Sorafenib/Regorafenib and Phosphatidyl Inositol 3 Kinase/Thymoma Viral Proto-Oncogene Inhibition Interact to Kill Tumor Cells

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ABSTRACT

The present studies were undertaken to determine whether the multikinase inhibitors sorafenib/regorafenib cooperated with clinically relevant, phosphatidyl inositol 3 kinase (PI3K)-thymoma viral proto-oncogene (AKT) inhibitors to kill tumor cells. In liver, colorectal, lung, breast, kidney, and brain cancer cells, at clinically achievable doses, sorafenib/regorafenib and the PI3K inhibitor acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[*a*]phenanthren-11-yl ester (PX-866) cooperated in a greater than additive fashion to kill tumor cells. Cells lacking phosphatase and tensin homolog were as sensitive to the drug combination as cells expressing the protein. Similar data were obtained using the AKT inhibitors perifosine and 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-*f*] [1,6]naphthyridin-3(2*H*)-one hydrochloride (MK2206). PX-866 treatment abolished AKT/glycogen synthase kinase 3 (GSK3) phosphorylation, and cell killing correlated with reduced activity of AKT and mammalian target of

rapamycin (mTOR). Expression of activated AKT and to a lesser extent activated mTOR reduced drug combination lethality. Expression of B-cell lymphoma-extra large or dominant negative caspase 9, but not cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein short, protected cells from the drug combination. Treatment of cells with PX-866 increased protein levels of p62, lysosome-associated membrane protein 2 (LAMP2), and microtubule-associated protein light chain (LC) 3 and LC3II that correlated with a large increase in LC3-green fluorescent protein (GFP) vesicle numbers. Exposure of PX-866 treated cells to sorafenib reduced p62 and LAMP2 levels, decreased the ratio of LC3 to LC3II, and reduced LC3-GFP vesicle levels. Knockdown of Beclin1 or autophagy-related 5 suppressed drug toxicity by ~40%. In vivo, sorafenib and PX-866 or regorafenib and MK2206 cooperated to suppress the growth of established HuH7 and HCT116 tumors, respectively. Collectively our data demonstrate that the combination of sorafenib family kinase inhibitors with inhibitors of the PI3K/AKT pathway kills tumor cells in vitro and in vivo.

Introduction

Elevated activity within the phosphatidyl inositol 3-kinase (PI3K) pathway is a well-recognized event in many tumor types (Rodon et al., 2013). Activation of the PI3K pathway can occur at multiple levels, including activating mutation of the

PI3K catalytic subunit p110, constitutive activation of upstream stimulatory molecules such as Harvey (H)/Kirsten (K)-RAS and growth factor receptors, and sporadic activating mutations in the enzymes phosphoinositide-dependent kinase-1 and thymoma viral proto-oncogene (AKT) (Hafsi et al., 2012). PI3K pathway signaling has been linked to antiapoptotic effects both at the level of death receptors and at the mitochondrion (Sancho-Martinez and Martin-Villalba, 2009; Fulda, 2013). PI3K signaling, through mammalian target of rapamycin (mTOR), has also been shown to regulate autophagic flux with elevated mTOR activity suppressing autophagy (Sun et al., 2012). As the PI3K pathway has been validated as a tumor

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ABBREVIATIONS: AKT, thymoma viral proto-oncogene; ATG5, autophagy-related 5; BAD, Bcl-2-associated death promoter; BAX, Bcl-2-associated X protein; BCL, B-cell lymphoma; BEZ235, 2-methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]quinolin-1-yl]phenyl}propanenitrile; BIM, Bcl-2-interacting mediator of cell death; c-FLIP-s, cellular FLICE (FADD-like IL-1 β -converting enzyme) inhibitory protein short; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; JNK-1/2, c-Jun N-terminal kinases 1/2; LC, light chain; MCL-1, myeloid cell leukemia-1; MEK, mitogen-activated extracellular regulated kinase; MK2206, 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-*f*] [1,6]naphthyridin-3(2*H*)-one hydrochloride; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphatidyl inositol 3 kinase; PX-866, acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[*a*]phenanthren-11-yl ester; REGOR, regorafenib; RTK, receptor tyrosine kinase; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase UTP nick end labeling; XL, extra large.

biomarker, several attempts have been made by pharmaceutical companies to inhibit pathway members, most notably the catalytic p110 subunit of PI3K and the plexstrin homology/allosteric domain of AKT. Examples of the former include the p110 inhibitor acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-[4-diallyl-aminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[*a*]phenanthren-11-yl ester (PX-866) and of the latter, perifosine or 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-*f*] [1,6]naphthyridin-3(2*H*)-one hydrochloride (MK2206) (Ihle et al., 2009; Locatelli et al., 2013). These agents are undergoing clinical evaluation in a variety of tumor types (Cho et al., 2012; Hong et al., 2012). PX-866 is a derivative of wortmannin, and acts as a *suicide inhibitor* of p110 $\alpha/\beta/\delta/\gamma$. Dual inhibition of the PI3K and extracellular signal-regulated kinase (ERK)1/2 pathways has been proposed as one approach to kill tumor cells and there are multiple clinical trials combining PI3K and mitogen-activated protein kinase (MEK)1/2 inhibitors; however, there are no *ongoing* studies examining the interaction of PI3K pathway inhibitors with a multikinase inhibitor such as sorafenib or regorafenib (see www.clinicaltrials.gov; Rahmani et al., 2009).

