



(51) International Patent Classification:

A61K 39/395 (2006.01) C07K 16/24 (2006.01)
A61K 45/06 (2006.01)

(21) International Application Number:

PCT/US2019/059877

(22) International Filing Date:

05 November 2019 (05.11.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/756,230 06 November 2018 (06.11.2018) US

(71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US];

Office of the General Counsel, Building 170, 3rd Floor, Main Quad, P.O. Box 20386, Stanford, California 94305-2038 (US).

(72) Inventors: PELTZ, Gary; 110 Danbury Lane, Redwood City, California 94061 (US). GUAN, Yuan; L212, Lane

building, 1291 Welch Road, Stanford, California 94305 (US).

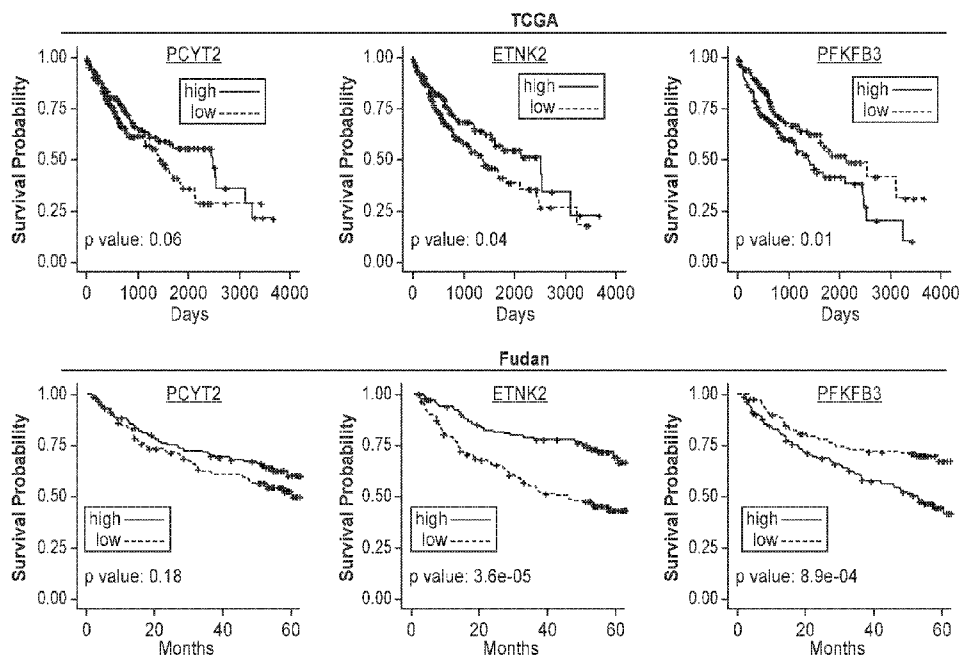
(74) Agent: DAVY, Brian E.; Bozicevic, Field & Francis LLP, 201 Redwood Shores Pkwy., Suite 200, Redwood City, California 94065 (US).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

(54) Title: COMBINATION THERAPY FOR CANCER

FIG. 6



(57) Abstract: Provided are methods of treating cancer. The methods include administering to an individual having cancer a therapeutically effective amount of a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor and a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor. Also provided are compositions and kits that find use, e.g., for practicing the methods of the present disclosure.

EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

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

COMBINATION THERAPY FOR CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/756,230, filed November 6, 2018, which application is incorporated herein by reference
5 in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with Government support under contract DK102182 awarded by the National Institutes of Health. The Government has certain rights in the invention.

SUMMARY

Provided are methods of treating cancer. The methods include administering to an individual having cancer a therapeutically effective amount of a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor and a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor. Also provided are compositions and kits that find use,
15 e.g., for practicing the methods of the present disclosure.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 scRNA-Seq profiling of the transcriptome of cells during their *in vitro* differentiation from iPSC into primary HO. scRNA-Seq was performed on cells obtained from iPSC, hepatoblast (HB) (day 9), and primary HO cultures prepared from control donor
20 1; and from primary human hepatocytes (PHH). Panel A: t-SNE visualization of the scRNA-Seq data. The developmental path of the iPSC as they differentiate into hepatoblasts and then into primary human organoids, whose transcriptome profile is similar to that of PHHs, is evident. There were two clusters of HBs: early HBs are those at the bottom-left whose transcriptome was close to that of iPSC; and late HBs at the upper-right whose
25 transcriptome was similar to that of PHHs and HO. The single cell clusters are visualized using t-SNE. Panels B and C: The fractional identity (FI) of the transcriptome of individual cells are superimposed upon the t-SNE plot shown in panel A. The similarity of the transcriptome of each cell relative to iPSCs, PHHs (panel B) or cholangiocytes (panel C) was plotted as the FI. This calculation was performed so that the three FIs for each cell
30 (each relative to iPSC, PHH or cholangiocytes) sums to 1. For example, the graph in panel B clearly shows a gradient in the relationship between the FI of the transcriptome of each

cell type and PHH: iPSCs have zero FI, early HB have a low FI (indicative of proximity to iPSCs), while late HBs and HOs have a high FI (indicating they have greater similarity to PHH). Also, the FI for each of the PHHs are all virtually one, as expected. Of note, the gradient of FI values matches perfectly with the developmental path observed in the tSNE plot. In panel C, a group of cells in the HO had a transcriptome profile that partially resembles that of cholangiocytes. The results in panels B and C indicate that the iPSC develop into cells resembling hepatocytes and cholangiocytes in a primary HO. Panels D and E: The relative similarities of the transcriptome profile of cultured cells to iPSCs and PHHs (panel D), or to that of adult or fetal hepatocytes (panel E). scRNA-Seq was performed on cells obtained from iPSC, hepatoblasts (HB), or hepatic organoid (HO) cultures prepared from 4 donors; or from PHHs. Quadratic programming was used to computationally infer their similarities, which was expressed as the FI relative to iPSC (FI = 0) and PHH (FI = 1) in panel D, or to fetal (FI = 0) and adult (FI = 1) hepatocytes in panel E. The cells were then ranked by their FI values relative to that of iPSC/PHH in panel D, or relative to fetal/adult hepatocytes in panel E. Each cell analyzed in this way is indicated by a colored dot that corresponds with the indicated cell type. Of note, most of the HBs had a FI relative to PHH that was <0.5 , which was below the majority of the HO cells. However, a small percent of HB cells had a higher FI, which was closer to that of PHHs. This result is consistent with presence of early and late populations of HBs. A similar trend was observed for the FI relative to fetal or adult hepatocytes: most HBs had a FI relative to mature adult hepatocytes that was virtually zero, but the majority ($\sim 2/3$) of the HO cells had a positive FI. However, a small percent of HBs had a higher inferred fractional identity, which was closer to that of PHHs.

FIG. 2 Panel A: A comparison of the transcriptomes of iPSC, HB, HO and PHH cells with human fetal and adult liver cells. scRNA-Seq was performed on cells obtained from iPSC, day 9 hepatoblast (HB), and primary HO cultures prepared from four donors, and from primary human hepatocytes (PHH). Previously obtained transcriptomic data for hepatocytes present in one fetal (gestation age 17.5 weeks) and three adult (ages 21-65) livers was used for the comparison. The t-SNE visualization indicates that: adult liver hepatocytes and PHH were tightly clustered, and the hepatocytes in fetal liver were clearly separated from this cluster; and the transcriptome of HO cells was more closely related to adult hepatocytes/PHH than to fetal hepatocytes. Panel B: The SLICER method was used to computationally infer a developmental trajectory (beginning from iPSC) for the cells shown in panel A. Each cell type is indicated by a different color, and this method generated a developmental path from iPSC to HB, and then to HO, and then to adult hepatocytes/PHH. This pattern is the same as in the t-SNE visualization. Of note, a small proportion of HB

cells diverged from the main path, indicating that they may have a different developmental fate. Most HO cells are located farther away from iPSCs, and are closer to adult hepatocytes/PHH than to fetal hepatocytes. In contrast, HBs were located closer to fetal hepatocytes, and were far away from the adult hepatocytes. Therefore, the transcriptomic profile of HO cells resembled that of mature hepatocytes, while that of HBs more closely resembled fetal hepatocytes.

FIG. 3 Panel A: The relative abundance of 494 dansyl-labeled metabolites was measured in day 9 hepatoblasts and in iPSCs, which were generated from three different individuals. The fold difference (shown as the ratio of the hepatoblast/iPSC) in the measured relative abundance (Y-axis) of each metabolite (numbered along the X-axis) is graphed. The arrow indicates the metabolite with highest abundance difference, which was phosphoethanolamine. The identities of the two other metabolites with a fold change > 10 could not be determined. Panel B: The CDP (phosphoethanolamine cytidylyltransferase)-ethanolamine (Etn) pathway for phosphatidylethanolamine (PE) biosynthesis is activated when iPSC differentiate into hepatoblasts (HBs). In the first step, ethanolamine kinase (ETNK1) phosphorylates (Etn to produce P-Etn. The second step is rate limiting, and it is catalyzed by the product of the PCYT2 gene. This step generates CDP-Etn, which is then converted to PE by CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase (SELENOI). The measured abundance of Etn and P-Etn in iPSC and HBs are shown below the pathway. Each measurement was the average of three independent measurements. The relative expression levels for ETNK1, PCYT2, and SELENOI mRNAs in iPSC and HBs, which were determined from bulk scRNA-Seq data, are shown above the pathway. There was a marked increase in P-Etn abundance (170-fold, $p=0.018$); and of ETNK1, PCYT2 and SELENOI mRNAs in HBs (75, 58 and 41 fold, respectively); which indicated that the CDP-Etn pathway for PE synthesis was activated when iPSC differentiated into HBs.

FIG. 4 Panel A: A *PCYT2* gene knockout (KO) reduces cell viability. Two Control (C1, C2) and three iPSC lines with a CRISPR-mediated *PCYT2* KO-which were prepared using three different sgRNAs (*PCYT2* sg1-3)-were examined in these experiments. Each iPSC line was differentiated into endoderm (EN) through day 3; then transferred into media to direct their differentiation into HBs; and the developing HB cultures were examined on day 4. Bright field images of HB cultures prepared from the iPSC lines. While the cells generated from all of the iPSC lines were abundant and viable at the EN stage, the cell density and number of viable cells in all *PCYT2* KO lines was dramatically reduced as they differentiated toward HBs. The scale bar is 50 μ m. The bar graph shows the results of a trypan blue assay for cell viability (% viable cells) in day 4 differentiating HB cultures. Each

bar is the mean ($\pm$ SD) of three independent determinations for each iPSC line. Panel B: Meclizine induces cell death in early hepatoblasts (HBs). Control iPSCs were differentiated for 3 days into endoderm. On day 4, the cells were placed in HB differentiation media containing: 50 μ M Meclizine (M), 50 ng FGF10 (F10), 50 ng HGF (H), HGF and meclizine (HGF+M) or FGF10 and meclizine (F10+M) for 24 hrs. The bright field images showing the morphology of the cells indicate the extensive amount of cell death in occurring in meclizine-treated cells. Scale bar: 50 μ m. The bar graph of the trypan blue assay for viable cells (% viability) in the day 4 cultures is shown. Each bar is the mean $\pm$ SD of two independent determinations for each culture condition. Panel C: Meclizine impairs organoid regeneration. The cells in HOs were dissociated into single cells, and then placed in growth media to induce the formation of secondary organoids in the presence of meclizine at concentrations ranging from 0 to 80 μ M. Top: Bright field images of cultures with 0, 4 or 8 μ M meclizine examined on day 7 after staining with Calcein AM. Bottom: The efficiency of secondary organoid formation after 7 days in growth medium was quantitated by fluorescence measurement. Each data point is the mean of 2 independent determinations, and the results are normalized relative to the fluorescence of wells where organoids were re-formed in the absence of meclizine. Panels D and E: Meclizine selectively induces cell death in early hepatoblasts (HBs). Panel D: Cell Survival was measured 24 hour after the cells in iPSC (day 0); early (day 4), mid (day 7) or fully differentiated HB (day 10) cultures were exposed to 0, 1, 2, 4, 5, 8, 10, 15, 20, 40, 50, or 80 μ M meclizine. Each point is the mean ($\pm$ SD) of three independent trypan blue assays performed 24 hours after exposure to meclizine. Panel E: Meclizine survival curves were also examined in induced cardiomyocytes (CM) on day 5 or day 20; human fibroblasts (FB), primary human hepatocytes (PHH), and the HepG2 and Huh7 hepatocarcinoma lines (right). The red line indicates the meclizine concentration causing 50% cell death (LD_{50}). Panel F: The culture media does not alter the susceptibility of iPSC to meclizine-induced cell death. iPSCs were incubated in either the iPSC or hepatoblast (HB) media containing the indicated concentrations of meclizine for 24 hours, and cell viability was then measured using the Prestoblu assay. Each bar is the mean $\pm$ SD of two independent measurements performed for each condition.

FIG. 5 Panel A: The SLICER method was used to examine the relationships between the transcriptomes of cells in developing organoid cultures (iPSC, HB and HO) cells and PHH, 271 HCCs in the TCGA dataset, and normal liver. Each cell type is indicated by a different color. The previously identified developmental pathway from iPSC $\rightarrow$ HB $\rightarrow$ HO was preserved in this analysis. Normal liver clustered with HO, and is distinct from the other cells types. HCC transcriptomes overlapped with HO and HB cells, and were distinct from

iPSCs and normal liver. These results indicate that the transcriptomic profile of HB and HO cells resembles that of liver cancer cells. Panel B: Meclizine and PFK158 synergistically inhibit cell growth. Within the endoplasmic reticulum (ER), meclizine inhibits the rate-limiting enzyme (PCYT2) in the PEBP, which increases cellular phosphoethanolamine. ATP is generated by glycolysis occurring in the cytoplasm. PFK158 inhibits an enzyme (PFKFB3) whose reaction product activates the rate-limiting step in glycolysis. By inhibiting glycolysis, PFK158 increases cellular dependence upon energy production by mitochondria. Phosphoethanolamine inhibits the mitochondrial electron transport chain (ETC). By this mechanism, treatment with the combination of PFK158 and meclizine synergistically inhibits cell growth. Panel C: Meclizine and PFK158 inhibit the growth of the HepG2 (top) and HuH7 (bottom) hepatocarcinoma cell lines. The cells were grown for 7 days in a standard high glucose medium, or in media where galactose and glutamine replaced the glucose. While meclizine (40 μ M) did not significantly affect the proliferation of either cell line in the glucose medium, it significantly reduced their proliferation in the galactose medium. Addition of a low concentration (2 or 5 μ M) of PFK158 had no effect on cell proliferation in either media. However, addition of a high PFK158 (5 or 10 μ M) concentration did impair cell growth. When meclizine was combined with a low concentration of PFK158, cell viability was inhibited. C: vehicle treated cells; M: Meclizine (40 μ M); P2, 2 μ M PFK158; P5, 5 μ M PFK158; P10, 10 μ M PFK; M+P*, 40 μ M Meclizine plus the indicated μ M concentration of PFK158. Each data point is the average of 8 independent measurements obtained from 2 different experiments. Panel D: Meclizine increases cellular phosphoethanolamine. HepG2 cells (2×10^6 cells per 100 mm dish) were grown for 2 days to ~70% confluence in a high glucose medium (DMEM with 1% FBS); and then treated with vehicle (0 μ M), 10 μ M meclizine, or 40 μ M meclizine for 24 hours. Then, the ethanolamine and phosphoethanolamine levels were measured by MS analysis. Each bar shows the average $\pm$ SEM of 3 independent measurements for each group. Phosphoethanolamine abundance was significantly increased (* $p=0.004$) by 40 (but not 10) μ M meclizine, while there was no significant change in ethanolamine abundance. Panel E: Meclizine decreases the basal and maximal oxygen consumption rate of a hepatocarcinoma cell line. HepG2 cells were cultured in the presence of water, vehicle (DMSO), or 50 μ M meclizine. The oxygen consumption rate was measured using the Mito Stress Test on a XFe96 Seahorse Analyser at 2 hours after compound or vehicle addition. The upper graph shows the measured basal oxygen consumption rate, and the lower graph shows the maximal respiration rate measured after addition of uncoupling agent carbonyl cyanide-4 trifluoromethoxy phenylhydrazone (FCCP). Each bar is the average $\pm$ SD of 16

independent measurements; and 50 μ M meclizine induced a significant decrease in basal ($p < 0.001$) and maximal ($p < 0.001$) oxygen consumption.

FIG. 6 Survival risk prediction in hepatocellular carcinoma based upon mRNA expression levels. Kaplan-Meier survival curves showing the survival time after hepatocellular cancer diagnosis in The Cancer Gene Atlas (TCGA) ($n=371$) and Fudan ($n=242$) cohorts based upon the level of PCYT2, ETNK2 or PFKFB3 mRNA expression in each HCC sample. The expression level for each mRNA was classified as high (or low) if it was above (or below) the median level in each cohort. The p values of a Cox proportional hazard model, which compared the survival times between the two groups within each cohort, are also displayed. A high level of ETNK2 mRNA or a low level of PFKFB3 mRNA expression was associated with significantly increased survival in both cohorts.

FIG. 7 Treatment with meclizine and PFK158 inhibits the growth of a human hepatocellular carcinoma line *in vivo*. Panel A: Diagram of the model. Hep G2 cells expressing a GFP-luciferase fusion protein were transplanted into the livers of NOG mice. One day later, the mice were treated with either vehicle (Veh) or 25 mg/kg Meclizine and 25 mg/kg PFK158 by oral gavage (OG) each day for 14 days ($n=7$ mice per group). Bioluminescent imaging was then used to measure the amount of human hepatocarcinoma cells within the liver of these mice. Panel B: Bioluminescent images show the tumor area within the livers of the vehicle and drug treated NOG mice on day 14. The luminescence scale is shown on the right. Of note, the 1st and 4th mouse shown have a very small area of bioluminescence within the splenic area; which represents residual cells present at the site of transplantation. The bioluminescence in the 6th mouse is also not in the liver and is within the colon (or bladder). The graph compares the tumor area within the livers of vehicle and drug treated mice. Each dot shows the measured bioluminescent signal (total flux in photons/second) within the liver region for each mouse; and the horizontal lines are the mean $\pm$ SEM for each group. The results of a t-test comparing the measured human tumor areas of the vehicle and P+M treated groups is shown. Panel C: The day 14 bioluminescent images and graph of the measurements obtained in an independently performed second experiment is shown. Panel D: Macroscopic tumors are formed within the livers of mice after xenotransplantation of human hepatocarcinoma cells. *Left and Center Panels:* Representative H/E stained livers section obtained 4 weeks after human hepatocarcinoma cells were transplanted into NOG mice that were treated with either vehicle (Veh) or meclizine and PFK-158 (M+P). While tumor is not present within the M+P treated liver a macroscopic tumor is seen in the vehicle treated liver. The scale bar is 50 μ M. Right Panel: Immunofluorescence image of a liver section obtained from a vehicle-treated mouse one month after transplantation of a human hepatocarcinoma cell line

expressing GFP. To visualize the areas with the human hepatocarcinoma, the slide was stained with an anti-GFP antibody. The scale bar is 250 μ M. In both the H/E and immunostained images, there is an extensive amount of human carcinoma within the liver of vehicle treated mice. Panel E: PFK158 and Meclizine treatment does not cause liver toxicity. The serum ALT and AST levels were measured on day 14 in the vehicle or drug (P+M) treated (n=7 mice/ group) mice shown in B. The measurement for each mouse is indicated by a dot; and the line indicates the average and SEM for each group. The average serum ALT and AST levels in the P+M group were not different from that of the vehicle treated mice (ALT: 30.9 ± 2.3 vs 38.6 ± 11.1 U/L, $p=0.1$; AST: 74.1 ± 26.5 vs 78 ± 29.7 U/L, $p=0.8$). Panel F: Three weeks after Hep G2 cells expressing a GFP-luciferase fusion protein were transplanted into the livers of NOG mice, the mice were treated with either vehicle (Veh) or 25 mg/kg Meclizine and 25 mg/kg PFK158 by oral gavage (OG) each day for 21 days (n=10 mice per group). The graph compares the tumor area within the livers of vehicle and drug treated mice, which was measured by bioluminescent imaging after 0 (day 21), 1, 2, or 3 weeks of drug treatment. Each dot shows the measured bioluminescent signal (total flux in photons/second) within the liver region for each mouse; and the horizontal lines are the mean $\pm$ SEM for each group. The results of a t-test comparing the measured human tumor areas of the vehicle and P+M treated groups is shown.

FIG. 8 Panel A: Primary AML blasts obtained from an AML patient at the time of diagnosis were cultured in media containing the indicated concentrations of meclizine in absence (left) or presence of 0.9 μ M PKF158 (middle). After 96 hours in culture, the number of live cells was determined by flow cytometry. In the right panel, the numbers of live cells after treatment with the various drug combinations at the indicated concentrations are shown; and the p-values (where significant) for assessing the effect of the different drug concentrations are indicated. The corresponding IC_{50} for the effect of meclizine on their growth is also indicated. Panel B: Primary AML blasts obtained from two AML patients were cultured in media containing 0 or 0.5 μ M meclizine in absence or presence of 0.9 μ M PKF158. After 96 hours in culture, the number of live cells was determined by flow cytometry. The p-values (where significant) for assessing the effect of the meclizine and PKF158 relative to meclizine alone are indicated. In all figures, each bar is the average $\pm$ SE of 2 independent measurements, and an unpaired t-test was performed on the replicate analyses.

