. Only a small proportion of HCC patients are diagnosed at an early stage,

which enables the use of curative treatments such as tumor resection or liver transplant. However, most patients go undiagnosed until the disease has progressed to an advanced stage where there is little hope. Sorafenib, a multikinase inhibitor, is currently the only approved drug used in treating such patients. Unfortunately, the average overall survival of patients treated with sorafenib is only extended by 2.8 months compared to untreated patients. Although sorafenib treatment was shown to extend the overall survival of HCC patients, only 2% of patients displayed partial response to therapy based on RECIST criteria (Response Evaluation Criteria in Solid Tumors). This low response rate is attributed to HCC tumors having an intrinsic resistance to sorafenib toxicity. Since there are no other FDA approved therapies for advanced HCC patients, novel therapeutic strategies were developed to sensitize HCC tumors to sorafenib toxicity.

Disclosed herein is a novel therapeutic combination of sorafenib and 2-deoxyglucose for the treatment of a cancer in human patients. In one embodiment, disclosed herein is a novel therapeutic combination of sorafenib and 2-deoxyglucose for the treatment of advanced stage hepatocellular carcinoma in human patients. Hepatocellular carcinoma (HCC) is the fifth most common cancer in men worldwide. Due to late diagnosis and lack of effective drugs for treatment, HCC is the 2nd highest cause of cancer related death in males. Sorafenib, a multi-kinase inhibitor, is currently the only FDA approved drug for the treatment of advanced stage HCC. Unfortunately, the average overall survival of patients treated with sorafenib is only extended by 2.8 months compared to untreated patients. Since there are no other FDA approved therapies for advanced HCC patients, we sought to develop a novel therapeutic strategy to sensitize HCC tumors to sorafenib. After screening several therapeutic combinations, it was determined that the combination of 2- deoxyglucose with sorafenib synergistically inhibits the growth of HCC cells. 2-deoxyglucose is a glucose analog which has been shown to inhibit glycolysis. Several experiments were performed to validate this synergy (see examples below). In some embodiments, the final product is a therapeutic product that is administered to HCC patients by mouth at least once per day. This therapeutic product contains a clinically relevant amount of sorafenib and 2-deoxyglucose. This therapeutic combination can also be formulated as two separate therapeutic products: one containing sorafenib and the other containing 2-deoxyglucose. This product can be prescribed by oncologists for the treatment of advanced stage HCC.

In one aspect, disclosed herein is a pharmaceutical composition comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.

In some embodiments, the composition further comprises an additional chemotherapeutic agent.

In one aspect, disclosed herein is a kit comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof. In some embodiments, sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation. In some
5 embodiments, sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.

In another aspect, disclosed herein is a method of treating or preventing a cancer in a subject in need thereof, comprising: administering to the subject a therapeutically effective
10 amount of sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.

In some embodiments, the cancer is selected from hepatocellular carcinoma, breast cancer, or chronic lymphocytic leukemia (CLL). In some embodiments, the cancer is hepatocellular carcinoma (HCC). In some embodiments, the cancer is resistant to sorafenib.

15 In another aspect, disclosed herein is a method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.

In some embodiments, the administration of sorafenib and 2-deoxyglucose provides a synergistic effect. In some embodiments, the administration of sorafenib and 2-deoxyglucose
20 inhibits ATP production.

In some embodiments, sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation. In some embodiments, sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.

25 In some embodiments, the method further comprises the administration of an additional chemotherapeutic agent.

In additional aspects of the invention, fostamatinib or dasatinib are used as therapeutic options for liver cancer, both as first line therapies and following relapse from sorafenib. In one embodiment, disclosed herein is the new use for existing drugs to reduce the huge time and
30 cost associated with the traditional drug discovery process. The use case disclosed herein is liver cancer (primary and sorafenib resistant) due to the increasing incidence of this cancer and lack of effective therapies that results in high rates of mortality. The drug repurposing analysis identified fostamatinib as a drug that could reverse the gene expression signature of primary liver cancer compared to normal liver tissue as well as sorafenib resistant liver cancer cell lines

generated by continuous long-term exposure to sorafenib compared to the parental sensitive cells. This finding has been validated in the lab using in vitro assays of cell growth. An important aspect of this finding is that the target of this drug (spleen tyrosine kinase) is not expressed in liver cancer cells. Currently no other targets of this drug are known. It thus appears that the drug is functioning through a novel mechanism. As a result, traditional drug discovery approaches that are based on specific targets would miss this drug as a potential candidate for therapy of liver cancer. One advantage of this discovery is that because this drug is already in clinical trials for autoimmune disorders and hematologic diseases, preliminary preclinical data is already available to expedite clinical trials for this new indication, liver cancer. Further, due to the lab validation approach, the chances of success in the clinic are very high.

In a further aspect, disclosed herein is a method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of fostamatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

In some embodiments, the method further comprises the administration of an additional chemotherapeutic agent. In some embodiments, the additional chemotherapeutic is sorafenib.

In one aspect, disclosed herein is a method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of dasatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

In some embodiments, the method further comprises the administration of an additional chemotherapeutic agent. In some embodiments, the additional chemotherapeutic is sorafenib.

In some embodiments, the composition is a combination of sorafenib and 2-deoxyglucose for the treatment of cancer. In some embodiments, the combination of sorafenib and 2-deoxyglucose for the treatment of hepatocellular carcinoma. In some embodiments, the combination of sorafenib and 2-deoxyglucose for the treatment of cancer following evidence of sorafenib resistance. In some embodiments, the combination of sorafenib and 2-deoxyglucose for the treatment of hepatocellular carcinoma following evidence of sorafenib resistance. In some embodiments, disclosed herein is a method of treating cancer, including hepatocellular carcinoma, resistant to sorafenib via administration of fostamatinib. In some embodiments, disclosed herein is a method of treating cancer, including hepatocellular carcinoma, resistant to sorafenib via administration of dasatinib. In some embodiments, the combination of therapeutic agents is described herein. In some embodiments, the combination of therapeutic agents following evidence of sorafenib resistance are described herein. In some embodiments, disclosed

herein is a method of treating disease in a patient as described herein. In some embodiments, disclosed herein is a method of treating cancer in a patient. In some embodiments, disclosed herein is a method of treating hepatocellular carcinoma in a patient.

FDA approved anti-neoplastic kinase inhibitors identified for the treatment of hepatocellular carcinoma in the repurposing studies described herein include, for example: dasatinib (targets SRC family kinases, ABL), nilotinib (targets BCR-ABL, PDGFR, KIT), palbociclib (targets CDK4, CDK6), vemurafenib (targets BRAF), enzalutamide (targets AR), paclitaxel (targets TUBB1, BCL2, NR1H2, MAP4, MAP2), pemetrexed (targets TYMS, ATIC, DHFR, GART), toremifene (targets ESR1), aminoglutethimide (targets CYP19A1, CYP11A1), anastrozole (targets CYP19A1), procarbazine (targets DNA), thiotepa (targets DNA), and verteporfin.

Investigational anti-neoplastic kinase inhibitors identified for the treatment of hepatocellular carcinoma in the repurposing studies described herein include, for example: bms-754807 (targets IGF1R, InsR), brivanib (targets VEGFR), enmd-2076 (targets Aurora kinases, non-specified TKs), enzastaurin (targets PKC β), fostamatinib (targets SYK), motesanib (targets VEGFR, PDGFR, KIT, Ret), pf-04217903 (targets MET), quizartinib (targets FLT3, CSFR, KIT, PDGFR), roscovitine (targets CDK2/E, CDK2/A, CDK7, CDK9), sns-314 (targets Aurora kinases), darinaparsin, orteronel (targets CYP17A1), and tipifarnib (targets FNTB).

Additional Therapeutics for Treating Hepatocellular Carcinoma (HCC)

Numerous additional therapeutic drugs (drugs useful for treating cancer, including hepatocellular carcinoma, resistant to sorafenib) are available for use alone or in combination with the present compositions and methods. The following is a non-exhaustive lists of therapeutic drugs for treating hepatocellular carcinoma, and may include: acadesine, acarbose, albendazole, alclometasone, alfuzosin, alpha-linolenic-acid, alprenolol, ambroxol, amcinonide, amiloride, aminoglutethimide, amoxapine, anastrozole, atorvastatin, azithromycin, bendroflumethiazide, benzthiazide, benzydamine, benzylpenicillin, betamethasone, biperiden, bisacodyl, bms-754807, brivanib, bromhexine, bromocriptine, brompheniramine, budesonide, calcitriol, canrenoic-acid, carbamazepine, carbidopa, carbinoxamine, cefaclor, cefdinir, chloroquine, chlorphenamine, chlorpromazine, cholic-acid, cilomilast, cimetidine, cisapride, citalopram, clomifene, cortisone, cycloentolate, dalcetrapib, danoprevir, darinaparsin, dasatinib, desipramine, desoximetason, dexamethasone, dexchlorpheniramine, diclofenac, dienestrol, diethylstilbestrol, diflunisal, dihydroxydine, diloxanide, diltiazem, dipyridamole, domperidone, dosulepin, doxepin, doxylamine, droperidol, duloxetine, enmd-2076,

enzalutamide, enzastaurin, epicatechin, equilin, estradiol, estriol, estrone, estropipate, etodolac, famciclovir, flavoxate, flecainide, flucloxacillin, fluconazole, flumazenil, flunisolide, flupirtine, fluvastatin, fostamatinib, fulvestrant, genistein, glibenclamide, glimepiride, goserelin, guanfacine, halcinonide, hydrocortisone, hypericin, iloperidone, ipratropium, ketoprofen, labetalol, lamivudine, lenalidomide, loperamide, lovastatin, mafenide, mebeverine, meloxicam, mephentermine, mepyramine, mesoridazine, metolazone, metoprolol, mianserin, mirtazapine, mometasone, motesanib, moxifloxacin, nafcillin, naltrexone, niacin, nicardipine, nicergoline, nicotinamide, nifedipine, nilotinib, norgestrel, nortriptyline, omeprazole, ondansetron, ornidazole, orteronel, ouabain, oxaprozin, oxybenzone, oxypheonium, paclitaxel, palbociclib, palonosetron, paroxetine, pemetrexed, peralide, pf-04217903, phenelzine, phentermine, pimozide, pindolol, pirfenidone, prazosin, prednicarbate, prednisolone, pregnenolone, primidone, procarbazine, progesterone, promazine, propranolol, prostaglandin, protriptyline, quinapril, quinidine, quizartinib, racecadotril, raloxifene, ranitidine, rescinamine, retinal, rilmenidine, riluzole, ritodrine, rizatriptan, rolipram, rolitetracycline, roscovitine, safinamide, salmeterol, saquinavir, sildenafil, simvastatin, sirolimus, sitagliptin, sns-314, sulforaphane, tacrolimus, tamoxifen, teicoplanin, terazosin, terconazole, testosterone, tg100-115, thiotepa, thiothixene, ticlopidine, tipifamib, tolazamide, toremifene, tramadol, trifluoperazine, triflupromazine, trimethobenzamide, troglitazone, tropisetron, valproic-acid, vardenafil, vecuronium, vemurafenib, verapamil, verteporfin, vinpocetine, voriconazole, vx-222, xaliproden, zileuton, ziprasidone, and zosuquidar.

Cancers

As contemplated herein, the cancer treated can be a primary tumor or a metastatic tumor. In one aspect, the methods described herein are used to treat a solid tumor, for example but not limited to, melanoma, lung cancer (including lung adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, large cell carcinoma, bronchioloalveolar carcinoma, bronchogenic carcinoma, non-small-cell carcinoma, small cell carcinoma, mesothelioma); breast cancer (including ductal carcinoma, lobular carcinoma, inflammatory breast cancer, clear cell carcinoma, mucinous carcinoma, serosal cavities breast carcinoma); colorectal cancer (colon cancer, rectal cancer, colorectal adenocarcinoma); anal cancer; pancreatic cancer (including pancreatic adenocarcinoma, islet cell carcinoma, neuroendocrine tumors); prostate cancer; prostate adenocarcinoma; ovarian carcinoma (ovarian epithelial carcinoma or surface epithelial-stromal tumor including serous tumor, endometrioid tumor and mucinous cystadenocarcinoma,

sex-cord-stromal tumor); liver and bile duct carcinoma (including hepatocellular carcinoma, cholangiocarcinoma, hemangioma); esophageal carcinoma (including esophageal adenocarcinoma and squamous cell carcinoma); oral and oropharyngeal squamous cell carcinoma; salivary gland adenoid cystic carcinoma; bladder cancer; bladder carcinoma; carcinoma of the uterus (including endometrial adenocarcinoma, ovarian, uterine papillary serous carcinoma, uterine clear-cell carcinoma, uterine sarcomas, leiomyosarcomas, mixed müllerian tumors); glioma, glioblastoma, medulloblastoma, and other tumors of the brain; kidney cancers (including renal cell carcinoma, clear cell carcinoma, Wilms' tumor); cancer of the head and neck (including squamous cell carcinomas); cancer of the stomach (gastric cancers, stomach adenocarcinoma, gastrointestinal stromal tumor); testicular cancer; germ cell tumor; neuroendocrine tumor; cervical cancer; carcinoids of the gastrointestinal tract, breast, and other organs; signet ring cell carcinoma; mesenchymal tumors including sarcomas, fibrosarcomas, haemangioma, angiomatosis, haemangiopericytoma, pseudoangiomatous stromal hyperplasia, myofibroblastoma, fibromatosis, inflammatory myofibroblastic tumor, lipoma, angiolipoma, granular cell tumor, neurofibroma, schwannoma, angiosarcoma, liposarcoma, rhabdomyosarcoma, osteosarcoma, leiomyoma, leiomyosarcoma, skin, including melanoma, cervical, retinoblastoma, head and neck cancer, pancreatic, brain, thyroid, testicular, renal, bladder, soft tissue, adrenal gland, urethra, cancers of the penis, myxosarcoma, chondrosarcoma, osteosarcoma, chordoma, malignant fibrous histiocytoma, lymphangiosarcoma, mesothelioma, squamous cell carcinoma; epidermoid carcinoma, malignant skin adnexal tumors, adenocarcinoma, hepatoma, hepatocellular carcinoma, renal cell carcinoma, hypernephroma, cholangiocarcinoma, transitional cell carcinoma, choriocarcinoma, seminoma, embryonal cell carcinoma, glioma anaplastic; glioblastoma multiforme, neuroblastoma, medulloblastoma, malignant meningioma, malignant schwannoma, neurofibrosarcoma, parathyroid carcinoma, medullary carcinoma of thyroid, bronchial carcinoid, pheochromocytoma, Islet cell carcinoma, malignant carcinoid, malignant paraganglioma, melanoma, Merkel cell neoplasm, cystosarcoma phylloides, salivary cancers, thymic carcinomas, and cancers of the vagina among others. In one embodiment, the cancer is melanoma.

In one embodiment, the compounds, compositions, and methods described herein are useful for the treatment of cancers or tumors or proliferative disorder such as, but not limited to: chronic lymphocytic leukemia (CLL), multiple myeloma; Diffuse large B cell lymphoma; Follicular lymphoma; Mucosa-Associated Lymphatic Tissue lymphoma (MALT); Small cell lymphocytic lymphoma; Mediastinal large B cell lymphoma; Nodal marginal zone B cell lymphoma (NMZL); Splenic marginal zone lymphoma (SMZL); Intravascular large B-cell

lymphoma; Primary effusion lymphoma; or Lymphomatoid granulomatosis; B-cell prolymphocytic leukemia; Hairy cell leukemia; Splenic lymphoma/leukemia, unclassifiable; Splenic diffuse red pulp small B-cell lymphoma; Hairy cell leukemia-variant; Lymphoplasmacytic lymphoma; Heavy chain diseases, for example, Alpha heavy chain disease, 5 Gamma heavy chain disease, Mu heavy chain disease; Plasma cell myeloma; Solitary plasmacytoma of bone; Extraosseous plasmacytoma; Primary cutaneous follicle center lymphoma; T cell/histiocyte rich large B-cell lymphoma; DLBCL associated with chronic inflammation; Epstein-Barr virus (EBV)+ DLBCL of the elderly; Primary mediastinal (thymic) large B-cell lymphoma; Primary cutaneous DLBCL, leg type; ALK+ large B-cell lymphoma; 10 Plasmablastic lymphoma; Large B-cell lymphoma arising in HHV8-associated multicentric; Castleman disease; B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma; or B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma.

In some embodiments, the cancer is selected from hepatocellular carcinoma (HCC), 15 breast cancer, or chronic lymphocytic leukemia (CLL). In some embodiments, the cancer is hepatocellular carcinoma (HCC). In one embodiment, the cancer is breast cancer. In some embodiments, the cancer is chronic lymphocytic leukemia (CLL).

Composition Components

20 Compositions, as described herein, comprising an active compound and an excipient of some sort may be useful in a variety of applications.

“Excipients” include any and all solvents, diluents or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form 25 desired. General considerations in formulation and/or manufacture can be found, for example, in *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), and *Remington: The Science and Practice of Pharmacy*, 21st Edition (Lippincott Williams & Wilkins, 2005). The pharmaceutically acceptable excipients may also include one or more of fillers, binders, lubricants, glidants, disintegrants, and the like.

30 Exemplary excipients include, but are not limited to, any non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some examples of materials which can serve as excipients include, but are not limited to, sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its

derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as Tween 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. As would be appreciated by one of skill in this art, the excipients may be chosen based on what the composition is useful for. For example, with a pharmaceutical composition or cosmetic composition, the choice of the excipient will depend on the route of administration, the agent being delivered, time course of delivery of the agent, etc., and can be administered to humans and/or to animals, orally, rectally, parenterally, intracisternally, intravaginally, intranasally, intraperitoneally, topically (as by powders, creams, ointments, or drops), buccally, or as an oral or nasal spray.

Exemplary diluents include calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, etc., and combinations thereof.

Exemplary granulating and/or dispersing agents include potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (Veegum), sodium lauryl sulfate, quaternary ammonium compounds, etc., and combinations thereof.

Exemplary surface active agents and/or emulsifiers include natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and Veegum [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol,

triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [Tween 20], polyoxyethylene sorbitan [Tween 60], polyoxyethylene sorbitan monooleate [Tween 80], sorbitan monopalmitate [Span 40], sorbitan monostearate [Span 60], sorbitan tristearate [Span 65], glyceryl monooleate, sorbitan monooleate [Span 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [Myrj 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and Solutol), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. Cremophor), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [Brij 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, Pluronic F 68, Poloxamer 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

Exemplary binding agents include starch (e.g. cornstarch and starch paste), gelatin, sugars (e.g. sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol, etc.), natural and synthetic gums (e.g. acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum), and larch arabogalactan), alginates, polyethylene oxide, polyethylene glycol, inorganic calcium salts, silicic acid, polymethacrylates, waxes, water, alcohol, etc., and/or combinations thereof.

Exemplary preservatives include antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and other preservatives.

Exemplary antioxidants include alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and sodium sulfite.

Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA) and salts and hydrates thereof (e.g., sodium edetate, disodium edetate, trisodium edetate, calcium disodium

edetate, dipotassium edetate, and the like), citric acid and salts and hydrates thereof (e.g., citric acid monohydrate), fumaric acid and salts and hydrates thereof, malic acid and salts and hydrates thereof, phosphoric acid and salts and hydrates thereof, and tartaric acid and salts and hydrates thereof. Exemplary antimicrobial preservatives include benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxymenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and thimerosal.

Exemplary antifungal preservatives include butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and sorbic acid.

Exemplary alcohol preservatives include ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and phenylethyl alcohol.

Exemplary acidic preservatives include vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and phytic acid.

Other preservatives include tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, Glydant Plus, Phenonip, methylparaben, Germall 115, Germaben II, Neolone, Kathon, and Euxyl. In certain embodiments, the preservative is an anti-oxidant. In other embodiments, the preservative is a chelating agent.

Exemplary buffering agents include citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium gluconate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, etc., and combinations thereof.

Exemplary lubricating agents include magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, etc., and combinations thereof.

5 Exemplary natural oils include almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, 10 nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary synthetic oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 15 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and combinations thereof.

Additionally, the composition may further comprise a polymer. Exemplary polymers contemplated herein include, but are not limited to, cellulosic polymers and copolymers, for example, cellulose ethers such as methylcellulose (MC), hydroxyethylcellulose (HEC), 20 hydroxypropyl cellulose (HPC), hydroxypropyl methyl cellulose (HPMC), methylhydroxyethylcellulose (MHEC), methylhydroxypropylcellulose (MHPC), carboxymethyl cellulose (CMC) and its various salts, including, e.g., the sodium salt, hydroxyethylcarboxymethylcellulose (HECMC) and its various salts, carboxymethylhydroxyethylcellulose (CMHEC) and its various salts, other polysaccharides and 25 polysaccharide derivatives such as starch, dextran, dextran derivatives, chitosan, and alginic acid and its various salts, carageenan, various gums, including xanthan gum, guar gum, gum arabic, gum karaya, gum ghatti, konjac and gum tragacanth, glycosaminoglycans and proteoglycans such as hyaluronic acid and its salts, proteins such as gelatin, collagen, albumin, and fibrin, other polymers, for example, polyhydroxyacids such as polylactide, polyglycolide, poly(lactide-co-glycolide) and poly(epsilon-caprolactone-co-glycolide)-, carboxyvinyl polymers and their salts 30 (e.g., carbomer), polyvinylpyrrolidone (PVP), polyacrylic acid and its salts, polyacrylamide, polyacilic acid/acrylamide copolymer, polyalkylene oxides such as polyethylene oxide, polypropylene oxide, poly(ethylene oxide-propylene oxide), and a Pluronic polymer, polyoxyethylene (polyethylene glycol), polyanhydrides, polyvinylalcohol, polyethyleneamine and

polypyrridine, polyethylene glycol (PEG) polymers, such as PEGylated lipids (e.g., PEG-stearate, 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-1000], 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000], and 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-5000]), copolymers and salts thereof.

Additionally, the composition may further comprise an emulsifying agent. Exemplary emulsifying agents include, but are not limited to, a polyethylene glycol (PEG), a polypropylene glycol, a polyvinyl alcohol, a poly-N-vinyl pyrrolidone and copolymers thereof, poloxamer nonionic surfactants, neutral water-soluble polysaccharides (e.g., dextran, Ficoll, celluloses), non-cationic poly(meth)acrylates, non-cationic polyacrylates, such as poly(meth)acrylic acid, and esters amide and hydroxyalkyl amides thereof, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and Veegum [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [Tween 20], polyoxyethylene sorbitan [Tween 60], polyoxyethylene sorbitan monooleate [Tween 80], sorbitan monopalmitate [Span 40], sorbitan monostearate [Span 60], sorbitan tristearate [Span 65], glyceryl monooleate, sorbitan monooleate [Span 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [Myrj 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and Solutol), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. Cremophor), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [Brij 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, Pluronic F 68, Poloxamer 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof. In certain embodiments, the emulsifying agent is cholesterol.

Liquid compositions include emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compound, the liquid composition may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and

emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Injectable compositions, for example, injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents for pharmaceutical or cosmetic compositions that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. Any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. In certain embodiments, the particles are suspended in a carrier fluid comprising 1% (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) Tween 80. The injectable composition can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Compositions for rectal or vaginal administration may be in the form of suppositories which can be prepared by mixing the particles with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol, or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the particles.

Solid compositions include capsules, tablets, pills, powders, and granules. In such solid compositions, the particles are mixed with at least one excipient and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc,

calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Tablets, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Compositions for topical or transdermal administration include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The active compound is admixed with an excipient and any needed preservatives or buffers as may be required.

The ointments, pastes, creams, and gels may contain, in addition to the active compound, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc, and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the active compound, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

Transdermal patches have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the nanoparticles in a proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the particles in a polymer matrix or gel.

EXAMPLES

The following examples are set forth below to illustrate the compounds, compositions, methods, and results according to the disclosed subject matter. These examples are not intended to be inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative methods and results. These examples are not intended to exclude equivalents and variations of the present invention which are apparent to one skilled in the art.

Example 1. Sorafenib and 2-Deoxyglucose Synergistically Inhibit Proliferation of both Sorafenib Sensitive and Resistant HCC Cells by Inhibiting ATP Production

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths globally ^{1,2}. Sorafenib is the only first-line systemic drug for advanced HCC, but it has very limited survival benefits because patients treated with sorafenib either suffer from side effects or show disease progression after initial response. Thus, there is an urgent need to develop novel strategies for first-line and second-line therapy. The association between sorafenib resistance and glycolysis prompted us to screen several drugs with known anti-glycolytic activity to identify those that will sensitize cells to sorafenib. In this example, it was demonstrated that the combination of glycolytic inhibitor 2-deoxy-D-glucose (2DG) and sorafenib drastically inhibits viability of sorafenib sensitive and resistant cells. However, the combination of other anti-glycolytic drugs like lonidamine, gossypol, 3-bromopyruvate and imatinib with sorafenib does not show synergistic effect. Cell cycle analysis revealed that the combination of 2DG and sorafenib induced cell cycle arrest at G₀/G₁. Mechanistic investigation suggests that the cell-cycle arrest is due to depletion of cellular ATP that activates AMP-activated protein kinase (AMPK), which, in turn, inhibits mammalian target of rapamycin (mTOR) to induce cell cycle arrest. This example discloses the unexpected therapeutic combination of sorafenib and 2-deoxyglucose for treating cancer, for example, HCC.

Background

Hepatocellular carcinoma (HCC) is the second most common cancer in men worldwide. Due to late diagnosis and lack of effective drugs for treatment, HCC is the second highest cause of death in males from cancer ³. Only a small proportion of HCC patients are diagnosed at an early stage, which enables the use of curative treatments such as tumor resection or liver transplant. Unfortunately, most patients go undiagnosed until the disease has progressed to an advanced stage when none of the available treatments are effective. Sorafenib, a multi-kinase

inhibitor, is currently the only FDA approved drug used in treating such patients⁴. Unfortunately, the average overall survival of patients treated with sorafenib is only extended by 2.8 months compared to untreated patients⁵. Although sorafenib treatment was shown to extend the overall survival of HCC patients, only 2% of patients displayed partial response to therapy based on
5 RECICT criteria (Response Evaluation Criteria in Solid Tumors)⁵. This low response rate is attributed to intrinsic resistance of HCC to sorafenib toxicity⁶. In view of the lack of other FDA approved therapies for advanced HCC patients, it is critical to develop novel therapeutic strategies to sensitize HCC tumors to sorafenib toxicity, which could extend the survival of HCC patients.

10 The mechanisms that mediate sorafenib resistance remain relatively unknown⁶. A handful of studies have demonstrated that a variety of mechanisms are involved in maintaining sorafenib resistance, which include CD44 overexpression¹, activation of PI3K/AKT signaling⁷ and increased MAPK14 activity⁸. Another group of studies has linked sorafenib sensitivity to cellular metabolism and glycolysis^{9,10}. These studies are interesting because sorafenib therapy
15 has been shown to inhibit oxidative phosphorylation and enhance glycolysis in a subset of HCC cell lines².

In order to elucidate further the most significant mechanism(s) of sorafenib resistance, the inventors have developed sorafenib resistant HCC cell lines. Here, it is demonstrated that rates of glycolysis are markedly higher in sorafenib resistant HCC cells than parental HCC cells
20 when treated with sorafenib. Glucose consumption and lactate production were measured in sorafenib sensitive and resistant cells and initially examined the combination of several anti-glycolytic agents/drugs and sorafenib in the resistant cell lines. This example unexpectedly shows that only one anti-glycolytic drug, 2-deoxyglucose (2DG), displayed synergy with sorafenib. 2DG is a structural analog of glucose, which inhibits glycolysis^{11,12}. In this example, drastic
25 inhibition of cell growth was demonstrated by combined treatment with these two drugs, elucidating the mechanisms underlying the remarkable synergistic effect of these drugs in sorafenib sensitive and resistant HCC cell lines, and offer a therapeutic strategy to treat hepatocellular carcinoma particularly at an advanced stage.