Sorafenib is a multikinase inhibitor designed to be an inhibitor of RAF-1 in the ERK1/2 pathway (Gollob et al., 2006). Many of the actions of sorafenib, including its antiangiogenic effects, could not be simplistically linked *only* to modulation of ERK1/2, and it was subsequently noted that sorafenib inhibited class III receptor tyrosine kinases (Matsuda and Fukumoto, 2011). The steady-state (7 days) $C_{\max}$ for sorafenib is $\sim 21 \mu\text{M}$ in plasma, with $>95\%$ of the drug protein-bound, based on in vitro human serum binding assays; nonetheless *patient data* would argue that at least $\sim 5\text{--}10 \mu\text{M}$ of the drug *has* to be bioavailable in a tumor based on its single-agent effects on decreasing both ERK1/2 phosphorylation and reducing myeloid cell leukemia-1 (MCL-1) protein expression in tumor cells that are not specifically oncogene-addicted (Elser et al., 2007, see below). Our in vitro and in vivo data have tended to argue, using several sorafenib plus “drug” combinations, that platelet-derived growth factor receptor β is a major target of sorafenib for its interactions with other agents, e.g., with histone deacetylase inhibitors (Park et al., 2008). A major biologic effect of sorafenib at in vitro concentrations in the $\sim 3 \mu\text{M}$ range is the induction of an endoplasmic reticulum stress/unfolded protein response, with reduced expression of proteins that have short half-lives, such as MCL-1 and B-cell lymphoma-extra large (BCL-XL) (Rahmani et al., 2005, 2007). Reduced MCL-1 levels due to sorafenib exposure have been linked in many tumor types to increased levels of apoptosis (Kim et al., 2008; Gores and Kaufmann, 2012). Studies by our group have also linked high-dose single-agent sorafenib exposure to an increase in the levels of autophagic markers, including increased numbers of light chain (LC) 3–green fluorescent protein (GFP) vesicles and elevated expression of Beclin1 and autophagy-related 5 (ATG5) (Park et al., 2008); however, lower sorafenib concentrations only caused a modest transient alteration in autophagy flux. Other studies from our groups have shown that based on the sorafenib dose the induction of endoplasmic reticulum stress may be a “protective” or a “toxic” event in the cellular response to the drug.

The regulation of the apoptotic machinery by the PI3K and ERK1/2–receptor tyrosine kinase (RTK) pathways is complex. Inhibition of the PI3K and/or the ERK1/2 pathways can lead

to activation of the c-Jun N-terminal kinases 1/2 (JNK1/2) pathway, which can control the activity of the proapoptotic BH3 domain proteins Bcl-2–associated X protein (BAX) and Bcl-2 homologous antagonist/killer (Yacoub et al., 2010). Reduced ERK1/2 activity can facilitate increased expression of the proapoptotic BH3 domain protein Bcl-2–interacting mediator of cell death (BIM) and dephosphorylation and activation of Bcl-2–associated death promoter (BAD) (Rahmani et al., 2013). Expression of the caspase 8 inhibitor cellular FLICE (FADD-like IL-1b–converting enzyme) inhibitory protein short (c-FLIP-s), as well as other caspase inhibitors such as X-linked inhibitor of apoptosis protein, can be regulated by the PI3K and ERK/RTK pathways (Sheridan et al., 2008). Thus an *a priori* prediction for the biologic effects of sorafenib combined with a PI3K inhibitor on tumor cells would be reduced expression of protective BCL-2 family proteins and increased activity/decreased expression of toxic BH3 domain proteins and caspase inhibitors.

The present studies were designed to determine whether inhibitors of RAF-1/RTKs (sorafenib, regorafenib) cooperated with inhibitors of the PI3K pathway (PX-866, perifosine, MK2206) to kill a variety of tumor cell types (liver, kidney, lung, colorectal, breast, brain).

Materials and Methods

Phospho-/total-antibodies were purchased from Cell Signaling Technologies (Danvers, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All drugs were purchased from Selleckchem (Houston, TX). Commercially available validated short hairpin RNA molecules to knockdown RNA/protein levels were from Qiagen (Valencia, CA). Antibody reagents, other kinase inhibitors, caspase inhibitors cell culture reagents, and noncommercial recombinant adenoviruses have been previously described (Caron et al., 2005; Park et al., 2008; Yacoub et al., 2010; Bareford et al., 2011; Cruickshanks et al., 2012). In vitro assays were performed using trypan blue exclusion and a Scepter hand-held mini-flow cytometer instrument (Millipore, Billerica, MA).

Cell Culture and In Vitro Exposure of Cells to Drugs

All established cancer lines were cultured at 37°C (5% v/v CO_2) in vitro using RPMI supplemented with 10% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. All primary human glioblastoma multiforme cells were initially cultured at 37°C (5% (v/v) CO_2) in vitro using RPMI supplemented with 2% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids at 37°C (5% v/v CO_2), then after cleansing of fibroblasts, cultured in vitro using RPMI supplemented with 5% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. For short-term cell killing assays and immunoblotting, cells were plated at a density of 3×10^3 per cm^2 and 24 hours after plating were treated with various drugs, as indicated. In vitro small molecule inhibitor treatments were from a 100 mM stock solution of each drug and the maximal concentration of vehicle (dimethylsulfoxide) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study.