FIG. 9 NOG mice (n=9 mice/group) were treated with vehicle or with meclizine (25 mg/kg PO qd) and PFK158 (25 mg/kg PO qd) for 26 days. The body weights of drug-treated (M+PFK) mice was not significantly different from that of vehicle-treated mice at the end of

the treatment period ($p=0.24$). Each bar shows the average $\pm$ SD of the measured body weight for each group.

DETAILED DESCRIPTION

Provided are methods of treating cancer. The methods include administering to an individual having cancer a therapeutically effective amount of a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor and a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor. Also provided are compositions and kits that find use, e.g., for practicing the methods of the present disclosure.

Before the methods, compositions and kits of the present disclosure are described in greater detail, it is to be understood that the methods, compositions and kits are not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the methods, compositions and kits will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the methods, compositions and kits. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the methods, compositions and kits, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the methods, compositions and kits.

Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods, compositions and kits belong. Although any methods, compositions and kits similar or equivalent to those described herein can also be used in the practice or testing of

the methods, compositions and kits, representative illustrative methods, compositions and kits are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the materials and/or methods in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present methods, compositions and kits are not entitled to antedate such publication, as the date of publication provided may be different from the actual publication date which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

It is appreciated that certain features of the methods, compositions and kits, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the methods, compositions and kits, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed, to the extent that such combinations embrace operable processes and/or compositions. In addition, all sub-combinations listed in the embodiments describing such variables are also specifically embraced by the present methods, compositions and kits and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present methods. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

METHODS

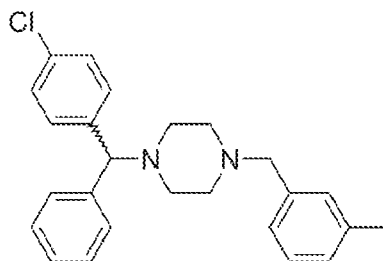
As summarized above, the present disclosure provides methods of treating cancer. The methods include administering to an individual having cancer a therapeutically effective amount of a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor and a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor. As demonstrated in the Experimental section below, the combined use of a PEBP inhibitor and a PFKFB3 inhibitor has an unexpected synergistic anti-cancer effect.

As used herein, an “inhibitor” may be a single agent or combination of agents. Non-limiting examples of an inhibitor that may be employed include a small molecule inhibitor, a short interfering RNA (siRNA), a microRNA (miRNA), a morpholino, and/or the like. By “small molecule” is meant a compound having a molecular weight of 1000 atomic mass units (amu) or less. In some embodiments, the small molecule is 750 amu or less, 500 amu or less, 400 amu or less, 300 amu or less, or 200 amu or less. In certain aspects, the small molecule is not made of repeating molecular units such as are present in a polymer. When the inhibitor is an siRNA, miRNA, morpholino, or the like, the inhibitor may be designed to target an mRNA encoding a PEBP enzyme, including any of the CDP-ethanolamine pathway enzymes or P-Ser decarboxylation pathway enzymes described below. Approaches for designing and delivering siRNAs, miRNAs, morpholinos, etc. for targeting a particular mRNA are known and described, e.g., in Monsoori et al. (2014) *Adv Pharm Bull.* 4(4):313-321; Xin et al. (2017) *Mol Cancer* 16:134; Chakraborty et al. (2017) *Mol Ther Nucleic Acids* 8:132-143; and Ahmadzada et al. (2018) *Biophys Rev.* 10(1):69-86.

As summarized above, the methods include administering to an individual having cancer a therapeutically effective amount of a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor. By “phosphatidylethanolamine biosynthetic pathway” or “PEBP” is meant a biosynthetic pathway that produces phosphatidylethanolamine (sometimes referred to herein as “P-Etn” or “PE”) that occurs in the individual. The PEBP inhibitor inhibits at least one component (e.g., an enzyme) of the PEBP, and the inhibitor may vary depending upon the particular PEBP to be inhibited. In some embodiments, the PEBP inhibitor is a cytidine diphosphate (CDP)-ethanolamine pathway inhibitor. In the CDP-ethanolamine pathway (also known as the “Kennedy pathway”), ethanolamine is converted to P-Etn by the sequential actions of ethanolamine kinase (EK), phosphoethanolamine cytidyltransferase (PCYT2), and finally ethanolaminephosphotransferase (EPT, Selenoi). The CDP-ethanolamine pathway is well characterized and described, e.g., in Bleijerveld et al. (2007) *J Biol Chem.* 282(39):28362-72.

In certain aspects, when the PEBP inhibitor is a CDP-ethanolamine pathway inhibitor, the inhibitor is selected from an ethanolamine kinase (EK) inhibitor, a

phosphoethanolamine cytidyltransferase (PCYT2) inhibitor, an ethanolaminephosphotransferase (EPT, Selenoi) inhibitor, and any combination thereof. For example, in some embodiments, the inhibitor is a PCYT2. Any suitable PCYT2 may be employed. A non-limiting example of a PCYT2 inhibitor which may be employed is
5 meclizine. Meclizine is an over-the-counter anti-histamine that is widely used for the treatment of motion sickness and vertigo, and was recently found to be a non-competitive inhibitor of PCYT2 enzyme activity. See, e.g., Gohil et al. (2013) *J Biol Chem.* 288:35387-35395. Meclizine has the following structure:



10 In some embodiments, the PEBP inhibitor is a phosphatidylserine (PS) decarboxylation pathway inhibitor. In mammalian cells, P-Etn is mainly synthesized via the CDP-ethanolamine pathway (Kennedy) pathway and by decarboxylation of phosphatidylserine (P-Ser). In the P-Ser decarboxylation pathway, P-Ser synthesized from phosphatidylcholine or P-Etn by phosphatidylserine synthase-1 and -2, respectively, is
15 decarboxylated by the enzyme phosphatidylserine decarboxylase (PSD) to generate P-Etn. In certain aspects, when the PEBP inhibitor is a phosphatidylserine (PS) decarboxylation pathway inhibitor, the inhibitor is a phosphatidylserine decarboxylase (PSD) inhibitor.

As summarized above, the methods of the present disclosure further include administering to the individual having cancer a therapeutically effective amount of a 6-
20 phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) inhibitor. PFKFB3 belongs to a family of bifunctional proteins that are involved in both the synthesis and degradation of fructose-2,6-bisphosphate, a regulatory molecule that controls glycolysis in eukaryotes. PFKFB3 has a 6-phosphofructo-2-kinase activity that catalyzes the synthesis of fructose-2,6-bisphosphate (F2,6BP), and a fructose-2,6-bisphosphatase activity that
25 catalyzes the degradation of F2,6BP. PFKFB3 is required for cell cycle progression and prevention of apoptosis. It functions as a regulator of cyclin-dependent kinase 1, linking glucose metabolism to cell proliferation and survival in tumor cells. Further information regarding PFKFB3 may be found, e.g., in Shi et al. (2017) *Signal Transduction and Targeted Therapy* 2:e17044.

In certain aspects, the PFKFB3 inhibitor is a small molecule inhibitor of PFKFB3. Non-limiting examples of small molecule PFKFB3 inhibitors that may be employed include 1-(pyridin-4-yl)-3-(7-(trifluoromethyl)quinolin-2-yl)prop-2-en-1-one (also known as “PFK158”), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (also known as “3PO”), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (also known as “PFK15”), and any combination thereof. In certain aspects, the PFKFB3 inhibitor is PFK158. See, e.g., Mondal et al. (2018) *Int J Cancer* Sep 18 PMID: 30226266. In some embodiments, the PFKFB3 inhibitor is an siRNA or miRNA inhibitor, examples of which are described in Shi et al. (2017) *Signal Transduction and Targeted Therapy* 2:e17044, the disclosure of which is incorporated herein by reference in its entirety.

According to the methods of the present disclosure, the individual has cancer. By “cancer” is meant the individual comprises cells and/or tissue exhibiting a neoplastic cellular phenotype, which may be characterized by one or more of, for example, abnormal cell growth, abnormal cellular proliferation, loss of density dependent growth inhibition, anchorage-independent growth potential, ability to promote tumor growth and/or development in an immunocompromised non-human animal model, and/or any appropriate indicator of cellular transformation. “Cancer cell” may be used interchangeably herein with “tumor cell”, “malignant cell” or “cancerous cell”, and encompasses cancer cells of tumor tissue. “Tumor tissue” includes tissue of a solid tumor, a semi-solid tumor, a primary tumor, a metastatic tumor, and the like. As used herein, “tumor tissue” not only includes a tissue made up exclusively of cancer cells, but also a tissue that includes cancer cells and one or more additional cell types, including but not limited to, immune cells (e.g., tumor associated macrophages (TAMs)) associated with (e.g., infiltrated within) the tissue.

In some embodiments, the individual has a cancer characterized by the presence of a solid tumor, a semi-solid tumor, a primary tumor, a metastatic tumor, or the like. In some embodiments, the individual has a cancer selected from breast cancer, melanoma, lung cancer, colorectal cancer, prostate cancer, glioma, bladder cancer, endometrial cancer, kidney cancer, leukemia (e.g., acute myeloid leukemia (AML)) liver cancer (e.g., hepatocellular carcinoma (HCC), such as primary or recurrent HCC), non-Hodgkin lymphoma, pancreatic cancer, thyroid cancer, any combinations thereof, and any sub-types thereof. In certain aspects, the individual has liver cancer. By way of example, the individual may have hepatocellular carcinoma (HCC), non-limiting examples of which include primary HCC and recurrent HCC. In other embodiments, the individual has a leukemia. In certain aspects, the leukemia is acute myeloid leukemia (AML).

The PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual. The administration may be performed by the individual (that is – self-administered, such as by

oral administration in tablet form, capsule form, liquid form, etc.) or the PEBP inhibitor and the PFKFB3 inhibitor may be administered to the individual by someone other than the individual, e.g., an employee of a healthcare provider (e.g., a physician, a nurse, or other healthcare provider employee). In certain aspects, the PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual by a route of administration independently selected from: oral administration, parenteral administration, and intranasal administration.

The PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual in a therapeutically effective amount. By “therapeutically effective amount” is meant a dosage of PEBP inhibitor and the PFKFB3 inhibitor sufficient to produce a desired result, e.g., an amount sufficient to effect beneficial or desired therapeutic (including preventative) results, such as a reduction in a symptom of the individual’s cancer (e.g., hepatocellular carcinoma (HCC), acute myeloid leukemia (AML), etc.), as compared to a control. An effective amount can be administered in one or more administrations. In some embodiments, a therapeutically effective amount of the PEBP inhibitor (e.g., meclizine) is an oral administration (e.g., tablet or liquid form) of 12.5 to 600 mg (e.g., 25 to 100 mg) per day, either as a single dose or divided doses (e.g., every 6 to 12 hours). In certain aspects, a therapeutically effective amount of the PFKFB3 inhibitor (e.g., PFK158) is 20 to 1000 mg/meter² (e.g., 25 to 700 mg/meter²).

The PEBP inhibitor and the PFKFB3 inhibitor may be administered to the individual according to any suitable administration regimen. According to certain embodiments, the PEBP inhibitor and the PFKFB3 inhibitor are administered according to a dosing regimen approved for individual use. In some embodiments, the administration of the PEBP inhibitor permits the PFKFB3 inhibitor to be administered according to a dosing regimen that involves one or more lower and/or less frequent doses, and/or a reduced number of cycles as compared with that utilized when the PFKFB3 inhibitor is administered without administration of the PEBP inhibitor. In certain aspects, the administration of the PFKFB3 inhibitor permits the PEBP inhibitor to be administered according to a dosing regimen that involves one or more lower and/or less frequent doses, and/or a reduced number of cycles as compared with that utilized when the PEBP inhibitor is administered without administration of the PFKFB3 inhibitor.

In some embodiments, one or more doses of the PEBP inhibitor and the PFKFB3 inhibitor are administered concurrently to the individual. By “concurrently” is meant the PEBP inhibitor and the PFKFB3 inhibitor are either present in the same pharmaceutical composition, or the PEBP inhibitor and the PFKFB3 inhibitor are administered as separate pharmaceutical compositions within 1 hour or less, 30 minutes or less, or 15 minutes or less.

In some embodiments, one or more doses of the PEBP inhibitor and the PFKFB3 inhibitor are administered sequentially to the individual. In certain aspects, the PFKFB3 inhibitor (e.g., PFK158) finds use in reprogramming cancer cell metabolism to render it susceptible to the cytotoxic effect of the PEBP inhibitor (e.g., meclizine). Accordingly, in some embodiments, the PFKFB3 inhibitor is administered to the individual prior to administration of the PEBP inhibitor to the individual.

In some embodiments, the PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual in different compositions and/or at different times. For example, the PEBP inhibitor may be administered prior to administration of the PFKFB3 inhibitor (e.g., in a particular cycle). Alternatively, the PFKFB3 inhibitor may be administered prior to administration of the PEBP inhibitor (e.g., in a particular cycle). The second agent to be administered may be administered a period of time that starts at least 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, or up to 5 days or more after the administration of the first agent to be administered.

In one example, the PFKFB3 inhibitor is administered to the individual for a desirable period of time prior to administration of the PEBP inhibitor. In certain aspects, such a regimen “primes” the cancer cells to potentiate the cytotoxic effect of the PEBP inhibitor. Such a period of time separating a step of administering the PFKFB3 inhibitor from a step of administering the PEBP inhibitor is of sufficient length to permit priming of the cancer cells, desirably so that the cytotoxic effect of the PEBP inhibitor is increased.

In some embodiments, administration of one inhibitor is specifically timed relative to administration of the other inhibitor. For example, in some embodiments, a first inhibitor is administered so that a particular effect is observed (or expected to be observed, for example based on population studies showing a correlation between a given dosing regimen and the particular effect of interest).

In certain aspects, desired relative dosing regimens for inhibitors administered in combination may be assessed or determined empirically, for example using *ex vivo*, *in vivo* and/or *in vitro* models; in some embodiments, such assessment or empirical determination is made *in vivo*, in a patient population (e.g., so that a correlation is established), or alternatively in a particular individual of interest.

In some embodiments, the PEBP inhibitor and the PFKFB3 inhibitor are administered according to an intermittent dosing regimen including at least two cycles. Where two or more inhibitors are administered in combination, and each by such an intermittent, cycling, regimen, individual doses of different agents may be interdigitated with one another. In certain aspects, one or more doses of the second inhibitor is administered a period of time after a dose of the first inhibitor. In some embodiments, each dose of the

second inhibitor is administered a period of time after a dose of the first inhibitor. In certain aspects, each dose of the first inhibitor is followed after a period of time by a dose of the second inhibitor. In some embodiments, two or more doses of the first inhibitor are administered between at least one pair of doses of the second inhibitor; in certain aspects, two or more doses of the second inhibitor are administered between at least one pair of doses of the first inhibitor. In some embodiments, different doses of the same inhibitor are separated by a common interval of time; in some embodiments, the interval of time between different doses of the same inhibitor varies. In certain aspects, different doses of the PEBP inhibitor and the PFKFB3 inhibitor are separated from one another by a common interval of time; in some embodiments, different doses of the different inhibitors are separated from one another by different intervals of time.

One exemplary protocol for interdigitating two intermittent, cycled dosing regimens may include: (a) a first dosing period during which a therapeutically effective amount the PFKFB3 inhibitor is administered to the individual; (b) a first resting period; (c) a second dosing period during which a therapeutically effective amount of the PEBP inhibitor is administered to the individual; and (d) a second resting period. A second exemplary protocol for interdigitating two intermittent, cycled dosing regimens may include: (a) a first dosing period during which a therapeutically effective amount the PEBP inhibitor is administered to the individual; (b) a first resting period; (c) a second dosing period during which a therapeutically effective amount of the PFKFB3 inhibitor is administered to the individual; and (d) a second resting period.

In some embodiments, the first resting period and second resting period may correspond to an identical number of hours or days. Alternatively, in some embodiments, the first resting period and second resting period are different, with either the first resting period being longer than the second one or, vice versa. In some embodiments, each of the resting periods corresponds to 120 hours, 96 hours, 72 hours, 48 hours, 24 hours, 12 hours, 6 hours, 30 hours, 1 hour, or less. In some embodiments, if the second resting period is longer than the first resting period, it can be defined as a number of days or weeks rather than hours (for instance 1 day, 3 days, 5 days, 1 week, 2, weeks, 4 weeks or more).

If the first resting period's length is determined by existence or development of a particular biological or therapeutic event, then the second resting period's length may be determined on the basis of different factors, separately or in combination. Exemplary such factors may include type and/or stage of a cancer against which the therapy is administered; properties (e.g., pharmacokinetic properties) of the first inhibitor, and/or one or more features of the patient's response to therapy with the first inhibitor. In some embodiments, length of one or both resting periods may be adjusted in light of pharmacokinetic properties

(e.g., as assessed via plasma concentration levels) of one or the other of the administered inhibitors. For example, a relevant resting period might be deemed to be completed when plasma concentration of the relevant inhibitor is below a pre-determined level, optionally upon evaluation or other consideration of one or more features of the individual's response.

5 In certain aspects, the number of cycles for which a particular inhibitor is administered may be determined empirically. Also, in some embodiments, the precise regimen followed (e.g., number of doses, spacing of doses (e.g., relative to each other or to another event such as administration of another therapy), amount of doses, etc.) may be different for one or more cycles as compared with one or more other cycles.

10 The PEBP inhibitor and the PFKFB3 inhibitor may be administered together or independently via any suitable route of administration. Such inhibitors may be administered via a route of administration independently selected from oral, parenteral (e.g., by intravenous, intra-arterial, subcutaneous, intramuscular, or epidural injection), topical, or intra-nasal administration. According to certain embodiments, the PEBP inhibitor and the
15 PFKFB3 inhibitor are both administered orally (e.g., in tablet form, capsule form, liquid form, or the like) either concurrently (in the same pharmaceutical composition or separate pharmaceutical compositions) or sequentially.

 In certain aspects, the methods include administering to the individual a further therapeutic agent in addition to the PEBP inhibitor and the PFKFB3 inhibitor. Such
20 administration may include concurrently administering the further therapeutic agent and one or both of the PEBP inhibitor and the PFKFB3 inhibitor, or administering the further therapeutic agent sequentially with respect to one or both of the PEBP inhibitor and the PFKFB3 inhibitor. In some embodiments, the individual has cancer, and the further therapeutic agent is an anti-cancer agent. Anti-cancer agents of interest include, but are
25 not limited to, anti-cancer antibodies, small molecule anti-cancer agents, or the like.

 In some embodiments, the methods further include, prior to administering the PEBP inhibitor and the PFKFB3 inhibitor, determining the expression level of PFKBP3 in cancer cells obtained from the individual. A variety of suitable approaches are available for determining the expression level of PFKBP3. In some embodiments, PFKBP3 protein
30 levels are determined, and the determining is based on an immunoassay. A variety of suitable immunoassay formats are available, including ELISA, flow cytometry assays, immunohistochemistry on tissue section samples, immunofluorescence on tissue section samples, Western analysis, and/or the like.

 In certain aspects, determining the expression level PFKBP3 in the cancer cells
35 comprises determining the abundance of *PFKBP3* mRNA in the cancer cells. Any suitable approach for determining mRNA abundance from a cellular sample may be employed. In

certain aspects, *PFKBP3* mRNA abundance is determined by quantitative reverse transcription PCR (qRT-PCR) or RNA sequencing. For example, in the case of RNA sequencing, the number of sequencing reads corresponding to an mRNA encoding a protein of interest may be used to determine the expression level of the protein. In certain aspects, the sequencing is performed using a next-generation sequencing system, such as on a sequencing platform provided by Illumina® (e.g., the HiSeq™, MiSeq™ and/or Genome Analyzer™ sequencing systems); Oxford Nanopore Technologies (e.g., a SmidgION™, MinION™, GridION™, or PromethION™ sequencing system); Ion Torrent™ (e.g., the Ion PGM™ and/or Ion Proton™ sequencing systems); Pacific Biosciences (e.g., the PACBIO RS II sequencing system); Life Technologies™ (e.g., a SOLiD sequencing system); Roche (e.g., the 454 GS FLX+ and/or GS Junior sequencing systems); or any other sequencing platform of interest. Protocols for isolating nucleic acids from tissue or fluid samples, as well as protocols for preparing sequencing libraries having sequencing adapters appropriate for the desired sequencing platform are readily available.

In some embodiments, methods that include determining the expression level of *PFKBP3* in cancer cells obtained from the individual further include obtaining the cancer cells from the individual. The sample obtained from the individual may be any sample suitable for determining the expression level of *PFKBP3*. In certain aspects, the sample is a fluid sample, such as whole blood, serum, plasma, or the like. In some embodiments, the sample is a tissue sample. Tissue samples of interest include, but are not limited to, tumor biopsy samples, and the like.

In some embodiments, the methods further include determining the severity of the individual's cancer based on the determined expression level of *PFKBP3* in the cancer cells. In some embodiments, the methods further include classifying the type of cancer based on the determined expression level of *PFKBP3* in the cancer cells.

COMPOSITIONS

The present disclosure also provides compositions. A composition of the present disclosure includes a PEBP inhibitor and a PFKFB3 inhibitor. Any suitable PEBP inhibitor and PFKFB3 inhibitor may be included in the compositions of the present disclosure. Suitable PEBP and PFKFB3 inhibitors include any of the PEBP and PFKFB3 inhibitors described in the Methods section hereinabove, which is incorporated but not reiterated herein for purposes of brevity. Non-limiting examples of PEBP and PFKFB3 inhibitors which may be included in a composition of the present disclosure include meclizine and PFK158, respectively. The compositions of the present disclosure find use, e.g., in practicing the methods of the present disclosure.