Materials and Methods

30 *Reagents and Antibodies*

Sorafenib (catalog #S-8502) was purchased from LC Laboratories (Woburn, MA, USA). Lonidamine (catalog # L5658), gossypol (Catalog # G5874) and imatinib (catalog # I-5577) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). 2-Deoxy-D-glucose (catalog

#D6134) and propidium iodide (catalog # 81845) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies used for western blotting were purchased from Cell Signaling (Danvers, MA, USA). All other reagents were of molecular biology grade.

Cell Culture

5 All cells were maintained in Minimum Essential Media supplemented with L-glutamine (2 mM), 10% FBS, sodium pyruvate (0.11 g/L) and penicillin/streptomycin (100 U/mL). Cell media for sorafenib resistant cell lines was also supplemented with sorafenib (6 μ M in DMSO with 0.1% final DMSO concentration). Sorafenib was withdrawn from the cell media of resistant Huh7 cells for 5-7 days prior to performing all experiments.

10 Hep3B cells were obtained from the ATCC. In this paper, “Huh7-S” refers to the originally sorafenib sensitive Huh7 cells that were generously provided by Dr. James Taylor (Fox Chase Center, PA, USA). Sorafenib resistant cells “Huh7-R-Pool” and “Huh7-R-A7” were generated in the inventors’ laboratory. In order to generate Huh7-R-Pool cells, Huh7-S cells grown in MEM media were pulsed with 10 μ M sorafenib for 4 hours every week for 6 weeks.

15 The cells were then maintained in low concentration of sorafenib. Media sorafenib concentration was slowly increased to a final concentration of 6 μ M after several months. Several individual clones were isolated from the Huh7-R-Pool cells. The “Huh7-R-A7” cell line is one such clone.

Cell Viability and ATP Assays

Cells were seeded into Eppendorf 96 well plates (~2,000 cells/well) and allowed to attach overnight. Cell media was then changed for media containing sorafenib or other therapeutics in DMSO with 1% final DMSO concentration. After 48 hours of incubation, the CellTiter-Glo® was added following the manufacture’s protocol (Promega: Madison, WI). The luminescent supernatant was transferred to an opaque luminometer 96-well plate prior to measuring luminescence. The same procedure was followed for the ATP measurement assay. For drug combinations, Combination Index was calculated using CompuSyn software. The assays with sorafenib and 2DG were performed in triplicates and repeated twice.

Glucose Consumption and Lactate Production

Cells were seeded into 6 well plates (50% confluency) and allowed to attach overnight. Cells were then treated for 48 hours with phenol-red free DMEM media containing therapeutics in DMSO with 1% final DMSO concentration. After 4 hours, cell media supernatant was removed and analyzed for glucose and lactate concentrations.

Media glucose concentration was measured using a ReliOn® ULTIMA glucometer (Alameda, CA). Cell media was diluted 1:1 with PBS prior to glucose measurement to bring it within the linear range of the instrument. Glucose concentrations were compared to “fresh”

media that was not exposed to cellular metabolism. Glucose consumption was determined by subtracting the cellular glucose concentration from that of the fresh media. Since 2-deoxyglucose is detected by the glucometer at the same sensitivity as D-glucose, media containing 2-deoxyglucose was compared to fresh media containing the same initial concentration of 2-deoxyglucose. The glucose consumption measured by these cells is equivalent to D-glucose + 2-deoxyglucose consumption. This method does not allow distinguishing D-glucose consumption from 2-deoxyglucose consumption.

Media lactate concentrations were measured using the L-Lactate Assay kit from ScienCell (Carlsbad, CA). Cell media was diluted 1:30 with the kit assay buffer prior to measurement to bring it within the linear measurement range. The assay was conducted following the manufacturer's recommendations.

The assays were performed in biological replicates. Two-tailed non-paired t-test was used to determine statistical significance.

Colony Formation Assay

Cells were seeded into 6 well plates (2,000-5,000 cells/well) and allowed to attach for 24-48 hours. Cells were then treated with a continuous dose of therapeutics in DMSO (final concentration 1%) for 14 to 18 days. Media was changed every three days. After colonies were of sufficient size, the cells were fixed with 3.7% paraformaldehyde (in PBS). Cells were then stained with a 0.05% crystal violet solution and imaged. The assay was performed in biological duplicates for each cell type and drug combination.

Cell Cycle Analysis

Cells were seeded into 6 well plates (50% confluency) and allowed to attach overnight. Cells were then treated for 48 hours with therapeutics in DMSO (final concentration 1%). After 48 hours, cells were collected via trypsinization and fixed in 75% Ethanol. After washing, cells were stained in a solution containing PI (0.5 mg/mL) and RNase A (10 mg/mL). Cells were filtered through a 70 μ M cell strainer immediately prior to flow cytometry. Flow cytometry was performed at the Ohio State University Comprehensive Cancer Center Analytical Cytometry Core Facility on a BD LSR II (San Jose, CA).

Western blotting

Proteins extracted from cells were immunoblotted with different antibodies following published protocol^{13,14}. Briefly, cells were seeded into 60 mm dish and allowed to grow overnight. Cells were then treated with drugs dissolved in DMSO (final concentration 1%) for 6 hours and an equal amount of protein lysates prepared in the lysis buffer (Cell Signaling) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto

nitrocellulose membrane. After blocking with blocking buffer (LI-COR, Lincoln, NE, USA) containing 0.1% Tween-20, the membrane was incubated with primary antibodies overnight at 4°C. Following incubation with appropriate secondary antibody (IRD-680 or IRD-800), the immunoreactive bands were visualized using LI-COR-Odyssey infra-red scanner (LI-COR). The blots were re-probed with β -actin to correct for differences in protein loading. Protein was estimated using a Bio-Rad protein assay kit (cat #500-0006) with bovine serum albumin as standard.

Results

Establishment of Sorafenib Resistant HCC Cell Lines

In order to study sorafenib resistance, sorafenib resistant cell lines were generated from the human HCC cell line Huh7. In brief, Huh7-S cells were initially pulsed with high dose of sorafenib followed by continuous exposure to increasing doses of sorafenib to induce resistance. From this pool of resistant cells (Huh7-R-Pool), individual resistant clones exhibiting high degrees of resistance were isolated. These cell lines demonstrated a remarkable resistance to sorafenib toxicity; the IC_{50} dose for the resistant cells was about 4-5 times higher than that of the parental cells (Figure 1A).

There have been several recent studies linking sorafenib toxicity and resistance to glycolytic flux. One study demonstrated that exposure of rat hepatocolangiocarcinoma cells to sorafenib induces increased rates of glycolysis¹⁰. Another study demonstrated that increased glycolytic utilization has a strong correlation with sorafenib resistance across several HCC cell lines⁹. We, therefore, sought to investigate the glycolytic flux of sorafenib sensitive and resistant cells exposed to sorafenib. Interestingly, the resistant cells demonstrated a large increase in glucose consumption and lactate production when exposed to increasing concentrations of sorafenib (Figure 1B, C). However, parental Huh7 cells show minimal change in glucose consumption and lactate production upon sorafenib exposure. Based on these observations, it was hypothesized that increased glycolytic flux is a key mechanism for the resistance of HCC cells to sorafenib-induced toxicity. Thus, combination of sorafenib with therapeutics that inhibit glycolysis could sensitize cells to sorafenib toxicity.

In Vitro Screening of Anti-Glycolytic Agents

To determine if the inhibition of glycolysis could sensitize HCC cells to sorafenib toxicity, therapeutics were identified that were known to inhibit glycolysis. To accelerate the

future clinical trial process of successful therapeutic combinations identified in this study, the initial focus was on drugs that are already FDA approved or undergoing clinical trials for another indication. Table 1 contains a list of anti-glycolytic drugs selected for this study.

5 **Table 1. Anti-Glycolytic Therapeutics tested in this example.**

Drug Name	Anti-glycolytic Mechanism	Development Stage	Clinical Use	Clinical Trial Citation
2-Deoxyglucose	Glucose Analog: Hexokinase Inhibition	Phase II	Prostate Cancer	19
Gossypol	GAPDH Inhibition	Phase II/III	Lung Cancer	22
Imatinib	Inhibition	FDA Approved	CML	24
Lonidamine	Hexokinase Inhibition	Phase III	Prostate Hyperplasia	-

Each therapeutic was used alone and in combination with sorafenib to generate dose-dependent viability curves in Huh7-R-Pool cells (Figure 2A-E). The degree of synergy between sorafenib and the anti-glycolytic drug was quantified using the widely accepted Chou-Talalay combination index (CI) method¹⁵. The CI for lonidamine could not be calculated accurately because it did not show any toxicity on its own (Figure 2E). A combination index value of less than 1 indicates that the drugs are acting synergistically; a lower CI value indicates a greater degree of synergy. Several of the key CI values for the combination of sorafenib and 2-deoxyglucose (2DG) were less than 1, demonstrating synergy (Figure 2A). This initial screening demonstrated that the anti-glycolytic agent (2DG) significantly potentiated sorafenib toxicity whereas 3-bromopyruvate, gossypol, imatinib, and lonidamine showed little or no synergy (Figure 2B-E).

The synergistic combination of sorafenib and 2DG was further confirmed in the Huh7-S cells, Huh7-R-A7 cells and another HCC cell line, Hep3B (Figure 2 F-H). Interestingly, 2DG treatment alone had very low toxicity whereas the combination of sorafenib and 2DG drastically inhibited cell growth. These experiments demonstrate that the combination of sorafenib and 2DG is more effective than sorafenib alone.

Combination of Sorafenib and 2DG Inhibits Colony Growth

In order to further validate the synergistic combination of sorafenib and 2DG, colony formation assays were performed in parental and resistant Huh7 cell lines (Figure 3). Treatment with sorafenib (2uM-4uM) alone showed a dose-dependent inhibition of colony formation while 2DG had little effect on colony formation in all cell lines even when used at 0.5 mM. However, combination of 2DG and sorafenib resulted in drastic inhibition of colony formation compared to independent drug treatments. Further investigation demonstrated that the number of colonies between all treatments was similar; however, the number of cells within each colony was dramatically different. Cells treated with a combination of high dose (4uM) of sorafenib and 2DG formed colonies of only 1-4 cells. These tiny colonies are hardly visible upon visual inspection, but can be seen in microscopic images (Figures 3, bottom panel). These data suggest that the combination of sorafenib and 2DG primarily results in inhibition of cell growth with minimal effect on cell death.

Combined treatment with 2DG and sorafenib inhibits cell cycle progression

Next, the mechanism driving the synergy between sorafenib and 2DG was further investigated. Based on the initial findings from the colony formation assay, it was hypothesized that the combination of sorafenib and 2DG is highly potent in inducing cell cycle arrest in HCC cells. To test this hypothesis, cell cycle analysis was performed on Huh7-R-Pool cells treated with sorafenib and 2DG, alone and in combination. Cells were synchronized overnight in serum-free medium and then treated with sorafenib, 2DG or the combination of both for 48 hours. After treatment, cells were stained with propidium iodide (PI) and analyzed via flow cytometry. Cells treated with the combination of sorafenib and 2DG demonstrated complete G₀/G₁ arrest, while treatments with individual drugs showed only minor cell cycle arrest (Figure 4A, B). Additionally, very few apoptotic cells were observed in all of the treatment groups, which correlated with lack of PARP cleavage in any of the treatment groups (data not shown). These data further confirm that the synergistic combination of sorafenib and 2DG results in reduced HCC cell growth without significant increase in cell death.

Combined treatment with Sorafenib and 2DG Depletes Cellular Energy

Next, it was hypothesized that combination therapy-induced cell cycle arrest was the result of cellular energy depletion leading to activation of AMPK and inhibition of mTOR¹⁶⁻¹⁸. To investigate this potential mechanism, ATP levels were measured in cells treated with sorafenib and 2DG alone and in combination. HCC cells treated with sorafenib or 2DG reduced

cellular ATP levels at comparable levels compared to untreated cells; however, the combination of both drugs dramatically reduced ATP levels (Figure 5A). This profound decrease in cellular-ATP level was observed in both parental and sorafenib resistant Huh7 cells. This extreme depletion of cellular energy could be the primary mechanism driving combination therapy-induced cell cycle arrest.

The inventors next examined how treatment with 2DG alone and in combination with sorafenib affects glycolytic flux in HCC cells. Parental and sorafenib resistant cells were treated with sorafenib, 2DG and a combination of both. After 48 hours, the cell culture supernatant was collected and analyzed for glucose and L-lactate concentrations. The results demonstrate that treatment with 2DG alone and in combination with sorafenib significantly reduced glucose consumption and lactate production at comparable levels in Huh7-S cells and prevented sorafenib induced increase in glucose consumption and lactate production in Huh7-R-Pool and Huh7-R-A7 cells (Figure 5B, C). The attenuation of glycolysis and oxidative phosphorylation may be a key mechanism driving the synergic action of sorafenib and 2DG.

To confirm the effects of ATP depletion on AMPK and mTOR signaling, immunoblot analysis was performed on whole cell extracts isolated from Huh7-S and Huh7-R-Pool cells treated with sorafenib and 2DG alone or in combination. The results showed that treatment with both drugs increased phosphorylation of AMPK(Thr172) but decreased phosphorylation of mTOR(Ser2448) as well as its downstream kinases p70S6K(Thr389) and 4E-Bp(Thr37/46) in Huh7-S cells (Figure 6A, B). Although basal levels of both p-mTOR and p70S6K were higher in Huh7 R-pool, combined treatment with sorafenib and 2DG also reduced their levels, albeit to a lower extent than in Huh7-S cells (Figure 6A, B). Notably, phosphorylation of 4E-Bp(Thr37/46) was completely blocked by this drug combination in both cell types. In contrast, 2DG alone minimally affected phosphorylation of any of these kinases whereas sorafenib alone modestly inhibited phosphorylation of these kinases in Huh-7S cells. Importantly, sorafenib treatment of Huh7-R-Pool cells did not inhibit phosphorylation of mTOR and its downstream kinases (Figure 6A, B) reinforcing the role of increased glycolytic flux in maintaining sorafenib resistance. Collectively, these results suggest that combination of 2DG and sorafenib depleted cellular ATP levels that could increase AMP:ATP ratio leading to activation of AMPK and thereby inhibition of mTOR.

Discussion

There is an urgent need to develop novel therapeutic strategies to extend the lives of patients with advanced HCC. Currently, sorafenib is the only FDA approved therapy for these patients. However, sorafenib extends the overall survival of HCC patients by only 2.8 months compared to untreated patients ⁵. This lack of clinical efficacy is attributed to an intrinsic resistance of HCC to sorafenib ⁶. In order to elucidate the mechanisms driving sorafenib resistance, the inventors developed sorafenib resistant HCC cell lines (Figure 1A). These initial studies demonstrated that sorafenib resistant cells display increased rates of glycolytic flux compared to non-resistant parental cells when treated with sorafenib (Figure 1B, C). Based on this observation, it was hypothesized that combining sorafenib with anti-glycolytic therapeutics would sensitize HCC cells to sorafenib toxicity. After screening several anti-glycolytic drug combinations, the combination of sorafenib and 2-deoxyglucose (2DG) was identified as the most synergistic therapeutic combination. The combination of 2DG and sorafenib drastically inhibited cell growth in resistant and sensitive cells (Figure 2).

These studies also demonstrated that the combination of 2DG and sorafenib significantly reduced colony formation (Figure 3) and potently induced G₀/G₁ cell cycle arrest in sorafenib resistant HCC cells (Figure 4A, B). Furthermore, combination therapy did not induce apoptosis in parental or sorafenib resistant HCC cells (data not shown). A previous study by Maher *et.al.* showed that the treatment of osteosarcoma cells with 2DG in hypoxic conditions resulted in cell cycle arrest. The mechanism behind this growth inhibition was attributed to the inhibition of ATP and macromolecule synthesis ¹⁹. Moreover, there is a strong correlation between cellular ATP levels and AMPK activation resulting in inhibition of mTOR ^{16,18,20}, a kinase that responds to altered energy levels to regulate cell cycle progression ²¹. It was hypothesized that the depletion of cellular ATP together with reduced mTOR activation could be the mechanism driving the combination therapy induced cell cycle arrest observed in these studies. However, unlike the study by Maher *et.al.* ¹⁹, the experiments herein were not conducted under hypoxic conditions. Thus, sorafenib treatment can mimic the effects of hypoxia by inhibiting oxidative phosphorylation and stimulating aerobic glycolysis. It has been shown that clinically relevant levels of sorafenib impair mitochondrial function in rat heart cells ²². Additionally, another study demonstrated that sorafenib treatment hinders oxidative phosphorylation and increases aerobic glycolysis in human HCC cell lines ². Taken together, these data suggest that the synergy observed between sorafenib and 2DG is due to the inhibition of oxidative phosphorylation by sorafenib and the inhibition of glycolytic flux by 2DG, which ultimately results in the depletion

of cellular energy (Figure 6C). This hypothesis is supported by the observation that the combination of 2DG and sorafenib significantly depletes cellular ATP level compared to independent drug treatments and untreated cells. Additionally, combination therapy with 2DG and sorafenib was shown to prevent sorafenib-induced stimulation of glycolytic flux. Combined treatment of HCC cells with sorafenib and 2DG displayed the equivalent levels of glucose consumption and lactate production compared to cells treated with 2DG alone. This suggests that 2DG treatment sets a firm limit on the rate of glycolytic flux of HCC cells. The relation between cellular ATP level and cell cycle inhibition is strengthened by the observed activation of AMPK that responds to alterations in cellular energy and inhibition of mTOR as well as its downstream kinases p70S6K and 4E-Bp (Figure 6A, B). Since these kinases are known to regulate cell cycle progression^{21,23}, this data suggests that alteration in the activation state of these kinases as a result of changes in cellular ATP leads to the observed cell cycle arrest (Figure 4A, B). The drastic reduction in activation of these kinases corroborated with significant inhibition of cell cycle progression following combined treatment compared to treatments with individual drugs.

In summary, the therapeutic combination of sorafenib and 2DG demonstrates unexpected synergy in sorafenib resistant and sensitive HCC cell lines. The synergy of 2DG with sorafenib was much greater than other anti-glycolytic therapeutics examined in this study. The mechanism driving this synergy appears to be the drastic inhibition of cell cycle progression due to reduction in cellular ATP levels leading to activation of AMPK and consequent reduction of mTOR activation.

References cited in this example

1. Fernando J, Malfettone A, Cepeda EB, Vilarrasa-Blasi R, Bertran E, Raimondi G, Fabra A, Alvarez-Barrientos A, Fernandez-Salguero P, Fernandez-Rodriguez CM and others. A mesenchymal-like phenotype and expression of CD44 predict lack of apoptotic response to sorafenib in liver tumor cells. *Int J Cancer* 2015;136(4):E161-72.
2. Fiume L, Manerba M, Vettraino M, Di Stefano G. Effect of sorafenib on the energy metabolism of hepatocellular carcinoma cells. *Eur J Pharmacol* 2011;670(1):39-43.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61(2):69-90.
4. Bruix J, Sherman M, American Association for the Study of Liver D. Management of hepatocellular carcinoma: an update. *Hepatology* 2011;53(3):1020-2.

5. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A and others. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359(4):378-90.
6. Villanueva A, Llovet JM. Second-line therapies in hepatocellular carcinoma: emergence
5 of resistance to sorafenib. *Clin Cancer Res* 2012;18(7):1824-6.
7. Chen KF, Chen HL, Tai WT, Feng WC, Hsu CH, Chen PJ, Cheng AL. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther* 2011;337(1):155-61.
8. Rudalska R, Dauch D, Longerich T, McJunkin K, Wuestefeld T, Kang TW, Hohmeyer
10 A, Pesic M, Leibold J, von Thun A and others. In vivo RNAi screening identifies a mechanism of sorafenib resistance in liver cancer. *Nat Med* 2014;20(10):1138-46.
9. Shen YC, Ou DL, Hsu C, Lin KL, Chang CY, Lin CY, Liu SH, Cheng AL. Activating oxidative phosphorylation by a pyruvate dehydrogenase kinase inhibitor overcomes sorafenib resistance of hepatocellular carcinoma. *Br J Cancer* 2013;108(1):72-81.
- 15 10. Tesori V, Piscaglia AC, Samengo D, Barba M, Bernardini C, Scatena R, Pontoglio A, Castellini L, Spelbrink JN, Maulucci G and others. The multikinase inhibitor Sorafenib enhances glycolysis and synergizes with glycolysis blockade for cancer cell killing. *Sci Rep* 2015;5:9149.
11. Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006;25(34):4633-46.
- 20 12. Scatena R, Bottoni P, Pontoglio A, Mastrototaro L, Giardina B. Glycolytic enzyme inhibitors in cancer treatment. *Expert Opin Investig Drugs* 2008;17(10):1533-45.
13. Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, Yadav A, Nuovo G, Kumar P, Ghoshal K. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* 2009;284(46):32015-27.
- 25 14. Wang B, Hsu SH, Frankel W, Ghoshal K, Jacob ST. Stat3-mediated activation of microRNA-23a suppresses gluconeogenesis in hepatocellular carcinoma by down-regulating glucose-6-phosphatase and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha. *Hepatology* 2012;56(1):186-97.
15. Chou TC. Drug combination studies and their synergy quantification using the Chou-
30 Talalay method. *Cancer Res* 2010;70(2):440-6.
16. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 2007;8(10):774-85.

17. Hahn-Windgassen A, Nogueira V, Chen CC, Skeen JE, Sonenberg N, Hay N. Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem* 2005;280(37):32081-9.
18. Hung CM, Garcia-Haro L, Sparks CA, Guertin DA. mTOR-dependent cell survival mechanisms. *Cold Spring Harb Perspect Biol* 2012;4(12).
19. Maher JC, Krishan A, Lampidis TJ. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. *Cancer Chemother Pharmacol* 2004;53(2):116-22.
20. Zhou H, Shang C, Wang M, Shen T, Kong L, Yu C, Ye Z, Luo Y, Liu L, Li Y and others. Ciclopirox olamine inhibits mTORC1 signaling by activation of AMPK. *Biochem Pharmacol* 2016;116:39-50.
21. Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 2004;23(18):3151-71.
22. Will Y, Dykens JA, Nadanaciva S, Hirakawa B, Jamieson J, Marroquin LD, Hynes J, Patyna S, Jessen BA. Effect of the multitargeted tyrosine kinase inhibitors imatinib, dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. *Toxicol Sci* 2008;106(1):153-61.
23. Liao G, Gao B, Gao Y, Yang X, Cheng X, Ou Y. Phycocyanin Inhibits Tumorigenic Potential of Pancreatic Cancer Cells: Role of Apoptosis and Autophagy. *Sci Rep* 2016;6:34564.
24. Dazert E, Colombi M, Boldanova T, Moes S, Adametz D, Quagliata L, Roth V, Terracciano L, Heim MH, Jenoe P and others. Quantitative proteomics and phosphoproteomics on serial tumor biopsies from a sorafenib-treated HCC patient. *Proc Natl Acad Sci U S A* 2016;113(5):1381-6.

Example 2. Transcriptomics-based drug repurposing approach identifies novel drugs against sorafenib-resistant hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is frequently diagnosed in patients with late-stage disease who are ineligible for curative surgical therapies. Furthermore, the majority of patients become resistant to the only approved therapy, sorafenib. Recently, computational methods for drug repurposing have shown great promise to accelerate the discovery of new uses for existing drugs. In order to identify novel drugs for use against sorafenib resistant (SR)-HCC, a transcriptomics-based drug repurposing method termed connectivity mapping was employed. A

comprehensive analysis was conducted of available *in vitro* and *in vivo* gene signatures of (SR)-HCC, and generated an *in vitro* model using the Huh7 HCC cell line. Coverage of SR-HCC gene signatures were compared across seven patient-derived HCC gene expression datasets, and observed that patients harboring the Huh7 SR-HCC gene signature had significantly reduced survival. Utilizing the Huh7 SR-HCC gene signature, connectivity mapping was applied to drug-induced gene expression profiles (n= 3,740 drugs) in the HepG2 HCC cell line from the LINCS database in order to find drugs that could oppose sorafenib resistance. The use of two non-receptor tyrosine kinase inhibitors, dasatinib and fostamatinib, were determined to reduce viability of sorafenib-resistant HCC cells and up-regulated activity of Src family kinases, the targets of dasatinib, in SR-HCC models. Cell line-specific and HCC etiology-specific (e.g. HBV, HCV, alcohol-induced) drug prediction patterns were also observed.

Background

HCC is the second most common cause of cancer-related death worldwide, in part due to its late stage diagnosis and poor prognosis ¹. It is estimated that 40,710 people will be diagnosed, and 28,920 patients will die from HCC in the United States in 2017 ². While a small proportion of HCC patients diagnosed at an early stage can be treated by tumor resection, cryoablation or liver transplant, these treatments are not effective in the majority of HCC patients diagnosed at an advanced stage of the disease. Sorafenib, a multi-kinase inhibitor, is currently the only approved drug used in treating such patients ³. However, the median overall survival of sorafenib treated patients is only extended by 2.8 months compared to untreated patients ⁴. This minimal therapeutic response is attributed to HCC tumors having an intrinsic resistance to the cytostatic effects of sorafenib ⁵. Thus, there is an urgent need to develop therapeutic strategies to overcome sorafenib resistance and discover new, more effective therapies.

Due to the heterogeneous molecular mechanisms underlying HCC tumor progression and sorafenib resistance, it is unlikely that targeting one major molecular mechanism will be sufficient to treat this disease ⁶. Furthermore, collecting tissues through biopsy is not standard of care for advanced HCC patients that relapse on sorafenib. As a result, the lack of available biomolecular data of HCC patients treated with sorafenib severely limits the ability to study fundamental mechanisms of resistance and potential targets for combination therapies. Therefore, sorafenib resistance was investigated in HCC in an unbiased way through global analysis of the transcriptome in experimental models of sorafenib resistance. Gene expression data was generated from an *in vitro* model of HCC sorafenib resistance in the Huh7 cell line, and conducted a comprehensive analysis of other publicly available gene expression data from

experimental models of sorafenib resistance (SR-HCC) and patient-derived HCC tumors. These SR-HCC gene expression models were evaluated for their coverage in human HCC tissue samples and their prognostic significance. Next, in order to discover drug-disease hypotheses, the aforementioned gene expression profiles were utilized as the basis for computational drug repurposing analyses via connectivity mapping (see Figure 7 for workflow used in this study). Connectivity mapping uses pattern-matching algorithms to compare genome-wide gene expression changes observed in cultured human cells treated with drugs to those of biological states of interest: e.g. tumor vs. normal ⁷. Connectivity scores quantify the drug-disease hypotheses through correlations between ranked gene lists of query gene signatures and drug reference gene signatures, commonly via the Kolgomorov-Smirnov statistic or modified gene set enrichment analysis method ^{7,8}. For instance, drug-induced gene signatures with negative connectivity scores are hypothesized to reverse or oppose the query gene signature characterizing a disease, and vice versa. Furthermore, the use of genome-wide expression profiles provides mechanistic insight into tumor biology and drug efficacy, which may be missed by other guilt-by-association approaches. In this example, it was hypothesized that transcriptomics data from experimental models of sorafenib-resistant HCC i) can enable validation of the in vitro models in the absence of tissue available from sorafenib resistant tumors, ii) can be applied in connectivity mapping studies to predict novel therapies to curb resistance to sorafenib in HCC, and iii) can reveal molecular mechanisms underlying sorafenib resistance in HCC tumors in the context of gene targets and enriched pathways.

Results

Comparison of gene signatures of experimental models of sorafenib resistance

Generation and microarray analysis of sorafenib-resistant HCC cell line

Sorafenib resistant HCC cell lines were generated from parental (sensitive) Huh7 cells (Huh7-S) following long-term exposure to sorafenib ⁹. Viability assays demonstrated that the IC₅₀ doses for resistant cells were approximately 5-fold higher than that of parental Huh-7 cells (Figure 11). Microarray analysis was performed on the parental cells (Huh7-S) and sorafenib-resistant cells (Huh7-R and Huh7-R-A7) (GSE94550). Comparing Huh7-R-A7 vs. Huh-S cells to define a HCC sorafenib resistance gene signature, it was determined that 368 genes were differentially expressed (adjusted $p < 0.001$). The top Gene Ontology (GO) molecular functions, biological processes and cellular components enriched in the sorafenib resistance gene signature (FDR<0.05) were also determined.