Cell Treatments, SDS-PAGE, and Western Blot Analysis

Cells were treated with various drug concentrations, as indicated in the figure legends. SDS-PAGE and immunoblotting was performed as described in references (Park et al., 2008; Yacoub et al., 2010; Bareford et al., 2011; Cruickshanks et al., 2012). For SDS-PAGE and immunoblotting, cells were plated at 5×10^5 cells/ cm^2 and treated with drugs at the indicated concentrations and after the indicated time of treatment, lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β -mercaptoethanol, 0.02%

bromophenol blue), and the samples were boiled for 30 minutes. The boiled samples were loaded onto 10–14% SDS-PAGE and electrophoresis was run overnight (10–100 $\mu\text{g}/\text{lane}$ based on the gel size). Proteins were electrophoretically transferred onto 0.22- μm nitrocellulose, and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized using an Odyssey infrared imager (Li-Cor, Lincoln, NE). For presentation, immunoblots were digitally assessed using the provided Odyssey imager software. The data sets presented are the $\pm$ -fold increase $\pm$ S.E.M. ($n = 3$) in expression of the indicated protein compared with glyceraldehyde-3-phosphate dehydrogenase loading control; for phosphoproteins the $\pm$ -fold increase $\pm$ S.E.M. (at least $n = 3$) is normalized to the total protein level of the indicated kinase or substrate. Errors are not numerically shown due to space restrictions in the figure panels; any indicated significant differences between the expression/phosphorylation levels of proteins are indicated by an asterisk or other annotation and have a $P < 0.05$. Images have their color removed and figures generated in Microsoft PowerPoint.

Recombinant Adenoviral Vectors; Infection In Vitro

We generated and purchased as previously noted recombinant adenoviruses as per references (Park et al., 2008; Yacoub et al., 2010; Bareford et al., 2011; Cruickshanks et al., 2012). Cells were infected with these adenoviruses at an approximate multiplicities of infection as indicated in the figure and legend. Cells were incubated for 24 hours to ensure adequate expression of transduced gene products prior to drug exposures.

Detection of Cell Death by Trypan Blue, Hoechst, Terminal Deoxynucleotidyl Transferase UTP Nick-End Labeling (TUNEL), and Flow Cytometric Assays

Cells were harvested by trypsinization with trypsin/EDTA for ~ 10 minutes at 37°C . Cell death assays were performed as described in references (Park et al., 2008; Yacoub et al., 2010; Bareford et al., 2011; Cruickshanks et al., 2012). Briefly, for in vitro analyses of short-term cell death effects, cells were treated with vehicle or drugs for the times indicated in the figure legends. For apoptosis assays where indicated, cells were isolated at the indicated times, and either subjected to trypan blue cell viability assay by counting in a light microscope or fixed to slides, and stained using a commercially available Diff Quick (Giemsa) assay kit or Hoechst stain (Polysciences, Inc., Warrington, PA). Alternatively, the cell volume assay using the Scepter system was carried out to determine cell viability out as per the manufacturer's instructions (Millipore).

Assessment of Autophagy

Cells were transfected with a plasmid to express a GFP-tagged form of LC3 (ATG8). For analysis of cells transfected with the GFP-LC3 construct, the GFP-LC3-positive vesicularized cells were examined under the $40\times$ objective of a Zeiss Axiovert fluorescent microscope (Park et al., 2008; Yacoub et al., 2010; Bareford et al., 2011; Cruickshanks et al., 2012).

Plasmid Transfection

Plasmids. Cells were plated as described above and 24 hours after plating, transfected. Plasmids (0.5 μg) expressing a specific mRNA or appropriate vector control plasmid DNA was diluted in 50 μl of serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 μl Lipofectamine 2000 (Invitrogen/Life Technologies, Grand Island, NY) was diluted into 50 μl of serum-free and antibiotic-free medium. Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 minutes. This mixture was added to each well/dish of cells containing 200 μl of serum- and antibiotic-free medium for a total volume of 300 μl and the cells were incubated for 4 hours at 37°C . An equal

volume of $2\times$ medium was then added to each well. Cells were incubated for 48 hours, then treated with drugs. To assess transfection efficiency of plasmids, we used a plasmid to express GFP and defined the percentage of cells being infected as the percentage of GFP+ cells. For all cell lines the infection efficiency was $>70\%$.

Small Interfering RNA. Cells were plated in 60-mm dishes from a fresh culture growing in log phase as described above, and 24 hours after plating, transfected. Prior to transfection, the medium was aspirated and 1 ml of serum-free medium was added to each plate. For transfection, 10 nM of the annealed small interfering RNA (siRNA), the positive sense control double-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines), were used (predominantly Qiagen; occasional alternate siRNA molecules were purchased from Ambion, Inc., Austin, TX). Ten nanomolar siRNA (scrambled or experimental) was diluted in serum-free media. Four microliters of Hiperfect (Qiagen) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temp for 10 minutes, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37°C for 2 hours. One ml of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37°C for 24–48 hours before replating (50×10^3 cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0–48 hours). Trypan blue exclusion assays and SDS-PAGE/immunoblotting analyses were then performed at the indicated time points.

Animal Studies. Athymic female NCr- ν/ν mice (National Cancer Institute at Frederick) weighing ~ 20 g were used for this study (Bareford et al., 2011). Mice were maintained under pathogen-free conditions in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, Washington, DC, and the National Institutes of Health, Bethesda, MD. Mice were injected with 1.0×10^7 HuH7/HCT116 cells (~ 40 mice per separate experiment to obtain at least four usable tumors per group) in 10 μl of growth medium. Fourteen days after tumor cell implantation mice were PO administered drugs or the drug combination QD for 3 days (see figure legends). Animals were monitored daily and tumor volume determined every fourth day. When the volume of the tumor reached >2000 mm^3 , animals were killed.