In certain aspects, a composition of the present disclosure includes the PEBP inhibitor and the PFKFB3 inhibitor present in a liquid medium. The liquid medium may be an aqueous liquid medium, such as water, a buffered solution, or the like. One or more additives such as a salt (e.g., NaCl, MgCl₂, KCl, MgSO₄), a buffering agent (a Tris buffer, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2-(N-Morpholino)ethanesulfonic acid (MES), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), 3-(N-Morpholino)propanesulfonic acid (MOPS), N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), etc.), a solubilizing agent, a detergent (e.g., a non-ionic detergent such as Tween-20, etc.), a nuclease inhibitor, a protease inhibitor, glycerol, a chelating agent, and the like may be present in such compositions.

Pharmaceutical compositions are also provided. The pharmaceutical compositions of the present disclosure include a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor, a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor, and a pharmaceutically acceptable carrier. Non-limiting examples of PEBP and PFKFB3 inhibitors which may be included in a composition of the present disclosure include meclizine and PFK158, respectively. The pharmaceutical compositions include an effective amount of the PEBP inhibitor and the PFKFB3 inhibitor. An "effective amount" is meant an amount sufficient to produce a desired result, e.g., treating cancer in an individual. An effective amount can be administered in one or more administrations.

The PEBP inhibitor and the PFKFB3 inhibitor can be incorporated into a variety of formulations for administration to an individual. More particularly, the PEBP inhibitor and the PFKFB3 inhibitor can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable excipients or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, injections, inhalants and aerosols.

Formulations of the PEBP inhibitor and the PFKFB3 inhibitor suitable for administration to an individual (e.g., suitable for human administration) are generally sterile and may further be free of detectable pyrogens or other contaminants contraindicated for administration to a patient according to a selected route of administration.

In pharmaceutical dosage forms, the PEBP inhibitor and the PFKFB3 inhibitor can be administered alone or in appropriate association, as well as in combination, with a pharmaceutically active compound, e.g., an anti-cancer agent (including but not limited to small molecule anti-cancer agents). The following methods and carriers/excipients are merely examples and are in no way limiting.

For oral preparations, the PEBP inhibitor and the PFKFB3 inhibitor can be used alone or in combination with appropriate additives to make tablets, powders, granules or

capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The PEBP inhibitor and the PFKFB3 inhibitor can be formulated for parenteral (e.g., intravenous, intra-arterial, intraosseous, intramuscular, intracerebral, intracerebroventricular, intrathecal, subcutaneous, etc.) administration. In certain aspects, the PEBP inhibitor and the PFKFB3 inhibitor are formulated for injection by dissolving, suspending or emulsifying the the PEBP inhibitor and the PFKFB3 inhibitor in an aqueous or non-aqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

Pharmaceutical compositions that include the PEBP inhibitor and the PFKFB3 inhibitor may be prepared by mixing the PEBP inhibitor and the PFKFB3 inhibitor having the desired degree of purity with optional physiologically acceptable carriers, excipients, stabilizers, surfactants, buffers and/or tonicity agents. Acceptable carriers, excipients and/or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid, glutathione, cysteine, methionine and citric acid; preservatives (such as ethanol, benzyl alcohol, phenol, m-cresol, p-chlor-m-cresol, methyl or propyl parabens, benzalkonium chloride, or combinations thereof); amino acids such as arginine, glycine, ornithine, lysine, histidine, glutamic acid, aspartic acid, isoleucine, leucine, alanine, phenylalanine, tyrosine, tryptophan, methionine, serine, proline and combinations thereof; monosaccharides, disaccharides and other carbohydrates; low molecular weight (less than about 10 residues) polypeptides; proteins, such as gelatin or serum albumin; chelating agents such as EDTA; sugars such as trehalose, sucrose, lactose, glucose, mannose, maltose, galactose, fructose, sorbose, raffinose, glucosamine, N-methylglucosamine, galactosamine, and neuraminic acid; and/or non-ionic surfactants such as Tween, Brij Pluronics, Triton-X, or polyethylene glycol (PEG).

The pharmaceutical composition may be in a liquid form, a lyophilized form or a liquid form reconstituted from a lyophilized form, wherein the lyophilized preparation is to be reconstituted with a sterile solution prior to administration. The standard procedure for reconstituting a lyophilized composition is to add back a volume of pure water (typically equivalent to the volume removed during lyophilization); however solutions comprising

antibacterial agents may be used for the production of pharmaceutical compositions for parenteral administration.

An aqueous formulation of the PEBP inhibitor and the PFKFB3 inhibitor may be prepared in a pH-buffered solution, e.g., at pH ranging from about 4.0 to about 7.0, or from about 5.0 to about 6.0, or alternatively about 5.5. Examples of buffers that are suitable for a pH within this range include phosphate-, histidine-, citrate-, succinate-, acetate-buffers and other organic acid buffers. The buffer concentration can be from about 1 mM to about 100 mM, or from about 5 mM to about 50 mM, depending, e.g., on the buffer and the desired tonicity of the formulation.

A tonicity agent may be included in the formulation to modulate the tonicity of the formulation. Example tonicity agents include sodium chloride, potassium chloride, glycerin and any component from the group of amino acids, sugars as well as combinations thereof. In some embodiments, the aqueous formulation is isotonic, although hypertonic or hypotonic solutions may be suitable. The term "isotonic" denotes a solution having the same tonicity as some other solution with which it is compared, such as physiological salt solution or serum. Tonicity agents may be used in an amount of about 5 mM to about 350 mM, e.g., in an amount of 100 mM to 350 mM.

A surfactant may also be added to the formulation to reduce aggregation and/or minimize the formation of particulates in the formulation and/or reduce adsorption. Example surfactants include polyoxyethylensorbitan fatty acid esters (Tween), polyoxyethylene alkyl ethers (Brij), alkylphenylpolyoxyethylene ethers (Triton-X), polyoxyethylene-polyoxypropylene copolymer (Poloxamer, Pluronic), and sodium dodecyl sulfate (SDS). Examples of suitable polyoxyethylenesorbitan-fatty acid esters are polysorbate 20, (sold under the trademark Tween 20™) and polysorbate 80 (sold under the trademark Tween 80™). Examples of suitable polyethylene-polypropylene copolymers are those sold under the names Pluronic® F68 or Poloxamer 188™. Examples of suitable Polyoxyethylene alkyl ethers are those sold under the trademark Brij™. Example concentrations of surfactant may range from about 0.001% to about 1% w/v.

A lyoprotectant may also be added in order to protect the PEBP inhibitor and the PFKFB3 inhibitor against destabilizing conditions during a lyophilization process. For example, known lyoprotectants include sugars (including glucose and sucrose); polyols (including mannitol, sorbitol and glycerol); and amino acids (including alanine, glycine and glutamic acid). Lyoprotectants can be included in an amount of about 10 mM to 500 mM.

In some embodiments, the pharmaceutical composition includes the PEBP inhibitor and the PFKFB3 inhibitor, and one or more of the above-identified agents (e.g., a surfactant, a buffer, a stabilizer, a tonicity agent) and is essentially free of one or more preservatives,

such as ethanol, benzyl alcohol, phenol, m-cresol, p-chlor-m-cresol, methyl or propyl parabens, benzalkonium chloride, and combinations thereof. In other embodiments, a preservative is included in the formulation, e.g., at concentrations ranging from about 0.001 to about 2% (w/v).

5 KITS

As summarized above, the present disclosure also provides kits. In certain aspects, a kit of the present disclosure includes a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor, a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor, and instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to
10 an individual in need thereof. Any suitable PEBP inhibitor and PFKFB3 inhibitor may be included in the kits of the present disclosure. Suitable PEBP and PFKFB3 inhibitors include any of the PEBP and PFKFB3 inhibitors described in the Methods section hereinabove, which is incorporated but not reiterated herein for purposes of brevity. Non-limiting examples of PEBP and PFKFB3 inhibitors which may be included in a kit of the present
15 disclosure include meclizine and PFK158, respectively. The kits of the present disclosure find use, e.g., in practicing the methods of the present disclosure.

Kits for practicing the subject methods may include a quantity of the PEBP and PFKFB3 inhibitors, present in unit dosages, e.g., ampoules, or a multi-dosage format. As such, in certain embodiments, the kits may include one or more (e.g., two or more) unit
20 dosages (e.g., ampoules) of a pharmaceutical composition that includes the PEBP inhibitor and one or more (e.g., two or more) unit dosages (e.g., ampoules) of a pharmaceutical composition that includes the PFKFB3 inhibitor. In some embodiments, a kit of the present disclosure includes one or more (e.g., two or more) unit dosages (e.g., ampoules) of a pharmaceutical composition that includes both the PEBP inhibitor and the PFKFB3 inhibitor
25 – e.g., as described in the Compositions section hereinabove.

The term “unit dosage”, as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the composition calculated in an amount sufficient to produce the desired effect. The amount of the unit dosage depends on various factors, such as the particular PEBP
30 and PFKFB3 inhibitors employed, the effect to be achieved, and the pharmacodynamics associated with the PEBP and PFKFB3 inhibitors, in the individual. In yet other embodiments, the kits may include a single multi dosage amount of the composition.

Components of the kits may be present in separate containers, or multiple components may be present in a single container. For example, in a kit that includes both
35 a PEBP inhibitor and a PFKFB3 inhibitor, the PEBP inhibitor and the PFKFB3 inhibitor may

be provided in the same composition (e.g., in one or more containers) or may be provided in separate compositions in separate containers. Suitable containers include individual tubes (e.g., vials), one or more wells of a plate (e.g., a 96-well plate, a 384-well plate, etc.), or the like.

5 As summarized above, a kit of the present disclosure includes instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to an individual in need thereof. In some embodiments, the instructions include instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to an individual having cancer. By way of example, the instructions may include instructions for administering the PEBP inhibitor and the PFKFB3
10 inhibitor to an individual having hepatocellular carcinoma (HCC) or acute myeloid leukemia (AML).

 The instructions may be recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container
15 of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g., portable flash drive, DVD, CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are
20 provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, the means for obtaining the instructions is recorded on a suitable substrate.

 Notwithstanding the appended claims, the present disclosure is also defined by the following embodiments.

25

1. A method of treating cancer, comprising administering to an individual having cancer a therapeutically effective amount of:

 a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor; and
 a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor.

30 2. The method according to embodiment 1, wherein the PEBP inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.

3. The method according to embodiment 2, wherein the PEBP inhibitor is a small molecule.

4. The method according to any one of embodiments 1 to 3, wherein the PEBP inhibitor is a cytidine diphosphate (CDP)-ethanolamine pathway inhibitor.
5. The method according to embodiment 4, wherein the CDP-ethanolamine pathway inhibitor is selected from the group consisting of: an ethanolamine kinase (EK) inhibitor, a phosphoethanolamine cytidyltransferase (PCYT2) inhibitor, an ethanolaminephosphotransferase (EPT, Selenoi) inhibitor, and any combination thereof.
6. The method according to embodiment 5, wherein the CDP-ethanolamine pathway inhibitor is a PCYT2 inhibitor.
7. The method according to embodiment 6, wherein the PCYT2 inhibitor is meclizine.
8. The method according to any one of embodiments 1 to 3, wherein the PEBP inhibitor is a phosphatidylserine (PS) decarboxylation pathway inhibitor.
9. The method according to embodiment 8, wherein the PS decarboxylation pathway inhibitor is phosphatidylserine decarboxylase (PSD).
10. The method according to any one of embodiments 1 to 9, wherein the PFKFB3 inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.
11. The method according to embodiment 10, wherein the PFKFB3 inhibitor is a small molecule.
12. The method according to embodiment 11, wherein the PFKFB3 inhibitor is selected from the group consisting of: 1-(pyridin-4-yl)-3-(7-(trifluoromethyl)quinolin-2-yl)prop-2-en-1-one (PFK158), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and any combination thereof.
13. The method according to embodiment 12, wherein the PFKFB3 inhibitor is PFK158.
14. The method according to any one of embodiments 1 to 13, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered concurrently to the individual.
15. The method according to embodiment 14, wherein the administering comprises administering to the individual a pharmaceutical composition comprising the PEBP inhibitor and the PFKFB3 inhibitor.
16. The method according to any one of embodiments 1 to 13, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered sequentially to the individual.
17. The method according to embodiment 16, wherein the PFKFB3 inhibitor is administered to the individual prior to administration of the PEBP inhibitor to the individual.

18. The method according to any one of embodiments 1 to 17, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual by a route of administration independently selected from: oral administration, parenteral administration, and intranasal administration.
- 5 19. The method according to any one of embodiments 1 to 18, wherein the cancer is liver cancer.
20. The method according to embodiment 19, wherein the liver cancer is hepatocellular carcinoma (HCC).
21. The method according to embodiment 20, wherein the HCC is primary HCC.
- 10 22. The method according to embodiment 20, wherein the HCC is recurrent HCC.
23. The method according to any one of embodiments 1 to 18, wherein the cancer is a leukemia.
24. The method according to embodiment 23, wherein the leukemia is acute myeloid leukemia (AML).
- 15 25. The method according to any one of embodiments 1 to 24, further comprising, prior to administering the PEBP inhibitor and the PFKFB3 inhibitor, determining the expression level of PFKBP3 in cancer cells obtained from the individual.
26. The method according to embodiment 25, wherein determining the expression level PFKBP3 comprises determining the abundance of *PFKBP3* mRNA in the cancer cells.
- 20 27. The method according to embodiment 26, wherein the abundance of *PFKBP3* mRNA in the cancer cells is determined by quantitative reverse transcription PCR (qRT-PCR) or RNA sequencing.
28. The method according to any one of embodiments 25 to 27, further comprising determining the severity of the individual's cancer based on the determined expression level
- 25 of PFKBP3 in the cancer cells.
29. The method according to any one of embodiments 25 to 28, further comprising classifying the type of cancer based on the determined expression level of PFKBP3 in the cancer cells.
30. A pharmaceutical composition comprising:
- 30 a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor;
a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor; and
a pharmaceutically acceptable carrier.

31. The pharmaceutical composition of embodiment 30, wherein the PEBP inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.
32. The pharmaceutical composition of embodiment 31, wherein the PEBP inhibitor is a small molecule.
- 5 33. The pharmaceutical composition of any one of embodiments 30 to 32, wherein the PEBP inhibitor is a cytidine diphosphate (CDP)-ethanolamine pathway inhibitor.
34. The pharmaceutical composition of embodiment 33, wherein the CDP-ethanolamine pathway inhibitor is selected from the group consisting of: an ethanolamine kinase (EK) inhibitor, a phosphoethanolamine cytidyltransferase (PCYT2) inhibitor, an
10 ethanolaminephosphotransferase (EPT, Selenoi) inhibitor, and any combination thereof.
35. The pharmaceutical composition of embodiment 34, wherein the CDP-ethanolamine pathway inhibitor is a PCYT2 inhibitor.
36. The pharmaceutical composition of embodiment 35, wherein the PCYT2 inhibitor is meclizine.
- 15 37. The pharmaceutical composition of any one of embodiments 30 to 32, wherein the PEBP inhibitor is a phosphatidylserine (PS) decarboxylation pathway inhibitor.
38. The pharmaceutical composition of embodiment 37, wherein the PS decarboxylation pathway inhibitor is phosphatidylserine decarboxylase (PSD).
39. The pharmaceutical composition of any one of embodiments 30 to 38, wherein the
20 PFKFB3 inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.
40. The pharmaceutical composition of embodiment 39, wherein the PFKFB3 inhibitor is a small molecule.
41. The pharmaceutical composition of embodiment 40, wherein the PFKFB3 inhibitor
25 is selected from the group consisting of: 1-(pyridin-4-yl)-3-(7-(trifluoromethyl)quinolin-2-yl)prop-2-en-1-one (PFK158), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and any combination thereof.
42. The pharmaceutical composition of embodiment 41, wherein the PFKFB3 inhibitor is PFK158.
- 30 43. A kit comprising:
a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor;
a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor; and

instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to an individual in need thereof.

44. The kit of embodiment 43, wherein the PEBP inhibitor and the PFKFB3 inhibitor are present in two or more unit dosages.

5 45. The kit of embodiments 43 or embodiment 44, comprising a pharmaceutical composition comprising the PEBP inhibitor and the PFKFB3 inhibitor.

46. The kit of any one of embodiments 43 to 45, wherein the instructions comprise instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to an individual having cancer.

10 47. The kit of embodiment 46, wherein the cancer is hepatocellular carcinoma (HCC) or acute myeloid leukemia (AML).

48. The kit of any one of embodiments 43 to 47, wherein the PEBP inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.

49. The kit of embodiment 48, wherein the PEBP inhibitor is a small molecule.

15 50. The kit of any one of embodiments 43 to 49, wherein the PEBP inhibitor is a cytidine diphosphate (CDP)-ethanolamine pathway inhibitor.

51. The kit of embodiment 50, wherein the CDP-ethanolamine pathway inhibitor is selected from the group consisting of: an ethanolamine kinase (EK) inhibitor, a phosphoethanolamine cytidyltransferase (PCYT2) inhibitor, an
20 ethanolaminephosphotransferase (EPT, Selenoi) inhibitor, and any combination thereof.

52. The kit of embodiment 51, wherein the CDP-ethanolamine pathway inhibitor is a PCYT2 inhibitor.

53. The kit of embodiment 52, wherein the PCYT2 inhibitor is meclizine.

54. The kit of any one of embodiments 43 to 49, wherein the PEBP inhibitor is a
25 phosphatidylserine (PS) decarboxylation pathway inhibitor.

55. The kit of embodiment 54, wherein the PS decarboxylation pathway inhibitor is phosphatidylserine decarboxylase (PSD).

56. The kit of any one of embodiments 43 to 55, wherein the PFKFB3 inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.

30 57. The kit of embodiment 56, wherein the PFKFB3 inhibitor is a small molecule.

58. The kit of embodiment 57, wherein the PFKFB3 inhibitor is selected from the group consisting of: 1-(pyridin-4-yl)-3-(7-(trifluoromethyl)quinolin-2-yl)prop-2-en-1-one (PFK158),

3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and any combination thereof.

59. The kit of embodiment 58, wherein the PFKFB3 inhibitor is PFK158.

The following examples are offered by way of illustration and not by way of limitation.

5 EXPERIMENTAL

Introduction

Organoids have provided a powerful model system for analyzing organogenesis and disease pathophysiology. A novel method was recently developed to direct the differentiation of induced pluripotent stem cells (iPSCs) into 3-dimensional human hepatic
10 organoids (HOs), which consist of sheets of hepatocytes, and cholangiocytes that are organized into epithelia that surround the lumina of duct-like structures. The HOs have biosynthetic and functional capabilities of human liver, and HOs with engineered mutations provide a unique model for characterizing the functional impact of human disease-causing genetic mutations. In response to changes in the growth factors added to the media, HOs
15 develop through stages that resemble human liver during embryonic development. Because of these properties, HOs could be used to identify the key pathways regulating various stages in liver development and disease pathogenesis.

However, at each differentiation stage, HO cultures contain different types of cells, and sub-populations of each cell type can be in various stages of differentiation, making it
20 difficult to identify critical transcriptional changes through analyses performed on the bulk population. To deal with the cellular complexity within dynamically changing organoid cultures at different developmental stages, single cell RNA sequencing (scRNA-Seq) can be used to simultaneously analyze global changes in the transcriptome of many individual cells. This analytic methodology has advanced our ability to analyze cellular fate
25 determination, cell ontogeny, and cell heterogeneity in various tissues, including liver. However, as when any 'omic' methodology is used in isolation, it can be difficult to determine which of many measured gene expression changes are critical for even a single differentiation step.

To identify the key pathways for early liver development, described herein is the
30 simultaneous characterization of metabolomic and transcriptomic changes that occur during an early stage of HO formation. The reasoning was that a change in the level of mRNA expression for a gene within a metabolic pathway with an altered metabolite is likely to be of importance. This integrative analysis identified a biosynthetic pathway that plays an important role in early liver development. Based upon this finding and an independently

performed study of mitochondrial drug effect Gohil et al. (2010) *Nat Biotechnol* 28:249-255, it was discovered that a commonly used over the counter medication targeting this pathway could be used as one component of a new treatment for primary liver cancer. Systemic therapies that are effective and well-tolerated are critically needed for primary liver cancer because it is one of the few cancers whose incidence has continued to rise in the US and worldwide, it has a dismal 5-year survival with little improvement in recent times (12.2% in 2001-2003 and 14.8% in 2004-2009), and limited treatment options are available.

Example 1 – scRNA-Seq Analysis of Developing HOs

To characterize the dynamic changes that occur as a control iPSC line (C1) differentiates into an organoid, scRNA-Seq was performed on the iPSC, day 9 hepatoblasts (HB), and day 21 HO cultures; and scRNA-Seq was also performed on primary human hepatocytes (PHHs) for comparison purposes. The t-Distributed Stochastic Neighbor Embedding (t-SNE) method was used to visualize this high dimensional data, which enables the relationships across each developmental stage to be evaluated. This analysis shows the developmental path as the iPSCs differentiate into hepatoblasts (HBs) and then into primary human organoids; and the transcriptome profile of many cells within the HO was similar to that of PHHs (FIG. 1, panel A). Unexpectedly, this analysis identified two clusters of HBs: (i) early HBs whose expression profile was close to that of iPSC; and (ii) late HBs whose expression profile was similar to that of PHHs and HO. This finding is consistent with prior gene expression results, which indicated that there is a 'resonant state' between pluripotent cells and their progeny when the cells are in an early stage of differentiation. The early HBs express more pluripotency-related genes, which are lost as the cells are further differentiated.