Evaluation of experimental models of HCC sorafenib resistance against HCC patient datasets

A comprehensive analysis of publicly available gene expression datasets was conducted characterizing sorafenib resistance in HCC, including an *in vitro* HepG2 cell line (HepG2-R)¹⁰, *in vitro* HCC patient culture (HCC-3sp-R)¹¹, and an *in vivo* xenograft model using transplanted
 5 Huh7 cells (Xeno-R)¹². Sorafenib resistance (SR) dataset features are described in Table 2, and gene signature overlap is shown in Figure 8.

Table 2. Description of HCC patient gene expression datasets and classification of HCC tumor vs. normal liver status by sorafenib resistance gene signatures

HCC patient dataset		GSE14323	GSE14520_GPL 571	GSE14520_GP L3921
Number HCC tumor/ normal liver samples		n=22 tumor / n=19 normal	n=38 tumor / n=19 normal	n=214 tumor / n=220 normal
Microarray platform		Affymetrix Human Genome U133A 2.0 Array	Affymetrix Human Genome U133A 2.0 Array	Affymetrix HT Human Genome U133A Array
HCC etiology		HCV	HBV	HBV
Huh7-R-A7 (n= 368 genes)	% SR+ samples	53.9%	55.8%	44.5%
	P value	< 0.0001	0.4225	0.019
	Sensitivity	0.56 (95% CI: 0.37 - 0.72)	0.53 (95% CI: 0.30 - 0.75)	0.47 (95% CI: 0.38 - 0.56)
	Specificity	1.0 (95% CI: 82 - 1.0)	0.67 (95% CI: 0.35 - 0.88)	0.70 (95% CI: 0.60 - 0.76)
HepG2-R (n= 1,147 genes)	% SR+ samples	32.2%	20.9%	22.5%
	P value	0.018	0.0405	< 0.0001
	Sensitivity	0.42 (95% CI: 0.26 - 0.59)	0.54 (95% CI: 0.29 - 0.77)	0.53 (95% CI: 0.45 - 0.60)
	Specificity	0.93 (95% CI: 0.70 - 1.0)	0.88 (95% CI: 0.64 - 0.98)	0.90 (95% CI: 0.85 - 0.94)
HCC-3sp-R (n= 847 genes)	% SR+ samples	31.0%	23.3%	25.6%
	P value	0.0007	0.0538	< 0.0001
	Sensitivity	0.45 (95% CI: 0.28 - 0.63)	0.50 (95% CI: 0.28 - 0.72)	0.46 (95% CI: 0.39 - 0.53)
	Specificity	1.0 (95% CI: 0.82 - 1.0)	0.87 (95% CI: 0.62 - 0.98)	0.83 (95% CI: 0.77 - 0.88)
Xeno-R (n= 175 genes)	% SR+ samples	25.2%	60.5%	50.1%
	P value	0.0686	< 0.0001	< 0.0001
	Sensitivity	0.33 (95% CI: 0.15 - 0.58)	0.82 (95% CI: 0.52 - 0.97)	0.86 (95% CI: 0.77 - 0.92)
	Specificity	0 (95% CI: 0 - 0.56)	1.0 (95% CI: 0.80 - 1.0)	0.99 (95% CI: 0.96 - 1.0)
HCC patient dataset		GSE45267	GSE62232	GSE6764

Number HCC tumor/ normal liver samples		n=31 tumor / n=24 normal	n=81 tumor / n=10 normal	n=35 tumor / n=10 normal
Microarray platform		Affymetrix Human Genome U133 Plus 2.0 Array	Affymetrix Human Genome U133 Plus 2.0 Array	Affymetrix Human Genome U133 Plus 2.0 Array
HCC etiology		n/a; HCC dx >40 yrs	AI, HBV, HCV	HCV
Huh7-R-A7 (n= 368 genes)	% SR+ samples	50.6%	58.2%	53.30%
	P value	0.5307	0.0397	0.073
	Sensitivity	0.43 (95% CI: 0.27 - 0.61)	0.42 (95% CI: 0.29 - 0.57)	0.61 (95% CI: 0.39 - 0.80)
	Specificity	0.69 (95% CI: 0.44 - 0.86)	1.0 (95% CI: 0.68 - 1.0)	0.86 (95% CI: 0.49 - 0.99)
HepG2-R (n= 1,147 genes)	% SR+ samples	24.1%	27.5%	30.7%
	P value	<0.0001	0.0212	0.1139
	Sensitivity	0.57 (95% CI: 0.41 - 0.72)	0.42 (95% CI: 0.31 - 0.55)	0.68 (95% CI: 0.46 - 0.85)
	Specificity	0.97 (95% CI: 0.84 - 1.0)	1.0 (95% CI: 0.87 - 1.0)	0.67 (95% CI: 0.35 - 0.88)
HCC-3sp-R (n= 847 genes)	% SR+ samples	31.0%	28.6%	26.7%
	P value	0.0168	0.0443	0.0443
	Sensitivity	0.49 (95% CI: 0.34 - 0.64)	0.40 (95% CI: 0.29 - 0.52)	0.57 (95%CI: 0.37 - 0.76)
	Specificity	0.79 (95% CI: 0.62 - 0.89)	1.0 (95% CI: 0.68 - 1.0)	0.86 (95% CI: 0.53 - 0.99)
Xeno-R (n= 175 genes)	% SR+ samples	62.1%	40.7%	40.0%
	P value	<0.0001	0.008	0.0049
	Sensitivity	0.86 (95% CI: 0.67 - 0.95)	0.6 (95% CI: 0.42 - 0.75)	0.93 (95% CI: 0.70 - 1.0)
	Specificity	1.0 (95% CI: 0.89 - 1.0)	1.0 (95% CI: 0.65 - 1.0)	0.80 (95% CI: 0.38 - 0.99)

For each of the six gene expression datasets from the Gene Expression Omnibus (GEO) database, the number of HCC tumor samples, normal liver samples, microarray platform type and HCC etiology for tumors are shown (AI= alcohol-induced, HBV= hepatitis B virus, HCV= hepatitis C virus). Fisher exact test P values, sensitivity and specificity measures are shown for each of the four sorafenib resistance (SR) gene signatures across the six gene expression datasets to classify tumor vs. normal liver tissue status: Huh7-R-A7 (Huh7 cell line), HepG2-R (HepG2 cell line), HCC-3sp-R (short term culture of HCC patient), Xeno-R (xenograft of Huh7 cells).

Interestingly, the distinct gene signatures showed very little overlap among up- and down-regulated genes. To assess the clinical prognostic relevance of each of the SR gene signatures, additional publicly available gene expression datasets characterizing HCC patient tumors of diverse etiologies were obtained from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. First, the presence of the SR gene signatures within the HCC patient datasets was detected using the nearest template method (weighted cosine similarity metric,

FDR<0.05). The ability for the gene signatures to distinguish between HCC tumor and normal liver tissue were comparable across datasets. However, the average frequency of SR+ HCC tumors defined by the SR gene signatures among the GEO datasets were highest in the Huh7-R-A7 (52.7%) Xeno-R (46.4%) signatures, while HepG2-R (26.3%) and HCC-3sp-R (27.7%) were lower (Table 2). Similarly, the Huh7-R-A7 and Xeno-R gene signatures detected a higher percentage of SR+ samples using RNAseq data from the TCGA liver hepatocellular carcinoma (LIHC) subset (Figure 8A). Second, the relationship between presence of sorafenib resistance gene signature and survival was assessed using the TCGA dataset. Of the four SR gene signatures, HCC patients with tumors harboring the Huh7-R-A7 sorafenib resistance gene signature (SR+) exhibited significantly reduced survival as compared to those that showed the opposite gene signature pattern (SR-) (log-rank $p=0.0086$; HR= 1.59, 95% CI: 1.13 – 2.45) (Figure 8B). Due to the lack of survival outcome data for HCC patients with gene expression data from the GEO database, the sensitivity and specificity by which the four SR gene signatures could distinguish HCC tumor vs. normal liver tissue was assessed. The average sensitivity and specificity for the SR signatures are as follows: Huh7-R-A7 (0.50, 0.82), HepG2-R (0.53, 0.89), HCC-3sp-R (0.48, 0.89), Xeno-R (0.73, 0.80).

Drug repurposing predictions to reverse sorafenib resistance in HCC

Library of Integrated Network-based Cellular Signatures (LINCS) analysis for drug repurposing predictions

To identify drugs that can reverse sorafenib resistance in HCC, connectivity mapping analyses were conducted via the Library of Integrated Network-based Cellular Signatures (LINCS) L1000 system. This database consists of 476,251 gene expression profiles of drug and genetic perturbation conditions across 77 cellular contexts. The initial focus was on drug-treated profiles from the HepG2 cell line, as it represented the HCC cell line with the greatest number of unique drugs ($n=3,740$). To determine whether HCC cell line context can affect the distribution of drug repurposing hypotheses, gene expression profiles for 18 HCC cell lines from the CellMiner HCC database were used to query HepG2 drug perturbation gene expression profiles from the LINCS database. Hierarchical clustering analysis of connectivity scores for drug predictions across the 18 HCC cell lines was performed. The heatmap shown in Figure 8C revealed two distinct clusters of drug connectivity scores. Interestingly, both HepG2 and Huh7 belonged to the same main cluster branch, suggesting that the LINCS HepG2 represents a suitable system to generate drug predictions from gene expression profiles originating in Huh7 cell line models. Next, the connectivity scores across the four HCC sorafenib resistance models were

compared via hierarchical clustering analysis (Figure 8D). It was observed that drug prediction patterns derived from the two cell lines (Huh7-R-A7 and HepG2-R) and short-term culture (HCC-3sp-R) were nearly opposite those derived from the mouse model (Xeno-R). The highest degree of similarity of drug prediction patterns was between HCC-3sp-R and HepG2-R. These results are consistent with the observed gene signature overlap shown in Figure 8. For instance, the maximum observed gene overlap between HCC-3sp-R and HepG2-R was 88 up-regulated and 189 down-regulated genes, while Xeno-R showed the least overlap with any other model. Additionally, the Huh7-R-A7 gene signature shared n= 29, n=32 and n=0 up-regulated genes with HepG2-R, HCC-3sp-R and Xeno-R signatures, respectively. Similarly, the Huh7-R-A7 gene signature shared n= 29, n=27 and n=3 down-regulated genes with HepG2-R, HCC-3sp-R and Xeno-R signatures, respectively.

Drug target analysis and candidate prioritization

Due to the superiority of the Huh7-R-A7 sorafenib resistance gene signature to distinguish significant patient survival patterns using the TCGA LIHC data (Figure 8B), validation efforts were prioritized for LINCS drug predictions from this gene signature. Amongst the drugs predicted to reverse sorafenib resistance from the LINCS analyses, the approval and investigational status from the DrugBank and Aggregate Analysis of ClinicalTrials.gov (AACT) databases was determined in order to prioritize those drugs that would be most feasible for preclinical and clinical testing.

Drug perturbations tested in the HEPG2 cell line within LINCS exhibiting negative connectivity scores to the query gene signature with a known approved/investigational status were prioritized. From these analyses, these approved/investigational drugs include: acadesine, acarbose, albendazole, alclometasone, alfuzosin, alpha-linolenic-acid, alprenolol, ambroxol, amcinonide, amiloride, aminoglutethimide, amoxapine, anastrozole, atorvastatin, azithromycin, bendroflumethiazide, benzthiazide, benzydamine, benzylpenicillin, betamethasone, biperiden, bisacodvl, bms-754807, brivanib, bromhexine, bromocriptine, brompheniramine, budesonide, calcitriol, canrenoic-acid, carbamazepine, carbidopa, carbinoxamine, cefaclor, cefdinir, chloroquine, chlorphenamine, chlorpromazine, cholic-acid, cilomilast, cimetidine, cisapride, citalopram, clomifene, cortisone, cvclooentolate, dalcetrapib, danoprevir, darinaparsin, dasatinib, desipramine, desoximetasone, dexamethasone, dexchlorpheniramine, diclofenac, dienestrol, diethylstilbestrol, diflunisal, dihydrexidine, diloxanide, diltiazem, dipyrindamole, domperidone, dosulepin, doxepin, doxylamine, droperidol, duloxetine, enmd-2076, enzalutamide, enzastaurin, epicatechin, equilin, estradiol, estriol, estrone, estropipate, etodolac, famciclovir, flavoxate, flecainide, flucloxacillin, fluconazole, flumazenil, flunisolide, flupirtine,

fluvastatin, fostamatinib, fulvestrant, genistein, glibenclamide, glimepiride, goserelin, guanfacine, halcinonide, hydrocortisone, hypericin, iloperidone, ipratropium, ketoprofen, labetalol, lamivudine, lenalidomide, loperamide, lovastatin, mafenide, mebeverine, meloxicam, mephentermine, mepyramine, mesoridazine, metolazone, metoprolol, mianserin, mirtazapine, 5 mometasone, motesanib, moxifloxacin, nafcillin, naltrexone, niacin, nifedipine, nicardipine, nicergoline, nicotinamide, nifedipine, nilotinib, norgestrel, nortriptyline, omeprazole, ondansetron, ornidazole, orteronel, ouabain, oxaprozin, oxybenzone, oxypheonium, paclitaxel, palbociclib, palonosetron, paroxetine, pemetrexed, peralide, pf-04217903, phenelzine, phentermine, pimozide, pindolol, pirfenidone, prazosin, prednicarbate, prednisolone, pregnenolone, 10 primidone, procarbazine, progesterone, promazine, propranolol, prostaglandin, protriptyline, quinapril, quinidine, quizartinib, racecadotril, raloxifene, ranitidine, rescinamine, retinal, rilmenidine, riluzole, ritodrine, rizatriptan, rolipram, rolitetracycline, roscovitine, safinamide, salmeterol, saquinavir, sildenafil, simvastatin, sirolimus, sitagliptin, sns-314, sulforaphane, tacrolimus, tamoxifen, teicoplanin, terazosin, terconazole, testosterone, tg100-115, thiotepa, 15 thiothixene, ticlopidine, tipifamib, tolazamide, toremifene, tramadol, trifluoperazine, triflupromazine, trimethobenzamide, troglitazone, tropisetron, valproic-acid, vardenafil, vecuronium, vemurafenib, verapamil, verteporfin, vinpocetine, voriconazole, vx-222, xaliproden, zileuton, ziprasidone, and zosuquidar.

For these drugs, the drug activity, target pathway and gene information from KEGG and 20 DrugBank databases was annotated. A filter was applied to focus on only those drugs with known anti-neoplastic activity for initial validation. Finally, a systematic search of genes associated with hepatocellular carcinoma was conducted using a publicly available literature mining tool ¹³ to determine whether the genes targeted by drug candidates had known roles in HCC. The final prioritized drug list is shown in Table 3.

25

Table 3. Prioritized LINCS drug predictions for reversing HCC sorafenib resistance using the Huh7-R-A7 gene signature.

Drug	Connectivity Score	Status	Target(s)	Drug Action(s)
dasatinib	-0.3073	Approved	ABL1, SRC, EPHA2, LCK, YES1, KIT,	antineoplastic, kinase inhibitor

			PDGFRB,STAT5B, ABL2,FYN	
enzalutamide	-0.3686	Approved	AR	antineoplastic, antiandrogen, receptor antagonist
paclitaxel	-0.2423	Approved	TUBB1,BCL2,NR1I2, MAP4,MAP2,MAPT	antineoplastic, antimicrotubule
palbociclib	-0.2387	Approved	CDK4,CDK6	antineoplastic, kinase inhibitor
pemetrexed	-0.2830	Approved	TYMS,ATIC,DHFR, GART	antineoplastic, antimetabolite, antifolate
toremifene	-0.3114	Approved	ESR1	antiestrogen, antineoplastic, receptor antagonist
amino- glutethimide	-0.3121	Approved	CYP19A1,CYP11A1	adrenocortical suppressant, antineoplastic, aromatase inhibitor
anastrozole	-0.3002	Approved	CYP19A1	antineoplastic, aromatase inhibitor
nilotinib	-0.2677	Approved	ABL1,KIT	antineoplastic, kinase inhibitor
procarbazine	-0.2573	Approved	DNA	antineoplastic, alkylating
thiotepa	-0.3158	Approved	DNA	antineoplastic, alkylating
vemurafenib	-0.3455	Approved	BRAF	antineoplastic, kinase inhibitor
verteporfin	-0.2223	Approved	n/a	antineoplastic, photosensitizer
brivanib	-0.2896	Investigational	VEGFR2,FGFR1,FGFR2	antineoplastic, kinase inhibitor
fostamatinib	-0.2388	Investigational	SYK	antineoplastic, anti-inflammatory, kinase inhibitor
darinaparsin	-0.3833	Investigational	n/a	antineoplastic, organic arsenical
enzastaurin	-0.2693	Investigational	PRKCB	antineoplastic, kinase inhibitor
orterone	-0.2719	Investigational	CYP17A1	antineoplastic, antiandrogen
quizartinib	-0.3080	Investigational	FLT3	antineoplastic, kinase inhibitor
tipifarnib	-0.4090	Investigational	FNTB	antineoplastic, farnesyltransferase inhibitor

LINCS drug predictions were prioritized if they had negative connectivity scores in the HEPG2 cell line against the query sorafenib-resistant HCC gene signatures, a known approval or investigational status from the DrugBank and/or ClinicalTrials.gov databases, and antineoplastic function described in the KEGG Drug and DrugBank

databases. Drugs in bold font targeted genes known to play a role in HCC from a systematic analysis of the biomedical literature.

Two drug candidates were selected: one representative from each approval status, for subsequent validation: 1) dasatinib, SRC family of kinases inhibitor (FDA-approved) and 2) fostamatinib, SYK inhibitor (under clinical investigation).

Additionally, a protein-protein interaction (PPI) network of drug target genes was analyzed, as shown in Figure 9. The initial drug targets were connected in a network of a total of 322 protein nodes through 1,029 total interactions (edges). The average node degree (interaction partners) is 6.39, and the number of observed edges is significantly higher than expected ($n=475$; PPI enriched p value < 0.05). PPI connections were recovered for 5 out of 7 dasatinib gene targets, including SRC. A community detection network algorithm was applied to the network, and both SRC and SYK were found in the largest PPI module 5 ($n=46$ total nodes). Notably, SRC was ranked with the third highest node degree ($n=33$ connections), while SYK has $n=8$ connections. SYK exhibited a clustering coefficient of 0.57, while SRC has a clustering coefficient of 0.14. Taken together, these results suggest SRC inhibition may have a broad impact on the overall network, while SYK appears to be involved with a more tightly regulated group of proteins. Finally, the top 100 central genes were selected based on the eigencentality measure, which included all dasatinib and fostamatinib gene targets that were in the network. These highly central drug target genes were altered (mutations, copy number variations, mRNA and protein expression levels) at a higher frequency in the SR+ TCGA patients (58%) as compared to the SR- patients (31%). The type and frequency of gene alterations of the top central genes in the PPI network in SR+ and SR- TCGA LIHC patient tumors were also identified.

Validation of SRC-inhibitor, dasatinib, and SYK-inhibitor, fostamatinib, alone and in combination with sorafenib

Dasatinib and fostamatinib were initially tested *in vitro* as single agents in HCC cell lines (parental Huh7-S, resistant pool Huh7-R and resistant clone Huh7-R-A7). Parental and sorafenib resistant Huh7 cells were treated with increasing concentrations of dasatinib and fostamatinib independently, and cellular viability was assessed after 48 hours. Sorafenib resistant Huh7 cells were significantly more sensitive to dasatinib toxicity than parental cells (Figure 9A). Parental cells displayed an IC_{50} of $>60 \mu M$, while the IC_{50} of resistant cells was $< 10 \mu M$. On the contrary, all three cell lines displayed similar sensitivity to fostamatinib toxicity with IC_{50} values between 20-35 μM (Figure 9B), indicating that fostamatinib may be useful both before and after resistance

to sorafenib occurs. It was next hypothesized that combining either dasatinib or fostamatinib with sorafenib would synergistically inhibit cell viability.

Thus, the effect of dasatinib and fostamatinib treatment, alone and in combination with sorafenib, on the reproductive ability of single cells using the clonogenic survival assay was assessed (Figure 9C and 9D). At the low (2 μ M) concentration tested, dasatinib completely inhibited colony formation in Huh7-R cells, while having little effect on parental cells. This observation is consistent with the higher sensitivity of resistant cells to dasatinib observed in viability assay (Figure 9A). Furthermore, addition of sorafenib did not further sensitize the Huh7-S cells to dasatinib. Similar to the viability assay, both parental and resistant cells were significantly sensitive to fostamatinib. The combination of fostamatinib and sorafenib appeared to additively inhibit colony formation in Huh7-S, Huh7-R and Huh7-R-A7 cells.

Since sorafenib resistant HCC cells appeared to be much more sensitive to long-term dasatinib toxicity than non-resistant HCC cells, it was hypothesized that the activity of SRC kinases would be up-regulated in the resistant cells. Kinase array analysis demonstrated that five out of seven SRC kinases (Src, Yes, Fyn, Lck and Lyn) were significantly hyper-phosphorylated in resistant cells as compared to parental cells (Figure 3E). On the contrary, SYK protein, the target of fostamatinb was not detected in the Huh7 cells. Therefore, in order to delineate possible mechanisms for the action of fostamatinib, the gene expression characterizing fostamatinib-treated HepG2 cells (10uM, 6 hr post-treatment) from the LINCS database was obtained, and performed Ingenuity pathway analysis. Several pathways were identified as significantly deregulated post-treatment: inhibition of oncogenic signaling through JAK/STAT ($p < 1.0E-8$), Protein Kinase A ($p < 1.0E-7$), PI3K/AKT ($p < 1.0E-4$), STAT3 ($p < 1.0E-4$) and ERK/MAPK ($p < 1.0E-3$); and activation of the tumor suppressor PTEN (Figure 9F). SYK mRNA expression was also analyzed for an impact on HCC patient survival. It was observed that HCC patients in the TCGA LIHC with tumors harboring the Huh7-R-A7 sorafenib resistance gene signature (SR+) and up-regulated SYK mRNA expression (≥ 2 -fold) had significantly reduced survival (log-rank P-value= 0.003) (Figure 10).

Analysis of clinical and demographic factors

Effect of etiology with drug repurposing hypothesis

Whether HCC etiology could influence drug repurposing predictions was assessed next. The GEO HCC patient gene expression datasets described in Table 2, were filtered to include patient tumors specific to a given HCC etiology: hepatitis B virus (HBV), hepatitis C virus (HCV) and alcohol-induced (AI). Etiology specific gene expression signatures were used in connectivity

mapping analysis. Using hierarchical clustering analysis of drug connectivity scores, it was found that all three HBV patient datasets clustered together, and that two of the three HCV patient datasets clustered together with the one AI patient dataset (Figure 10A).

5 *Association of clinical and demographic factors with sorafenib resistance signature*

Whether clinical factors (etiology, clinical stage, Child Pugh class) and patient demography (race, gender) influenced sorafenib resistance gene signature status was examined next. It was observed that HCC etiology could affect the proportion of tumors harboring SR+ and SR- gene signatures in the TCGA LIHC dataset (Chi-square test, $p=0.0279$), as shown in
10 Figure 10B. Two etiologies that exhibited significant differences in proportion of SR+/SR- HCC tumors included HBV (Fisher exact test, $p=0.0444$) and non-alcoholic fatty liver disease (NAFLD) (Fisher exact text, $p=0.0180$). The proportion of SR+ and SR- HCC tumors among different stages, Child Pugh class, race and gender was also examined (Figure 10C-F). HCC patient race exhibited a significant trend (Chi-square test, $p=0.004$), as shown in Figure 10E,
15 where a higher proportion of SR+ and SR- HCC tumors were found in white and Asian patients, respectively. However, no significant trend was observed for clinical stage, Child Pugh class or gender among SR+ vs. SR- HCC tumor status.

Discussion

20 In this example, we address how different publicly available gene expression datasets derived from *in vitro* and *in vivo* models of sorafenib resistance in HCC may be reliably assessed in HCC patient tissue in regards to their prognostic significance and ability to derive drug repurposing predictions. This example also provides evidence for the successful use of the computational drug repurposing approach in the context of sorafenib-resistant HCC. Similarly,
25 differences in drug repurposing hypotheses with respect to HCC etiology and cell line source were examined. Although connectivity mapping has been applied across diverse domains, this is the first example that has sought to explicitly identify drugs for sorafenib-resistant HCC using drug-induced gene expression signatures from the Library of Integrated Network-based Cellular Signatures (LINCS) database.

30 Several previous studies have applied HCC gene signatures to the Connectivity Map (CMap) database, which consists of a collection of gene expression profiles of five human cancer (non-HCC) cell lines treated with 1,309 compounds⁷. Two studies used HCC gene expression signatures to query against the CMap database, and validated several drug candidates *in vitro* and *in vivo*^{14,15}. Another group used a combination of CMap and LINCS to discover novel HCC

drugs, and validated three anthelmintics in primary hepatocytes and two mouse models¹⁶. *Lv et al* queried CMap using the HCC-3sp-R gene expression data evaluated in this study, and generated 6 drug predictions to reverse resistance to sorafenib in HCC; however, these predictions were not validated in any context in this publication and no information regarding drug mechanisms was presented⁶. While these previous studies have demonstrated the feasibility of validating connectivity mapping predictions in HCC, all studies to date have been mostly limited to the use of the CMap drug reference database. Two distinct advantages of this LINCS-based study include the ability to gauge the effect of drug perturbations on liver cancer cells (HepG2), as well as the increased number of perturbagens tested in the LINCS systems (n=3,740 total in HepG2 cell line).

In this study, fostamatinib was discovered to be a potentially useful first-line therapy or following resistance to sorafenib, either alone or in combination with sorafenib. Fostamatinib is an inhibitor of spleen tyrosine kinase (SYK), and is currently under investigation for the treatment of several autoimmune diseases¹⁷. Fostamatinib has been shown to have anti-cancer properties for hematological malignancies^{18,19}, and this is the first study investigating its use in HCC and sorafenib resistance. SYK is a non-receptor cytoplasmic tyrosine kinase involved in signal transduction in cells of hematopoietic origin, and more recently, implicated both as a tumor suppressor and promoter of cell survival in various hematopoietic and epithelial cancers^{20,21}. Reduction of SYK expression has been described as a potential prognostic biomarker in several cancers, including HCC²²⁻²⁵.

In contrast to previous findings, the inventors' survival analysis of patients in TCGA LIHC showed that those with high expression of SYK had poorer survival. However, the role of SYK in HCC may be variant-dependent. One recent study by Hong *et al* demonstrated that SYK was down-regulated in 38% of HCC tissue specimens, and that the SYK(S) variant isoform was found in 40% of these samples and not in non-cirrhotic normal liver samples. Furthermore, SYK(S) was found to be associated with clinical and pathologic parameters, including decreased survival, increased tumor number, vascular invasion as well as promotion of tumor growth, suppression of apoptosis and inducing epithelial-mesenchymal transition. Conversely, patients with the SYK(L) variant isoform exhibited increased overall survival and time to recurrence, and SYK(L) was shown to inhibit the metastatic phenotypes that SYK(S) promoted²⁶. Although SYK mRNA has prognostic significance in HCC, the lack of its expression at protein level in some HCC cell lines that are sensitive to fostamatinib suggest that the drug functions through other targets that remain to be discovered.

Dasatinib was confirmed to have a unique role in inhibiting cell growth of sorafenib-resistant HCC cells. The Src family kinase inhibitor dasatinib is approved for the treatment of Ph⁺ chronic myeloid leukemia (CML) in chronic phase and imatinib-resistant disease, Ph⁺ acute lymphoblastic leukemia with resistance to prior therapy, and is under clinical investigation for solid cancers. Src family kinase activity has been implicated in several oncogenic processes, including cellular proliferation, survival, migration and angiogenesis, and increased activity has been demonstrated in HCC *in vitro*²⁷⁻²⁹. These experiments demonstrated that dasatinib, a Src family kinase inhibitor, was effective in reducing HCC cell viability and colony formation alone and in combination with sorafenib in sorafenib-resistant HCC cells. Additionally, it was shown that Src family kinases were significantly activated in the sorafenib-resistant HCC cells as compared to sorafenib-sensitive HCC cells, consistent with the known mechanism of action of dasatinib.