Immunohistochemistry and Staining of Fixed Tumor Sections

Postsacrifice, tumors were fixed in optimum cutting temperature compound (Tissue Tek; Sakura, Torrance, CA) and cryostat sectioned (Leica) as 10- μm sections. Nonspecific binding was blocked with a 2% (v/v) rat sera, 1% (v/v) bovine sera, 0.1% (v/v) Triton X100, 0.05% (v/v) Tween-20 solution, then sections were stained for cell signaling pathway markers: anti-Ki67, anticlaved caspase 3, antiphospho-ERK1/2. For staining of sectioned tumors, primary antibodies were applied overnight, sections washed with phosphate buffer solution, and secondary antibodies applied for detection. Apoptotic cells with double-stranded DNA breaks were detected using the Upstate TUNEL Apoptosis Detection Kit (Lake Placid, NY) according to the manufacturer's instructions. Slides were applied to high powered light/confocal microscopes (Zeiss LSM 510 Metaconfocal scanning microscope; Zeiss HBO 100 microscope with Axio Cam MRm camera) at the indicated magnification in the figures and legends ($40\times$). Data shown are representative slides from several sections from the same tumor, with multiple tumors (from multiple animals and multiple experiments) having been examined ($n =$ at least 3–6 animals, tumors).

Data Analysis

Comparison of the effects of various treatments was performed using one way analysis of variance and a two-tailed Student's t test.

Differences with a P value of <0.05 were considered statistically significant. Statistical examination of in vivo animal survival data used log rank statistical analyses between the different treatment groups. Experiments shown are the means of multiple individual points from multiple experiments ($\pm$ S.E.M.).

Results

Initial studies examined the toxic interaction of sorafenib and PX-866 in hepatoma, colon, kidney, lung, breast, and

brain cancer cells. PX-866 (0.5 μ M) did not exhibit significant toxicity over 24 hours in the tumor cells used in our studies (Fig. 1). Sorafenib (1.0–3.0 μ M) and PX-866 cooperated in a dose-dependent fashion to enhance sorafenib lethality in multiple hepatoma cell lines (Fig. 1, A–C). As judged by DNA condensation/Hoechst staining, sorafenib and PX-866 also cooperated to kill cells (Fig. 1D). Sorafenib and PX-866 enhanced the radiosensitivity of hepatoma cells (Fig. 1E). Similar data to that in hepatoma cells combining sorafenib and PX-866 was obtained in kidney cancer, breast cancer,

Fig. 1. Sorafenib (SOR) and PI3K pathway inhibitors interact to kill multiple tumor cell types. (A) HEPG2 cells were treated with vehicle (dimethylsulfoxide; DMSO), sorafenib (sor, 1.0–3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (B) HEP3B cells were treated with vehicle (DMSO), sorafenib (sor, 1.0–3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (C) HuH7 cells were treated with vehicle (DMSO), sorafenib (1.0–3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (D) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by levels of nuclear DNA condensation ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. Upper inset: Images of HEP3B and HuH7 cells treated with vehicle, sorafenib, and PX-866. (E) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (3 μ M), and PX-866 (0.5 μ M) (PX+SOR). Thirty minutes after drug treatment, cells were irradiated (4 Gy, XRT). Cells were isolated 12 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than corresponding unirradiated control}$. (F) A498, UOK121LN, and CAKI cells were treated with vehicle (DMSO), sorafenib (2.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (G) BT549, BT474, and MCF7 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (H) Glioblastoma multiforme (GBM)5 and GBM14 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (I) A549, H460, and H1975 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M) as indicated. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $\#P < \text{greater than vehicle control}$. (J) HEPG2, HEP3B, and HuH7 cells were plated (250 single cells/well) in six well plates. Cells were permitted to attach and after 12 hours treated with drugs. Cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (PX, 0.5 μ M) as indicated for 24 hours. Media was removed and replaced with drug-free media and cells permitted to grow and form colonies for the next 14 days ($n = 3$ in sextuplicate, $\pm$ S.E.M.). $\#P < \text{less than vehicle control}$.

lung cancer, and brain cancer cells, with similar levels of killing observed in cells regardless of phosphatase and tensin homolog expression (Fig. 1, F–I). Notably, the $\sim$ IC₅₀ of sorafenib in our system, with cells cultured with 10% FCS to reduce phosphorylation of wild-type vascular endothelial growth factor 2 and ERK2, was $\sim$ 2 μ M and $\sim$ 2 μ M, respectively. Sorafenib and PX-866 cooperated to suppress colony formation of multiple liver cancer cell lines *regardless of CD95 expression status* (Fig. 1J; n.b. HuH7 cells lack expression of CD95). The interaction between the agents was synergistic with Combination Index values below 0.70 (Table 1). Of note, in vitro the drug combination did not kill primary human hepatocytes or peripheral mononuclear cells (data not shown).

Based on our effects with PX-866 and sorafenib we next determined, using other kinase inhibitors and molecular tools, the effect of drug exposure on tumor cell viability. The AKT inhibitor perifosine enhanced sorafenib toxicity (Fig. 2A). In hepatoma and colon cancer cells, PX-866 enhanced the lethality of the novel fluorinated inhibitor derived from sorafenib and regorafenib (Fig. 2, B and C) (Carr et al., 2013). Perifosine enhanced the lethality of regorafenib in hepatoma cells (Fig. 2D). The AKT inhibitor MK2206 enhanced regorafenib toxicity in hepatoma cells (Fig. 2E; Table 1). The AKT inhibitor MK2206 enhanced regorafenib toxicity in colon cancer cells; cells expressing an H-RAS V12 mutant that specifically activates RAF-ERK were more sensitive to regorafenib, whereas cells expressing a mutant H-RAS V12 that specifically activates PI3K-AKT were more sensitive to MK2206 (Fig. 2F). Of note, HCT116 cells deleted for K-RAS D13 but still expressing a mutant active p110 α PI3K were less sensitive to MK2206 (Caron et al., 2005). Expression of dominant negative AKT or combined knockdown of p110 α / β expression enhanced cell killing by sorafenib (Fig. 2, G and H).