Transcriptome analysis using the fractional identify method (FI) revealed that HOs contain cells whose transcriptomes are similar to hepatocytes, and others that are similar to cholangiocytes (FIG. 1, panels B-E). The validity of the scRNA-Seq data is confirmed by the fact that mRNAs – which are characteristically expressed in iPSC (*NANOG*, *OCT4*), PHHs (*A1AT*, *ALB*), or cholangiocytes (*CFTR*) – were expressed in the appropriate cell type. Analysis of this data (and from organoids formed from other iPSC lines) with another statistical method confirmed that cells within the different groupings were clustered, and the same developmental relationships were apparent.

The transcriptome of these cells was also compared with previously obtained transcriptomic data for hepatocytes present in one fetal (gestation age 17.5 weeks) and three adult (ages 21-65) livers using t-SNE visualization. As expected, the transcriptomes of hepatocytes in adult liver and PHHs were tightly clustered, while the transcriptomes of hepatocytes in fetal liver were separated from that of the adult liver hepatocytes/PHH cluster

(FIG. 2, panel A). Of importance, the transcriptome of HO cells was more closely related to adult hepatocytes/PHH than to fetal hepatocytes. The SLICER method was also used to computationally infer a developmental trajectory for the cells starting from iPSC. As with the t-SNE visualization, this method generated a developmental path from iPSC to HB, and then to HO, and then to adult hepatocytes/PHH (FIG. 2, panel B). However, a small proportion of HB cells (~15 out of 149) diverged from the main path, which indicates that a subset of the HBs could have a different developmental fate. Of importance, most HO cells are located farther away from iPSCs, and are closer to adult hepatocytes/PHH than to fetal hepatocytes (FIG. 2, panel B). Also, HBs were located closer to fetal hepatocytes, and were far away from the adult hepatocytes. Therefore, the transcriptomic profile of HO cells resembled that of mature hepatocytes, while the HBs had a closer resemblance to fetal hepatocytes. This analysis also identified many genes that were expressed at specific stages of HO development, and several were also associated with liver or liver cancer development.

Example 2 – A Phosphatidylethanolamine (PE) Biosynthesis Pathway is Essential for HB Differentiation

To identify changes in mRNA levels that are essential for early liver development, a semi-targeted metabolomic method was used to characterize metabolomic changes occurring when iPSC differentiate into HBs. Of the 494 metabolites evaluated, only eleven had a >3-fold abundance difference ($P < 0.05$), and only three had a >10-fold difference in abundance between iPSC and HBs (FIG. 3, panel A). Of these metabolites, phosphoethanolamine (P-Etn) had the greatest differential abundance; its abundance was >170-fold increased in HBs ($p = 0.018$) relative to that in iPSCs. In contrast, there was no difference in ethanolamine abundance in iPSC vs. hepatoblasts (FIG. 3, panel B). The increased P-Etn abundance in HBs was of interest, since P-Etn is required for the biosynthesis of phosphatidylethanolamine (PE), which accounts for 25% of all cellular phospholipids and is critical for cellular membrane formation. Although it can be produced via four different biosynthetic pathways, the CDP-ethanolamine pathway is thought to be the major de novo PE biosynthetic pathway (PEBP) in the liver. Of note, analysis of bulk sample RNA-Seq data indicated that mRNAs encoding all three enzymes in this pathway (ENTK1, PCYT2 and SELENOI) were increased in HBs (75, 58 and 41 fold, respectively) (FIG. 3, panel B). PCYT2 encodes the rate-limiting second enzymatic step in the PEBP pathway, and ethanolamine is not synthesized in mammalian cells. These transcriptomic and metabolomic changes indicate that the mRNAs for the enzymes and a key metabolite in the PEBP are coordinately increased at an early stage of liver development.

To determine if the PEBP is essential for early liver development, three different sgRNAs were used to prepare iPSCs with a CRISPR-mediated PCYT2 gene knockout from control iPSC lines (PCYT2 KO1-3). Two control iPSC (C1, C2) and three PCYT2 KO lines were differentiated into endoderm on day 3, and then transferred into the media that directs their differentiation into HBs; and the cultures were examined on day 4. The control iPSC lines could differentiate into endoderm, and remained viable in the HB media. While each of three PCYT2 KO cells could differentiate into endoderm, >95% of these cells were dead within 24 hrs after transfer to the HB media (FIG. 4, panel A). This was an unexpected result, since mice with a hepatocyte-specific conditional *Pcyt2* gene deletion had liver abnormalities, but were viable and fertile. Consistent with our metabolomic results, it was previously observed that when physiologic ethanolamine concentrations are present, increased ENTK1 expression in cell lines produces a marked increase in cellular P-Etn, but not in CDP-Etn abundance.

Example 3 – Early HBs are Selectively Sensitive to PEBP Inhibition

Meclizine is an over the counter anti-histamine that is widely used for the treatment of motion sickness and vertigo. Meclizine was recently found to be a non-competitive inhibitor of PCYT2 enzyme activity (Gohil et al. (2013) *J Biol Chem* 288:35387-35395). Therefore, it was examined whether meclizine would have the same effect on early HBs as a CRISPR-mediated PCYT2 KO. Within 24 hours after exposure to meclizine, virtually all cells in the day 4 cultures, which were in the early stage of differentiating toward HBs, were killed (FIG. 4, panel B). Growth factor (FGF10 or HGF) addition did not reduce their sensitivity to meclizine-induced cell death. It was previously demonstrated that HOs contained a population of SOX9+CK7+ cells that resemble liver progenitor cells, and that after HOs were formed, they could be dissociated into single cells, which could re-form hepatic organoids when placed in growth medium. Therefore, it was examined whether meclizine addition to the growth medium could inhibit organoid re-formation. Meclizine was a potent inhibitor of organoid reformation, as addition of 4 μ M meclizine inhibited organoid regeneration by 50% (FIG. 4, panel C).

To better define the time period for sensitivity to meclizine-induced cell death, cell survival was examined after meclizine was added to iPSC cultures (day 0), or when it was added to early (day 4), mid (day 7) or fully differentiated HB (day 10) cultures. Only the early HBs (day 4) were sensitive to meclizine-induced cell death. 4 μ M meclizine killed 50% of the cells at this stage. In contrast, the day 7 and day 10 HB cultures were relatively insensitive to meclizine-induced cell death (FIG. 4, panel D). Human iPSCs were far less susceptible (LD50 ~60 μ M), and human fibroblasts (LD50 >80 μ M) and adult PHHs (LD50 >80 μ M) were resistant to meclizine-induced cell death. Unlike the early HBs, human iPSC

in the early stage of differentiating into cardiomyocytes (day 5) were no more sensitive to meclizine-induced cell death than were iPSCs, and fully differentiated induced cardiomyocytes (day 20) were not sensitive to meclizine-induced cell death (FIG. 4, panel E). Moreover, HUH7 and Hep G2 cell lines, which represent well differentiated hepatocellular carcinomas, were also resistant to meclizine-induced cell death (FIG. 4, panel E). Taken together, these results indicate that there is a time window when cells in the early stage of organoid formation (and possibly liver development) are extremely sensitive to PEBP inhibition.

Example 4 – PEBP in Hepatocellular Cancer

iPSCs express high levels of mRNAs encoding CD24, CD90, PROM1 (CD133) and EPCAM. Based upon expression within a subset of cells within tumor samples that will form tumors in xenotransplantation models, CD133, EpCAM, CD24 and CD90 have been identified as markers for cells contributing to the growth and maintenance of primary liver cancer. Moreover, DLK1, which is expressed in HBs, has been identified as a potential therapeutic target for treatment of liver cancer. To investigate the possibility that the PEBP could be a therapeutic target for liver cancer, two gene expression datasets were downloaded from The Cancer Gene Atlas. One contained 374 primary (HCC1) or recurrent hepatocellular carcinomas (HCC2) and 50 normal liver samples (N1), and the other had 31 cholangiocarcinomas (CC) and 8 normal liver samples (N2). The SLICER method was used to examine the relationship between the transcriptomes of cells HCC, normal liver tissue, and the cells in developing HO cultures. This analysis revealed that the transcriptome of HCC clusters with that of HO and HB cells; and was distinct from that in of normal liver tissue (FIG. 5, panel A). Also compared was the expression level for mRNAs encoding PEBP pathway genes (*ETNK1*, *ETNK2*, *PCYT2*, and *SELENOI*) in the HCC, CC and normal liver samples. Of importance, *PCYT2* mRNA was significantly increased in primary HCC (1.7-fold, p value 1×10^{-7}), and its expression was further increased in recurrent HCC (3.3-fold, p value 7×10^{-9}) relative to normal liver.

Many types of cancer cells have very high rates of glycolysis, which results from oncogene-induced increases in glucose uptake and in the enzymes involved in glucose metabolism. Rapidly growing hepatoma cell lines are metabolically adapted to grow in high glucose media, and they derive the bulk of their energy from glycolysis (FIG. 5, panel B). Although this pathway produces only 5% of the potential energy that could be generated from a glucose substrate by mitochondrial oxidative phosphorylation (OXPHOS), the flux through this pathway can be greatly accelerated when glucose is abundant. Since conversion of galactose to pyruvate generates no net ATP, when mammalian cells are switched to a media where galactose is the sole sugar source, they use OXPHOS as their

energy source. Also, cancer cells use glutamine metabolism via the tricarboxylic acid cycle as the major pathway for producing the molecules required for proliferation. Since HCC may exist within a metabolically constrained environment in the liver, examined was the effect of meclizine on the growth of two hepatoma cell lines (Hep G2 and HuH7) in media containing galactose and glutamine. In this media, 40 μ M meclizine inhibited the growth of both hepatoma lines at 24 and 48 hours (FIG. 5, panel C). Thus, meclizine could inhibit the growth of hepatocarcinoma cell lines that are dependent upon mitochondrial OXPHOS for energy production.

Recently, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) inhibitors have been produced, and a third generation inhibitor (PFK158) is now in clinical testing as an anti-cancer agent. PFKFB3 stimulates glycolysis because the product of its enzymatic activity (fructose-2,6-bisphosphate F2,6P2) is an allosteric activator of the rate-limiting enzyme (phosphofructokinase-1, PFK-1) in the glycolysis pathway (FIG. 5, panel B). PFKFB3 expression is induced by hypoxia, and it is overexpressed and highly phosphorylated in many different types of cancer. Therefore, it was hypothesized that PFKFB3 inhibition, which would increase cellular dependence upon mitochondrial OXPHOS, could inhibit the growth of HCC when used in combination with meclizine. The combination of meclizine and PFK158 indeed inhibited the growth of both hepatoma cell lines (FIG. 5, panel C). Although meclizine alone did not inhibit their growth in the glucose media, the mitochondrial membrane potential of the HepG2 hepatocellular carcinoma line in the glucose media (and also in the galactose/glutamine media) was altered by meclizine. This effect is consistent with meclizine's known mechanism of action (discussed below). To more fully characterize the effect of this drug combination, examined was the effect of exposure to 24 different combinations of meclizine and PFK158 concentrations for 24 and 48 hrs on HepG2 viability. This data indicates that there is a very significant interaction between the two drugs, which appeared at both time points ($p < 2.2 \times 10^{-16}$ for both exposure periods). Specifically, in the presence of a high dose of meclizine (40 μ M) and PFK158 (2 or 5 μ M), a significant synergistic effect appeared at both time points: this drug combination reduced cell viability by 1.3-1.4 fold more than expected by simply adding the effect of each individual drug at the same dose. A lesser, but consistent effect on cell viability, was also observed with the combination of 20 μ M meclizine and 2 (or 5) μ M PFK158. The synergistic effect of this drug combination was also observed at lower drug concentrations: this effect was especially apparent at the 48 hour time point. 5 μ M concentrations of both drugs induced an additional 1.52 fold decrease in cell viability relative to total additive effect of each of the two drugs. Low concentrations of meclizine (2 or 5 μ M) altered the mitochondrial

membrane potential of HepG2 cells. This effect is consistent with these meclizine concentrations having a synergistic effect when combined with PFK158.

Because of these results, investigated was whether mRNA levels for PEBP mRNAs or PFKFBK3 could predict survival risk after HCC diagnosis. Kaplan-Meier survival curves showing the survival time after primary HCC diagnosis in The Cancer Gene Atlas (TCGA) (n=371) cohort were prepared based upon the level of PCYT2, ETNK2 or PFKFB3 mRNA expression in each HCC sample (FIG. 6). The expression level for each mRNA was classified as high (or low) if it was above (or below) the median level in the respective cohort. To confirm this finding, the same analysis was performed using the data obtained from 242 HCC subjects in the Fudan cohort. Subjects whose HCCs had a higher level of ETNK2 mRNA expression had a significantly longer median survival time than those with a lower level in both cohorts (TCGA $p=0.04$, Fudan $p=3 \times 10^{-5}$) (FIG. 6). Also, HCC with a lower level of PFKFB3 mRNA expression had a significantly longer survival time (TCGA $p=0.01$, Fudan $p=9 \times 10^{-4}$). In contrast, the level of PCYT2 mRNA expression was not associated with a significant survival difference in either cohort (FIG. 6). The fact the ETNK2 and PFKFB3 mRNA expression level predicted survival in both cohorts was unexpected, especially since prior genome-wide mRNA profile clustering failed to identify TCGA subgroups with different survival periods. However, since PCYT2 is the rate-limiting step for the PEBP, the fact that subjects with a lower PFKFB3 mRNA expression level had longer survival is not surprising. Cancers with a lower level of PFKB3 enzyme activity will have a lower rate of glycolysis and will be more dependent upon mitochondrial OXPHOS as an energy source. This will make them more sensitive to the mitochondrial effect of the elevated P-Etn concentration caused by meclizine-mediated inhibition of PCYT2.

Example 5 – Drug Efficacy in a Human Hepatocellular Carcinoma Liver Xenotransplantation Model

To assess the efficacy of meclizine and PFK158 treatment *in vivo*, developed was a novel xenotransplantation model in which a well differentiated human hepatocarcinoma cell line (Hep G2) was directly transplanted into the liver of immunocompromised NOG mice. The transplanted Hep G2 cells stably expressed a GFP-luciferase fusion protein, which enabled a bioluminescence-based assessment of the human tumor burden within the liver. One day after transplantation of 1×10^6 human cells, the mice with similar signal intensities in their livers ($p=0.98$) and they were randomly separated into two groups. Each group was then treated for a two-week period (orally) each day with either vehicle, or with the combination of 25 mg/kg meclizine and 25 mg/kg PFK158. The liver tumor burden was substantially reduced in the drug-treated mice relative to vehicle treated mice ($P=0.02$) (FIG. 7, panel A). Of note, three drug-treated mice had no detectable human tumor cells

within their livers, while all vehicle treated mice had significant levels of human tumor cells. The efficacy of this combination in reducing the liver tumor burden in this model was confirmed in an independent experiment ($p=0.037$) (FIG. 7, panel B). Also of note, the drug-treated mice had a normal appearance, and no liver toxicity was apparent by serologic testing ($P=0.8$) (FIG. 7, panel C). The results obtained with this xenotransplantation model demonstrate that meclizine and PFK158 can reduce the growth of a human hepatocarcinoma cell line *in vivo*.

Example 6 – Efficacy for Acute Myeloid Leukemia (AML)

An association was also found between the level of ETNK2 mRNA expression and survival in the AML TCGA cohort ($p = 0.008$). Since a high level of ETNK2 mRNA expression was associated with lower survival, the more aggressive forms of AML could have increased PE biosynthesis. This suggests that the PEBP pathway activity could play a role in the progression of a broader range of cancers than was previously known. To investigate this, examined was the effect of meclizine on primary cultures of AML blasts generated from 3 AML patients at the time of their diagnosis. Interestingly, after 96 hours in culture, AML blasts obtained from 1 subject were somewhat sensitive to meclizine alone ($IC_{50}=17.7 \mu M$). However, addition of $0.9 \mu M$ PFK-158 resulted in elimination of $>95\%$ of the blasts in these cultures in the presence of $10 \mu M$ meclizine, and the effect of the drug combination was significantly increased relative to that of $10 \mu M$ meclizine alone ($p=0.005$) (FIG. 8, panel A). The synergistic effect of this drug combination was also observed in AML blast cultures prepared from the two other subjects. When $0.9 \mu M$ PFK158 was combined with $0.5 \mu M$ meclizine, $>95\%$ of the AML blasts from both subjects were eliminated; and the drug combination effect was significantly increased relative to that of meclizine alone (AML SU582, $p=0.0001$; AML SU839, $p=0.0001$) (FIG. 8, panel B). These results indicate that the combination of meclizine and PFK158 had a synergistic effect on AML blasts.

Materials and Methods

Hepatic organoid (HO) preparation

The three control (C1, C2, C3) and two ALGS (ALGS1, ALGS2) iPSC lines used in this study, and the methods for directing their differentiation into HOs were as described in Guan, Y. et al. (2017) JCI Insight 2, pii: 94954.

scRNA-seq data processing and analysis

Single cells from iPSC (day 0), day 9 HB, and day 30 HO cultures, and PHHs were prepared using the C1 Single-Cell Auto Prep System (Fluidigm, South San Francisco, CA). The captured cells across the 96 wells were manually inspected as a quality control

measure to remove empty wells, doublets or debris-containing wells. For quality control (normalization and estimation of technical noise), 0.05 µl of 1:200,000 dilution of External RNA Controls Consortium (ERCC) RNA spike-in Mix1 (Ambion, AM1780) was added to lysis buffer. High-quality cDNA libraries for single cell transcriptome analysis were prepared using the SMART-Seq v4 Ultra Low RNA Kit for the Fluidigm C1 System (Takara Bio USA, 635025). The size distribution and the concentration of single-cell cDNA were assessed using a Agilent 2100 Bioanalyzer with the high sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, 5067). Sequencing libraries were constructed in 96-well plates using the Nextera XT DNA Sample Preparation kit according to the manufacturer's protocol (Illumina, San Diego, CA, FC-131-1096) with 96 dual barcoded indices (Illumina; FC-131-1002). Library cleanup and pooling was performed using AMPure XP beads (Agencourt Biosciences; A63880). Up to 200 quantified single-cell libraries were pooled, and these were sequenced 150 bp paired-end on one lane of Illumina HiSeq 2000 to a depth of 2.1 million reads per cell.

For data analysis, a quality-control step was first applied to the paired-end sequence reads using the FastQC tool. Adapter sequences and low-quality bases at the end of the reads were then removed using the Cutadapt software. The reads were then aligned to human exon sequences (Build GRCh38) using STAR. The expression level of each gene for each cell were then quantified using the FeatureCounts tool. Samples that passed the following criteria were retained for subsequent analysis: 1) the total number of reads should be at least 105, 2) the number of genes detected in the sample should >3000, and 3) <50% of the reads were mapped to the 37 mitochondrial genes. In the 1st sample set, 114 samples passed this QC step (25 iPSC, 25 HB, 50 HO and 14 PHH samples). In the 2nd sample set, 310 samples passed this QC step (16 iPSC, 124 HB and 170 HO samples). Therefore, 424 samples were subsequently analyzed (65, 71, 185 and 89 samples for A1, A2, C1 and C2, respectively, and 14 PHH samples). The expression matrix (rows for genes and columns for samples) was then analyzed using the scater package; and the Fragments Per Kilobase of Exon Per Million (FPKM) values were calculated for each gene in each sample. Next, the ComBat method was used for batch correction.

The Log2-transformed FPKM values (offset by 1 to avoid using the logarithm of zero values) were used for the analyses. Principle component analysis (PCA) and tSNE methods were used to visualize the high-dimensional transcriptome profiles of single cells. PCA identifies orthogonal directions that explain the largest proportion of variance in the data, and a linear axis rotation is performed accordingly. On the other hand, the tSNE method performs a non-linear transformation of the original (high-dimensional) data in order to convert it into lower dimensional manifolds, which preserve the relationships between

neighboring data points. For PCA analysis and tSNE visualization, the top 500 most variable genes (i.e. those with the largest Median Absolute Deviation (MAD) values) were used. The MAD is defined as: $MAD(\vec{x}) = \text{median}(|\vec{x} - \text{median}(\vec{x})|)$, where $\vec{x}$ is a vector composed of expression values of a gene across samples. The `prcomp` function in R was used for PCA analysis, and the tSNE visualization was performed using the `Rtsne` package. The consensus clustering analysis was performed using `SC3` package in R, and the number of clusters was estimated using the Tracy-Widom theory on random matrices. To better capture subtle differences among genes that were less variable, the top 2000 most variable genes were used for the consensus clustering analysis. Next, a computational method was implemented to express a single cell transcriptome as a linear combination of the transcriptomes of the three extremes (i.e. iPSC, PHH and cholangiocyte), using established methods. In brief, the average $\log_2(\text{RPKM})$ values of the iPSC cells and PHH cells was calculated. The RNA-seq data for cholangiocytes were obtained from GEO (GSM1416801), and was analyzed using the same pipeline to generate the transcriptome profile for cholangiocyte. Next, genes that had (i) an average $\log_2(\text{RPKM})$ value > 1 in at least one of the three cell types; and b) a $\log_2$ fold change > 1 were identified. This identified 13,572 genes. Using these genes, the fractional identities of each single cell was calculated using quadratic programming (as implemented in the `quadprog` package in R). The resulting fractional PHH or cholangiocyte identities of the cells were then visualized on the tSNE plot.

scRNA-seq data for adult and fetal hepatocytes were obtained from GEO (GSE96981). Only cells within groups 3 and 6, which correspond with adult and fetal hepatocytes, respectively, were used here. To avoid introducing un-necessary variation due to the different donors and developmental stages of the tissues, only 85 (of 88 total) cells in group 6 were analyzed, since they were obtained from one fetal donor (gestation week 17.5). The raw sequences were downloaded and filtering using the same criteria as described above. After filtering, 20 and 79 scRNA-seq samples from adult/fetal hepatocytes were selected and used for subsequent analysis. The above procedures were then used to analyze the sequences and to obtain the corresponding FPKM value for each gene in the samples. The mean and SEM of the $\log_2(1 + \text{FPKM})$ values in adult and fetal hepatocytes were then evaluated and plotted for each gene. The top 500 genes with the largest MAD values were used to construct the tSNE visualization. To evaluate the fractional fetal/adult hepatocyte identity of cells in the iPSC, HB, HO and PHH samples, genes that were differentially expressed between adult and fetal hepatocytes (fold change > 1.5 and adjusted p value < 0.05) were first selected, which were identified using the `limma` package in R. Among these 5726 genes identified by this method, 34 genes were removed due to insufficient presence in our scRNA-seq samples. The fractional identities of each single cell

relative to fetal/adult hepatocytes were then calculated using the same method for the remaining 5692 genes. The SLICER method was then used to computationally infer a developmental trajectory (beginning from iPSC) for the cells. To do this, the ComBat algorithm was applied to normalize the scRNA-seq data across different experiments. Next, the top 500 genes with the largest MAD values were selected and SLICER was applied using these selected genes.