Recently, dasatinib was shown to be successful in reducing HCC cell proliferation, adhesion, migration and invasion *in vitro* via inhibiting Src and several downstream signaling pathways, including PI3K/PTEN/Akt and SFK/FAK³⁰. Another study found that phosphorylation of Src was inhibited in a panel of HCC cells that were sensitive and resistant to dasatinib, and that cell proliferation was not affected by knocking down Src and p-Src in dasatinib-sensitive cells. The authors concluded that dasatinib-mediated inhibition of Src alone is not sufficient to induce its anti-proliferative or pro-apoptotic effects, and that dasatinib may mediate its effects via other targets in addition to Src³¹. Finally, dasatinib was tested in patients with advanced HCC in a recent phase II clinical trial (NCT00459108), but was terminated early due to futility. The primary objectives were to determine the progression-free survival (PFS) rate and response rate at 4 months in patients with unresectable advanced HCC treated with dasatinib. Several factors that may have influenced the results of this clinical trial include compromised liver status in advanced HCC patients and the use of RECIST criteria to determine response rate, which is known to be ineffective in evaluating cytostatic agents, including sorafenib³². Furthermore, these results suggest that dasatinib may be most useful for an enriched HCC patient population with transcriptomic biomarkers characteristic of sorafenib resistance.

It was also found that HCC etiology may influence sorafenib resistance and drug repurposing hypothesis generation using the transcriptomics-based LINCS system. Interestingly, sorafenib was previously observed to be more effective for HCC patients with an underlying HCV infection compared to HBV infection or alcoholic cirrhosis³³. In another study, dasatinib was shown to be most effective in a group of HCC patients with a “progenitor molecular subtype”, as assessed by gene expression profiling³¹. Taken together, these findings highlight

the importance of considering HCC patient etiology and other molecular features in drug prediction studies.

In summary, this example discloses the feasibility of the drug repurposing workflow by validating novel drugs to use alone and in combination with sorafenib in HCC.

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Methods

Drug reagents

Dasatinib (S1021) and Fostamatinib (S2206) were obtained from Selleckchem (Houston, TX, USA). Sorafenib (catalog #S-8502) was purchased from LC Laboratories (Woburn, MA, USA).

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Cell culture and acquired sorafenib resistance

All cells were maintained in Minimum Essential Media supplemented with L-glutamine (2 mM), 10% FBS, sodium pyruvate (0.11 g/L) and penicillin/streptomycin (100 U/mL). Cell media for sorafenib resistant cell lines was also supplemented with sorafenib (6 μ M and 0.1% DMSO). Sorafenib was withdrawn from the cell media of resistant Huh7 cells for 5-7 days prior performing all experiments. The HCC cell line Huh7 cells that were generously provided by Dr. James Taylor (Fox Chase Center, PA, USA). Sorafenib resistant (Huh7-R) and resistant clone (Huh7-R-A7) cell lines were derived from parental Huh7 (Huh7-S) cells. To generate the resistant cell lines, parental cells were initially pulsed with high concentration of sorafenib (10 μ M) in MEM media for 4 hours once a week for 6 weeks. These cells were then continuously exposed to sorafenib, beginning at a low dose of 1.5 μ M and gradually increasing to 6 μ M. The resistant clone Huh7-R-A7 was isolated from an individual colony of Huh7-R cells. See Reyes, R., Wani, N. A., Ghoshal, K., Jacob, S. T. & Motiwala, T. Sorafenib and 2-Deoxyglucose Synergistically Inhibit Proliferation of Both Sorafenib-Sensitive and -Resistant HCC Cells by Inhibiting ATP Production. *Gene expression* 17, 129-140 (2017). In addition to Huh7-R-A7, several other sorafenib resistant colonies were isolated from the pool of Huh-R cells (Figure 7). Huh-R-A7 was randomly selected to represent sorafenib resistant clones for all subsequent experiments.

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Cell Viability Assay

Cells were seeded into 96-well plates (~2,000 cells/well) and allowed to incubate overnight. The next day, cell media was replaced with MEM media containing specified concentration of sorafenib, fostamatinib or dasatinib (with 1% final DMSO concentration). After 48 hours of treatment, the CellTiter-Glo® luminescent viability assay was utilized following the

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manufacturer's instructions. Prior to measuring viability with a luminometer, the luminescent supernatant was transferred to an opaque luminometer 96-well plate.

Colony Formation Assay

Cells were seeded into 6-well plates (~2,000-5,000 cells/well) and allowed to incubate for 24-48 hours. Cells were then treated with a continuous dose of therapeutics (with 1% final DMSO concentration) for 14-18 days. Media was replaced every 2-3 days. After colonies grew to a sufficient size, cells were fixed with 3.7% paraformaldehyde (in PBS) and stained with a 0.05% crystal violet solution.

Differential gene expression analysis

i) Sorafenib resistance gene expression signatures

Microarray analysis was performed on the parental cells (Huh7-S), a pool of sorafenib-resistant cells (Huh7-R) and sorafenib-resistant clone A7 (Huh7-R-A7) using the Affymetrix GeneChip Human Transcriptome Array 2.0 platform. A differential gene expression signature was defined by comparing microarray data from Huh7-R-A7 vs. Huh7-S cells. Signal intensities were analyzed by Affymetrix Expression Console software. Gene expression levels were RMA-normalized and log-transformed³⁴. A filtering method based on percentage of arrays above noise cutoff was applied to filter out low expression genes, and a linear model was employed to detect differentially expressed genes. In order to improve the estimates of variability and statistical tests for differential expression, a variance smoothing method with fully moderated t-statistic was employed for this study³⁵. The significance level was adjusted for multiple hypothesis testing by controlling the mean number of false positives, and a threshold p-value <0.0001 was maintained to determine statistical significance³⁶. Gene expression values were averaged for multiple probeset ID's mapping to the same gene. A fold-change cutoff of >3 for up-regulated genes and <0.25 for down-regulated genes was imposed. Raw and normalized data were deposited in the Gene Expression Omnibus (GEO) database (accession: GSE94550).

ii) HCC tumor gene expression data

A systematic search of the Gene Expression Omnibus (GEO) database was conducted for datasets containing human HCC and normal liver tissue. Raw (.CEL files) gene expression data from six GEO microarray datasets was obtained: GSE14323 (GPL571), GSE14520 (GPL571), GSE14520 (GPL3921), GSE45267 (GPL570), GSE62232 (GPL570) and GSE6764 (GPL570). Signal intensity values were RMA-normalized and gene expression values were log-transformed. Differential gene expression analysis (tumor vs. normal) was conducted via the *limma* (Linear Models for Microarray Analysis) R package³⁷, which employs an empirical Bayes method to moderate the standard errors of estimated log-fold changes. Gene expression values for multiple

probeset ID's mapping to the same gene were averaged. Benjamini-Hochberg FDR correction was applied to adjust for multiple hypotheses testing, and significance cutoff was set at adjusted $p < 0.0001$ ³⁸. Gene overlap Venn diagrams were generated using Venny 2.1.0³⁹.

Library of Integrated Network-based Cellular Signatures (LINCS) analyses

Connectivity mapping analyses were conducted via the Library of Integrated Network-based Cellular Signatures (LINCS) system (database version A2) using the web-based platform (<http://www.lincscloud.org/>). Connectivity scores were calculated using the weighted Kolmogorov-Smirnov (KS) statistic to rank predictions from the LINCS database, as previously described⁷. LINCS compound perturbations tested exclusively in the HepG2 liver cancer cell line were selected. Connectivity scores were averaged for individual LINCS compound perturbations tested at different concentrations and time points in the HepG2 cell line. Individual LINCS gene signatures were obtained from the Broad LINCS Cmap C3 Cloud Compute platform using the slice_slice_tool command.

Database access: DrugBank, AACT, KEGG, Cell Miner, Beegle

The “Approved” and “Investigational” external drug link files from the DrugBank database (v 4.5.0, released date April 20, 2016) was downloaded and searches were conducted in the DrugBank web-interface to categorize drug approval status⁴⁰. The Aggregate Analysis of ClinicalTrials.gov (AACT) database was downloaded, which is a publicly available resource produced by the Clinical Trials Transformation Initiative (CTTI), for drugs under clinical investigation (AACT accessed: June 17, 2016). KEGG DRUG database ID's mapped to drugs in DrugBank were used to extract drug activity and target pathway information in KEGG Drug (KEGG DRUG accessed: June 20, 2016)⁴¹. Drug target genes were obtained from DrugBank and KEGG Drug databases, and assessed for association with HCC using the Beegle literature-mining tool¹³. Gene expression signatures for 18 HCC cell lines vs. a pool of 19 normal liver samples were obtained via the CellMinerHCC database⁴².

Bioinformatics analysis

Hierarchical clustering using the Euclidean distance of gene expression and drug connectivity scores was performed using the heatmap.2 function from the “gplots” R package. The nearest-template prediction (NTP) method was applied to normalized, log-transformed gene expression data to classify tumor samples as SR+ or SR- ($FDR < 0.05$)⁴³. Gene mutation, copy-number and mRNA expression data for SR+/SR- liver HCC (LIHC) patients in the TCGA analysis were obtained via the cBioPortal (v 1.2.4) tool⁴⁴. The drug target protein-protein interaction (PPI) network was generated using the STRING (v 10.0) database⁴⁵. Only high confidence interaction scores (0.700 and above) from experiments, databases, neighborhood and

gene fusion sources were included in the final network. Network analysis of the drug target PPI network was performed using Gephi 0.9.1 software, including algorithms to detect the following network features: degree, modularity class, eigencentrality and clustering coefficient. Enriched gene ontology functions in the sorafenib resistance gene signature were obtained via functional enrichment analysis using the ToppGene Suite ⁴⁶. Fostamatinib-treated HepG2 gene expression data from LINCS was analyzed via QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Survival analysis

Clinical data and RNAseq data (Level 3, v2, RSEM-normalized) from 377 liver HCC patients contained in the TCGA database were obtained via the Broad Institute Firebrowse tool (<http://firebrowse.org/>; TCGA data version 2016_01_28). Survival analysis comparing SR+ and SR- patients was conducted using Prism 7 software. Survival analysis of HCC patients for SYK based on gene expression data was conducted using the cBioPortal (v 1.2.4) tool for TCGA data ⁴⁴. Death was selected as the survival measure, and the median was chosen as the bifurcation point to define "high" vs. "low" gene expression. Survival curves were generated using the Kaplan-Meier method, and the log-rank test was used for statistical comparison.

References cited in this example

- 1 Ferlay, J. *et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer* 136, E359-386, doi:10.1002/ijc.29210 (2015).
- 2 Motiwala, T., Kite, B., Regan, K., Gascon, G. M. & Payne, P. R. Domain Analysis of Integrated Data to Reduce Cost Associated with Liver Disease. *Studies in health technology and informatics* 216, 414-418 (2015).
- 3 Bruix, J. & Sherman, M. Management of hepatocellular carcinoma: an update. *Hepatology (Baltimore, Md.)* 53, 1020-1022, doi:10.1002/hep.24199 (2011).
- 4 Llovet, J. M. *et al.* Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine* 359, 378-390, doi:10.1056/NEJMoa0708857 (2008).
- 5 Villanueva, A. & Llovet, J. M. Second-line therapies in hepatocellular carcinoma: emergence of resistance to sorafenib. *Clinical cancer research : an official journal of the American Association for Cancer Research* 18, 1824-1826, doi:10.1158/1078-0432.ccr-12-0151 (2012).

- 6 Lv, J., Zhu, B., Zhang, L., Xie, Q. & Zhuo, W. Detection and screening of small molecule agents for overcoming Sorafenib resistance of hepatocellular carcinoma: a bioinformatics study. *International journal of clinical and experimental medicine* 8, 2317-2325 (2015).
- 7 Lamb, J. *et al.* The Connectivity Map: using gene-expression signatures to connect small
5 molecules, genes, and disease. *Science (New York, N.Y.)* 313, 1929-1935, doi:10.1126/science.1132939 (2006).
- 8 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 102, 15545-15550, doi:10.1073/pnas.0506580102
10 (2005).
- 9 Reyes, R., Wani, N. A., Ghoshal, K., Jacob, S. T. & Motiwala, T. Sorafenib and 2-Deoxyglucose Synergistically Inhibit Proliferation of Both Sorafenib-Sensitive and -Resistant HCC Cells by Inhibiting ATP Production. *Gene expression* 17, 129-140, doi:10.3727/105221616x693855 (2017).
- 15 10 van Malenstein, H. *et al.* Long-term exposure to sorafenib of liver cancer cells induces resistance with epithelial-to-mesenchymal transition, increased invasion and risk of rebound growth. *Cancer letters* 329, 74-83, doi:10.1016/j.canlet.2012.10.021 (2013).
- 11 van Zijl, F. *et al.* A human model of epithelial to mesenchymal transition to monitor drug efficacy in hepatocellular carcinoma progression. *Molecular cancer therapeutics* 10, 850-
20 860, doi:10.1158/1535-7163.mct-10-0917 (2011).
- 12 Tovar, V. *et al.* Tumour initiating cells and IGF/FGF signalling contribute to sorafenib resistance in hepatocellular carcinoma. *Gut*, doi:10.1136/gutjnl-2015-309501 (2015).
- 13 ElShal, S. *et al.* Beegle: from literature mining to disease-gene discovery. *Nucleic acids research* 44, e18, doi:10.1093/nar/gkv905 (2016).
- 25 14 Woo, H. G. *et al.* Identification of potential driver genes in human liver carcinoma by genomewide screening. *Cancer research* 69, 4059-4066, doi:10.1158/0008-5472.can-09-0164 (2009).
- 15 Chen, M. H. *et al.* Gene expression-based chemical genomics identifies potential therapeutic drugs in hepatocellular carcinoma. *PloS one* 6, e27186, doi:10.1371/journal.pone.0027186
30 (2011).
- 16 Chen, B. *et al.* Computational Discovery of Niclosamide Ethanolamine, A Repurposed Drug Candidate That Reduces Growth of Hepatocellular Carcinoma Cells in Vitro and in Mice by Inhibiting CDC37 Signaling. *Gastroenterology*, doi:10.1053/j.gastro.2017.02.039 (2017).

- 17 Singh, R., Masuda, E. S. & Payan, D. G. Discovery and development of spleen tyrosine kinase (SYK) inhibitors. *Journal of medicinal chemistry* 55, 3614-3643, doi:10.1021/jm201271b (2012).
- 18 Friedberg, J. W. *et al.* Inhibition of Syk with fostamatinib disodium has significant clinical
5 activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* 115, 2578-2585, doi:10.1182/blood-2009-08-236471 (2010).
- 19 Herman, S. E. *et al.* Fostamatinib inhibits B-cell receptor signaling, cellular activation and tumor proliferation in patients with relapsed and refractory chronic lymphocytic leukemia. *Leukemia* 27, 1769-1773, doi:10.1038/leu.2013.37 (2013).
- 10 20 Geahlen, R. L. Getting Syk: spleen tyrosine kinase as a therapeutic target. *Trends in pharmacological sciences* 35, 414-422, doi:10.1016/j.tips.2014.05.007 (2014).
- 21 Coopman, P. J. & Mueller, S. C. The Syk tyrosine kinase: a new negative regulator in tumor growth and progression. *Cancer letters* 241, 159-173, doi:10.1016/j.canlet.2005.11.004 (2006).
- 15 22 Yuan, Y., Mendez, R., Sahin, A. & Dai, J. L. Hypermethylation leads to silencing of the SYK gene in human breast cancer. *Cancer research* 61, 5558-5561 (2001).
- 23 Yuan, Y. *et al.* Frequent epigenetic inactivation of spleen tyrosine kinase gene in human hepatocellular carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12, 6687-6695, doi:10.1158/1078-0432.ccr-06-0921
20 (2006).
- 24 Ogane, S. *et al.* Spleen tyrosine kinase as a novel candidate tumor suppressor gene for human oral squamous cell carcinoma. *International journal of cancer* 124, 2651-2657, doi:10.1002/ijc.24237 (2009).
- 25 25 Bailet, O. *et al.* Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest. *Cancer research* 69, 2748-2756, doi:10.1158/0008-5472.can-08-2690 (2009).
- 26 Hong, J. *et al.* Expression of variant isoforms of the tyrosine kinase SYK determines the prognosis of hepatocellular carcinoma. *Cancer research* 74, 1845-1856, doi:10.1158/0008-5472.can-13-2104 (2014).
- 30 27 Yeatman, T. J. A renaissance for SRC. *Nature reviews. Cancer* 4, 470-480, doi:10.1038/nrc1366 (2004).
- 28 Ito, Y. *et al.* Activation of c-Src gene product in hepatocellular carcinoma is highly correlated with the indices of early stage phenotype. *Journal of hepatology* 35, 68-73 (2001).

- 29 Lau, G. M. *et al.* Expression of Src and FAK in hepatocellular carcinoma and the effect of Src inhibitors on hepatocellular carcinoma in vitro. *Digestive diseases and sciences* 54, 1465-1474, doi:10.1007/s10620-008-0519-0 (2009).
- 30 Chang, A. Y. & Wang, M. Molecular mechanisms of action and potential biomarkers of growth inhibition of dasatinib (BMS-354825) on hepatocellular carcinoma cells. *BMC cancer* 13, 267, doi:10.1186/1471-2407-13-267 (2013).
- 31 Finn, R. S. *et al.* Molecular subtype and response to dasatinib, an Src/Abl small molecule kinase inhibitor, in hepatocellular carcinoma cell lines in vitro. *Hepatology (Baltimore, Md.)* 57, 1838-1846, doi:10.1002/hep.26223 (2013).
- 32 Takada, J. *et al.* Modified response evaluation criteria in solid tumors is superior to response evaluation criteria in solid tumors for assessment of responses to sorafenib in patients with advanced hepatocellular carcinoma. *BMC research notes* 8, 609, doi:10.1186/s13104-015-1565-2 (2015).
- 33 Bruix, J. *et al.* Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *Journal of hepatology* 57, 821-829, doi:10.1016/j.jhep.2012.06.014 (2012).
- 34 Irizarry, R. A. *et al.* Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)* 4, 249-264, doi:10.1093/biostatistics/4.2.249 (2003).
- 35 Yu, L. *et al.* Fully moderated T-statistic for small sample size gene expression arrays. *Statistical applications in genetics and molecular biology* 10, doi:10.2202/1544-6115.1701 (2011).
- 36 Schrod, S. J. The Use of Multiplicity Corrections, Order Statistics and Generalized Family-Wise Statistics with Application to Genome-Wide Studies. *PloS one* 11, e0154472, doi:10.1371/journal.pone.0154472 (2016).
- 37 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* 43, e47, doi:10.1093/nar/gkv007 (2015).
- 38 Solari, A. & Goeman, J. J. Minimally adaptive BH: A tiny but uniform improvement of the procedure of Benjamini and Hochberg. *Biometrical journal. Biometrische Zeitschrift*, doi:10.1002/bimj.201500253 (2016).
- 39 Cai, H. *et al.* VennPlex--a novel Venn diagram program for comparing and visualizing datasets with differentially regulated datapoints. *PloS one* 8, e53388, doi:10.1371/journal.pone.0053388 (2013).

- 40 Wishart, D. S. *et al.* DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic acids research* 34, D668-672, doi:10.1093/nar/gkj067 (2006).
- 41 Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M. & Hirakawa, M. KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic acids research* 38, D355-360, doi:10.1093/nar/gkp896 (2010).
- 42 Staib, F. *et al.* CellMinerHCC: a microarray-based expression database for hepatocellular carcinoma cell lines. *Liver international : official journal of the International Association for the Study of the Liver* 34, 621-631, doi:10.1111/liv.12292 (2014).
- 43 Hoshida, Y. Nearest template prediction: a single-sample-based flexible class prediction with confidence assessment. *PloS one* 5, e15543, doi:10.1371/journal.pone.0015543 (2010).
- 44 Gao, J. *et al.* Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science signaling* 6, pl1, doi:10.1126/scisignal.2004088 (2013).
- 45 Szklarczyk, D. *et al.* STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic acids research* 43, D447-452, doi:10.1093/nar/gku1003 (2015).
- 46 Chen, J., Bardes, E. E., Aronow, B. J. & Jegga, A. G. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic acids research* 37, W305-311, doi:10.1093/nar/gkp427 (2009).

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

CLAIMS

We claim:

1. A pharmaceutical composition comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.
2. The composition of claim 1, further comprising an additional chemotherapeutic agent.
3. A kit comprising sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.
4. The kit of claim 3, wherein sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation.
5. The kit of claim 3, wherein sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.
6. A method of treating or preventing a cancer in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of sorafenib and 2-deoxyglucose, or pharmaceutically acceptable salts, solvates, and/or prodrugs thereof.
7. The method of claim 6, wherein the cancer is selected from hepatocellular carcinoma, breast cancer, or chronic lymphocytic leukemia.
8. The method of claim 7, wherein the cancer is hepatocellular carcinoma (HCC).
9. The method of any one of claims 6 to 8, wherein administration of sorafenib and 2-deoxyglucose provides a synergistic effect.
10. The method of any one of claims 6 to 9, wherein the cancer is resistant to sorafenib.
11. The method of any one of claims 6 to 10, wherein administration of sorafenib and 2-deoxyglucose inhibits ATP production.
12. The method of any one of claims 6 to 11, wherein sorafenib and 2-deoxyglucose are provided as a single pharmaceutical formulation.
13. The method of any one of claims 6 to 11, wherein sorafenib and 2-deoxyglucose are provided as separate pharmaceutical formulations.
14. The method of any one of claims 6 to 13, wherein the method further comprises the administration of an additional chemotherapeutic agent.
15. A method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of fostamatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.
16. The method of claim 15, wherein the method further comprises the administration of an additional chemotherapeutic agent.

17. The method of claim 16, wherein the additional chemotherapeutic agent is sorafenib.
18. The method of claim 17, wherein administration of sorafenib and fostamatinib provides a synergistic effect.
19. The method of any one of claims 15 to 18, wherein the hepatocellular carcinoma is resistant to sorafenib.
20. A method of treating or preventing hepatocellular carcinoma (HCC) in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of dasatinib, or a pharmaceutically acceptable salt, solvate, or prodrug thereof.
21. The method of claim 20, wherein the method further comprises the administration of an additional chemotherapeutic agent.
22. The method of claim 21, wherein the additional chemotherapeutic agent is sorafenib.
23. The method of claim 22, wherein administration of sorafenib and dasatinib provides a synergistic effect.
24. The method of any one of claims 20 to 23, wherein the hepatocellular carcinoma is resistant to sorafenib.

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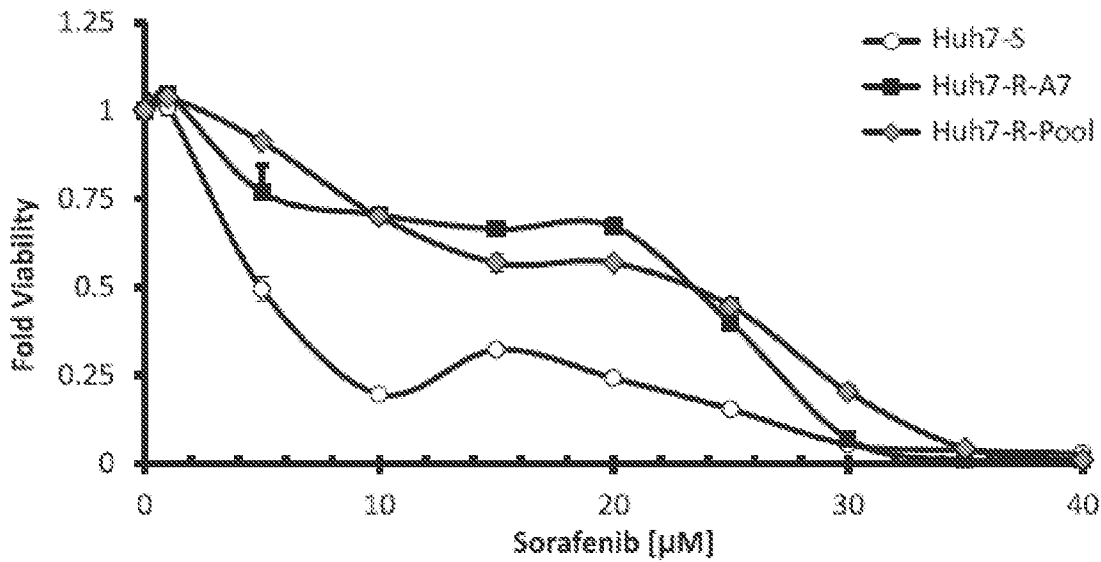