Studies next defined changes in the activity of signal transduction pathways and apoptosis regulator mediators. Treatment of cells with PX-866 abolished AKT phosphorylation (Fig. 3A). Combined, but not individual, treatment of cells with PX-866 and sorafenib reduced phosphorylation of mTOR, ERK1/2, and p70 S6K. Drug combination treatment either had no effect or *decreased* JNK1/2 pathway activity. Drug treatment increased the levels of BAX and BIM, decreased the

phosphorylation of BAD, and decreased the expression of MCL-1 and BCL-XL.

Expression of an activated form of AKT strongly suppressed the ability of PX-866 to enhance sorafenib toxicity (Fig. 3B). This correlated with reduced activation of BAX (Fig. 3B, inset). Expression of an activated form of mTOR but not of p70 S6K more modestly suppressed drug combination toxicity (Fig. 3, C and D). Expression of an activated form of MEK1 did not suppress drug combination toxicity (Fig. 3E). Activated MEK1 prevented the drug-induced expression of BIM. Expression of dominant negative glycogen synthase kinase 3 (dnGSK3) suppressed drug toxicity to a similar extent as activated AKT, which correlated with less activation of BAX and a weaker reduction in the expression of MCL-1 (Fig. 3F, graph and upper inset left). We have previously noted that sorafenib increased SRC Y416 phosphorylation, indicative of activation, and we noted that the drug combination also increased GSK3 tyrosine phosphorylation in an SRC-dependent manner (Fig. 3F, upper inset right). Knockdown of BAX, but not BIM, protected cells from the drug combination (Fig. 3G). Overexpression of BCL-XL or dominant negative caspase 9, but not c-FLIP-s, suppressed drug combination toxicity (Fig. 3, H–J).

In prior studies we have shown that sorafenib can modestly and transiently increase the number of LC3-GFP vesicles in hepatoma cells; others have shown that PX-866 can also increase the numbers of autophagosomes/vesicles in cells (Gwak et al., 2011). Treatment of hepatoma cells with sorafenib, and to a much greater extent PX-866, increased the numbers of LC3-GFP vesicles in hepatoma cells (Fig. 4A). Combined treatment of hepatoma cells with sorafenib and PX-866 reduced LC3-GFP vesicle levels to near those observed in cells treated with sorafenib alone. Expression of ca-mTOR suppressed the reduction in vesicle numbers comparing PX-866 and PX-866 + sorafenib treatments (Fig. 4B). Sorafenib treatment appeared to enhance the levels of a faster migrating form of regulatory-associated protein of mTOR (Raptor) on SDS-PAGE, and combined exposure of cells to PX-866 and sorafenib abolished the interaction between Raptor and mTOR (Fig. 4B, upper inset blot). Treatment with sorafenib weakly reduced p62 levels, whereas PX-866 strongly increased expression of both p62 and LC3 and LC3II (Fig. 4C). At later times the changes in expression of p62 and LAMP2 confirmed that PX-866 was stalling autophagy flux, an effect that did not occur in sorafenib- and PX-866-treated cells (Fig. 4D). In prior studies combining sorafenib and the histone deacetylase inhibitor vorinostat in hepatoma cells, the induction of autophagy was shown to be a protective event against CD95-induced caspase 8 activation (Zhang et al., 2008). As expected from our prior data and those of others, knockdown of Beclin1 or ATG5 abolished the induction of autophagy as judged by formation of LC3-GFP vesicles (data not shown). In the present studies we noted that knockdown of ATG5 or Beclin1 reduced but did not abolish sorafenib and PX-866 toxicity in our hepatoma cell lines (Fig. 4E).

We determined whether sorafenib combined with PX-866 or regorafenib combined with MK2206 cooperated in vivo to suppress tumor growth and increase animal survival. In preformed HuH7 tumors both sorafenib and PX-866 reduced tumor growth (Fig. 5A). Combined exposure to sorafenib and PX-866 reduced tumor growth to a greater extent than either individual drug. Isolation of tumors at day 15 revealed that the drug combination caused morphologic alterations in the tumor (H&E staining), reduced proliferation (Ki67 staining),

TABLE 1

Sorafenib/regorafenib cooperate with PI3K/AKT inhibitors to kill tumors cells

HuH7 and HCT116 cells (250–1500 cells/well) were plated in sextuplicate and allowed to attach for 12 hours. Cells, HuH7 [sorafenib + P-866] and HCT116 [regorafenib + MK2206], were treated with sorafenib (SOR, 2.0–4.0 μ M), regorafenib (REGOR, 0.5–1.0 μ M), PX-866 (0.5–1.0 μ M), MK2206 (MK, 1.0–2.0 μ M) for 24 hours. Media was removed, plates washed, and cells cultured for the next 10–14 days in media lacking drugs. After $\sim$ 10 days media was removed, plates washed, and cells fixed and stained. The number of colonies per plate ($>$ 50 cells per colony) was counted, plating efficiencies determined, and the relative changes in colony formation entered into the CalcuSyn for Windows program (Biosoft, Cambridge, UK) to determine synergy ($n = 3$). A combination index (CI) of less than 0.70 indicates a strong level of synergy.