The expression data for the Liver Hepatocellular Carcinoma (TCGA-LIHC) and Cholangiocarcinoma (TCGA-CHOL) projects were obtained from the Cancer Genome Atlas. These two datasets were then combined with our own organoid culture scRNA-seq data. Genes with a raw FPKM value that was < 0.5 in > 90% of the samples were filtered (17,674 genes left) and then the ComBat method 29 was applied to remove potential batch effects. The SLICER method was then used to computationally infer a developmental trajectory (beginning from iPSC) for the cells using the top 2000 genes with the largest MAD.

Metabolomic Analysis

The semi-targeted metabolomic method (STMM) uses Dansyl [5-(dimethylamino)-1-naphthalene sulfonamide] derivatization coupled with LC/MS analysis. Dansylation increases metabolite detection sensitivity by 10-1000 fold, which enables changes in many metabolites with primary or secondary amino (and other) groups to be evaluated in an unbiased fashion. LCMS grade acetonitrile; methanol and water were purchased from Honeywell Burdick and Jackson (Muskegon). High purity formic acid was purchased from Thermo Scientific (Rockford, IL). A Folch extraction was performed on three independent samples of iPSC and hepatoblast cultures, each containing about 1 million cells. An aliquot of the aqueous layer containing the polar compounds were labeled with Dansyl [5-(dimethylamino)-1-naphthalene sulfonamide], and the LCMS analysis was performed using our previously described methods. The identity of phosphoethanolamine was confirmed by its retention time and LC/MS/MS pattern relative to that of a chemical standard (Sigma). Of note, the abundance of phosphoethanolamine in iPSCs was below the minimal Signal to Noise ratio of 10; so its abundance was set to a baseline abundance of 1000 that was used for the fold abundance calculation.

PCYT2 gene knockout experiments

The sequences of three site-specific guide RNAs (sgRNAs) that target exons 8 and 12 of the PCYT2 gene, which were determined using the CRISPR Design Tool, are: (i) gccctggtgggcggaacccc, (ii) gatgactgtctcccctggct, and (iii) ggacgatgaggtctgtggtg. Oligonucleotides with these sequences were cloned into the lentiCRISPR v2 vector

(Addgene #52961, Cambridge, MA), which uses the Puromycin selection to enrich for cells with a PCYT2 KO. Control iPSCs (C1, C2 and C3) were pelleted and mixed with 5~10 µg CRISPR sgRNA expression vectors in 100 µl of human ES cell solution 1 (Lonza), and nucleofection program A23 was applied. On day 3, puromycin was added to select for cells with a PCYT2 KO; and after 48 hrs, the iPSCs lines were split and placed into media to direct their differentiation into HB for 3 days, and the cells were transferred into another media to direct their differentiation into HBs (using previously described methods 22). On day 4, it was noted that the PCYT2 KO cells were dying, and the Trypan Blue dye exclusion assay was performed to assess cell viability. In brief, 100 µl of the cell suspension was mixed with 100 µl 0.4% Trypan Blue stock solution (T8154 Sigma), and cell counts were performed immediately under bright field microscopy.

Cell survival after meclizine exposure

Meclizine (2HCl) was obtained from (Selleckchem, S1986, Houston, TX), and an 80 mM stock solution was freshly prepared in DMSO. Control iPSCs were differentiated for 3 days into endoderm. On day 4, the cells were exposed to hepatoblast differentiation media containing 50 µM Meclizine (M), 50 ng FGF10 (F10), 50 ng HGF (H), or F10 and meclizine (F10+M) for 24 hrs. Then, the PrestoBlue® Cell Viability assay (Invitrogen) was performed to assess the percentage of viable cells in each culture. Huh7 and Hep G2 cells were cultured to 90% confluence, and then exposed to meclizine concentrations ranging from 0 to 80 µM for 24 hrs, before the PrestoBlue® Cell Viability assay was performed. Induced cardiomyocytes were prepared from human iPSC using the methods described in 44. The human fibroblast cell line was obtained from Dr. Julian Sage. The primary human hepatocytes were purchased from Lonza (Basel, Switzerland).

For the combination drug experiments, HepG2 and HuH7 cells were cultured in DMEM and 10% FBS, which had either glucose (5.55 mM), or galactose (10 mM) and glutamine (2 mM) for 7 days. The cells were cultured in 96 wells plates; each well had 200 µl of medium; and the cells were ~50% confluent vehicle (DMSO), meclizine (40 µM) and/or PFK158 (Medchemexpress, NJ) (0, 2, 5 or 10 µM) were added to the medium. After 24 and 48 hours of drug exposure, cell viability was measured with PrestoBlue cell viability assay (Invitrogen, A13261), and the mitochondrial membrane potential was measured with JC-10 (Enzolifesciences, ENZ-52305) according the manufacture's protocol. All data were collected using a FLUOstar® Omega multi-mode microplate reader

Analysis of the combined effect of Meclizine and PFK158 on cell viability

The measured cell viability values were log-transformed so that the fold-reduction in cell number was analyzed. For this analysis, the concentration of each of the two drugs was

treated as a factor, such that the individual drug concentration was represented by a different factor. This analysis method does not introduce any additional bias by artificially enforcing dose-response curves for two drugs. A two-way ANOVA model was then evaluated: $\log(\text{cell viability}) \sim \text{Meclizine} + \text{PFK158} + \text{Meclizine} * \text{PFK158}$. The interaction terms provided the estimated combined effect of the two drugs at different concentrations of the two drugs.

Effect of mRNA expression levels on survival after HCC diagnosis

The survival and gene expression data for the 371 TCGA subjects with HCC were downloaded from The Cancer Genome Atlas. The data for the 242 HCC subjects in the Fudan cohort was obtained from the Gene Expression Omnibus (Accession GSE14520). The expression values a gene of interest were transformed into a binary low/high representation by comparing the values to the corresponding median. The survival probability was estimated using the Kaplan-Meier estimation, and a Cox proportional hazard model was used to compare the survival time between groups with high and low levels of expression of the gene of interest. Interestingly, the *ETNK2* expression level decreased ($p=0.033$ and 0.00002 , respectively) and the *PFKFB3* expression level increased ($p=0.047$ and 0.001 , respectively) with tumor stage in both TCGA and Fudan cohorts (fig. S13). This is consistent with the proposed roles of these genes. But this also raised the concern that the observed association with survival rate could be because of the correlation with tumor stage or other confounding factors. Therefore, also assessed was whether the observed association remained significant after adjustment for potential confounding factors, which were available in the database: age, gender and tumor stage (I, II, III or IV). In the TCGA HCC cohort, *ETNK2* and *PFKFB3* were only weakly associated after the adjustment ($p = 0.07$ and 0.11 , respectively). However, the level of expression of these two genes was still strongly associated with HCC survival in the Fudan cohort ($p = 0.011$ and 0.018 , respectively). Therefore, even after adjusting for these known potential confounding factors, the level of *ETNK2* and *PFKFB3* mRNA expression still was significantly associated with cancer survival even among individuals with tumors at the same stage.

A similar analysis was also performed for the TCGA-AML cohort, which had survival and gene expression data in TCGA. The association between cancer survival and the expression levels of *PCYT2*, *ETNK2* and *PFKFB3* mRNA (for primary blood derived cancer cells obtained from 145 subjects) were similarly analyzed using a Cox proportional hazard model. *ETNK2* was the only gene that showed a significant association ($p = 0.008$) with survival after diagnosis. This association remained significant even after adjusting for gender and age. However, since stage-related information was not available for this dataset, it could not be included with the adjustment analysis.

Animal studies

The Stanford University Administrative Panel on Laboratory Animal Care (APLAC) approved the animal protocol and the experimental procedures. For these studies, highly immunocompromised NOG mice were housed in a specific pathogen-free room, which was
5 equipped with a HEPA filter system with 12 hours light/dark cycle.

Generation of Hep G2 expressing GFP-Luciferase

Early passage Hep G2 cells were transduced with a lentivirus encoding a GFP-Luciferase fusion protein (MOI > 10). After the cells were cultured for 4 days, Hep G2 cells expressing a high level of GFP-Luciferase were selected by FACS sorting. The sorted cells
10 were then cultured to 80~90% confluence before another round of FACS sorting was performed, and all sorted cells expressed a high level of GFP.

In vivo effect on tumor growth

For this *in vivo* model, 1×10^6 Hep G2 cells stably expressing a luciferase gene were re-suspended in Williams E medium and injected into the spleen of NOG mice using our
15 previously described procedures. PFK158 (2.5 mg/ml) and Meclizine (2.5 mg/ml) were mixed with a 0.5% methyl cellulose solution to produce a drug suspension. Beginning on the 1st day after transplantation, the mice were dosed with either vehicle; or with 25 mg/kg Meclizine and 25 mg/kg PFK158 orally each day for two weeks. Mice have been treated with meclizine doses up to 100 mg/kg, but the meclizine dose was lowered to avoid toxicity.
20 The PFK158 dose was selected based upon the dose of a PFKBP3 inhibitor (PFK15, 25 mg/kg IP) with a similar structure that was used in a human lung carcinoma cell mouse xenotransplant model. To enable quantitation of the cells, D-luciferin (150 mg/kg IP) (Perkin Elmer, Richmond, California) was injected 5 minutes before imaging. The mice were then imaged using a bioluminescent optical imaging system (IVIS Spectrum In Vivo Imaging
25 System, PerkinElmer) according to the manufacturer's instructions. The liver region was defined for data acquisition; the radiance was measured and the data was exported as photons/second (p/s). This enabled the tumor cells levels to be compared between the different groups. Initially, eight mice were in each treatment group. However, two mice (one each in the vehicle and one in the drug-treated group) were excluded from the study
30 because they had a bioluminescent signal that mostly emanated from bladder and rectum (but not from the liver area). Hence, seven animals from each group were included in the final analysis. A t-test was performed to compare signal difference between the vehicle and drug-treated groups.

Patient samples and human ethics approval

The primary peripheral blood samples were obtained from subjects with acute myeloid leukemia prior to treatment, and after informed consents were obtained. The study was performed according to institutional guidelines that were approved by the Stanford University Institutional Review Board No. 6453. Patient details are provided in Supplemental Table 1.

Culture of primary AML cells

Vials containing > 95% AML blasts were thawed in 20% fetal bovine serum + IMDM with DNase; and plated at 1×10^5 - 1×10^6 cells/ml in IMDM with 0.5% serum and 20 ng/ml stem cell factor, Flt3L, thrombopoietin, interleukin-6, and interleukin-3. Various concentrations of meclizine and/or PFK148 were also added to the culture medium. After 96 hours in culture, the number of live cells was determined by flow cytometry using bead:cell ratios to count absolute numbers of propidium iodide-negative CD45 mid/side scatter blast gate cells.

Accordingly, the preceding merely illustrates the principles of the present disclosure. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein.

WHAT IS CLAIMED IS:

1. A method of treating cancer, comprising administering to an individual having cancer a therapeutically effective amount of:
 - a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor; and
 - 5 a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor.
2. The method according to claim 1, wherein the PEBP inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.
- 10 3. The method according to claim 2, wherein the PEBP inhibitor is a small molecule.
4. The method according to any one of claims 1 to 3, wherein the PEBP inhibitor is a cytidine diphosphate (CDP)-ethanolamine pathway inhibitor.
- 15 5. The method according to claim 4, wherein the CDP-ethanolamine pathway inhibitor is selected from the group consisting of: an ethanolamine kinase (EK) inhibitor, a phosphoethanolamine cytidyltransferase (PCYT2) inhibitor, an ethanolaminephosphotransferase (EPT, Selenoi) inhibitor, and any combination thereof.
- 20 6. The method according to claim 5, wherein the CDP-ethanolamine pathway inhibitor is a PCYT2 inhibitor.
7. The method according to claim 6, wherein the PCYT2 inhibitor is meclizine.
- 25 8. The method according to any one of claims 1 to 3, wherein the PEBP inhibitor is a phosphatidylserine (PS) decarboxylation pathway inhibitor.
9. The method according to claim 8, wherein the PS decarboxylation pathway inhibitor is phosphatidylserine decarboxylase (PSD).
- 30 10. The method according to any one of claims 1 to 9, wherein the PFKFB3 inhibitor is selected from the group consisting of: a small molecule, an siRNA, and an miRNA.

11. The method according to claim 10, wherein the PFKFB3 inhibitor is a small molecule.

12. The method according to claim 11, wherein the PFKFB3 inhibitor is selected from the group consisting of: 1-(pyridin-4-yl)-3-(7-(trifluoromethyl)quinolin-2-yl)prop-2-en-1-one (PFK158), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and any combination thereof.

13. The method according to claim 12, wherein the PFKFB3 inhibitor is PFK158.

14. The method according to any one of claims 1 to 13, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered concurrently to the individual.

15. The method according to claim 14, wherein the administering comprises administering to the individual a pharmaceutical composition comprising the PEBP inhibitor and the PFKFB3 inhibitor.

16. The method according to any one of claims 1 to 13, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered sequentially to the individual.

17. The method according to claim 16, wherein the PFKFB3 inhibitor is administered to the individual prior to administration of the PEBP inhibitor to the individual.

18. The method according to any one of claims 1 to 17, wherein the PEBP inhibitor and the PFKFB3 inhibitor are administered to the individual by a route of administration independently selected from: oral administration, parenteral administration, and intranasal administration.

19. The method according to any one of claims 1 to 18, wherein the cancer is liver cancer.

20. The method according to claim 19, wherein the liver cancer is hepatocellular carcinoma (HCC).

21. The method according to claim 20, wherein the HCC is primary HCC.

22. The method according to claim 20, wherein the HCC is recurrent HCC.

23. The method according to any one of claims 1 to 18, wherein the cancer is a leukemia.

24. The method according to claim 23, wherein the leukemia is acute myeloid leukemia (AML).

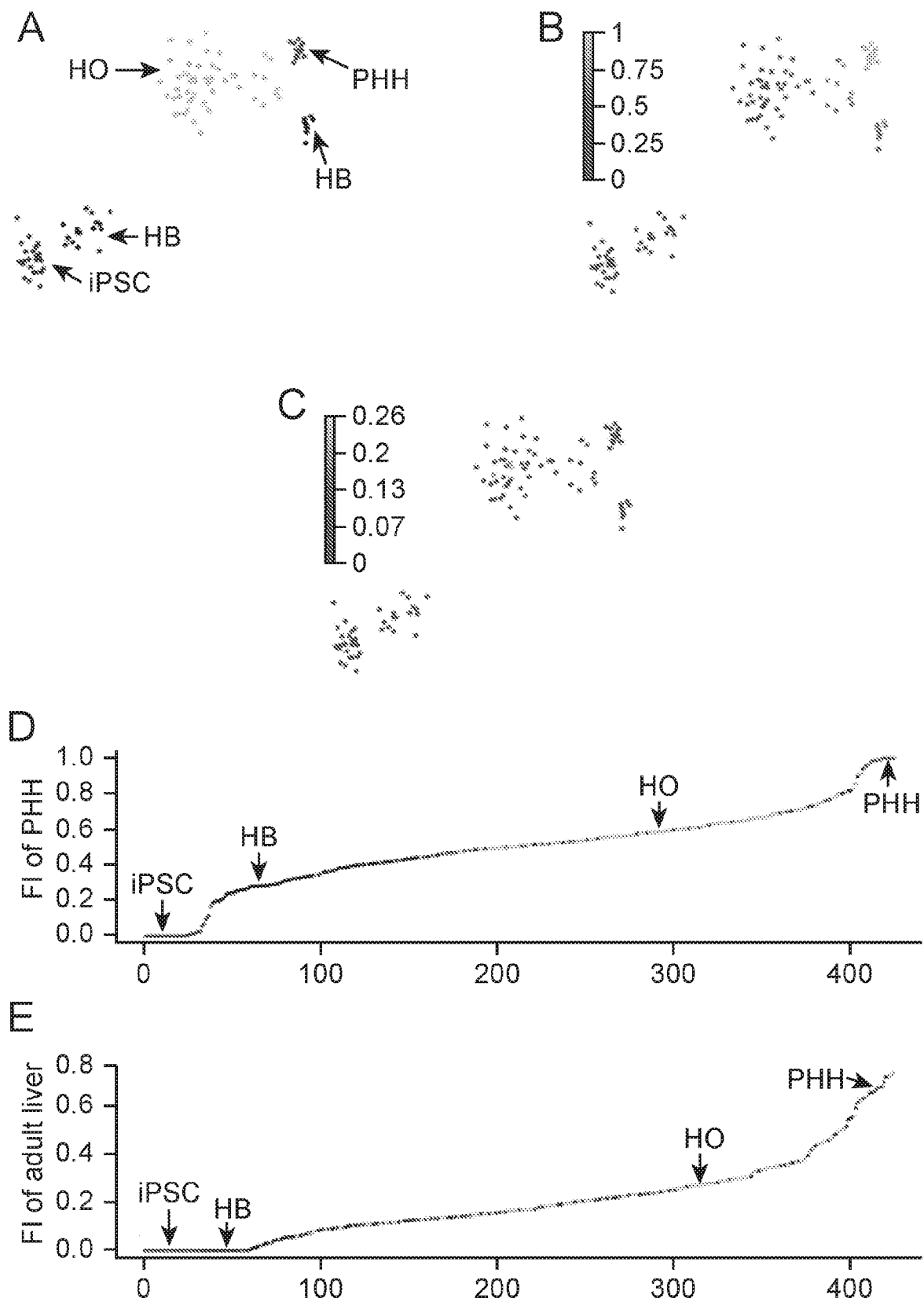
25. The method according to any one of claims 1 to 24, further comprising, prior to administering the PEBP inhibitor and the PFKFB3 inhibitor, determining the expression level of PFKBP3 in cancer cells obtained from the individual.

26. A pharmaceutical composition comprising:
a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor;
a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor; and
a pharmaceutically acceptable carrier.

27. A kit comprising:
a phosphatidylethanolamine biosynthetic pathway (PEBP) inhibitor;
a 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) inhibitor; and
instructions for administering the PEBP inhibitor and the PFKFB3 inhibitor to an individual in need thereof.