FIG. 1A

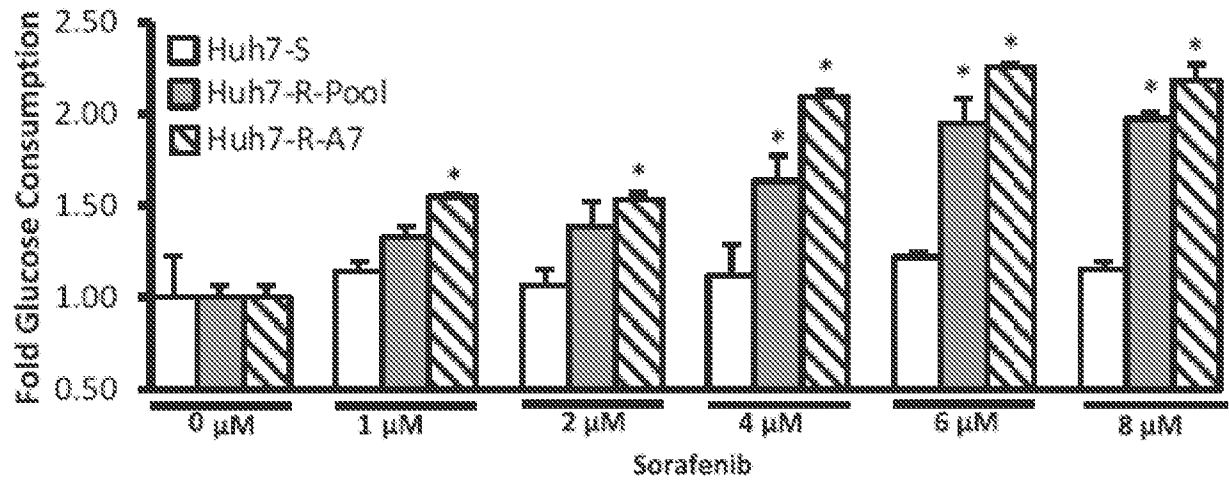


FIG. 1B

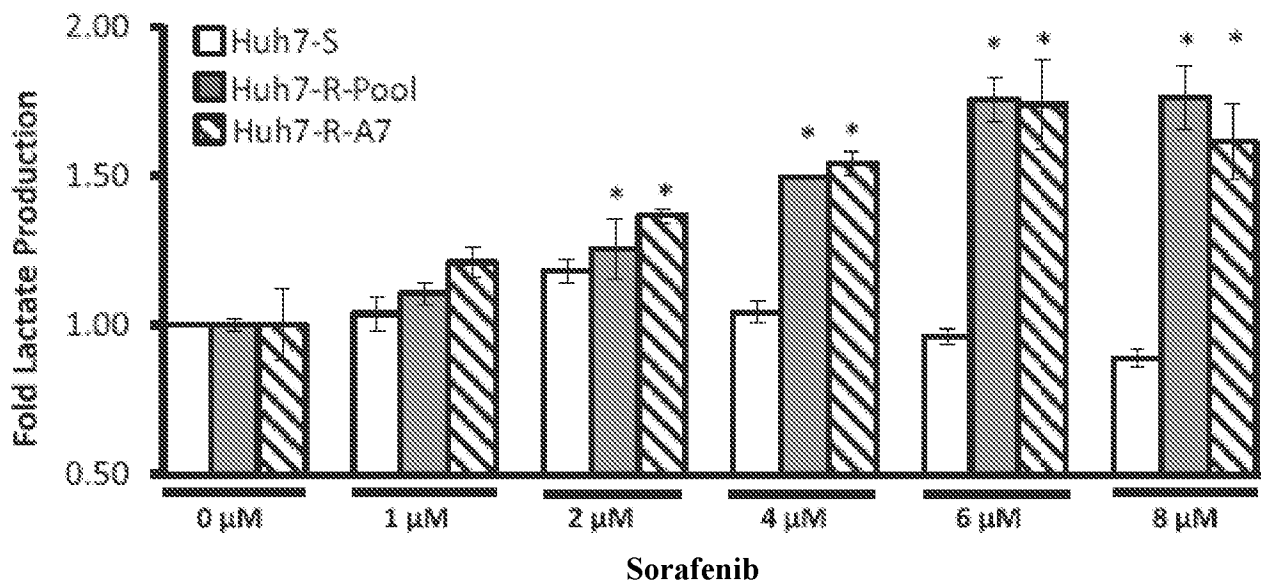


FIG. 1C

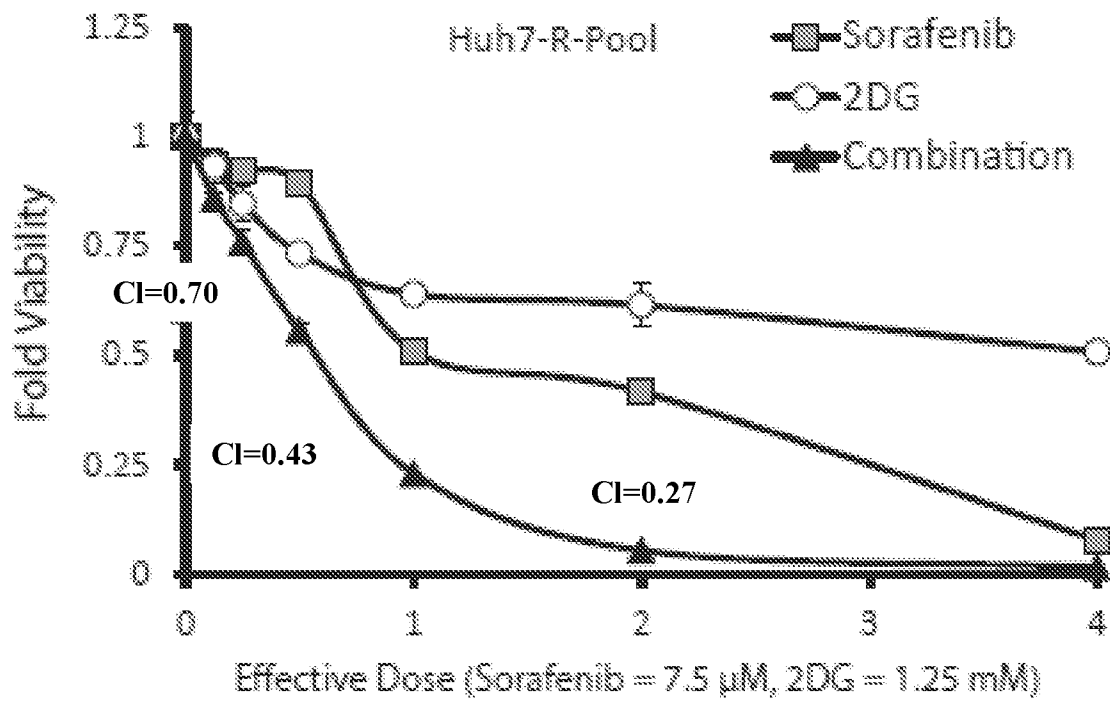


FIG. 2A

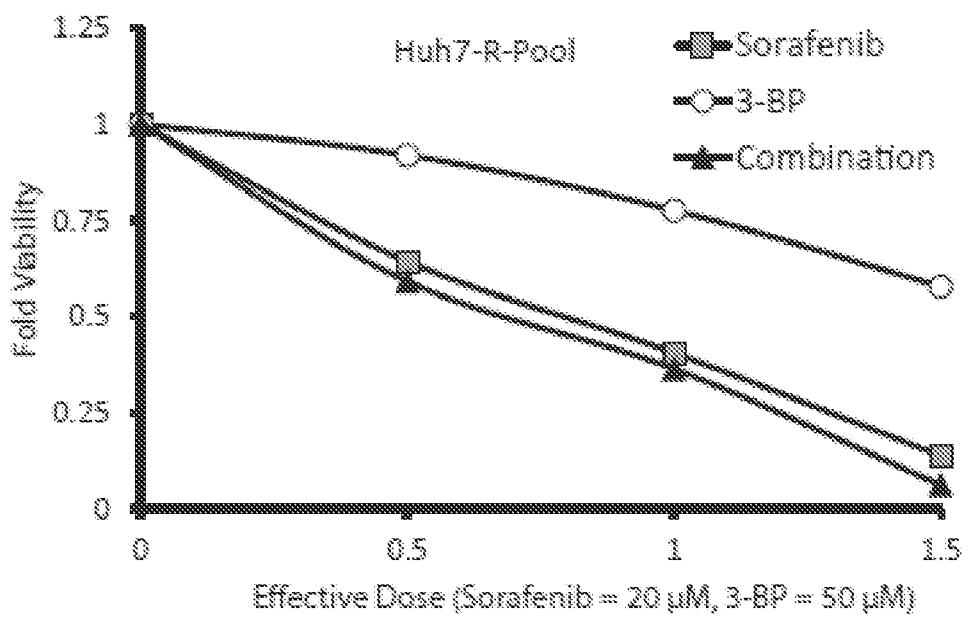


FIG. 2B

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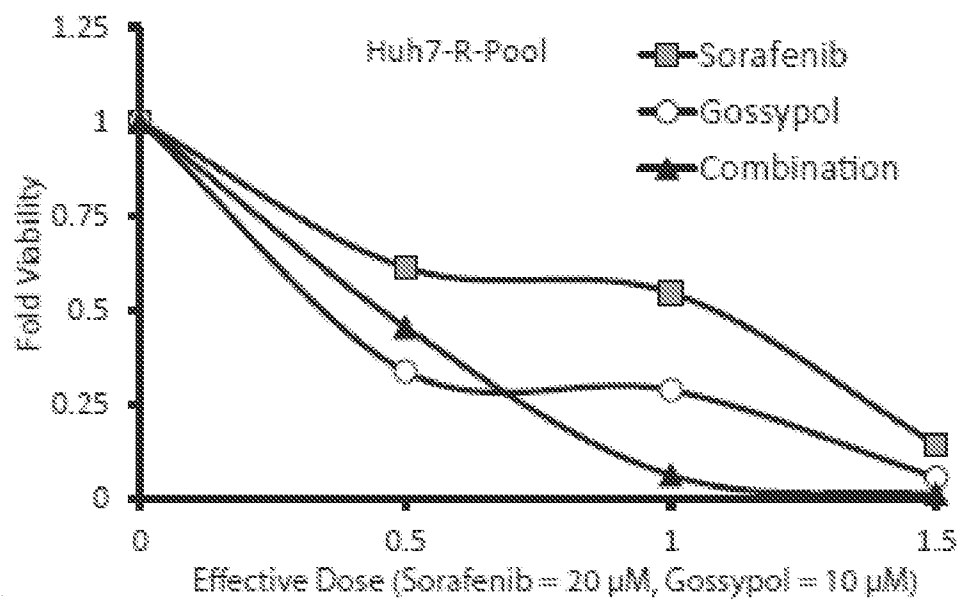


FIG. 2C

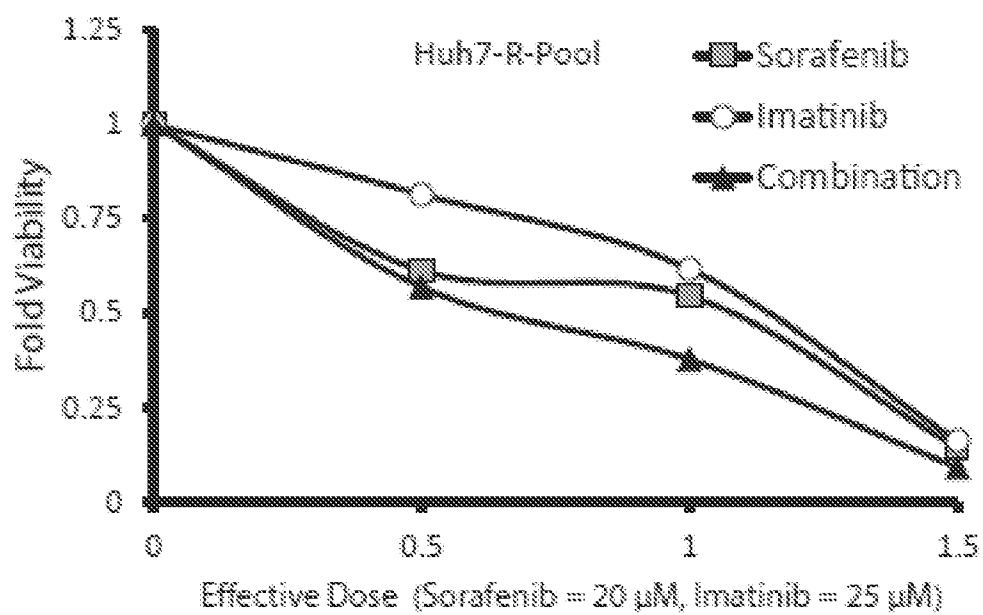


FIG. 2D

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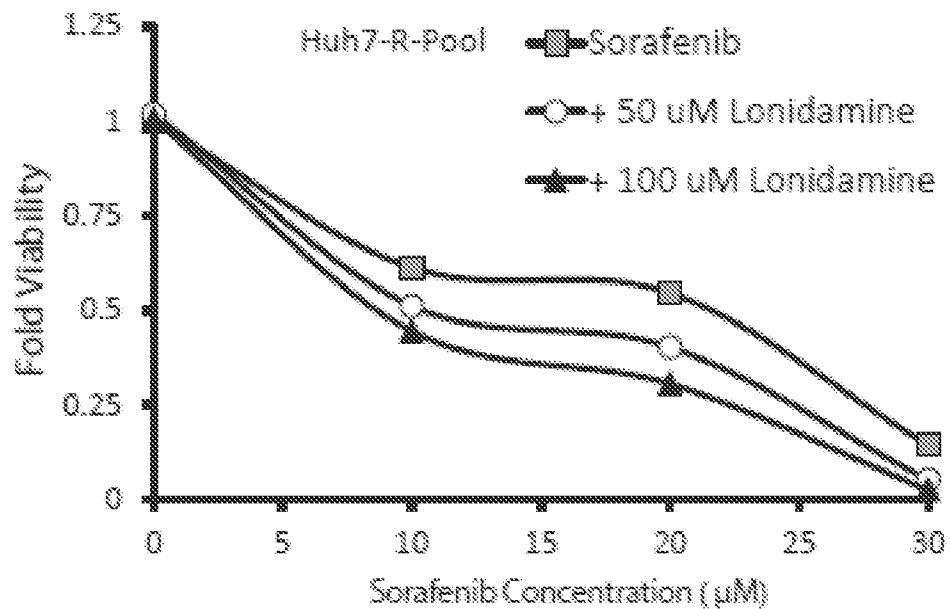


FIG. 2E

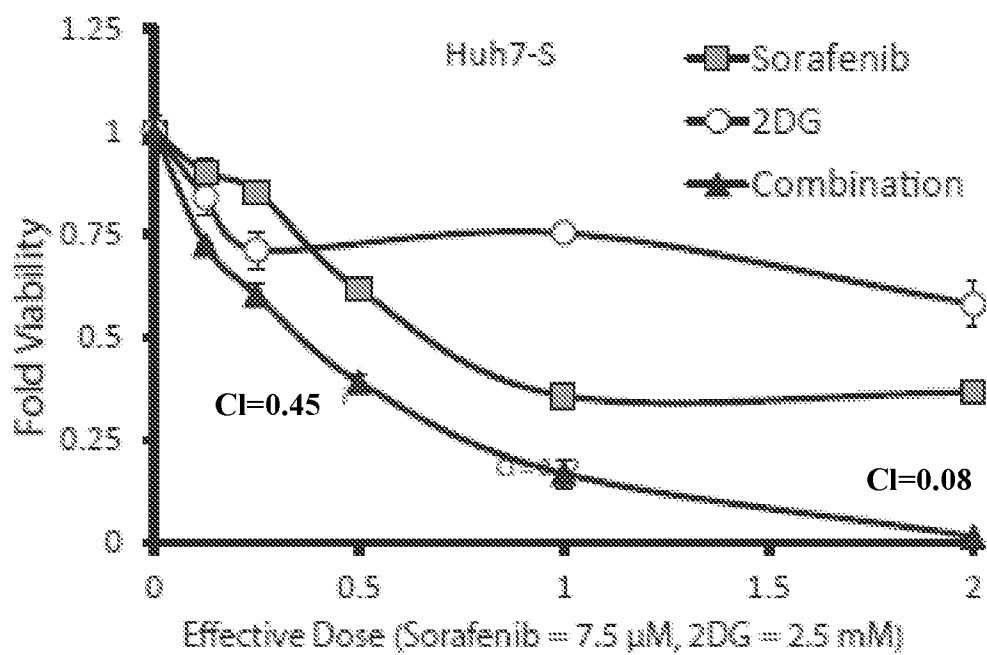


FIG. 2F

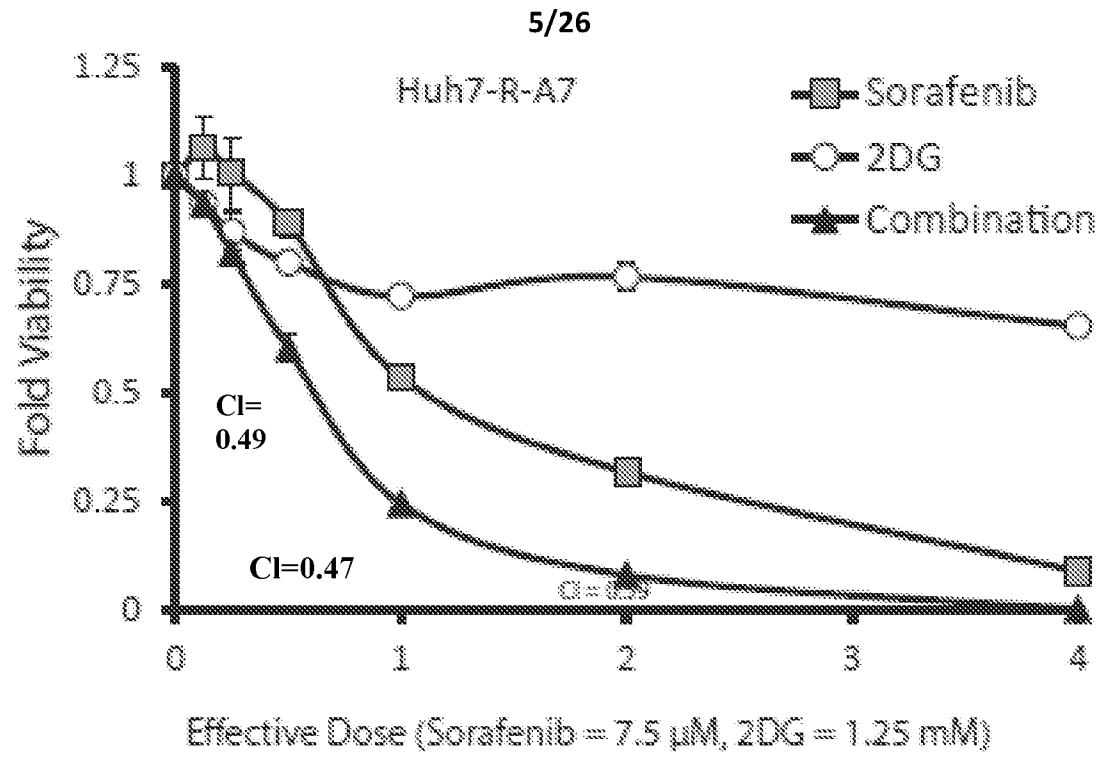


FIG. 2G

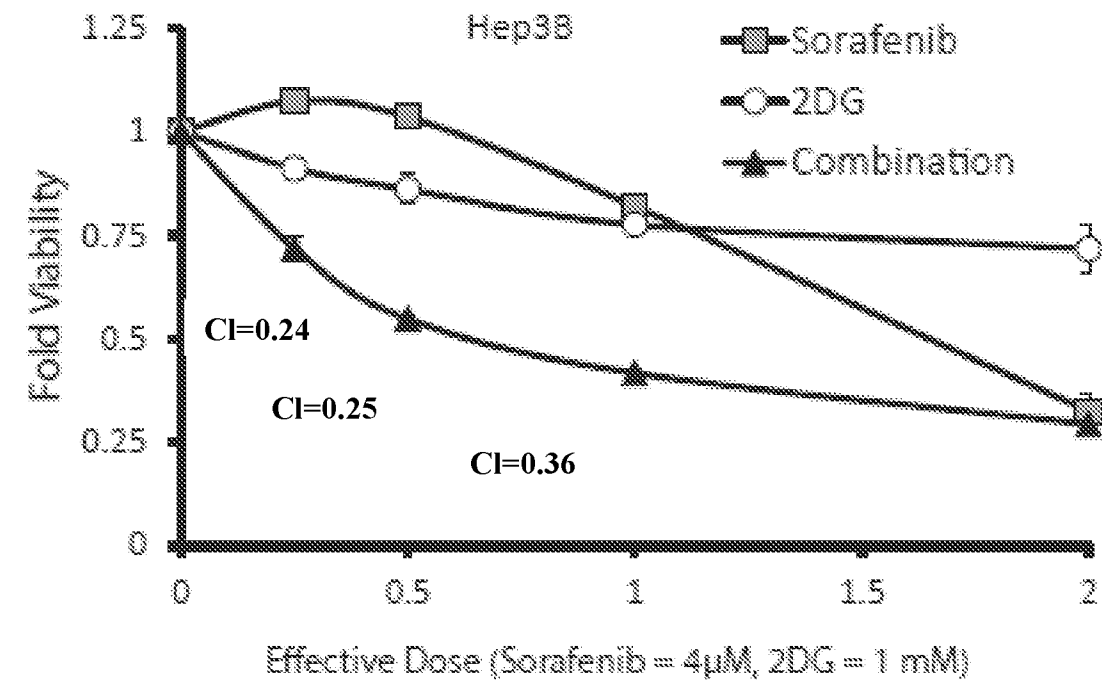


FIG. 2H

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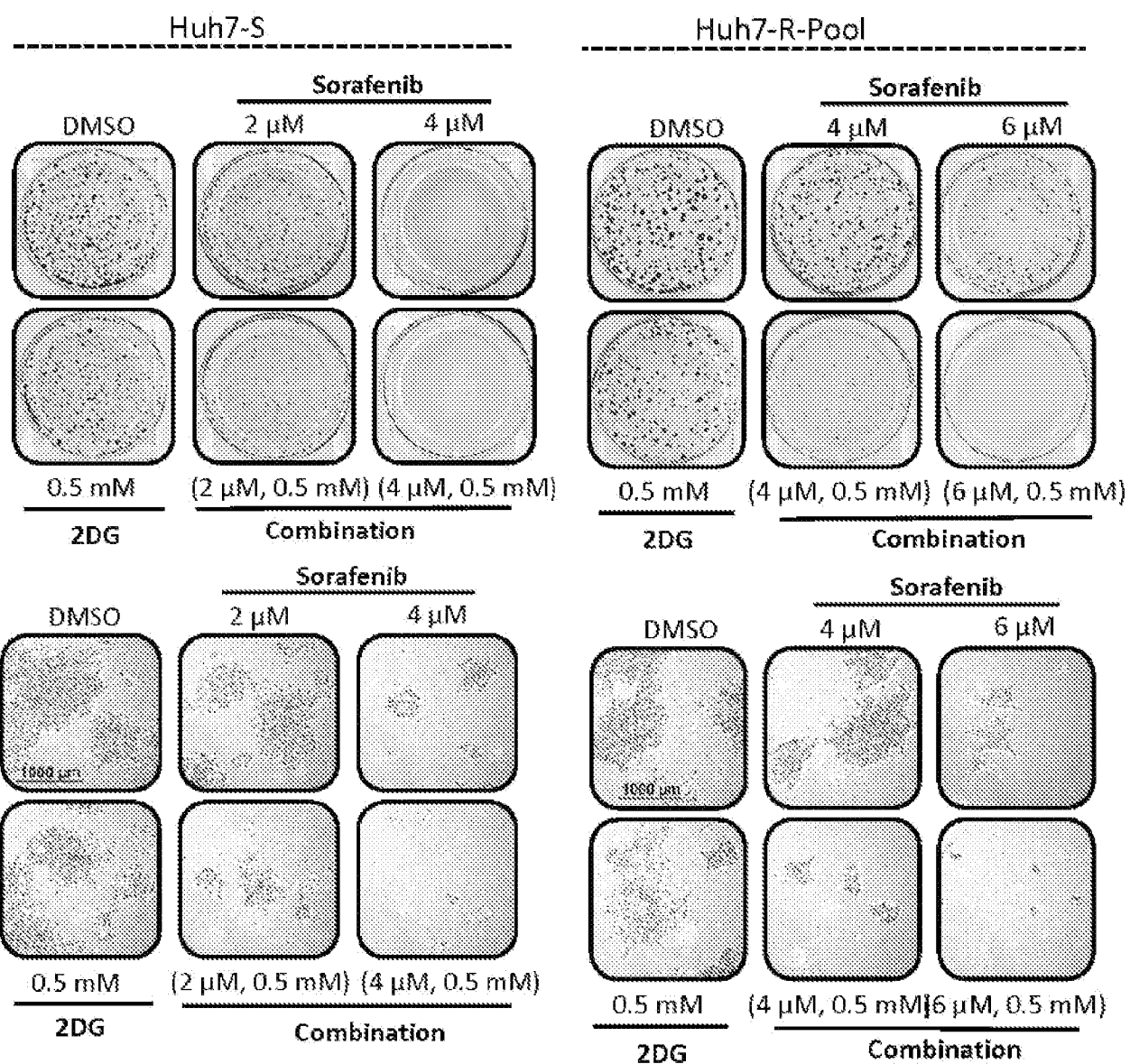


FIG. 3A

FIG. 3B

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Huh7-R-A7

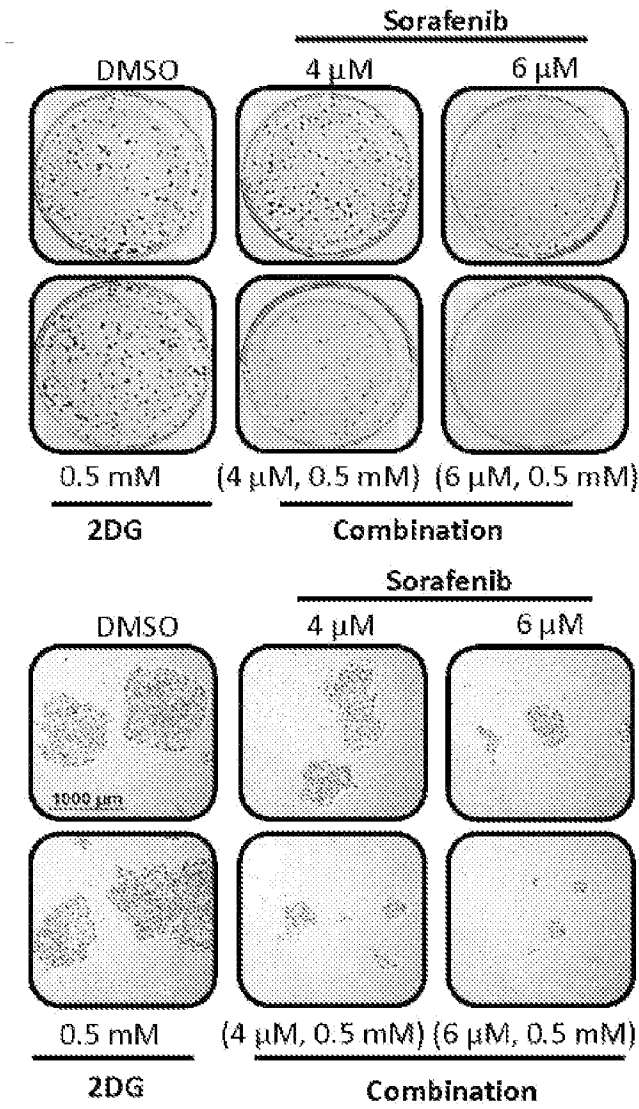


FIG. 3C

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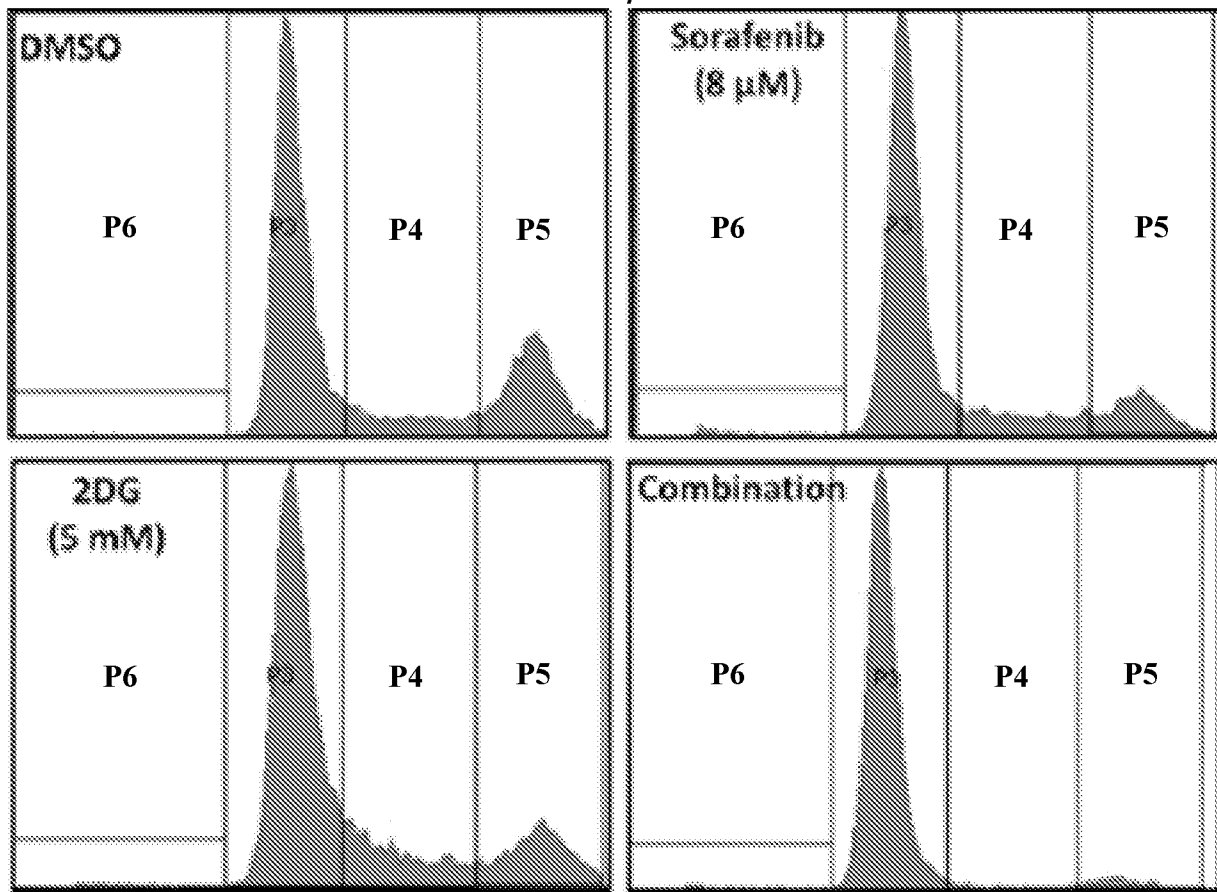