HuH7			HCT116		
PX-866	SOR	CI	MK2206	REGOR	CI
μ M	μ M		μ M	μ M	
0.50	2.0	0.51	1.0	0.50	0.65
0.75	3.0	0.46	1.5	0.75	0.54
1.00	4.0	0.48	2.0	1.00	0.51

Fig. 2. Inhibitors of RAF and the PI3K pathway interact to kill hepatoma cells. (A) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (dimethylsulfoxide; DMSO), sorafenib (sor, 3.0 μ M), and/or perifosine (Peri, 1.0 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (B) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), regorafenib (Reg, 0.5 μ M), and/or PX-866 (PX, 0.5 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (C) SW620, SW480, HT29, and HCT116 cells were treated with vehicle (DMSO), regorafenib (0.5 μ M), and/or PX-866 (0.5 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (D) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), regorafenib (0.5 μ M), and/or perifosine (1.0 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (E) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (2.0 μ M), and/or MK2206 (MK, 1.0 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (F) Wild-type HCT116, K-RAS D13 null HCT116 (CMV), K-RAS D13 null + H-RAS V12 HCT116 (V12), K-RAS D13 null + H-RAS V12 S35 HCT116 (V12 35-Raf), K-RAS D13 null + H-RAS V12 R37 HCT116 (V12 37-Ral), and K-RAS D13 null + H-RAS V12 C40 HCT116 (V12 40-PI3K) cells (Caron et al., 2005) were treated with vehicle (DMSO), regorafenib (REGOR, 0.5 μ M), and/or MK2206 (1.0 μ M) as indicated for 24 hours. Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control; $^{\ast}P < 0.05$ less than corresponding value in wild-type cells; $^{\ast\ast}P < 0.05$ greater than corresponding value in wild-type cells. (G) HEPG2 cells were infected with empty vector control virus (CMV) or a virus to express dominant negative AKT (dnAKT). Twenty-four hours after infection cells were treated with vehicle (DMSO) or sorafenib (3.0 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control. (H) HEPG2 cells were transfected with scrambled siRNA (siSCR) or with siRNA molecules to knockdown p110 α and p110 β (si-p110). Thirty-six hours after transfection cells were treated with vehicle (DMSO) or sorafenib (3.0 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than corresponding value in siSCR cells.

decreased phospho-ERK1/2 staining, and increased levels of tumor cell apoptosis (cleaved caspase 3) (Fig. 5, B and C). Normal tissue effects, as judged by altered H&E staining of liver, kidneys, and heart were not evident at day 15 (Fig. 5D). Of particular note, in tumors treated with PX-866 there was a rebound effect observed, with tumors exhibiting more phospho-AKT staining than vehicle or sorafenib-alone

tumors. In tumors treated with PX-866 and sorafenib, the rebound in phospho-AKT levels was not observed. Finally, we determined whether regorafenib, which is approved for the treatment of colon cancer, cooperated with the AKT inhibitor MK2206 to suppress colon tumor growth. The growth of HCT116 tumor cells was not altered by MK2206 treatment, whereas regorafenib significantly suppressed growth. The

Fig. 3. Regulation of cell signaling and cell survival by sorafenib and PX-866 treatment. (A) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μ M), and/or PX-866 (PX, 0.5 μ M) as indicated for 12 hours. Cells were isolated, lysed, and immunoblotting performed to determine the expression and/or phosphorylation of the indicated proteins. (B) HEPG2, HEP3B, and HuH7 cells were infected with empty vector control virus (CMV) or a virus to express constitutively active AKT (caAKT). Twenty-four hours after infection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in CMV cells. Inset: expression of activated AKT prevents BAX activation. (C) HEPG2, HEP3B, and HuH7 cells were transfected with empty vector plasmid (CMV) or a plasmid to express constitutively active mTOR (ca-mTOR). Twenty-four hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in CMV cells. (D) HEPG2, HEP3B, and HuH7 cells were transfected with empty vector control plasmid (CMV) or a plasmid to express constitutively active p70 S6K (ca-p70). Twenty-four hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in CMV cells. (E) HEPG2, HEP3B, and HuH7 cells were infected with empty vector control virus (CMV) or a virus to express constitutively active MEK1 (caMEK1). Twenty-four hours after infection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). Upper inset: Expression of activated MEK1 suppresses the increase in BIM expression. (F) HEPG2, HEP3B, and HuH7 cells were transfected with empty vector control plasmid (CMV), a plasmid to express dnGSK3 or a plasmid to express dominant negative SRC (dnSRC), as indicated. Twenty-four hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in CMV cells. Upper inset panels: expression of dominant negative GSK3 suppresses the decrease in MCL-1 expression and activation of BAX; dominant negative SRC prevents tyrosine phosphorylation of GSK3. (G) HEPG2, HEP3B, and HuH7 cells were transfected with scrambled control siRNA (siSCR) or siRNAs to knockdown expression of BIM or of BAX. Thirty-six hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in siSCR cells. (H–J) HEPG2, HEP3B, and HuH7 cells were infected with empty vector control virus (CMV) or viruses to express c-FLIP-s, BCL-XL, or dominant negative caspase 9. Twenty-four hours after infection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). * $P < 0.05$ less than corresponding value in CMV cells.

drug combination abolished growth, and tumor mass slightly declined compared with vehicle (Fig. 5E).