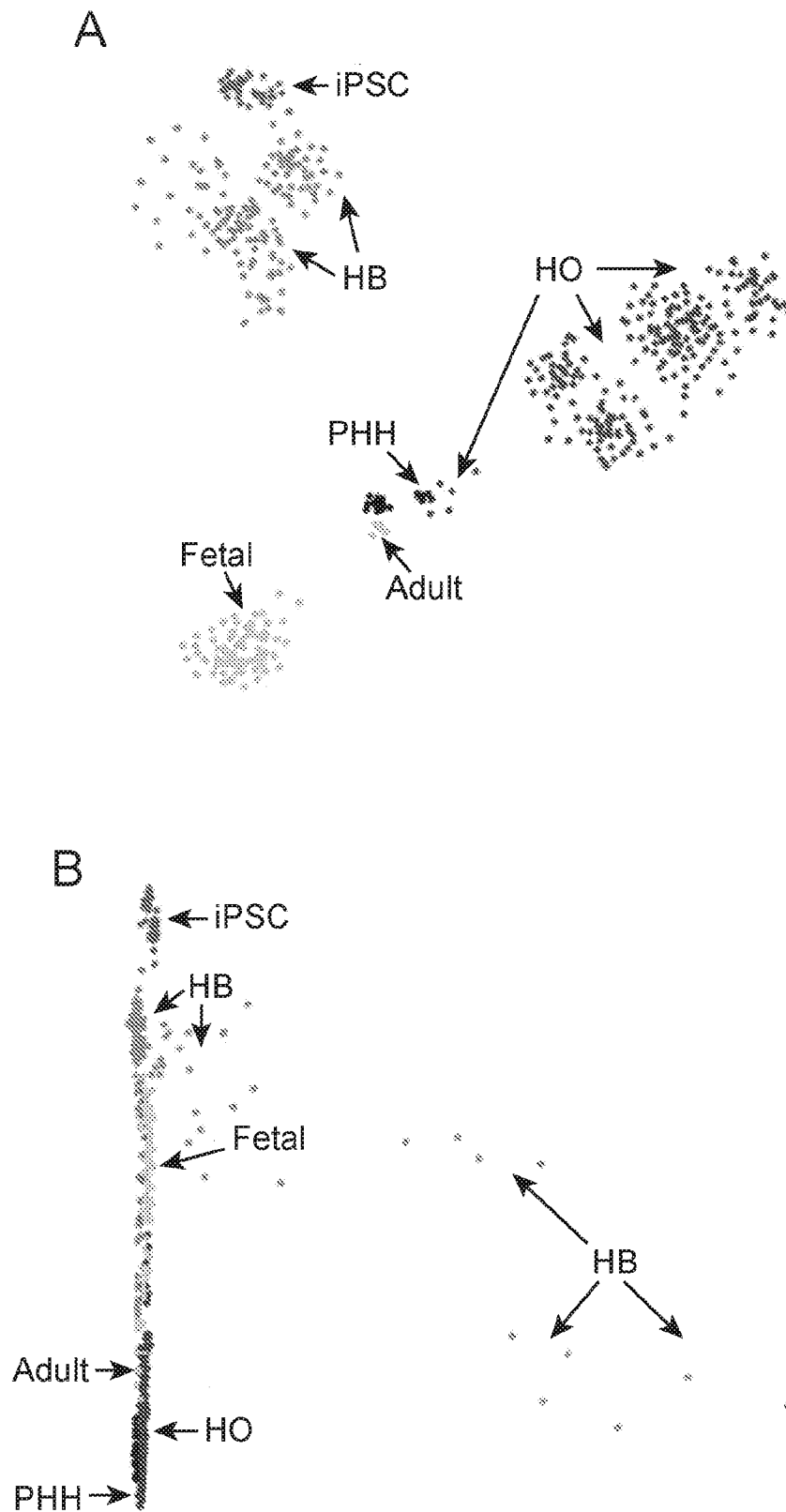
1/17

FIG. 1



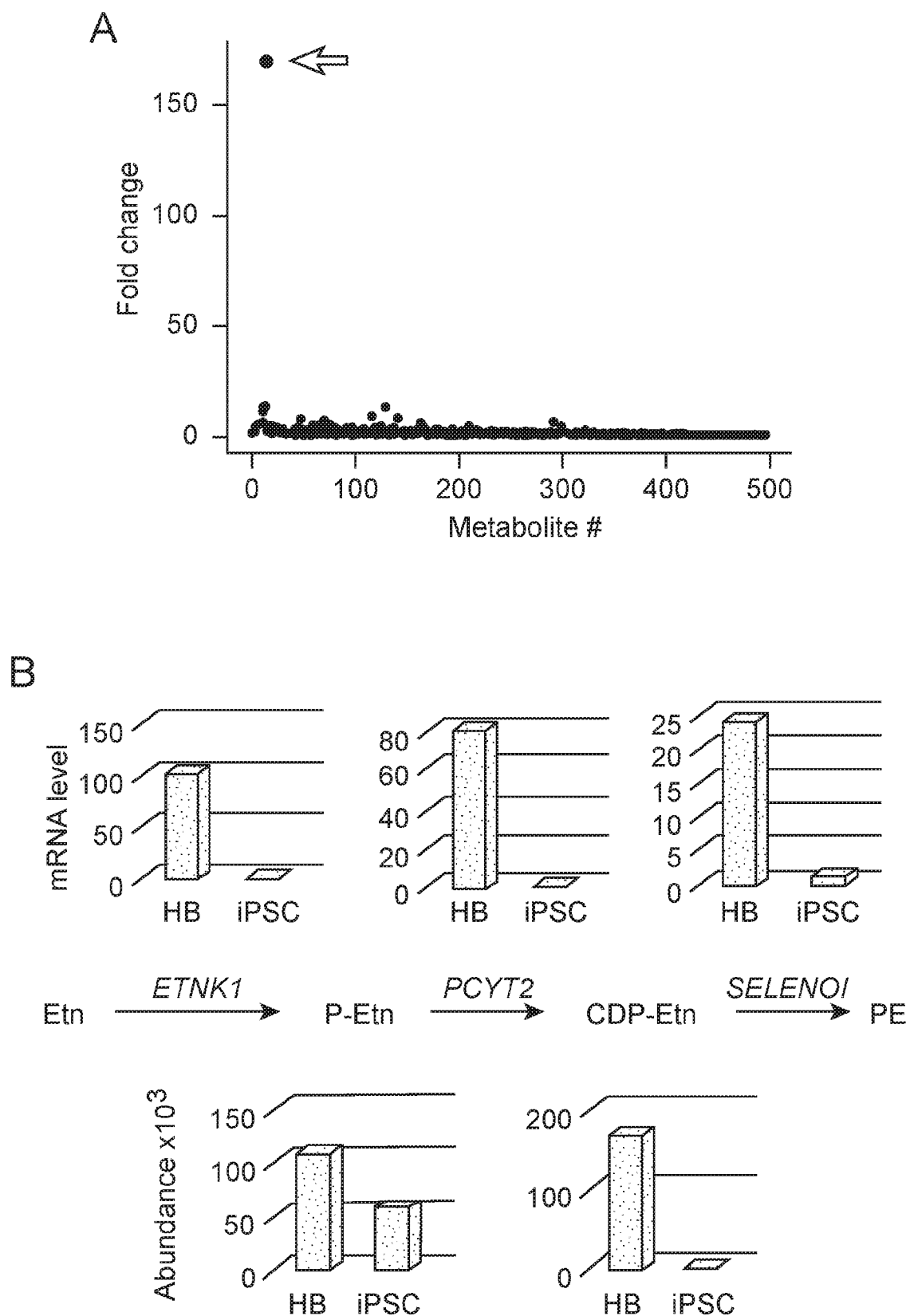
2/17

FIG. 2



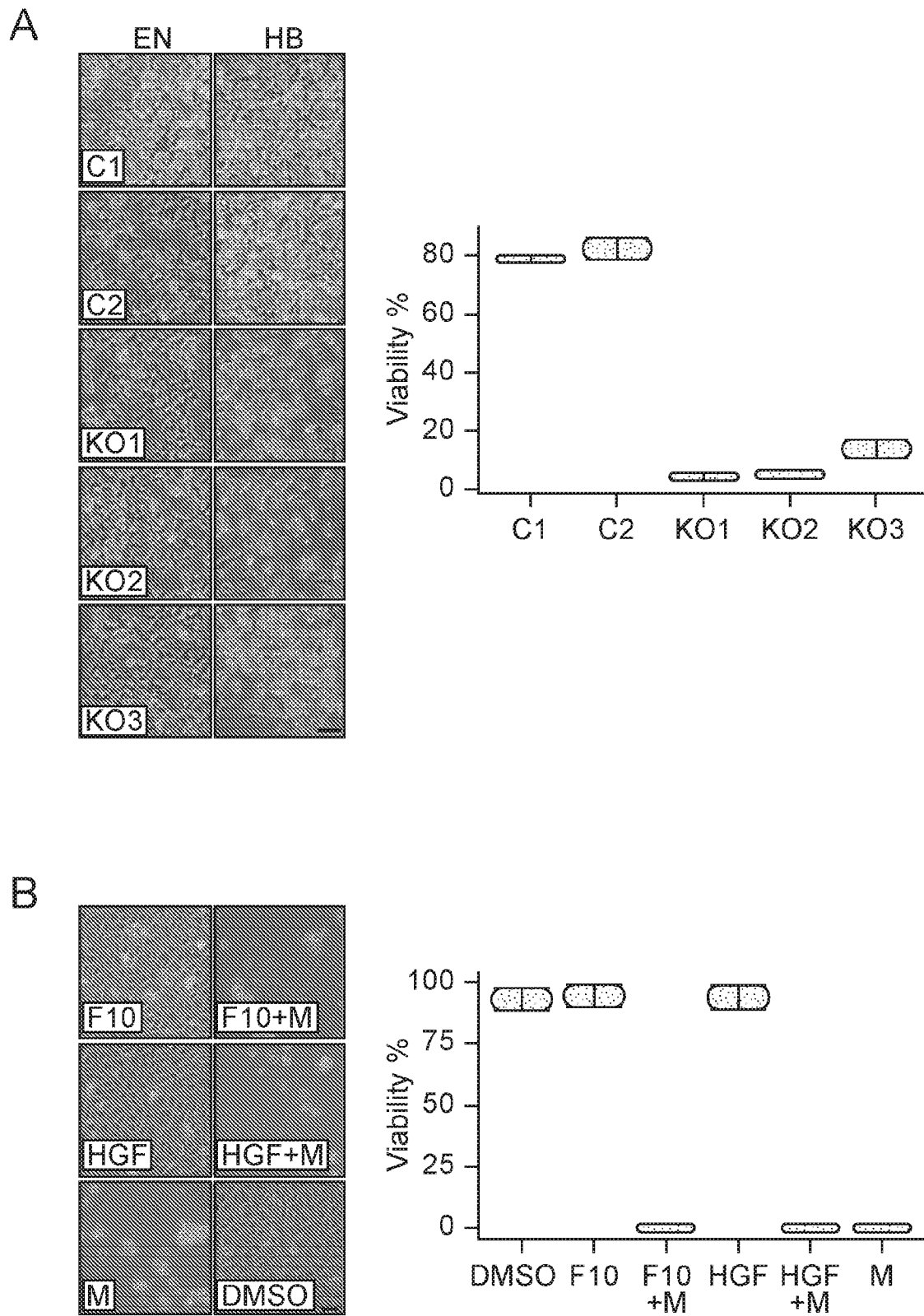
3/17

FIG. 3



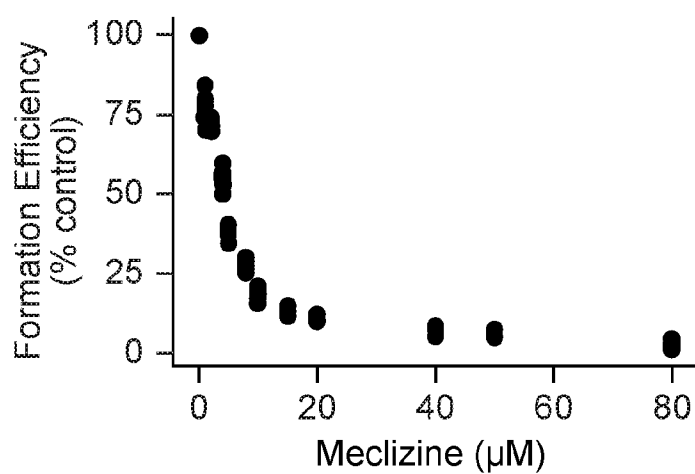
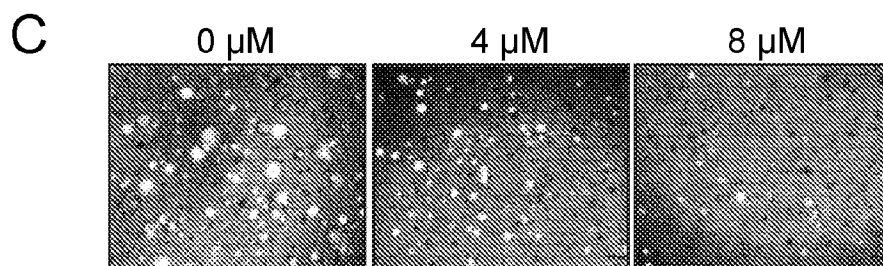
4/17

FIG. 4

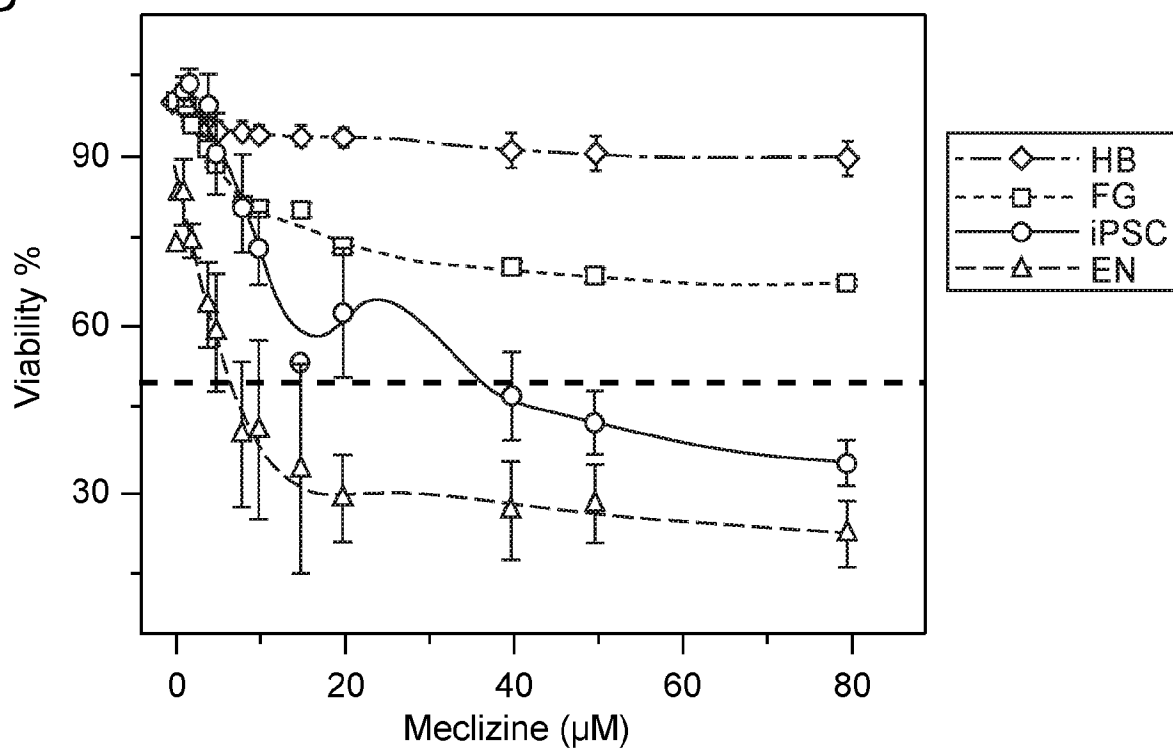


5/17

FIG. 4 (Cont.)

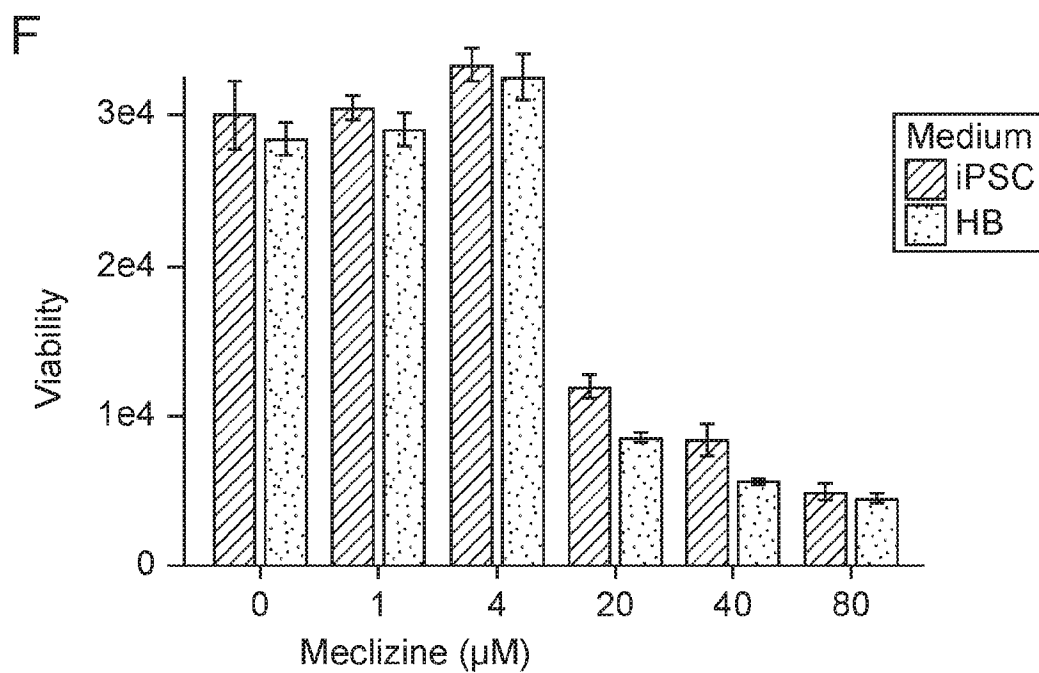
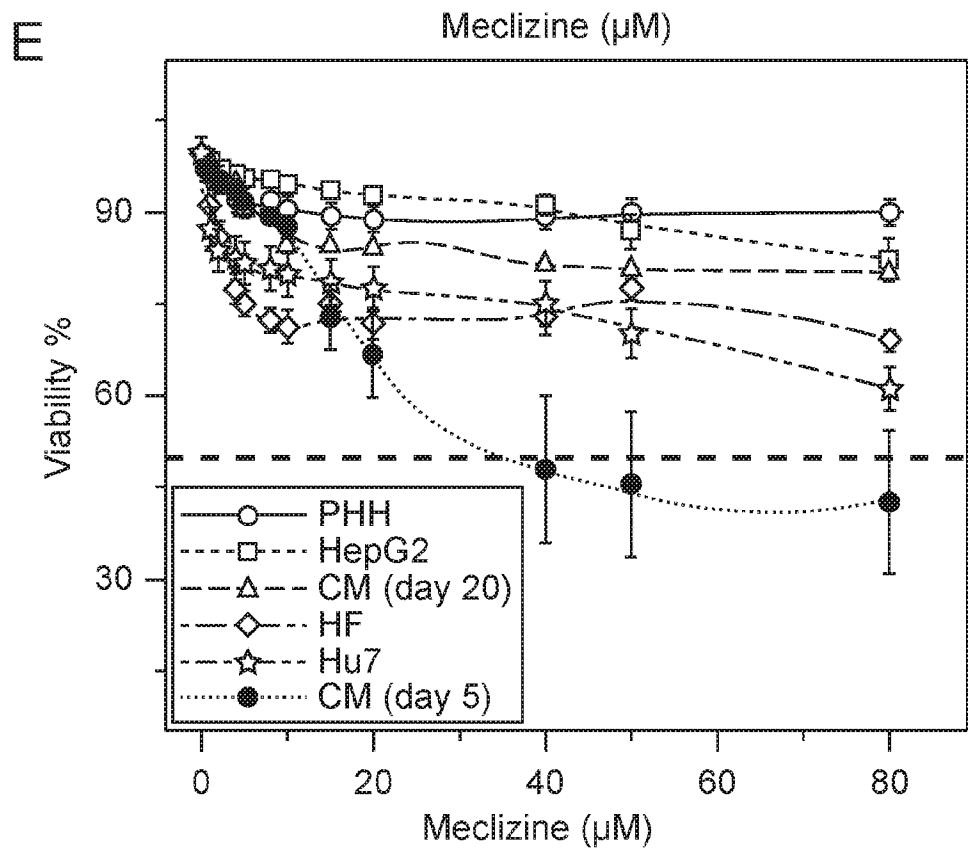


D



6/17

FIG. 4 (Cont.)