FIG. 4A

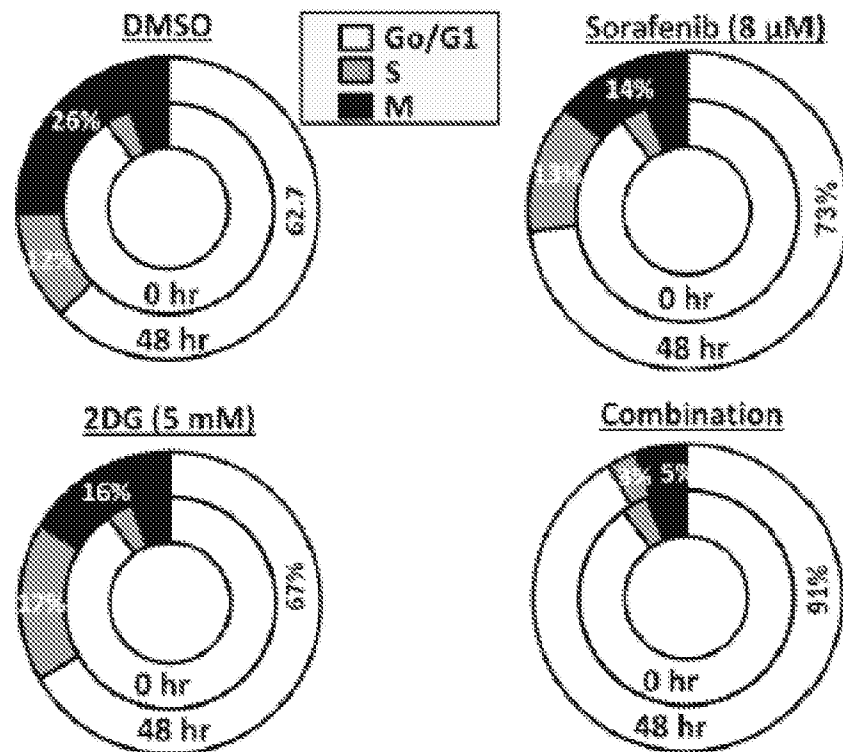


FIG. 4B

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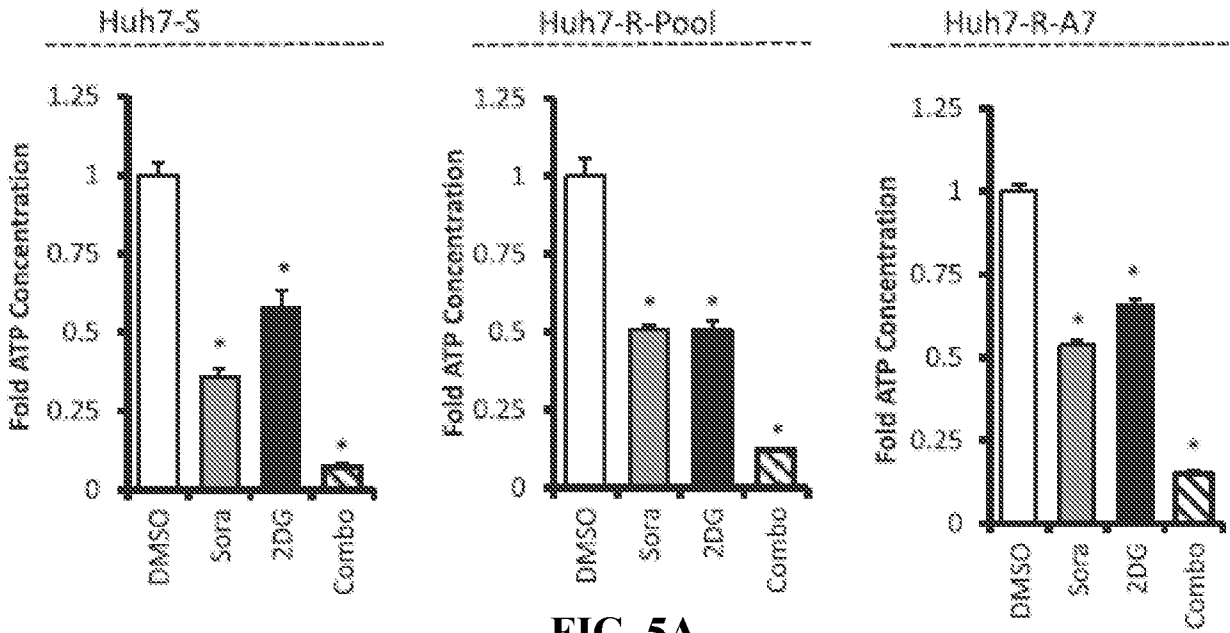


FIG. 5A

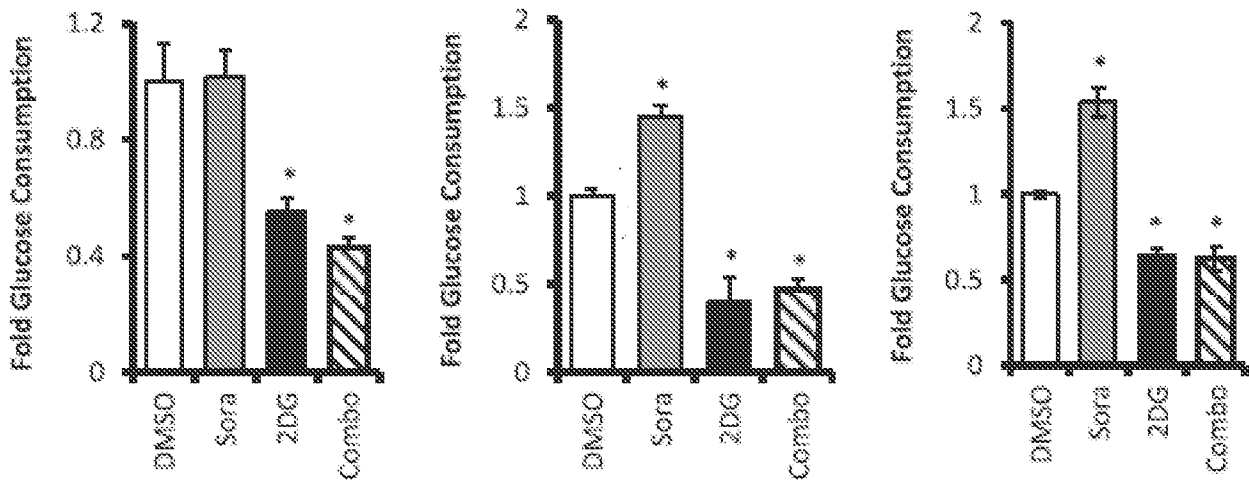


FIG. 5B

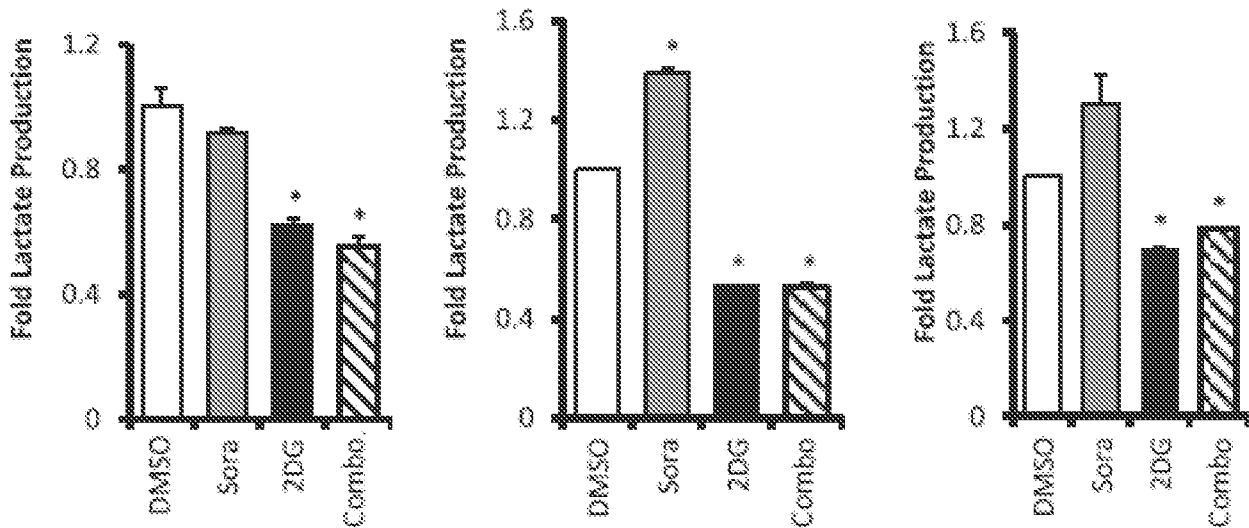


FIG. 5C

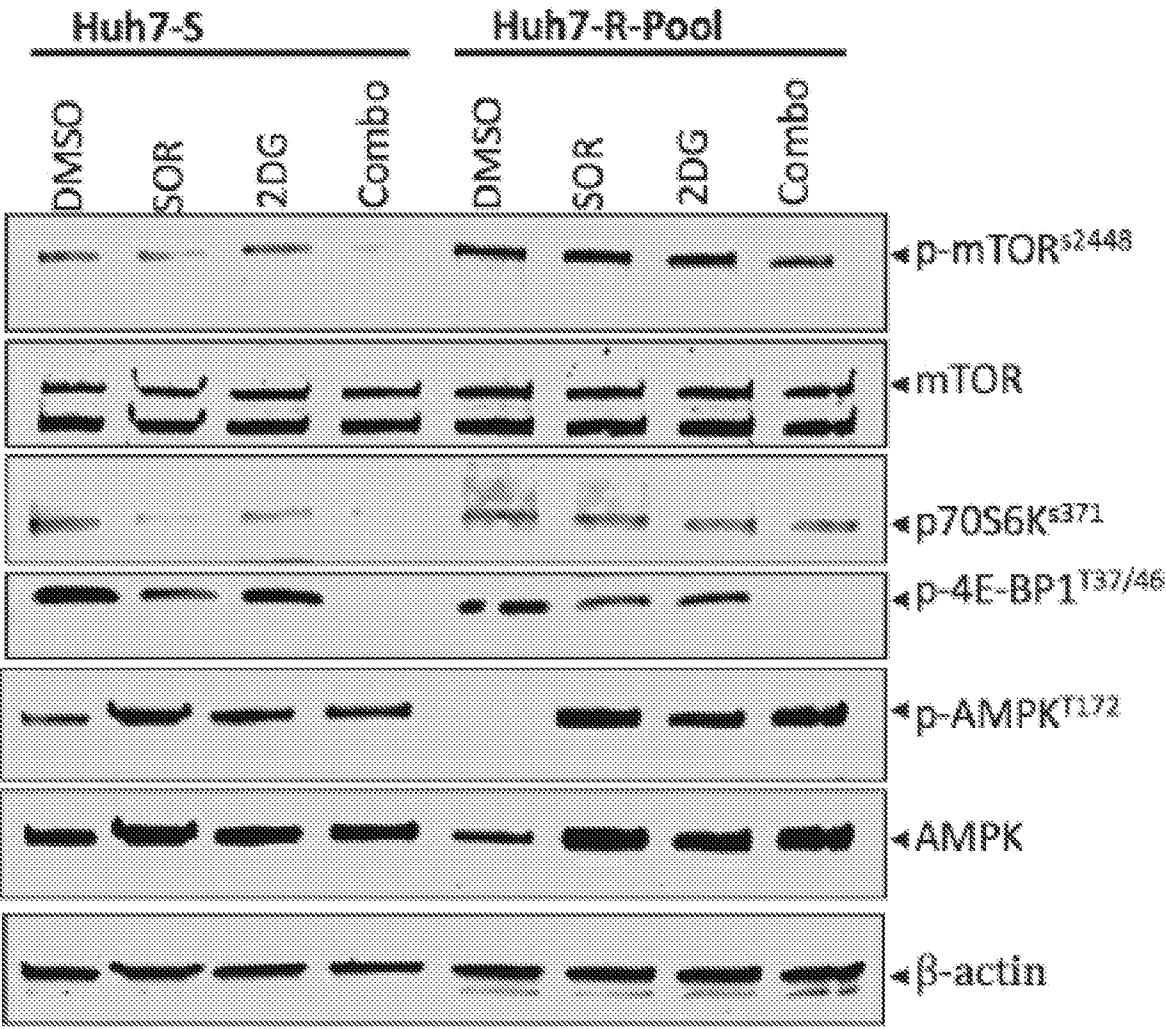


FIG. 6A

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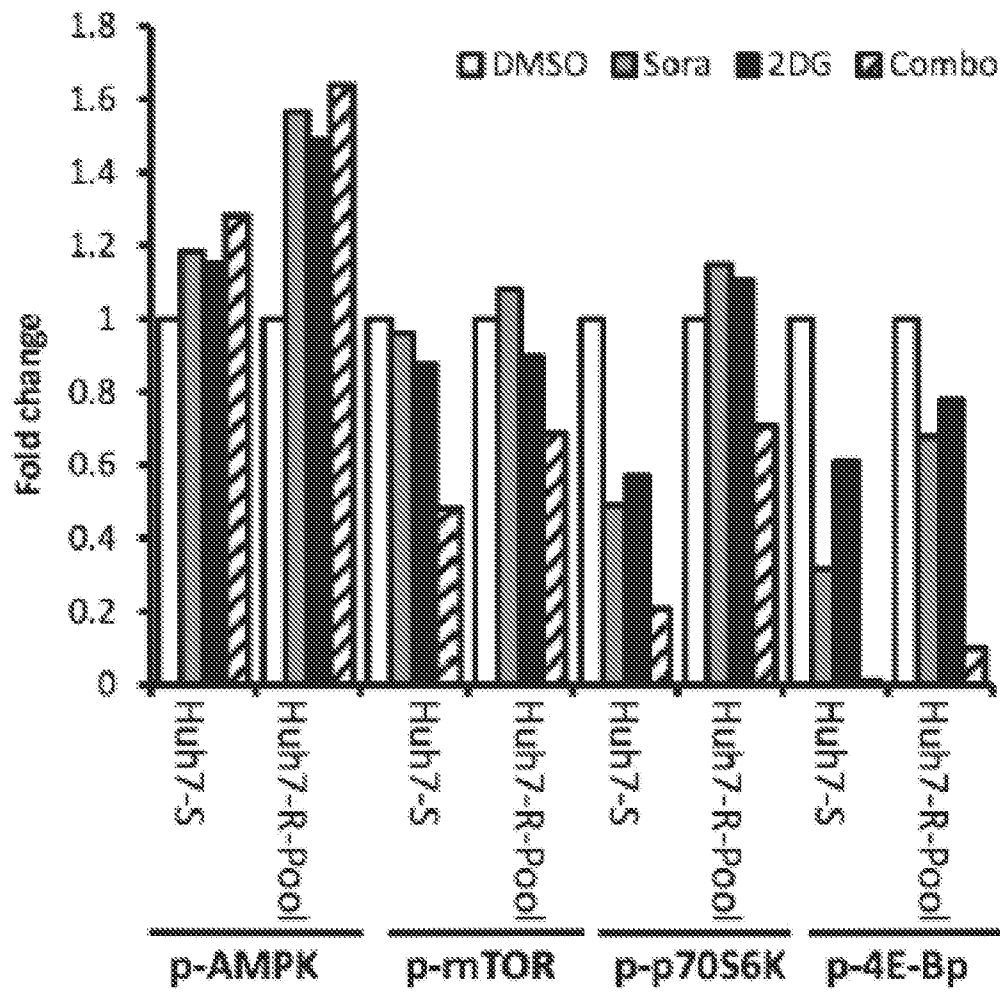


FIG. 6B

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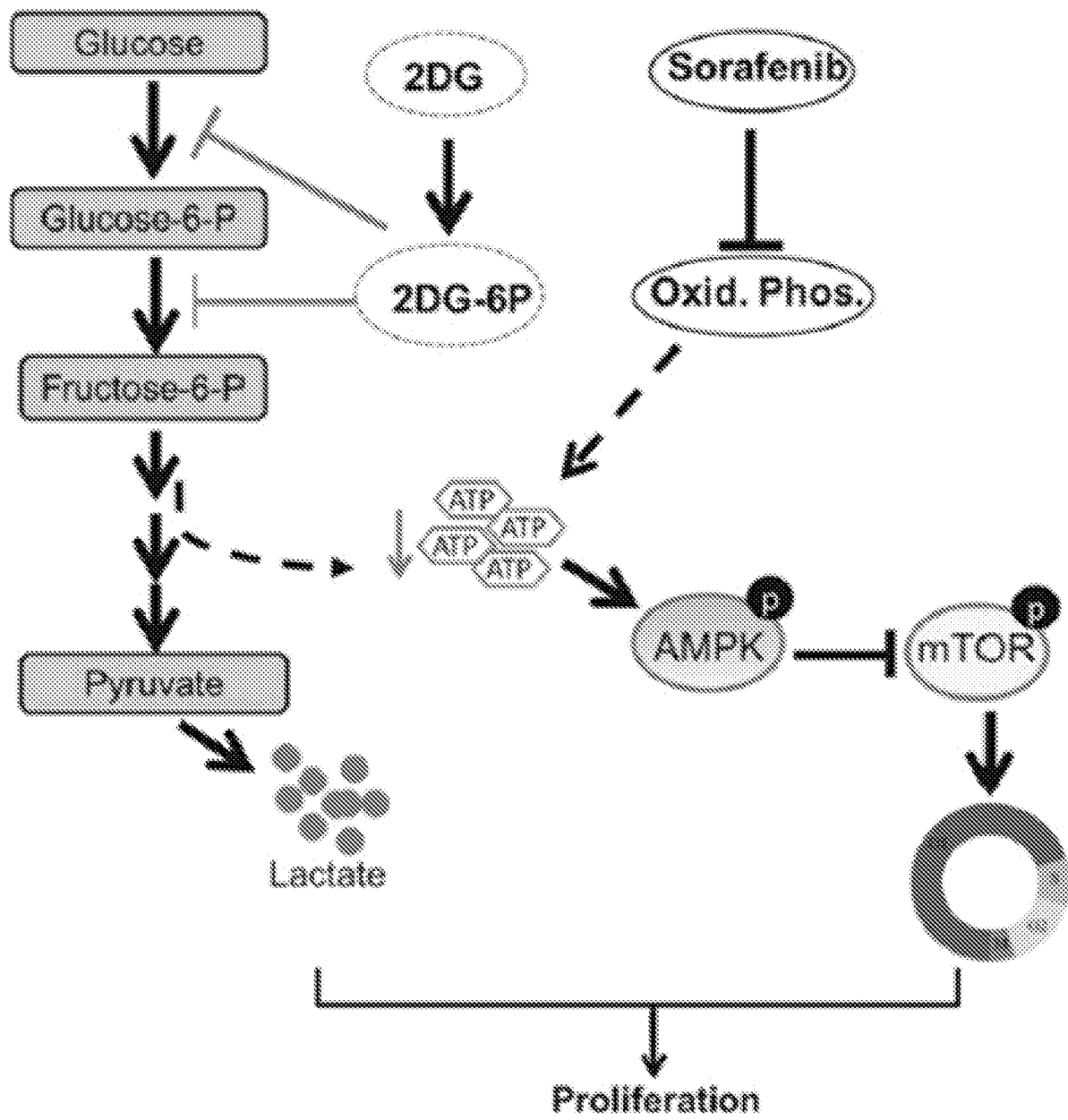


FIG. 6C

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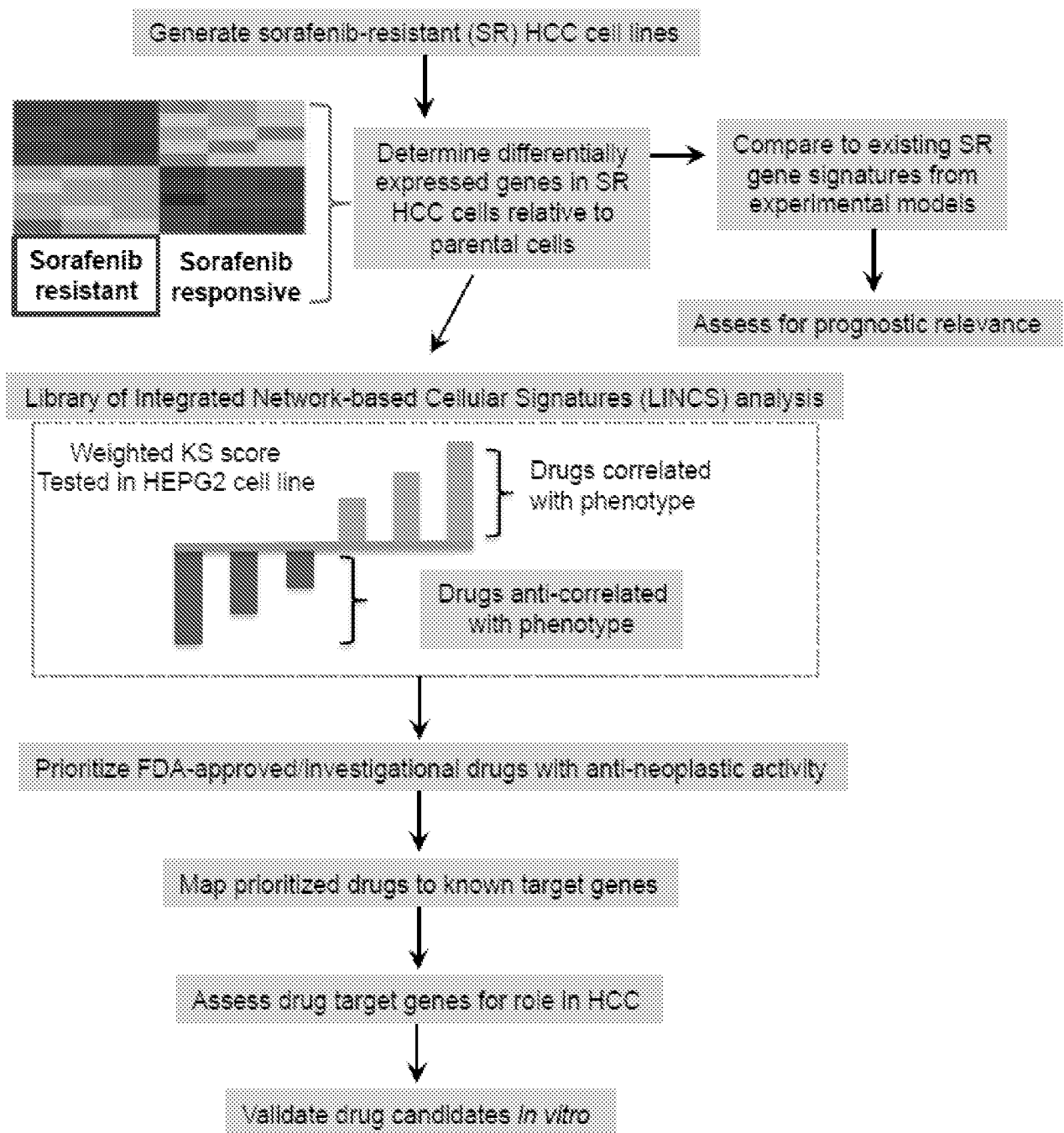