Discussion

The present studies were initiated to determine whether inhibitors of the PI3K/AKT pathway cooperated with

sorafenib/regorafenib to kill tumor cells. Inhibitors of PI3K p110 or of the AKT pleckstrin homology or kinase domains cooperated with sorafenib/regorafenib to kill hepatoma cells in a greater than additive fashion. The PI3K inhibitor PX-866 cooperated with sorafenib to kill breast and brain tumor cells. In vivo PX-866/MK2206 and sorafenib/regorafenib cooperated to kill hepatoma and colorectal tumor cells and to suppress

Fig. 4. The regulation of autophagy by sorafenib and PX-866. (A) HEPG2, HEP3B, and HuH7 cells were transfected with a plasmid to express LC3-GFP. Twenty-four hours after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μ M), and/or PX-866 (PX, 0.5 μ M). Cells were microscopically examined 12 hours after exposure and the number of GFP punctae determined in at least 40 random cells per condition ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than vehicle control; $^{##}P < 0.05$ greater than SOR value; $^{*}P < 0.05$ less than PX-866 value. (B) HEP3B cells were transfected with a plasmid to express LC3-GFP, and plasmids to express nothing (CMV) or to express activated mTOR (ca-mTOR). Twenty-four hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were microscopically examined 12 hours after exposure and the number of GFP punctae determined in at least 40 random cells per condition ($n = 3$, $\pm$ S.E.M.). Inset: cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Twelve hours after treatment cells were isolated and mTOR immunoprecipitated. The amount of Raptor coprecipitating with mTOR was assessed. The input of mTOR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) total protein loading are also presented. (C) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 3/6/9 hours after drug exposure and subjected to immunoblotting against the indicated proteins ($n = 3$, a representative is shown). (D) HEPG2, HEP3B, and HuH7 cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 15 hours after drug exposure and subjected to immunoblotting against the indicated proteins ($n = 3$, a representative is shown). (E) HEP2G, HEP3B, and HuH7 cells were transfected with scrambled control siRNA (siSCR) or siRNA molecules to knockdown (Knk Dwn) expression of Beclin1 (siB1) or ATG5 (siA5). Thirty-six hours after transfection cells were treated with vehicle (DMSO), sorafenib (3.0 μ M), and/or PX-866 (0.5 μ M). Cells were isolated 24 hours after exposure and viability determined by trypan blue exclusion ($n = 3$, $\pm$ S.E.M.). $^{\#}P < 0.05$ greater than corresponding value in CMV cells; $^{*}P < 0.05$ less than corresponding value in siSCR cells.

tumor growth. As sorafenib and regorafenib are FDA approved agents, and as PX-866/MK2206 have progressed into Phase II trials and perifosine into Phase III trials, it is hoped that a Phase I clinical trial could be opened combining some of these agents in hepatoma, colorectal, and renal carcinoma patients.

Simplistically PX-866 should inhibit many pathways downstream of PI3K, including AKT, p70 S6K, and mTOR. In our system, only PX-866 as a single agent suppressed AKT activity to any appreciable extent. Treatment with sorafenib modestly reduced the phosphorylation of ERK1/2 and in combination with a dose of PX-866, which by itself did not alter phospho-ERK1/2 levels, further reduced phospho-ERK1/2. A similar effect was observed for p70 S6K. In the case of mTOR in HEPG2 cells, neither sorafenib nor PX-866 altered phospho-S2448. However, combined exposure to both drugs almost abolished mTOR phosphorylation. Expression of activated AKT, and to a lesser extent activated mTOR but not activated MEK1, protected cells from drug combination toxicity. Drug combination treatment activated BAX; expression

of activated AKT or dominant negative GSK3 or knockdown of BAX suppressed activation of BAX and apoptosis.

In a recent article by Roulin et al. (2011) the authors argued that very high concentrations of sorafenib cooperated with the PI3K/mTOR inhibitor 2-methyl-2-[4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-1-yl]phenyl]propanenitrile (BEZ235) to kill renal carcinoma cells; by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay no interaction was observed; by DNA fragmentation there was an additive killing effect; by caspase 3 cleavage there was a greater than additive killing effect observed. Based on our prior experience with the suicide PI3K inhibitors wortmannin and PX-866, we have found physiologic concentrations of BEZ235 to be a much weaker inhibitor of PI3K signaling pathways. This relative lack of effect of BEZ235 may be because wortmannin and PX-866 are suicide inhibitors of PI3K enzymes, with a greater ability to inhibit AKT for prolonged periods.

Downstream of signal transduction pathways, e.g., AKT/GSK3, are proteins that regulate the apoptotic threshold, e.g.,