7/17

FIG. 5

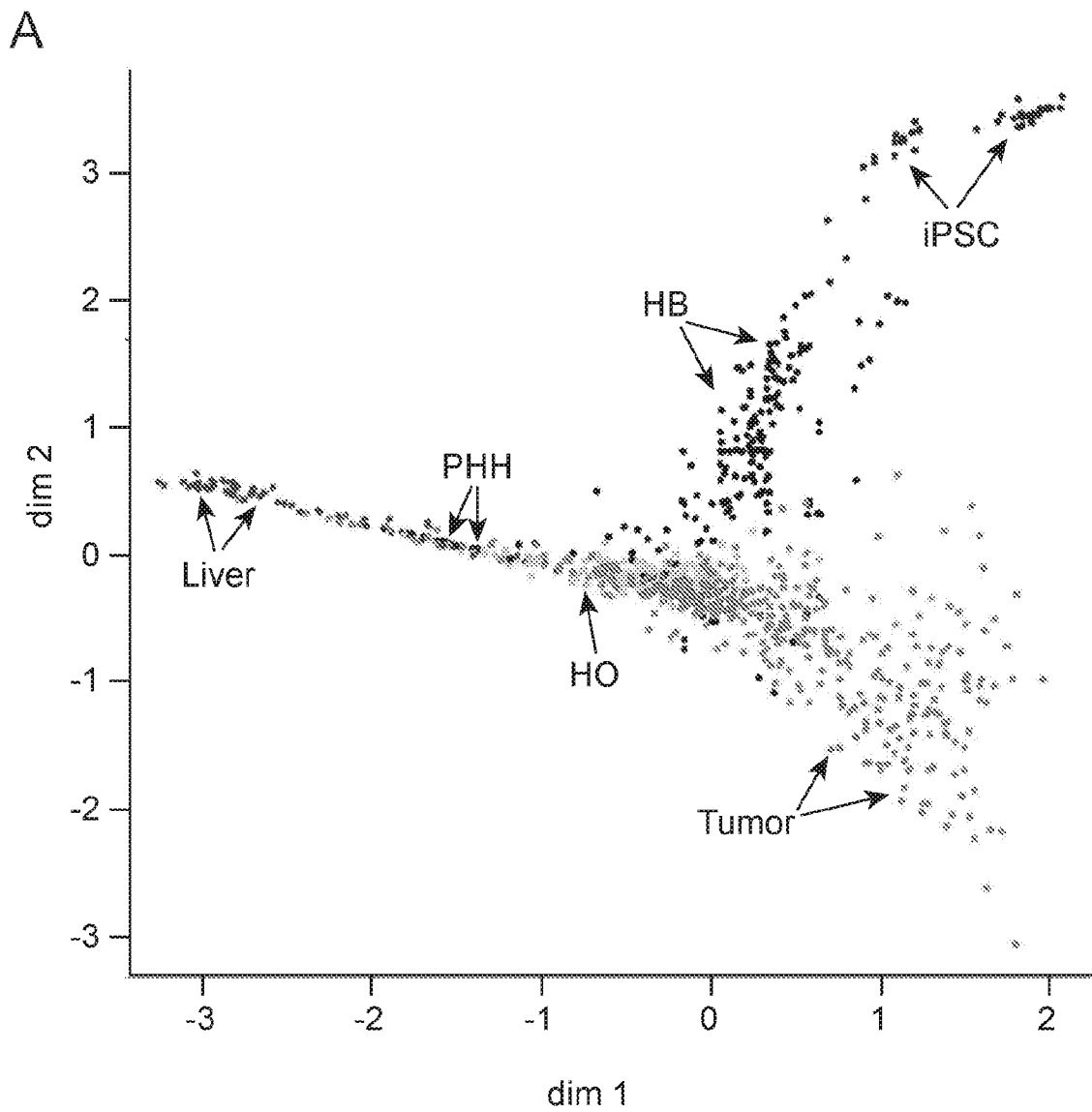
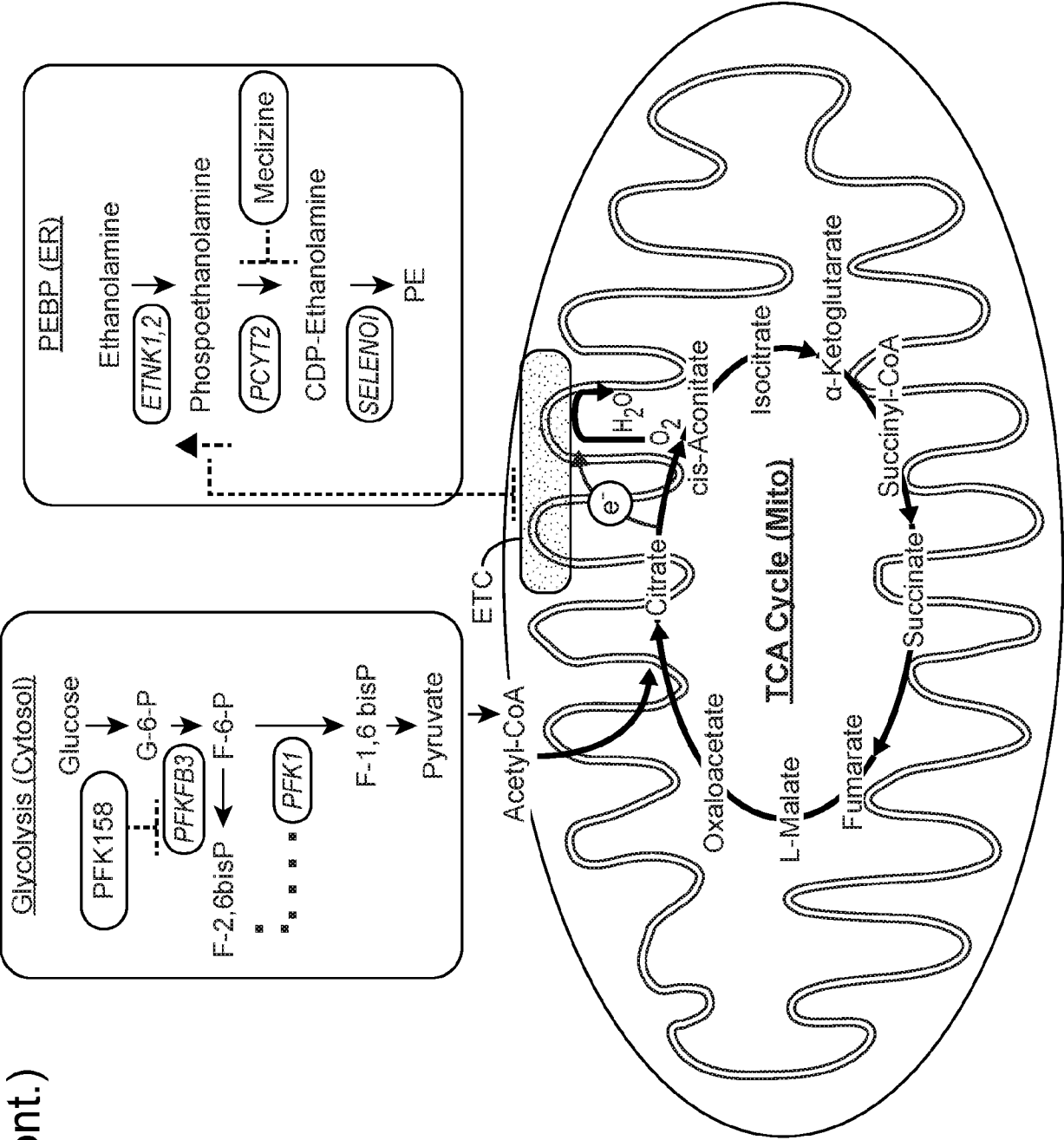


FIG. 5 (Cont.)

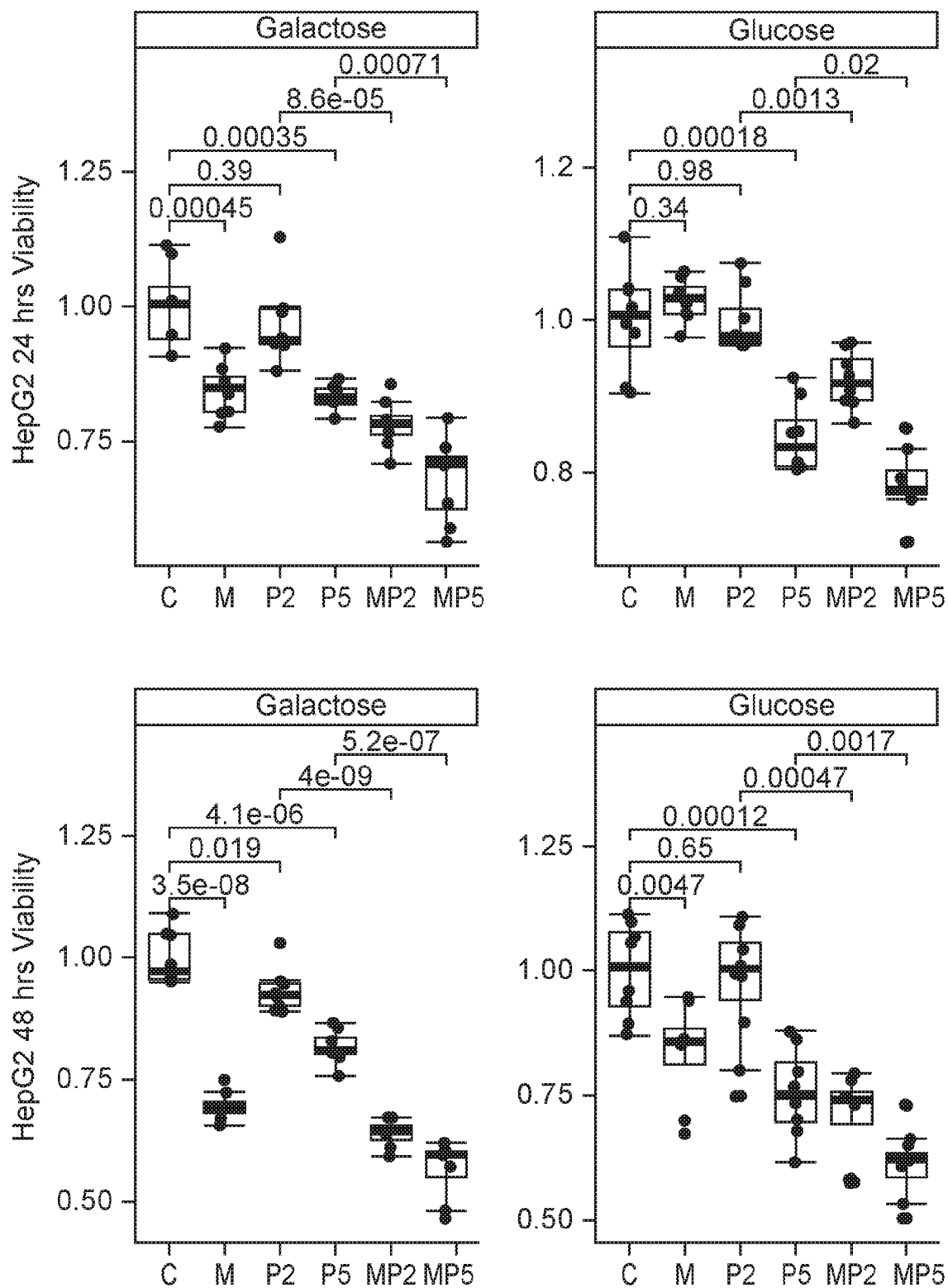
B



9/17

FIG. 5 (Cont.)

C



10/17

FIG. 5 (Cont.)

C (Cont.)

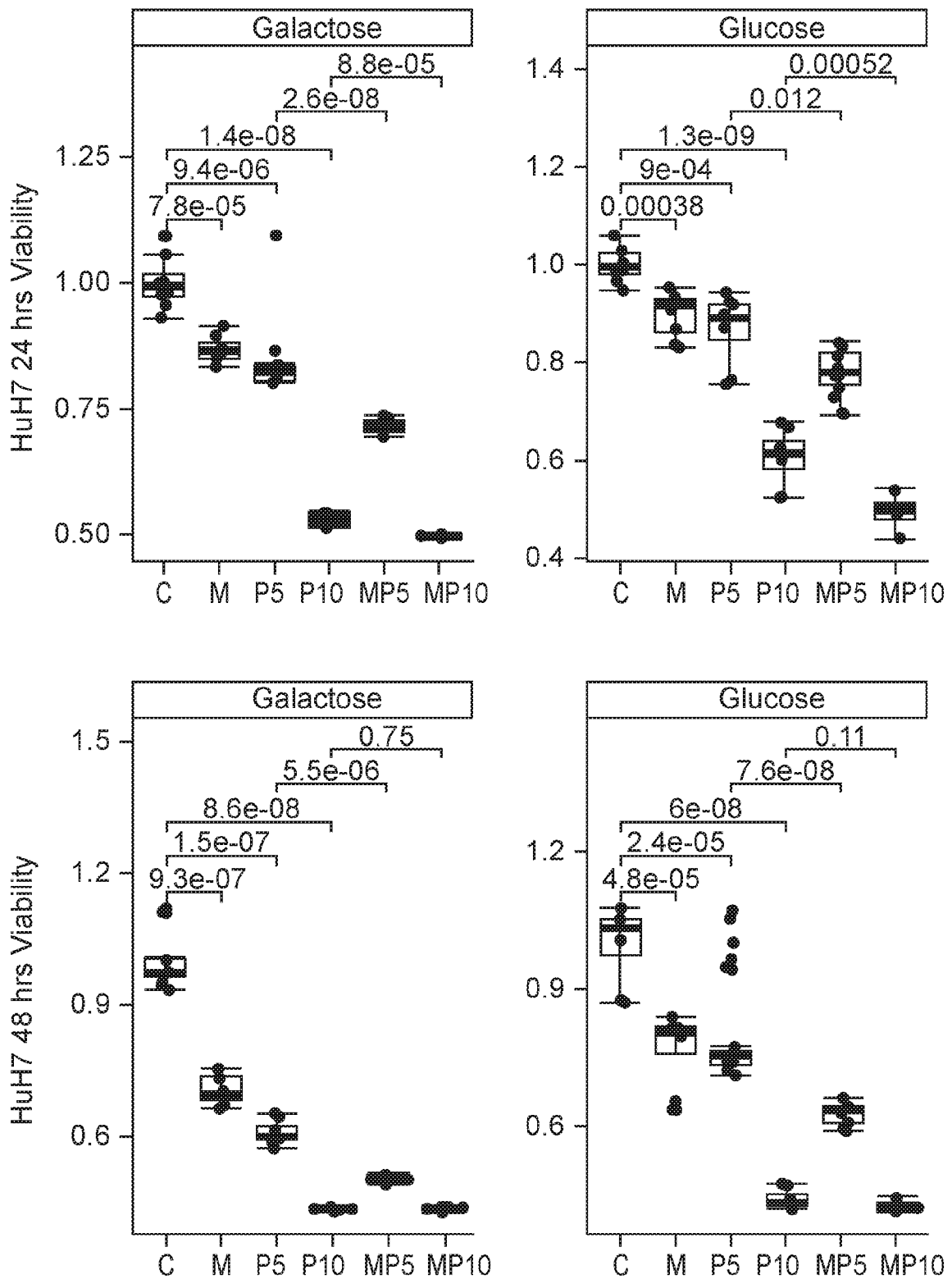
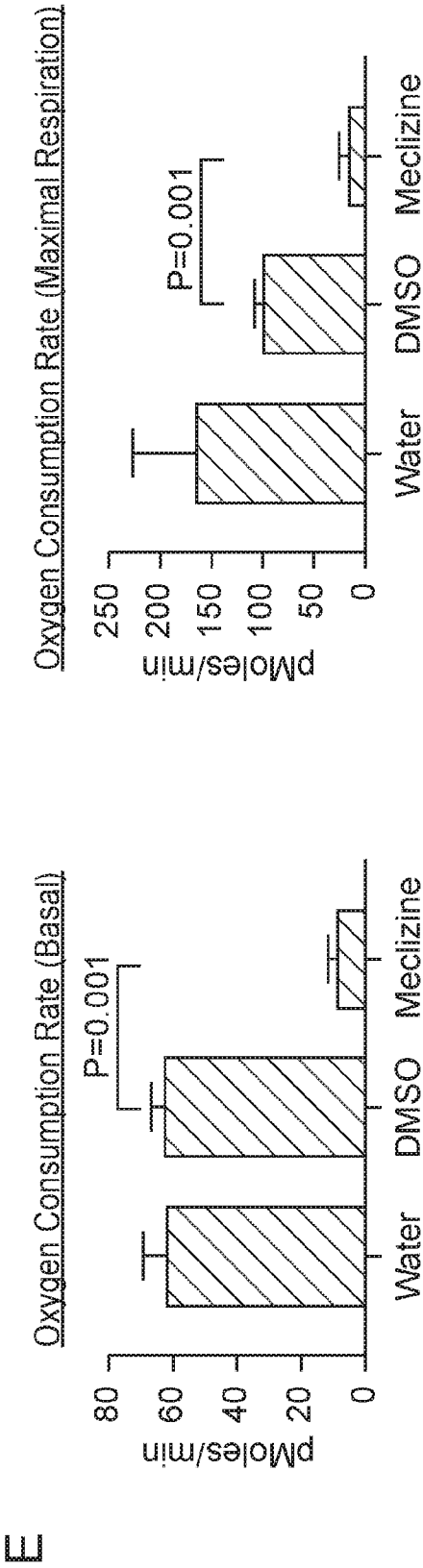
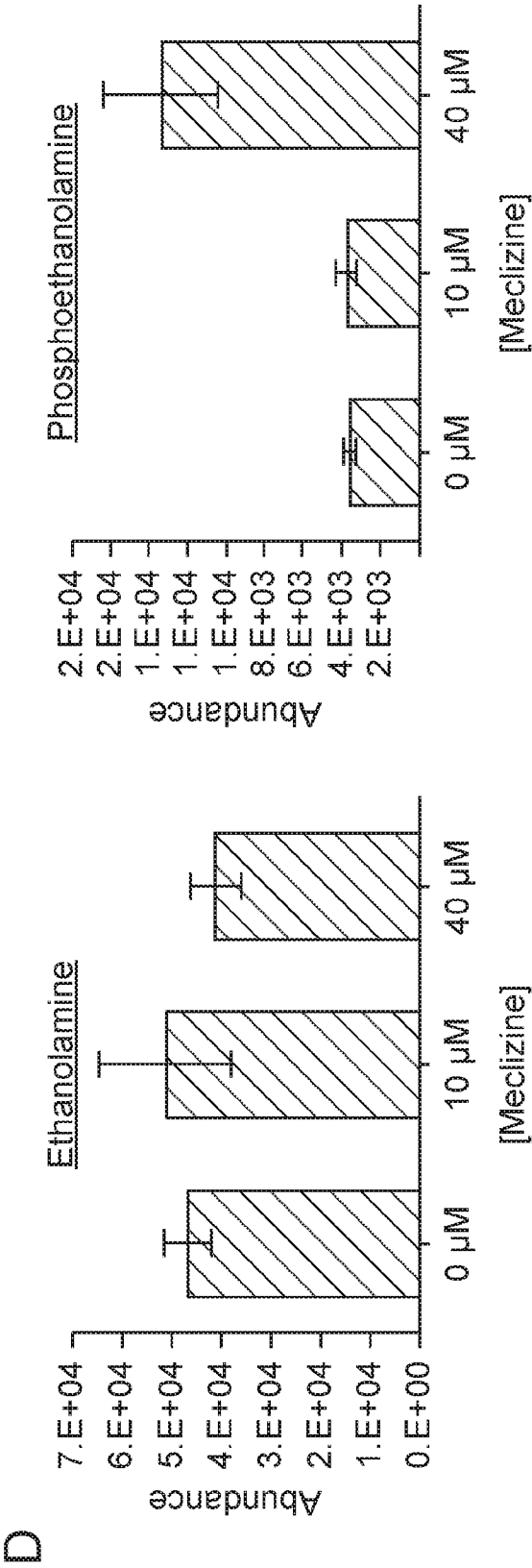
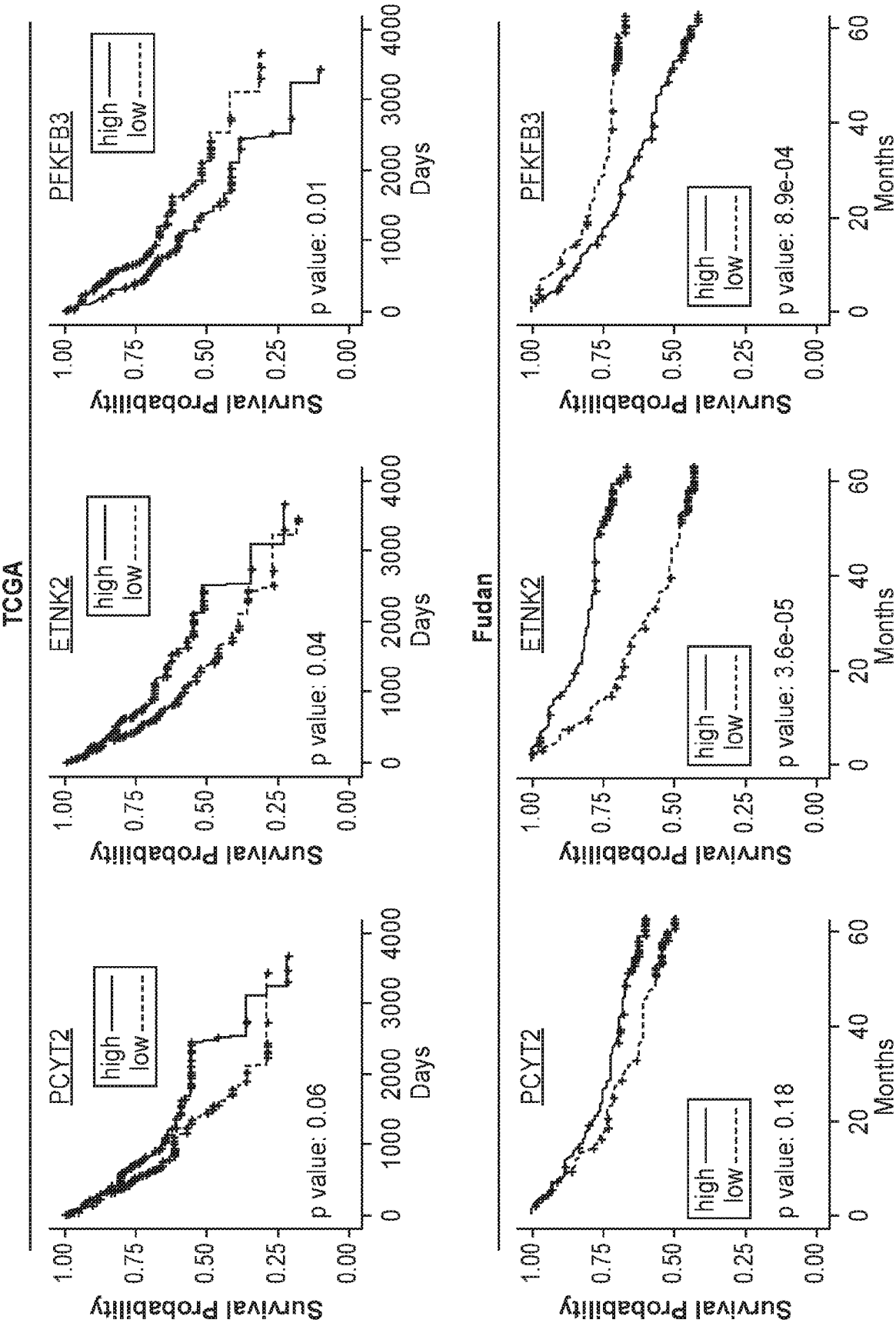


FIG. 5 (Cont.)