FIG. 7

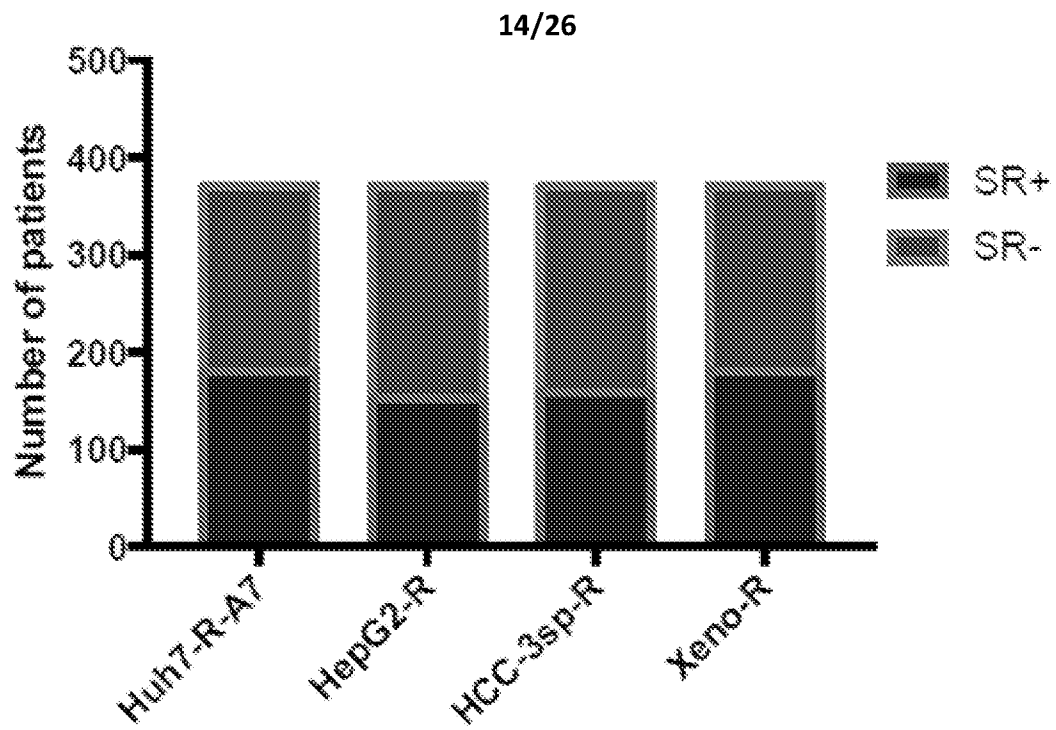


FIG. 8A

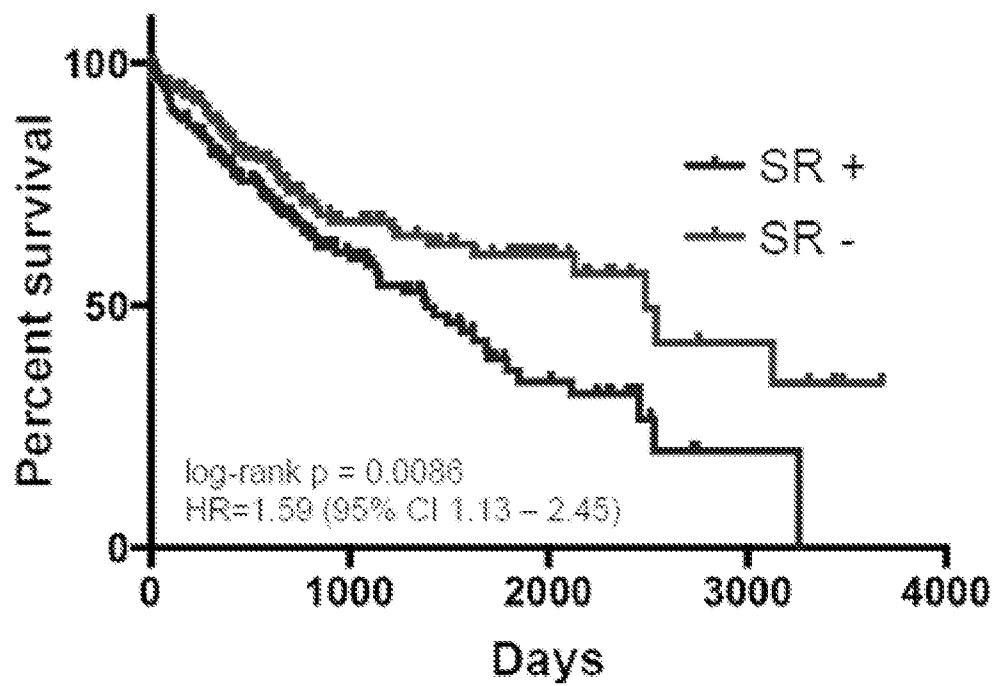


FIG. 8B

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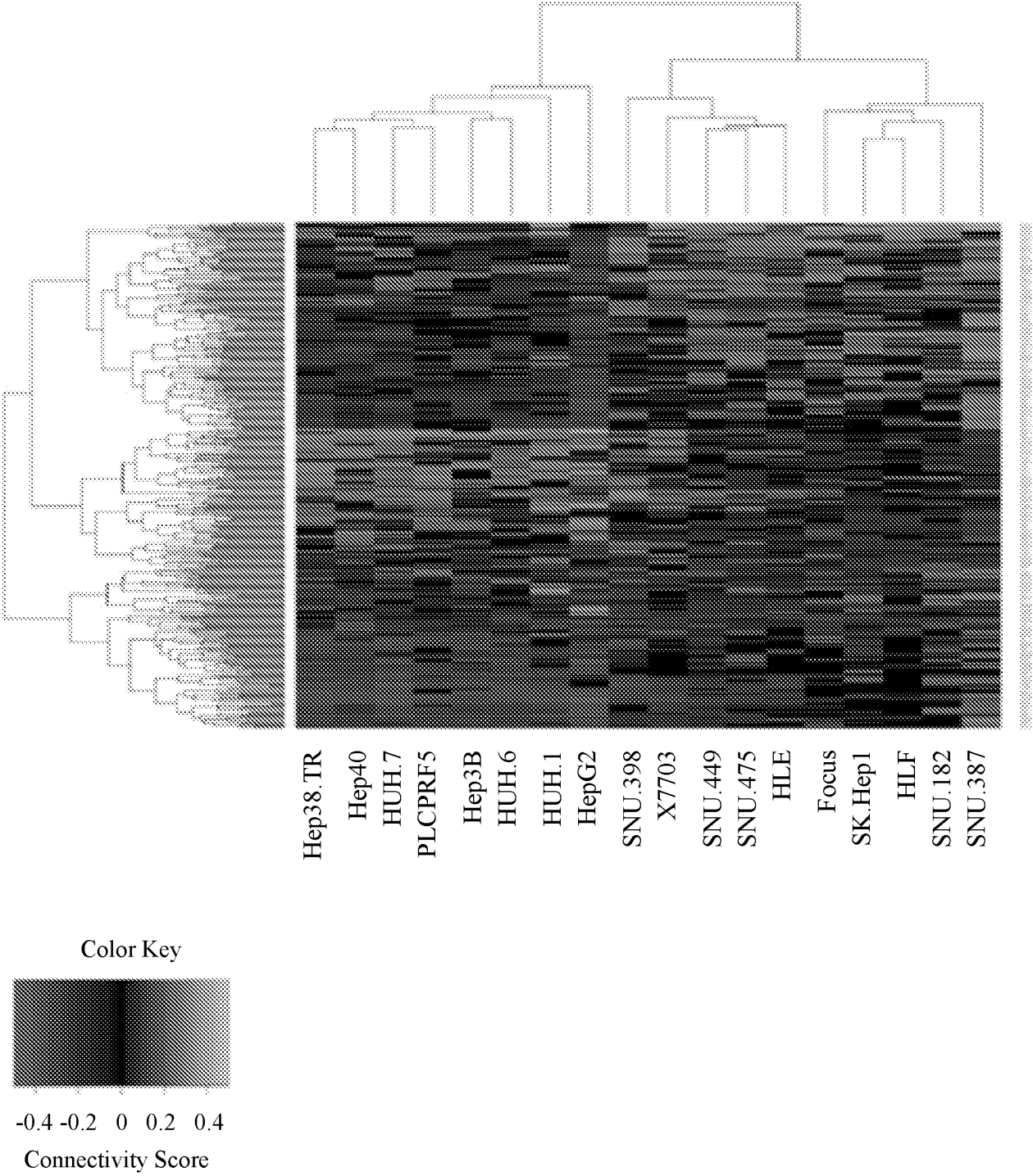
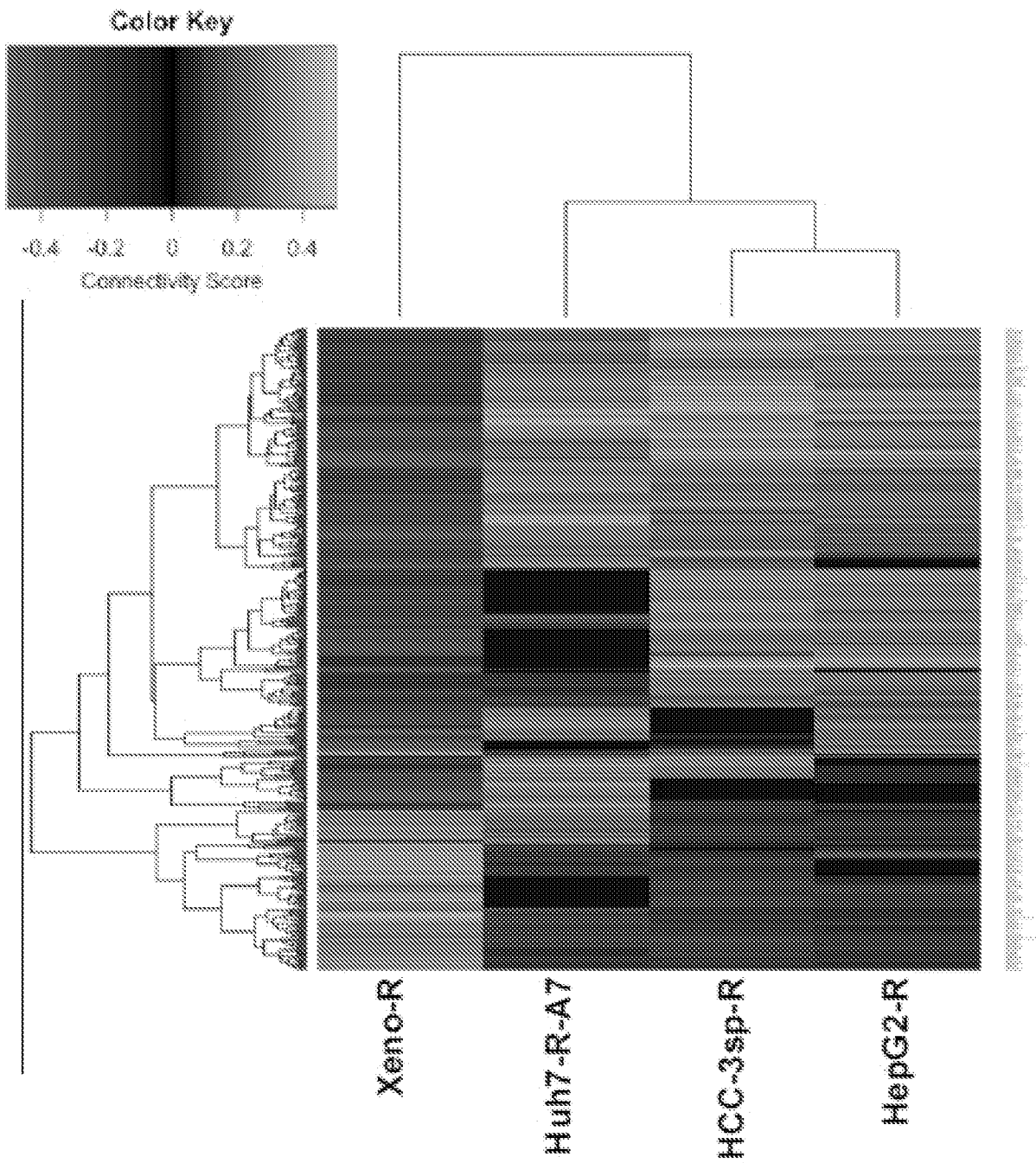


FIG. 8C



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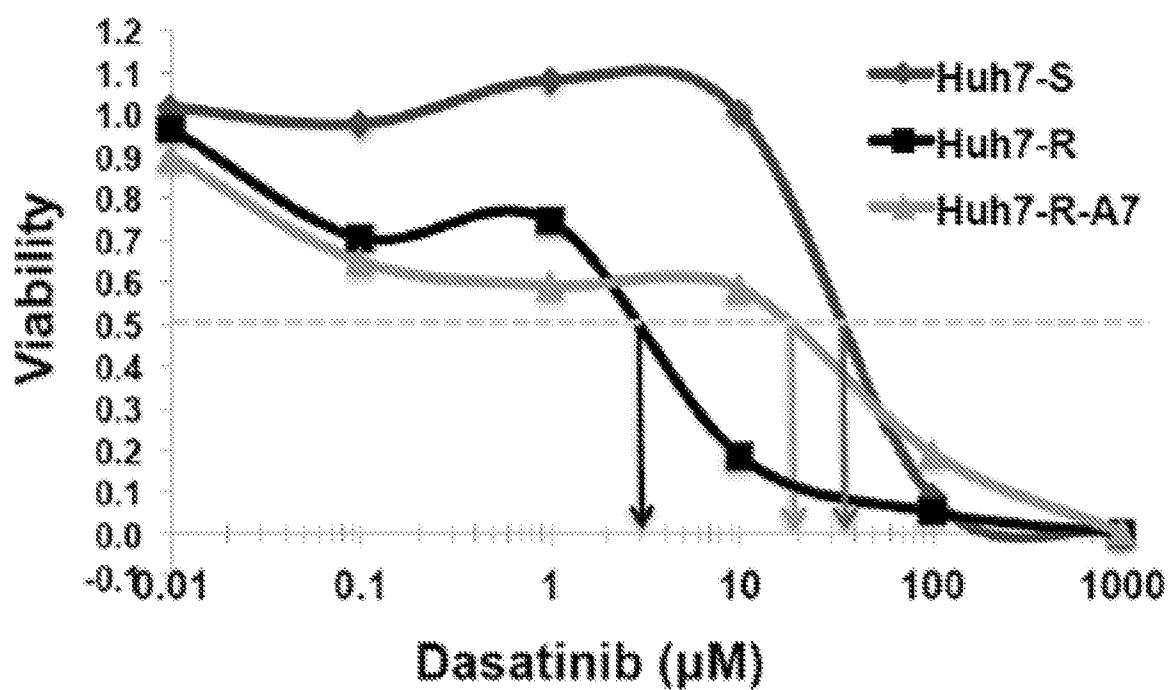


FIG. 9A

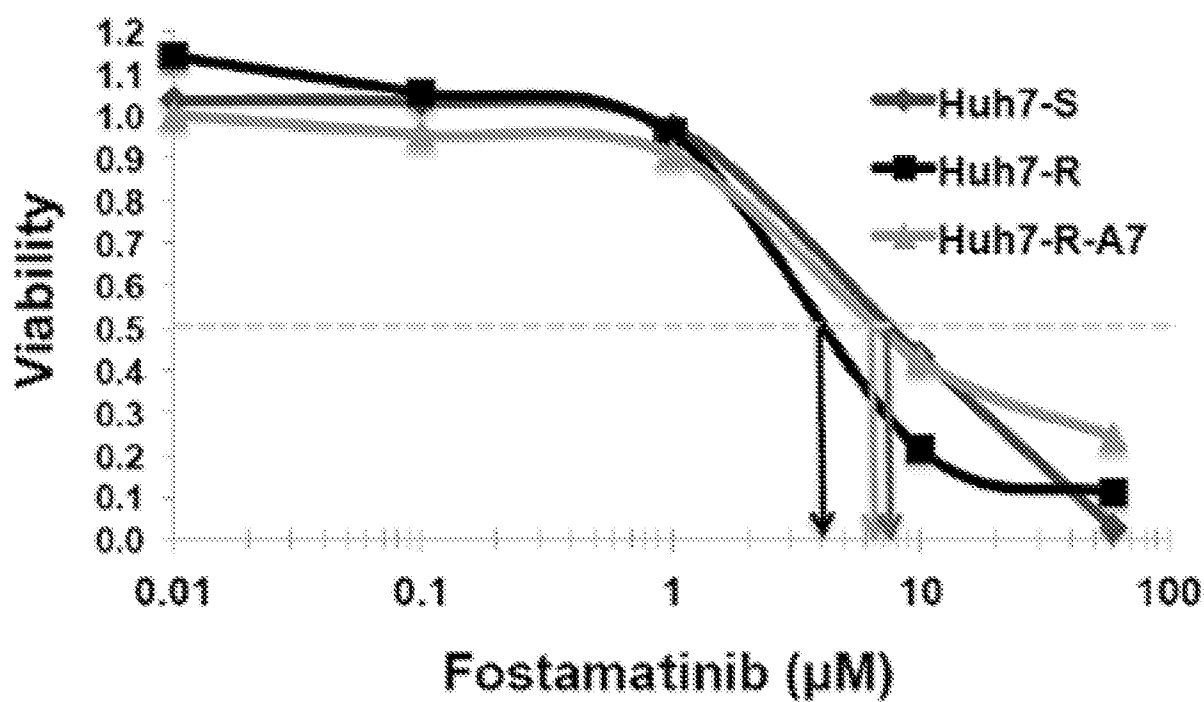


FIG. 9B

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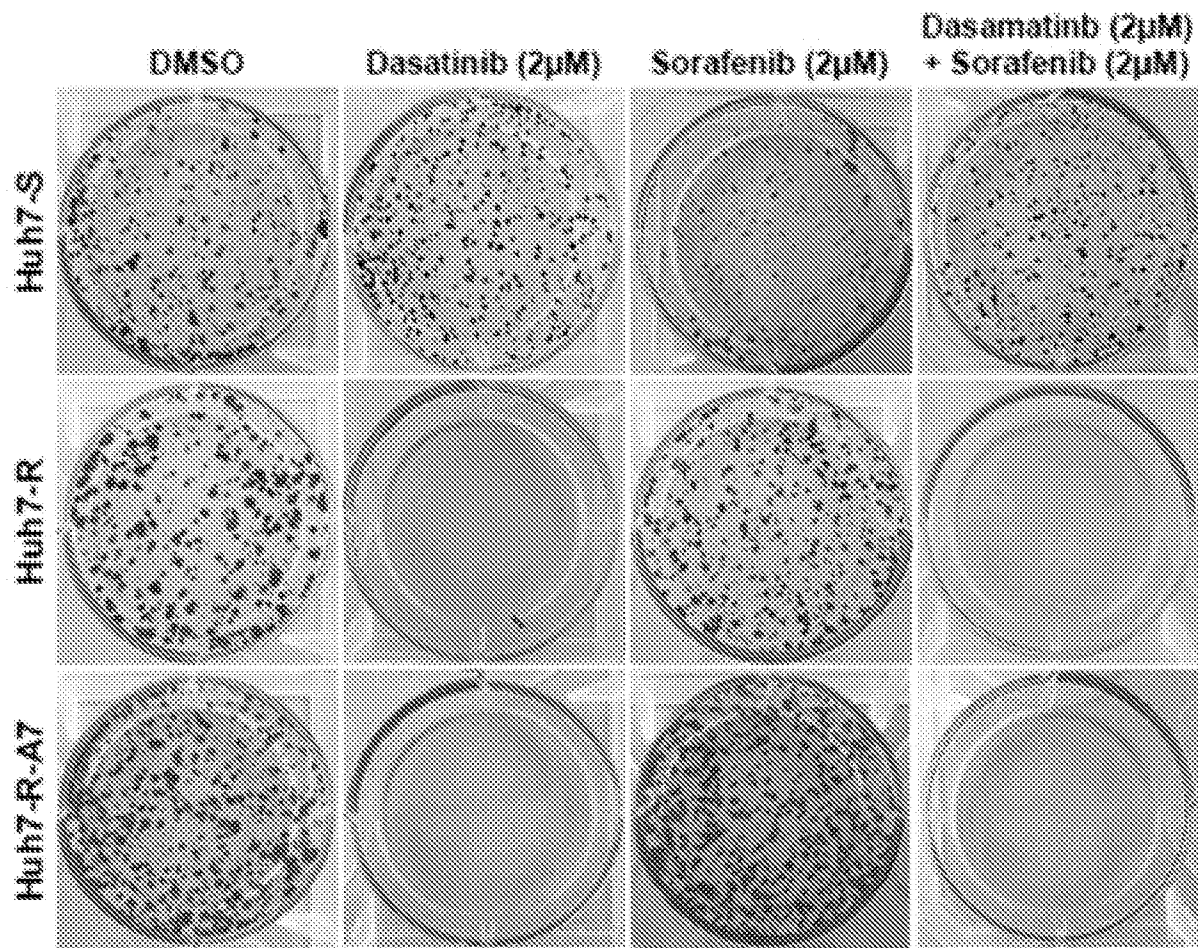


FIG. 9C

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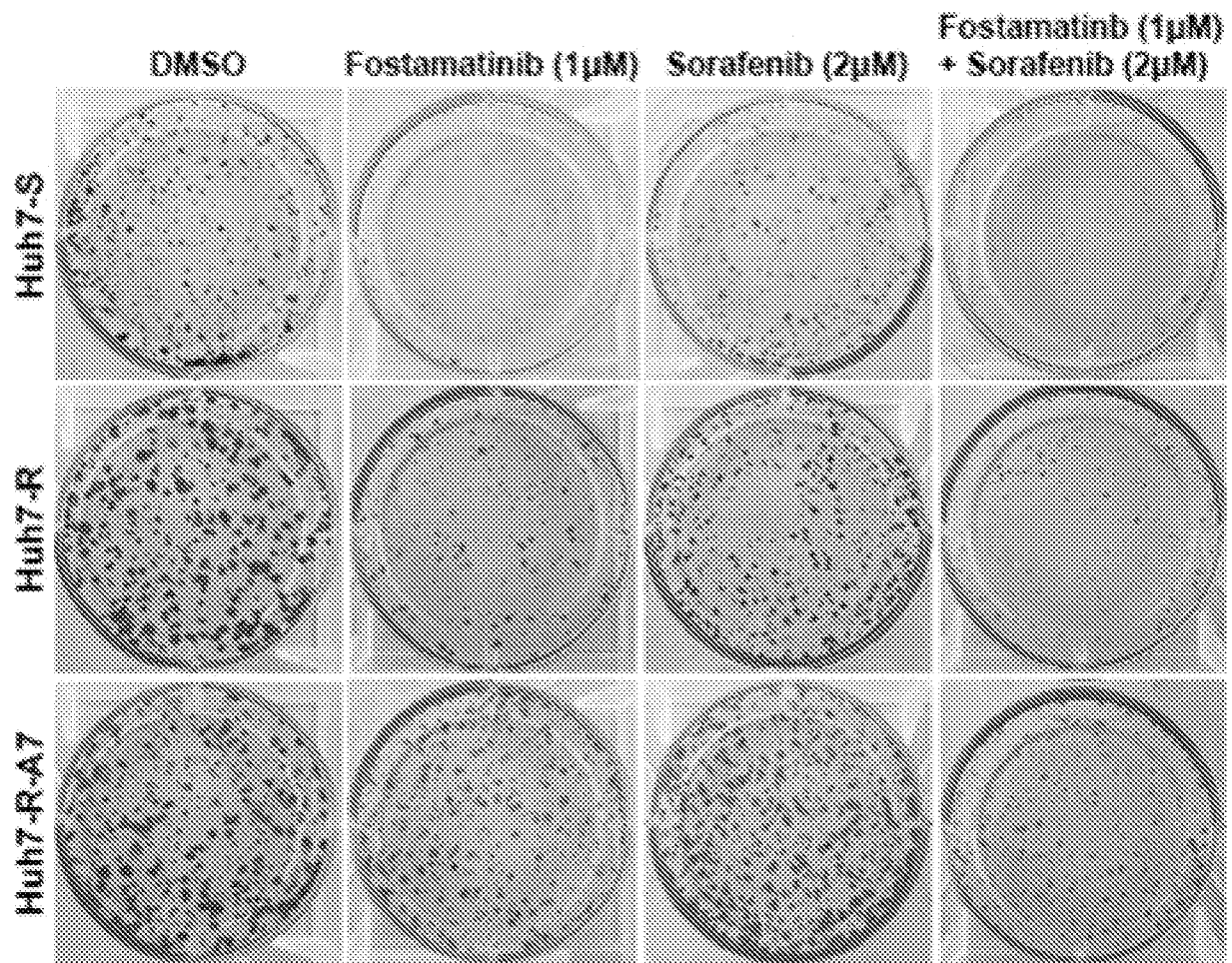


FIG. 9D

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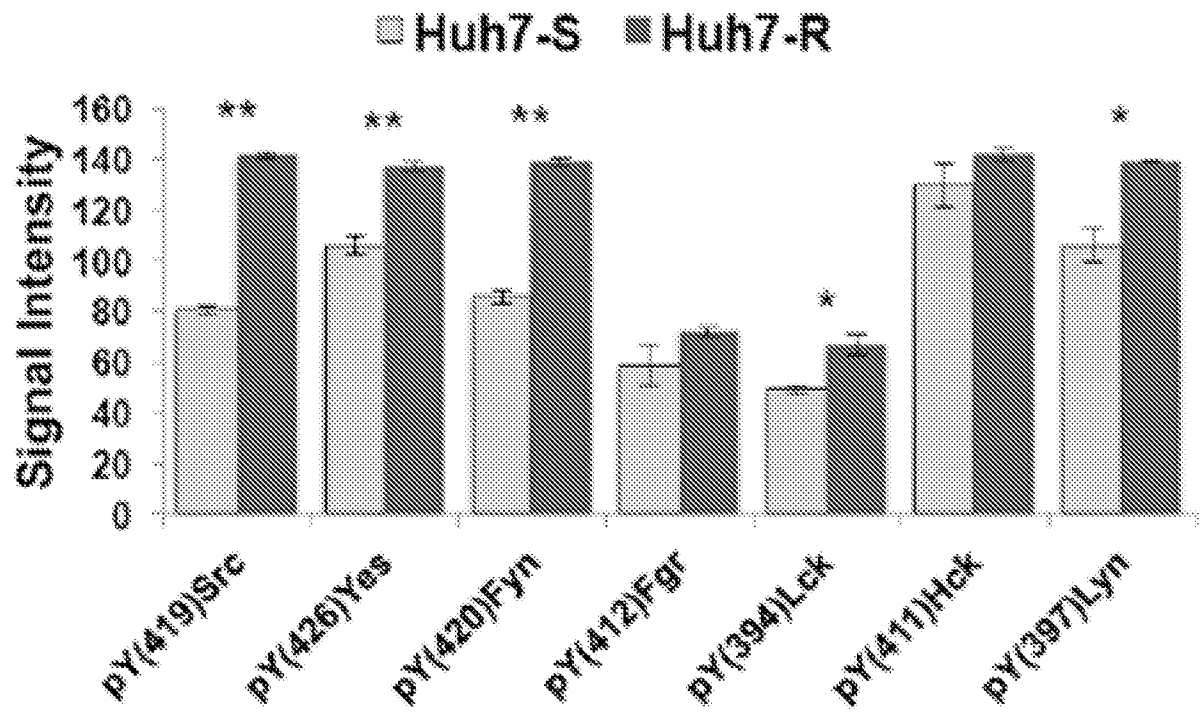


FIG. 9E

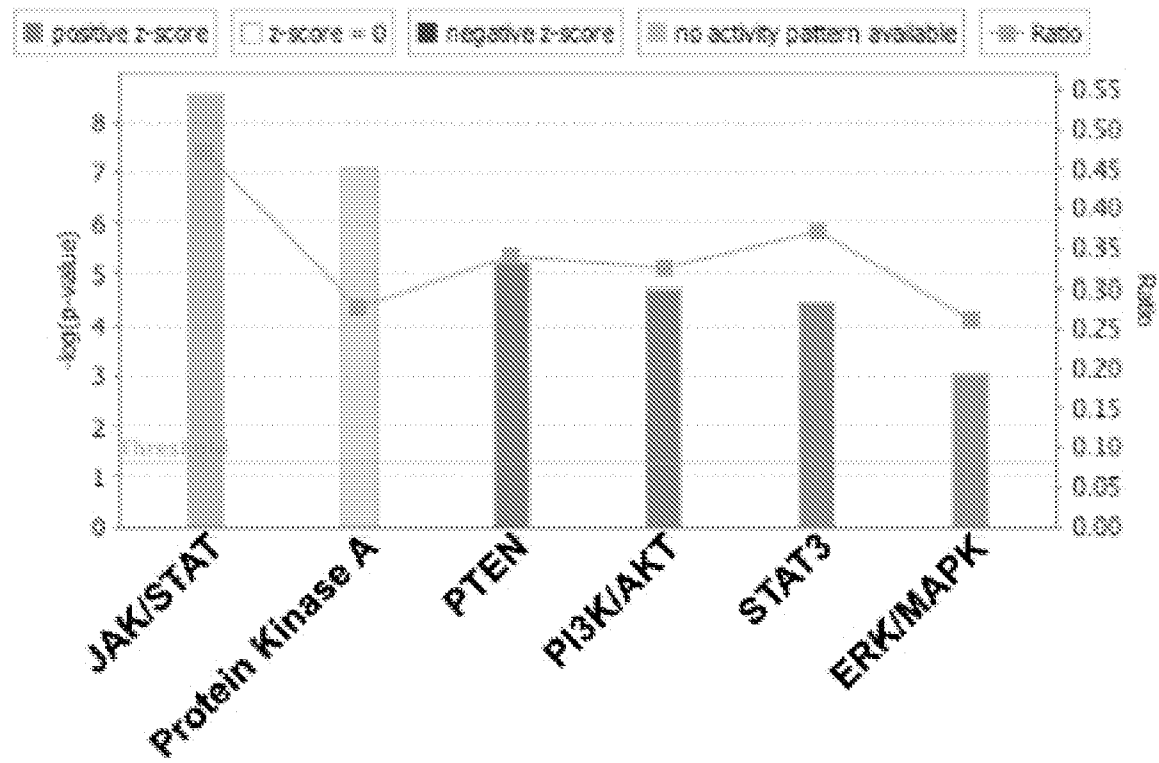


FIG. 9F

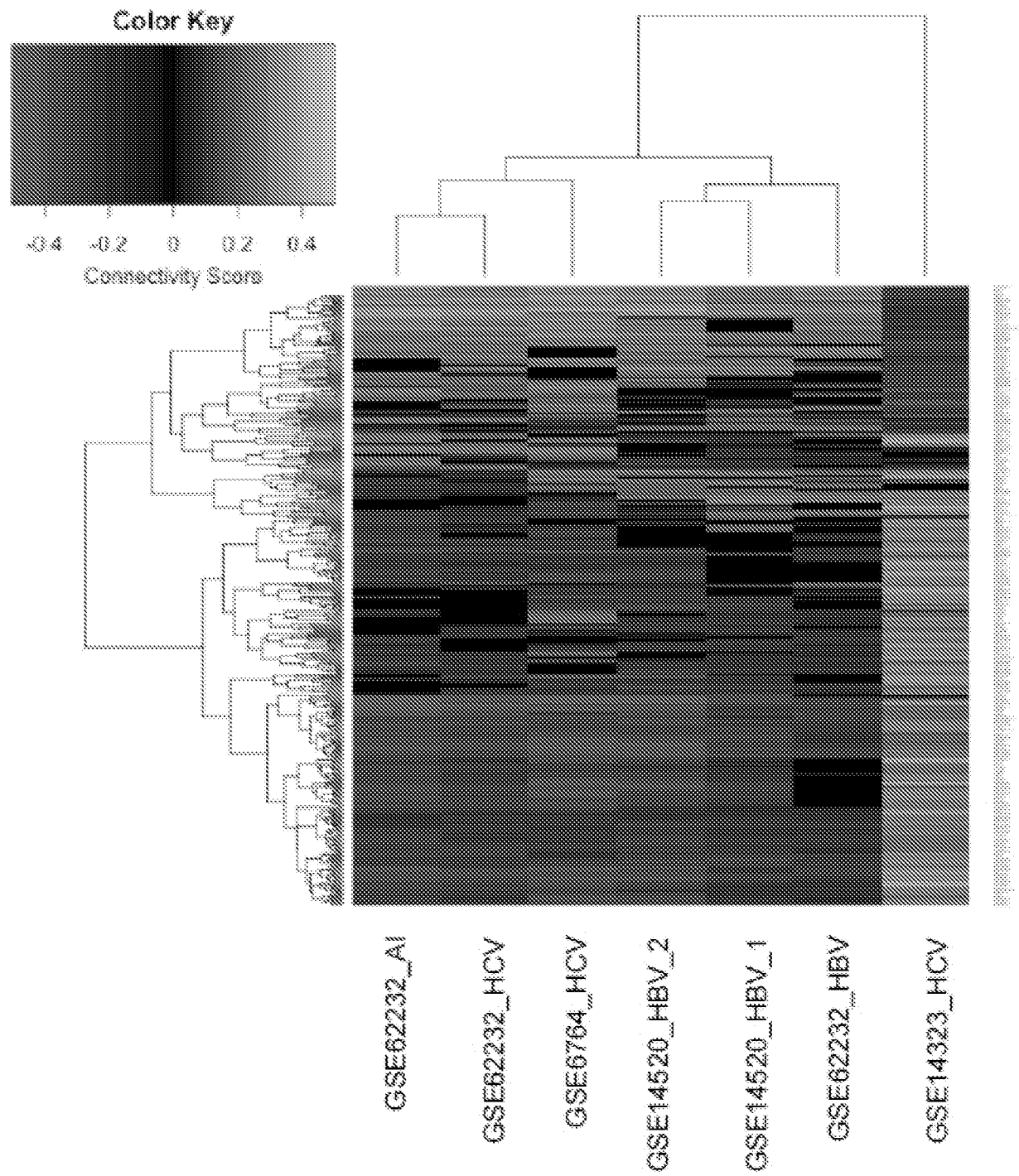


FIG. 10A

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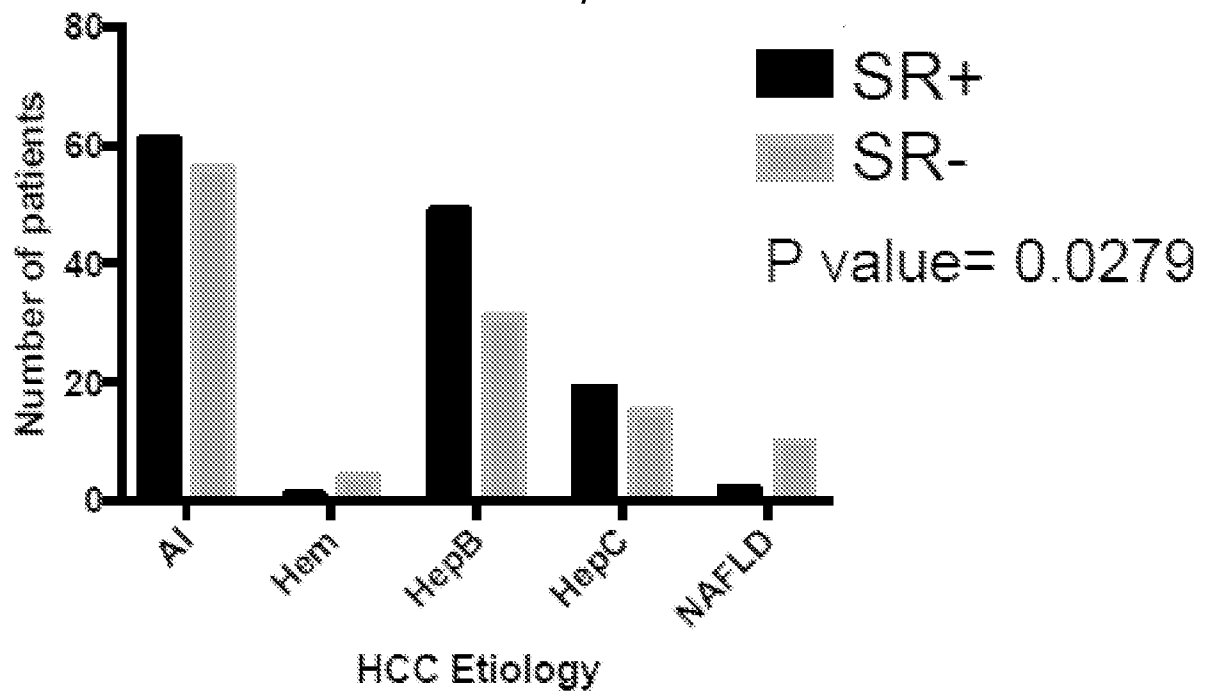