Fig. 5. Sorafenib and PX-866 interact to suppress tumor growth in vivo. (A) HuH7 cells (1×10^7) were implanted into the rear flank and tumors permitted to form for 14 days. The mean initial tumor volume in each group was: vehicle ($155 \pm 25 \text{ mm}^3$), sorafenib ($60 \pm 18 \text{ mm}^3$), PX-866 ($80 \pm 39 \text{ mm}^3$), sorafenib+PX-866 ($123 \pm 69 \text{ mm}^3$). Mice were PO administered vehicle diluent, sorafenib (25 mg/kg), PX-866 (2 mg/kg), or the drug combination QD for 3 days. Animals were monitored daily and tumor volume determined every fifth day, which is plotted as the -Fold change in tumor volume ($\pm$ S.E.M., $n = 8$, two separate studies). $^{\#}P < 0.05$ less than vehicle-treated animals; $^{\#\#}P < 0.05$ less than PX-866 or sorafenib-treated animals. (B and C) Tumors from animals were isolated at day 15 and fixed, sectioned (10- μm), and stained against proliferation (Ki67 staining), phospho-ERK1/2 and phospho-AKT staining, the levels of tumor cell apoptosis/cleaved caspase 3, as well as with H&E and 4',6-diamidino-2-phenylindole (DAPI). (D) Normal tissues from treated animals (liver, kidneys, heart) were isolated at day 15 and fixed, sectioned (10- μm), and stained against H&E and DAPI. (E) Wild-type parental HCT116 cells (1×10^7) were implanted into the rear flank and tumors permitted to form for 14 days. The mean initial tumor volume in each group was: vehicle ($133 \pm 21 \text{ mm}^3$), regorafenib ($79 \pm 15 \text{ mm}^3$), MK2206 ($102 \pm 28 \text{ mm}^3$), regorafenib+MK2206 ($109 \pm 37 \text{ mm}^3$). Mice were PO administered vehicle diluent, regorafenib (25 mg/kg), MK2206 (40 mg/kg), or the drug combination QD for 3 days. Animals were monitored daily and tumor volume determined every fifth day, which is plotted as the -fold change in tumor volume ($\pm$ S.E.M., $n = 6$, two separate studies). $^{\#}P < 0.05$ less than vehicle treated animals; $^{\#\#}P < 0.05$ less than MK2206 treated animals.

MCL-1. We discovered that drug combination treatment lowered BCL-XL and MCL-1 expression, reduced BAD S112 phosphorylation, and increased the expression of BAX and BIM. Over-expression of BCL-XL protected cells as did knockdown of BAX, but not overexpression of c-FLIP-s. Expression of activated MEK1 suppressed drug-induced BIM expression, but did not significantly protect the cells from drug toxicity, implying that BAX was a more important proapoptotic effector in our system than BIM. These findings would argue that inactivation of the ERK1/2 pathway through increased BIM levels does not play a pivotal role in drug combination toxicity. Thus inactivation of ERK1/2 and AKT facilitate activation of toxic BH3 domain proteins, e.g., BAX and reduced expression of protective BCL-2 family proteins, e.g., MCL-1.

Prior studies by our group have shown that sorafenib increases the levels of LC3-GFP vesicles in tumor cells, suggestive of autophagy, and knockdown of Beclin1 or ATG5 increased sorafenib toxicity (Zhang et al., 2008). It is also well known that reduced mTOR activity increases autophagic flux (Alers et al., 2012). In hepatoma cells, both individual sorafenib and PX-866 treatments increased LC3-GFP vesicle

levels. PX-866 treatment also increased p62 and LC3II levels, collectively indicative of a stalled autophagy process. This suggests PX-866 may be able to inhibit the Class III PI3K protein Vps34, Vps34 being required for the progression of autophagic vesicle maturation. Combined treatment of cells with sorafenib and PX-866 reduced the levels of LC3-GFP vesicles p62 and PC3II. As expected from our prior data and those of others, knockdown of Beclin1 or ATG5 abolished the induction of autophagy as judged by formation of LC3-GFP vesicles. Knockdown of Beclin1 or ATG5 moderately reduced sorafenib and PX-866 toxicity by $\sim 30\%$. Thus in our system autophagy appeared to be a "toxic" event. Induction of autophagy followed by "release" and completion of autophagic flux was required for killing. Expression of activated mTOR reduced the levels of autophagy and also modestly protected cells from sorafenib and PX-866 toxicity. Thus in addition to modulation of ERK1/2, AKT, and JNK1/2 signaling, reduced mTOR activity results in both elevated levels of autophagy and a modestly lowered threshold for apoptosis.

Sorafenib is an FDA approved agent for the treatment of renal carcinoma and hepatoma. Regorafenib is an FDA

approved agent for the treatment of colon cancer (Ibrahim et al., 2012; Carr et al., 2013). A major in vivo antitumor effect of these drugs are the inhibition of class III receptor tyrosine kinases, e.g., platelet-derived growth factor receptor β , and a reduction in tumor neo-angiogenesis (Park et al., 2008). Combined exposure to sorafenib/regorafenib and PX-855/MK2206 significantly reduced the growth of tumors growing in the animal flank. Reduced tumor growth correlated with decreased Ki67 staining, decreased phospho-ERK1/2 staining, and increased cleaved caspase 3 and TUNEL+ staining (not shown). Tumors treated with PX-866 exhibited a rebound effect with elevated phospho-AKT staining compared with vehicle or sorafenib-alone tumors. In tumors treated with PX-866 and sorafenib, the rebound in phospho-AKT levels was not observed. This suggests use of PI3K inhibitors in vivo as single agents could have unforeseen delayed effects, including reactivation of the PI3K pathway. Our findings in the HuH7/HCT116 models argue that hepatoma growth and tumor cell survival are reduced by combined drug treatment.

In conclusion, we have shown that sorafenib cooperates with inhibitors of the PI3K pathway to kill a wide range of tumor cell types. The signaling mechanisms underpinning the toxic drug interaction is complex, involving inactivation of ERK1/2 and multiple enzymes downstream of PI3K, particularly AKT, leading to mitochondrial dysfunction. Treatment of established hepatoma and colorectal carcinoma tumors with sorafenib and PX-866 or regorafenib and MK2206 resulted in slower tumor growth and increased levels of cell death within the tumor.

Authorship Contributions

Participated in research design: Grant, Poklepovic, Dent.

Conducted experiments: Sajithlal, Hamed, Cruickshanks, Booth, Tavallai, Syed.

Performed data analysis: Dent.

Wrote or contributed to the writing of the manuscript: Sajithlal, Dent.

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