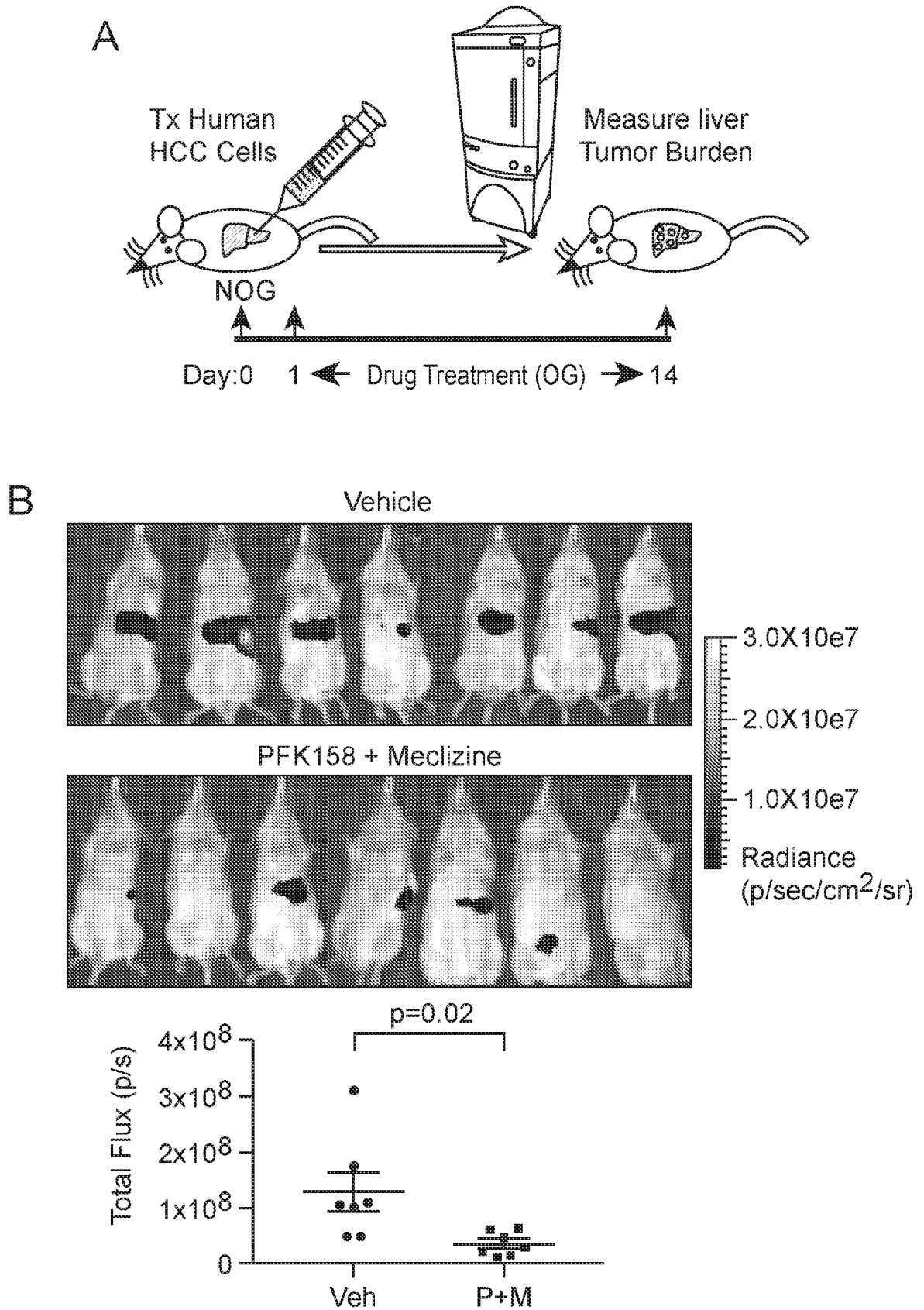
12/17

FIG. 6



13/17

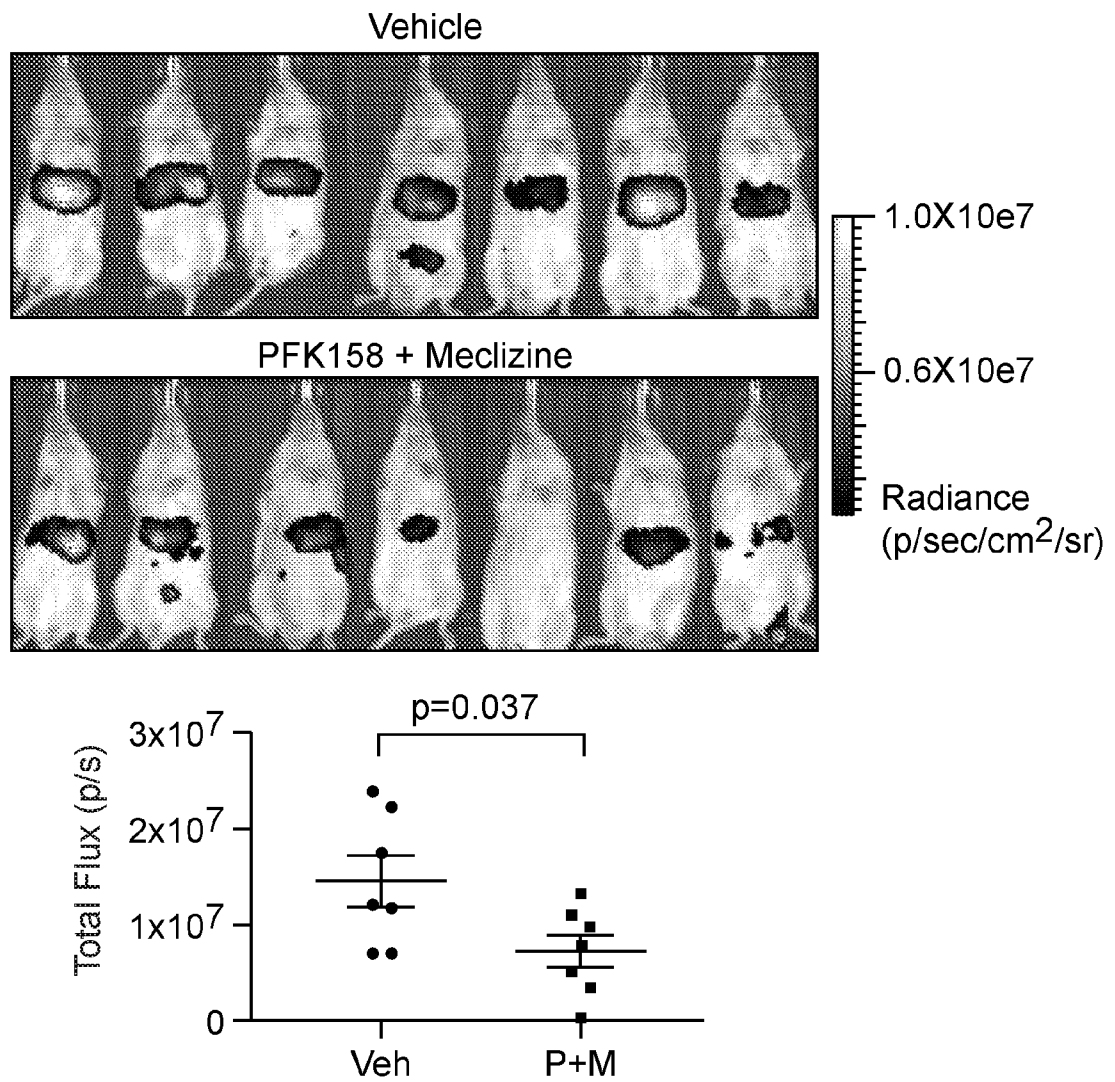
FIG. 7



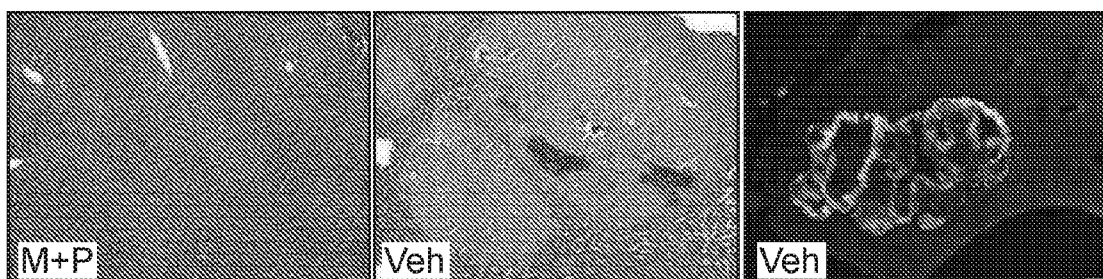
14/17

FIG. 7 (Cont.)

C



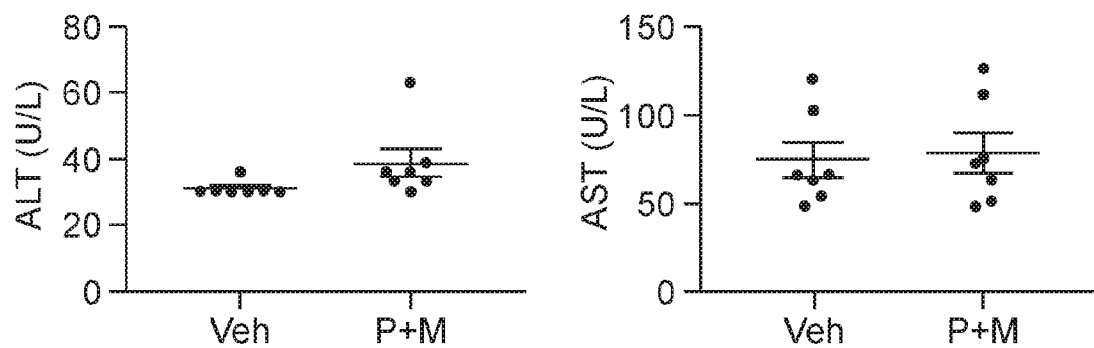
D



15/17

FIG. 7 (Cont.)

E



F

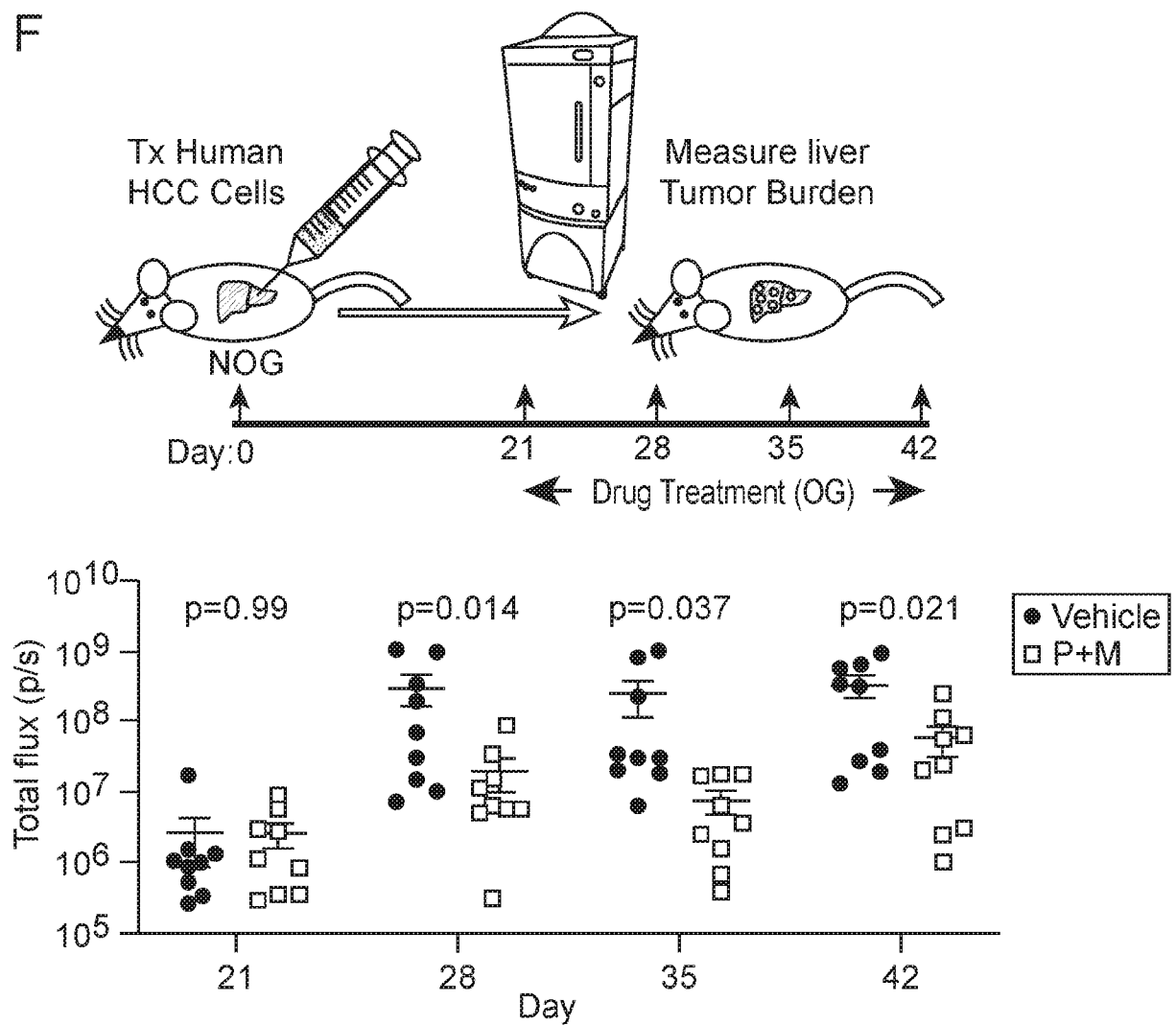


FIG. 8

Primary AML SU903

A

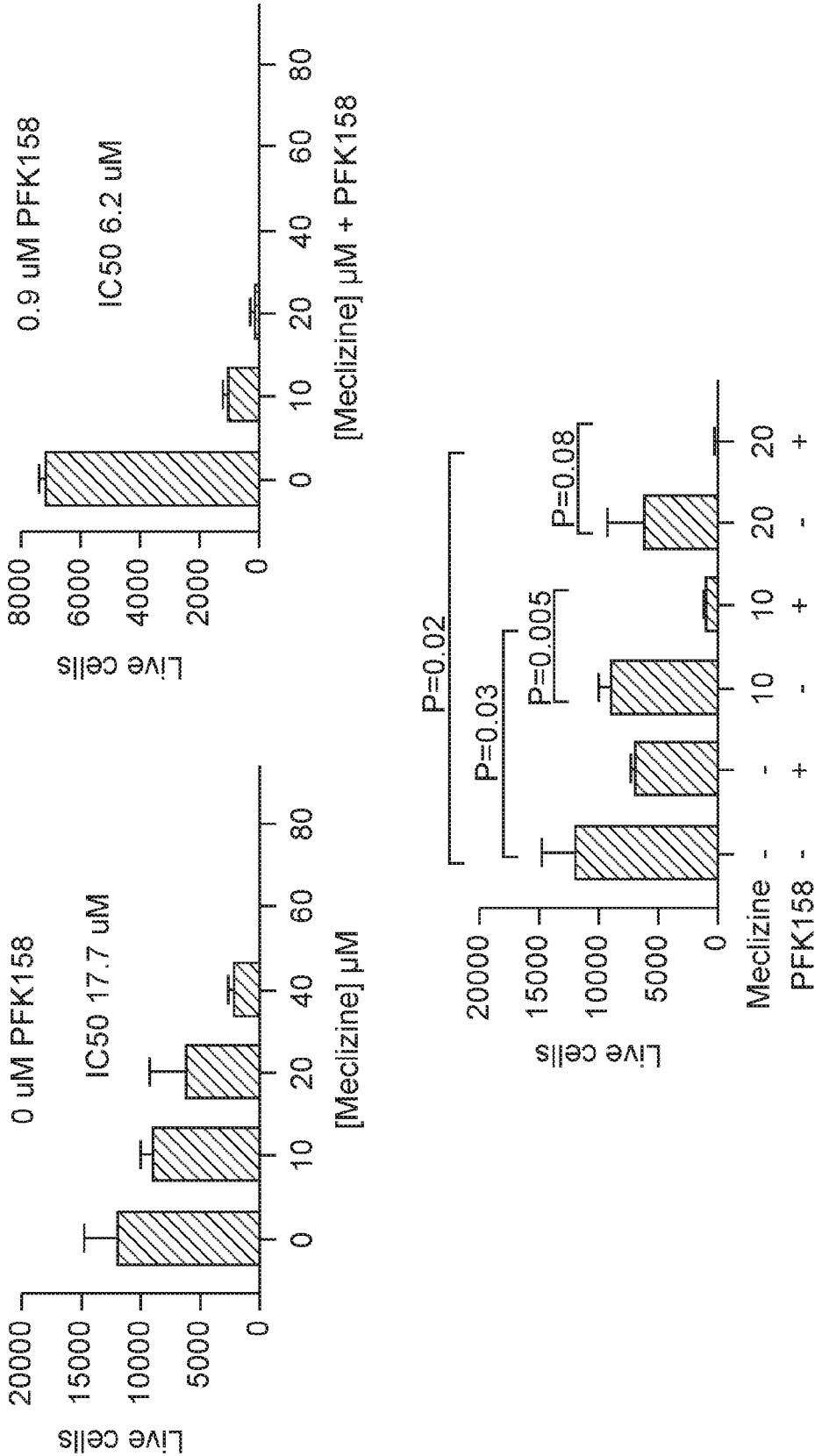


FIG. 8 (Cont.)

B

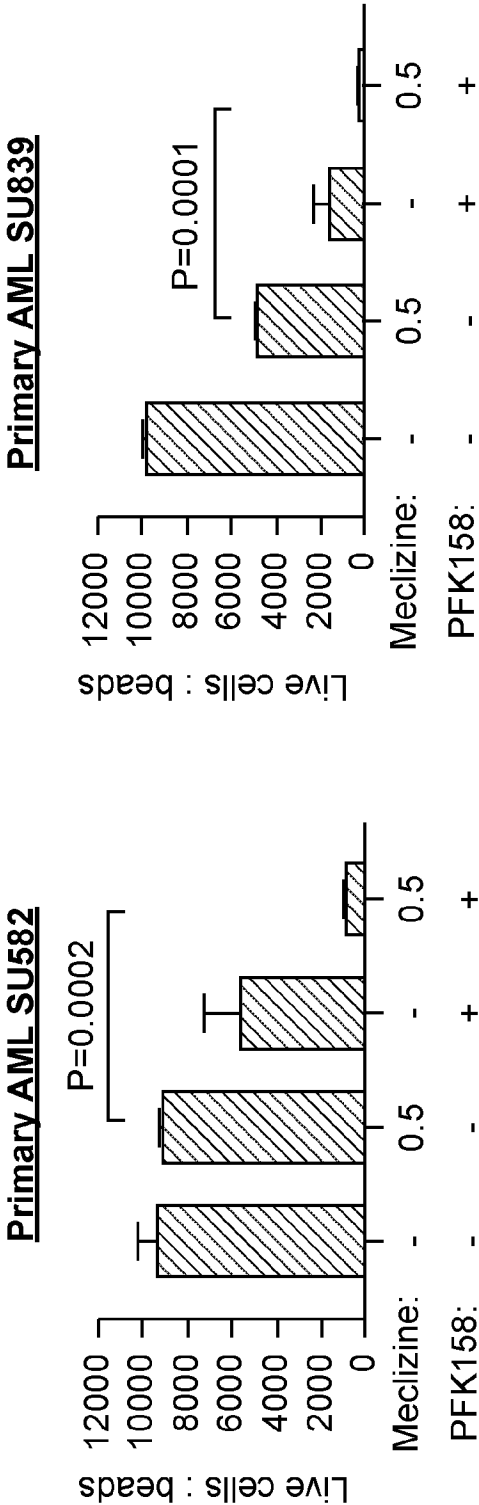
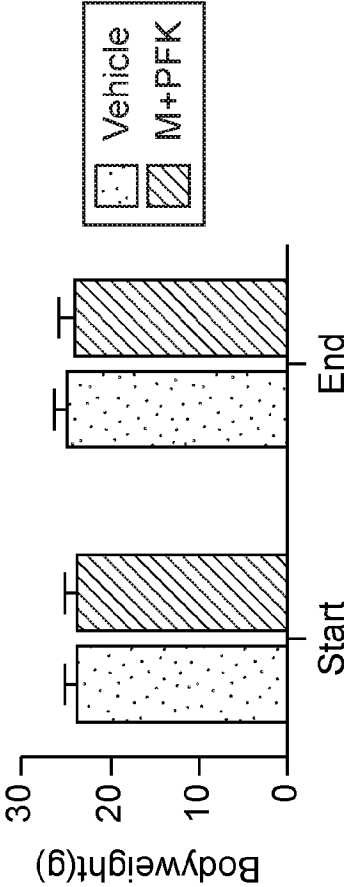


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/59877

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 39/395; A61K 45/06; C07K 16/24 (2020.01)

CPC - A61K 31/18; A61K 31/341; A61K 31/366; A61K 31/436; A61K 45/06; C12N 15/1135

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.
Y	US 2018/0071279 A1 (The General Hospital Corporation) 15 March 2018 (15.03.2018); para [0002], [0004], [0008]-[0009], [0023], [0025], [0028], [0146], [0158], [0245], abstract	1-9, 26-27
Y	WO 2017/095751 A1 (PARTIKULA LLC) 08 June 2017 (08.06.2017); pg. 2, ln 30, pg. 20, ln 7, pg. 11, ln 24-25	1-9, 26-27
A	WO 2012/040727 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 29 March 2012 (29.03.2012); para [00313]	1-9, 26-27
A	WO 2017/161096 A1 (TARVEDA THERAPEUTICS, INC.) 21 September 2017 (21.09.2017); entire document	1-9, 26-27
A	US 2018/0207273 A1 (Dranoff et al.) 26 July 2018 (26.07.2018); entire document	1-9, 26-27
A	US 2012/0177749 A1 (Tapolsky et al.) 12 July 2012 (12.07.2012); entire document	1-9, 26-27

☐ 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

"D" document cited by the applicant in the international application

"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

22 January 2020

Date of mailing of the international search report

06 FEB 2020

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

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/59877

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.: 10-25
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.