FIG. 10B

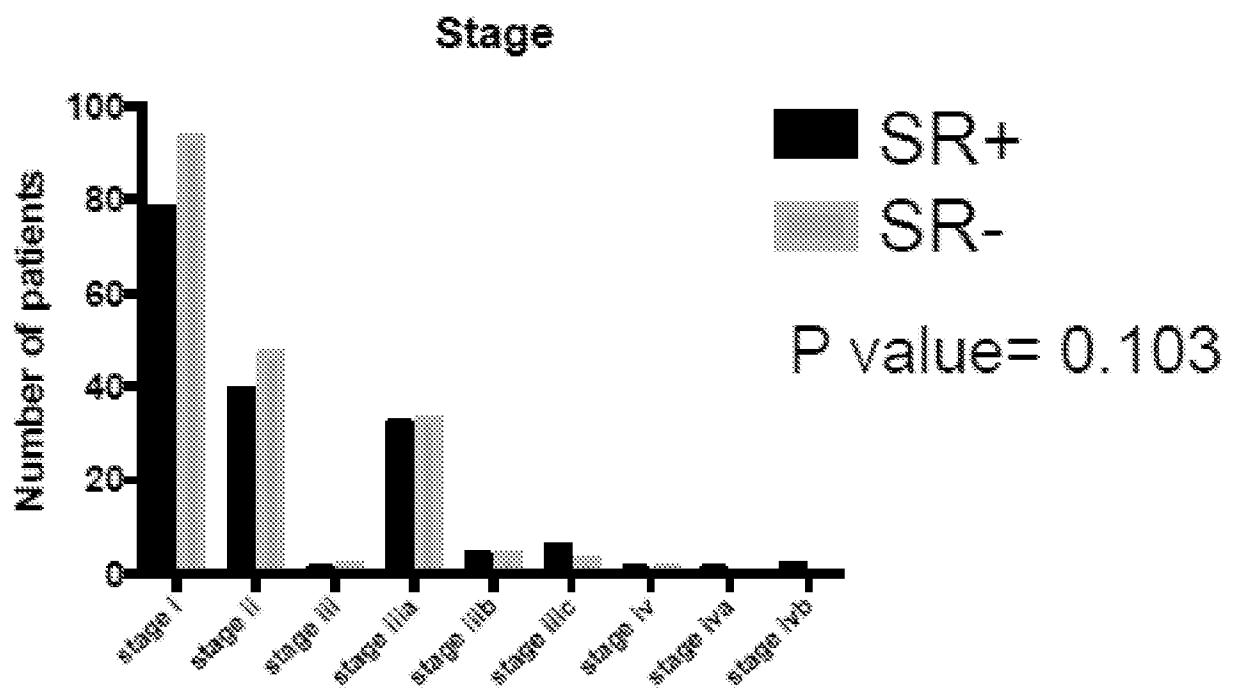


FIG. 10C

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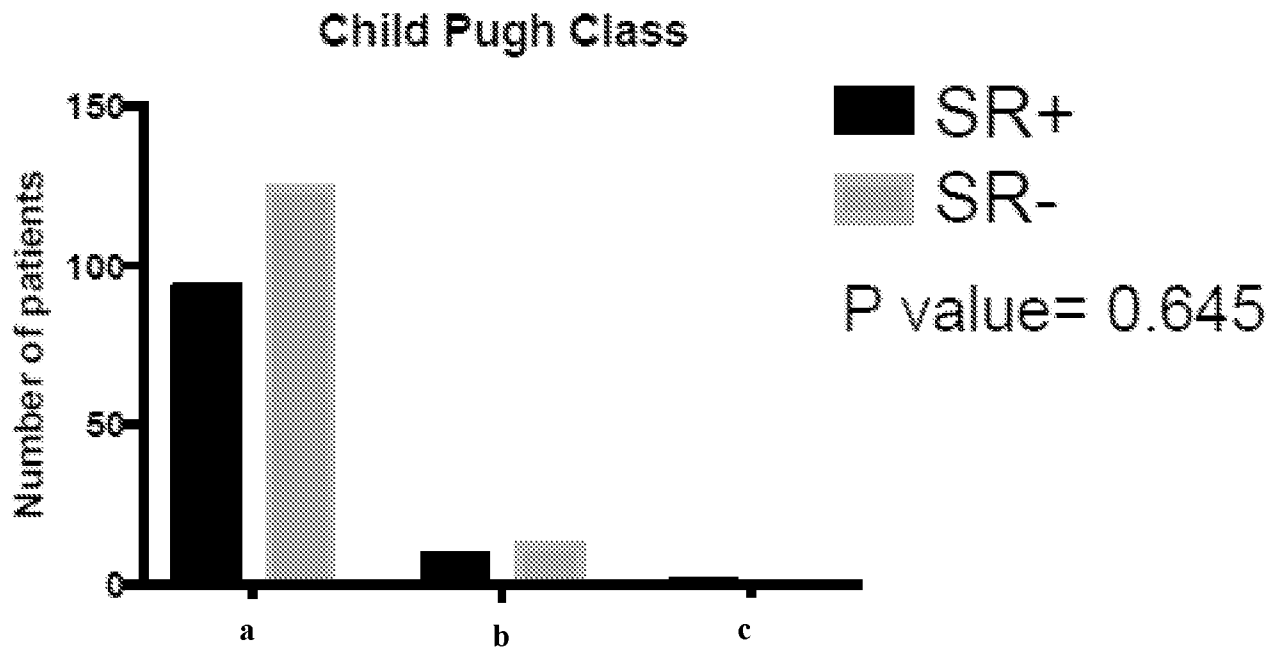


FIG. 10D

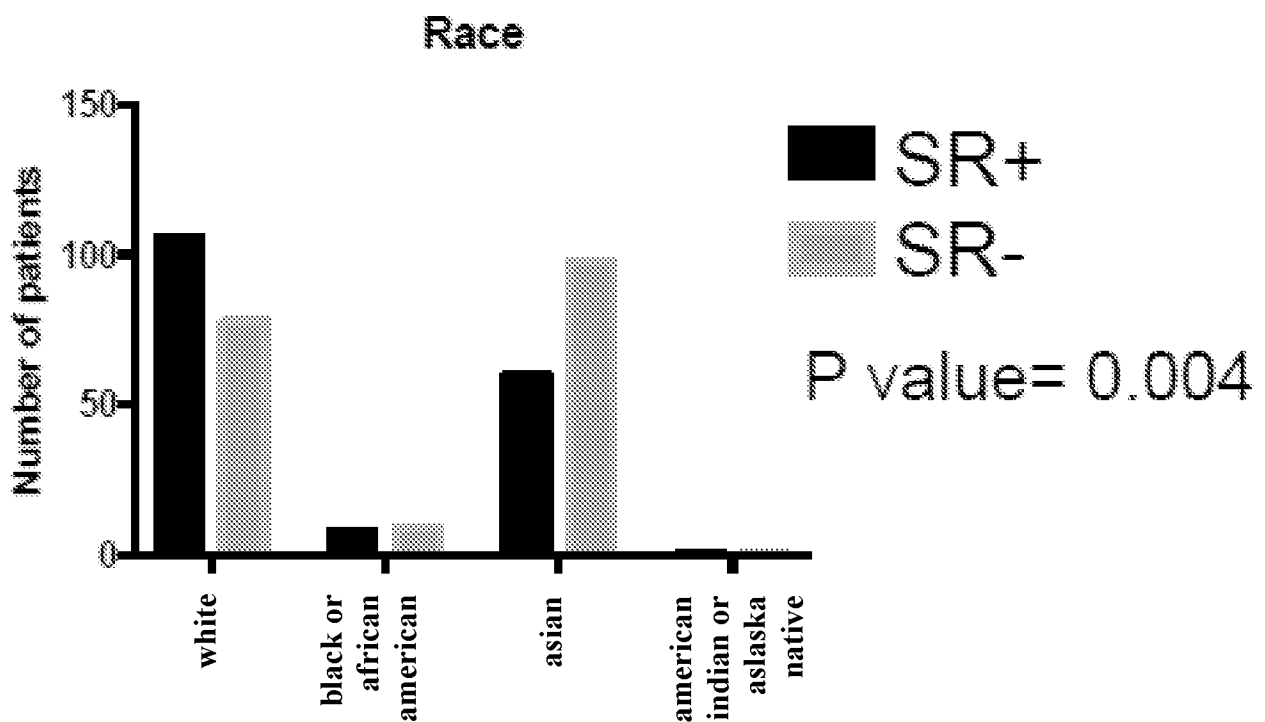


FIG. 10E

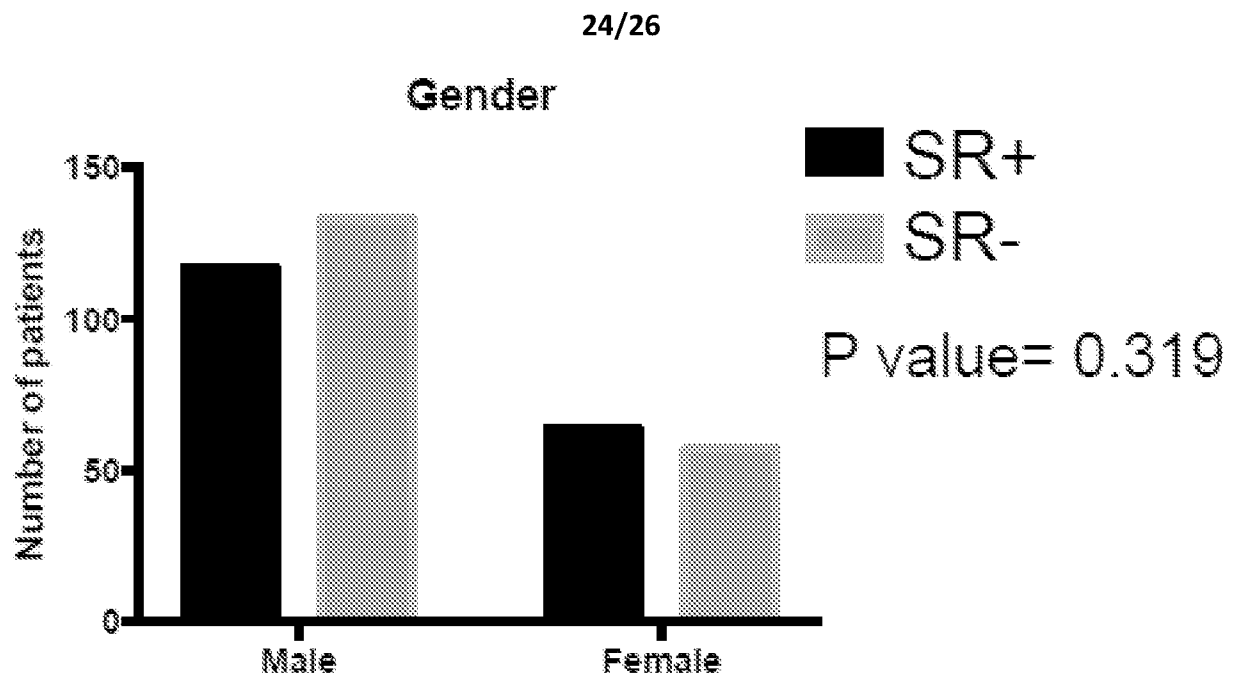


FIG. 10F

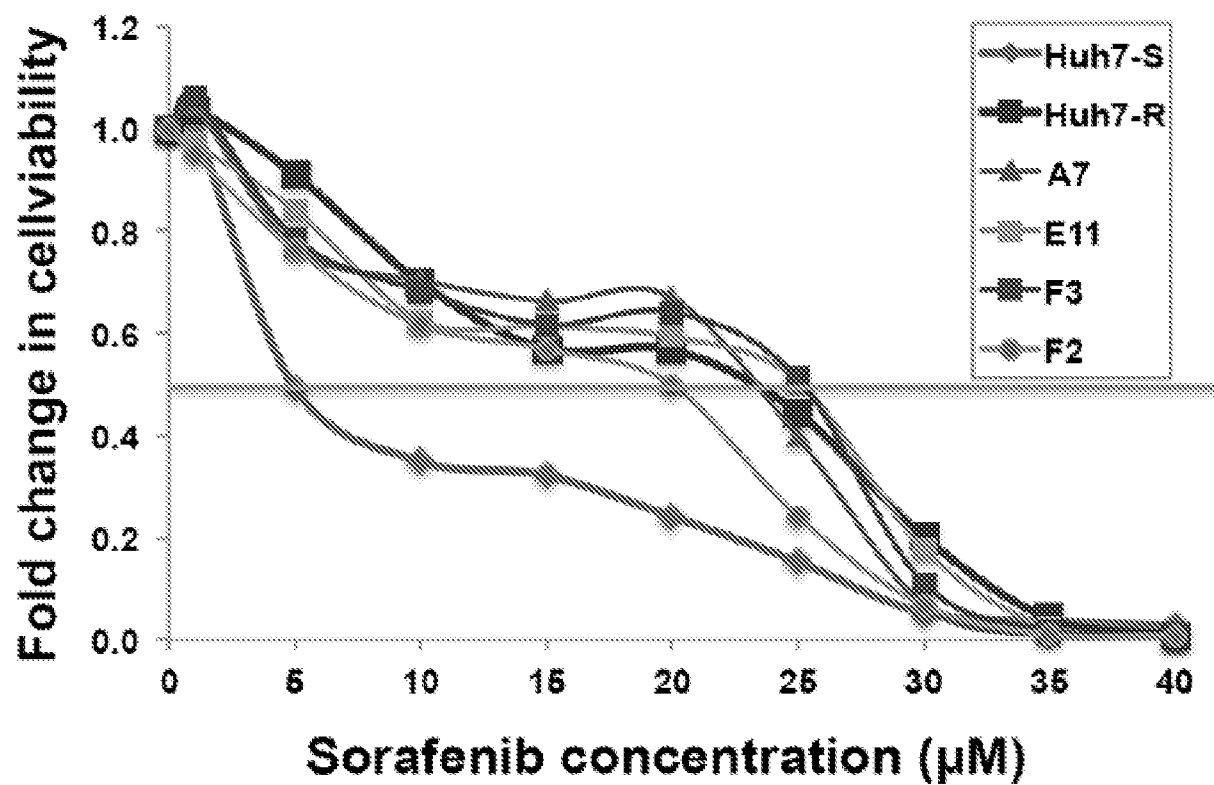


FIG. 11

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HCC-3sp-R

HepG2-R

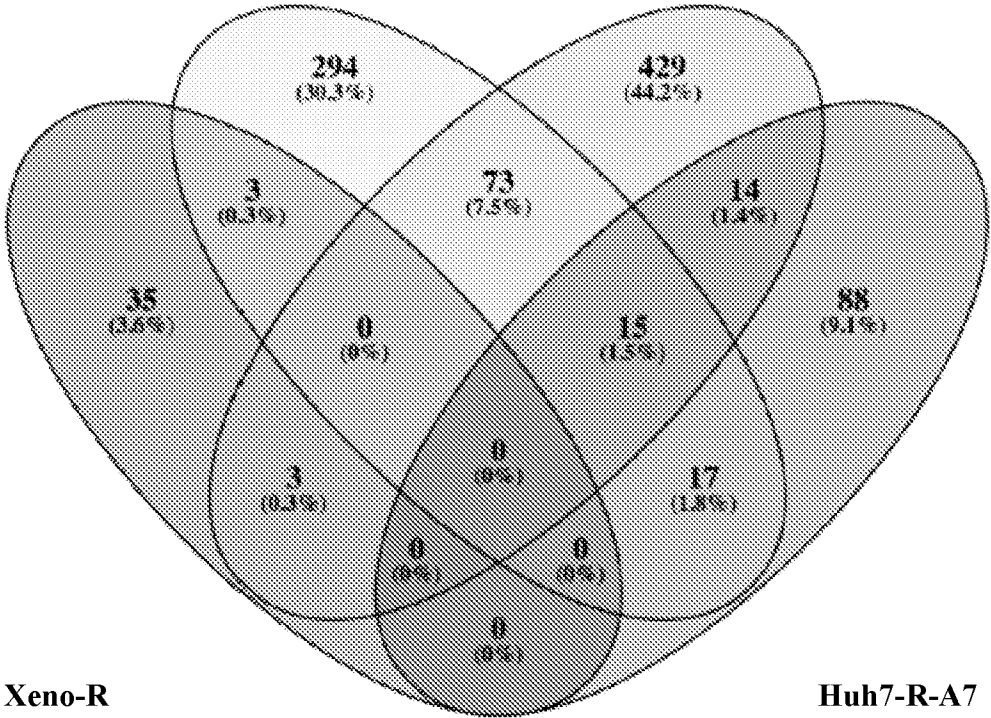


FIG. 12A

HCC-3sp-R

HepG2-R

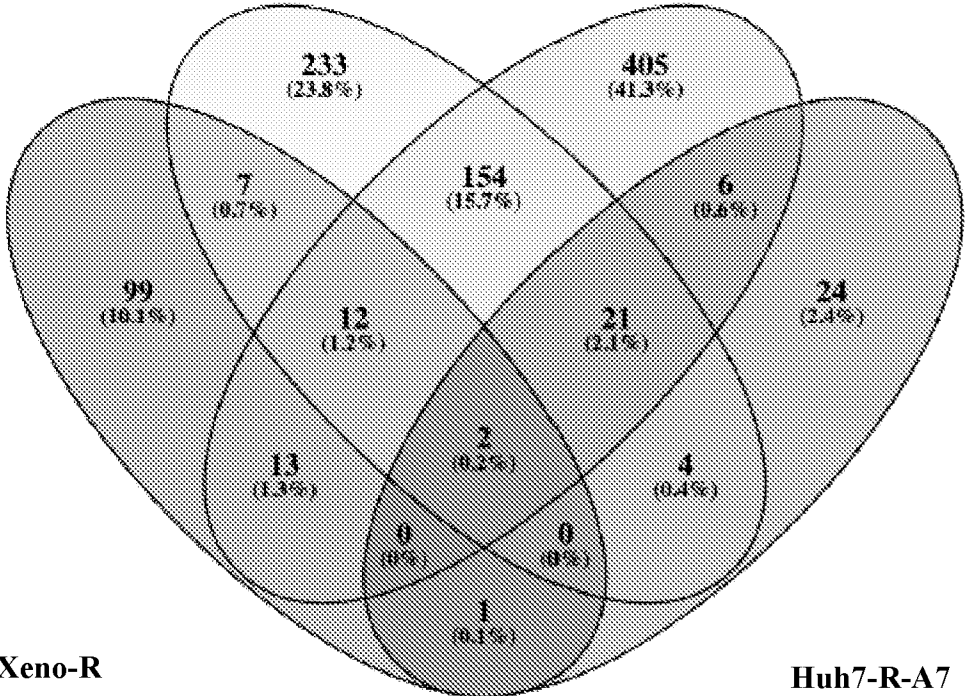


FIG. 12B

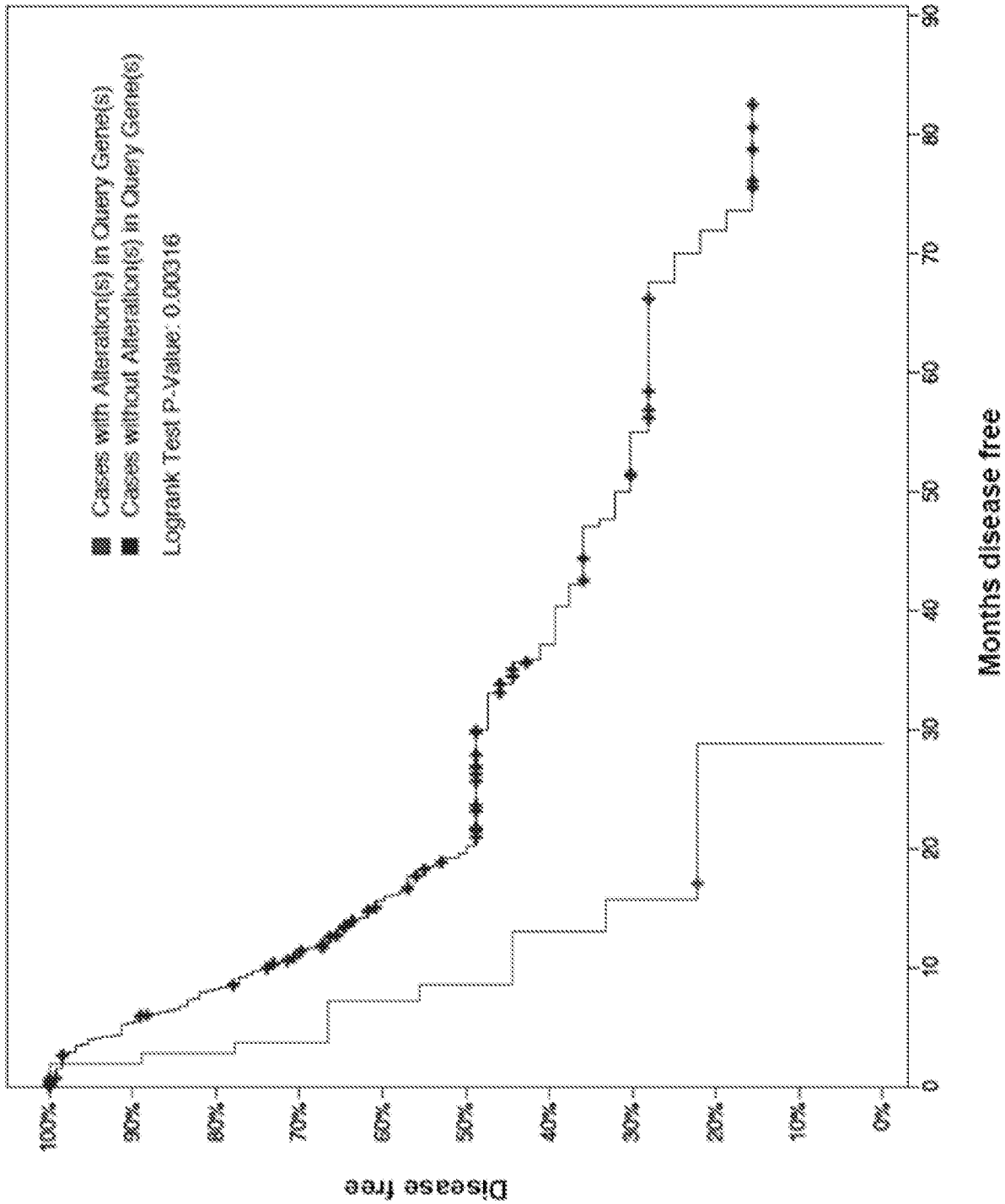


FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/38978

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/48, A61K 38/54 (2017.01)

CPC - A61K 38/4826, A61K 38/47, A61K 45/06, A61K 31/21

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/117396 A1 (Novoty Therapeutics) 7 September 2012 (07.09.2012) Abstract, pg 4 para 4, pg 5 para 5; claims, pg 42 para 5; pg 4 para 1; pg 7, pg 42 para 3	1-9
X	US 2011/0206661 A1 (Zhang et al.) 25 August 2011 (25.08.2011) para [0002], para [0003], para [00110], para [0099], para [0004]	15-19
X ---	US 2010/0278776 A1 (Hangauer) 4 November 2010 (04.11.2010) Abstract, para [0095], para [0087], para [00359], para [0070]	20,21,24/(20-21) ----- 22,23,24/(22-23)
Y	US 2015/0342896 A1 (Amritha Vishwa Vidyapeetham University) 3 December 2015 (03.12.2015) Abstract, para [0009], para [0081], [0087],	22,23,24/(22-23)
Y	WO 2016/025635 A1 (Epizyme) 18 February 2016 (18.02.2016) Abstract, para [0203]-[0205], [177]	1-9,15-24
Y	US 2015/0056215 a1 (Sukhatme et al.) 26 February 2015 (26.02.2015) Abstract, para [0041], para [0044]	1-9
Y	WO 2015/126903 A1 (Health Research Inc) 27 August 2015 (27.08.2015) Abstract, claim 1	1-9

☐ 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

01 September 2017 (01.09.2017)

Date of mailing of the international search report

28 SEP 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

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Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/38978

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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.:
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:
3. ☒ Claims Nos.: 10-14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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 additional fees, this Authority did not invite payment of 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.