Molecular Pharmacology Fast Forward. Published on July 22, 2013 as DOI: 10.1124/mol.113.088005
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MOLPHARM#88005

Sorafenib/Regorafenib and PI3K/AKT inhibition interact to kill tumor cells.

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Running Title: Sorafenib and PI3K

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Number of text pages: 34
Number of Tables: 1
Number of Figures: 5
Words in Abstract: 233
Words in Introduction: 818
Words in Discussion: 1016

Abbreviations: ERK: extracellular regulated kinase; MEK: mitogen activated extracellular regulated kinase; PI3K: phosphatidyl inositol 3 kinase; MAPK / ERK: mitogen activated protein kinase; ca: constitutively active; dn: dominant negative; CMV: empty vector plasmid or virus; si: small interfering; SCR: scrambled; Ad: adenovirus; TUNEL: Terminal deoxynucleotidyl transferase UTP nick end labeling; VEH: vehicle; SOR: sorafenib; PX: PX-866; MK: MK2206; REGOR: regorafenib.

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Abstract

The present studies were to determine whether the multi-kinase inhibitors sorafenib/regorafenib cooperated with clinically relevant PI3K-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 PX-866 cooperated in a greater than additive fashion to kill tumor cells. Cells lacking PTEN were as sensitive to the drug combination as cells expressing the protein. Similar data were obtained using the AKT inhibitors perifosine and MK2206. PX-866 treatment abolished AKT / GSK3 phosphorylation and cell killing correlated with reduced activity of AKT and mTOR. Expression of activated AKT and to a lesser extent activated mTOR reduced drug combination lethality. Expression of BCL-XL or dominant negative caspase 9, but not c-FLIP-s, protected cells from the drug combination. Treatment of cells with PX-866 increased protein levels of p62, LAMP2 and LC3 and LC3II that correlated with a large increase in LC3-GFP vesicle numbers. Exposure of PX-866 treated cells to sorafenib reduced p62 and LAMP2 levels, decreased the ratio of LC3:LC3II and reduced LC3-GFP vesicle levels. Knock down of Beclin1 or ATG5 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.

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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 H/K-RAS and growth factor receptors; and sporadic activating mutations in the enzymes PDK-1 and AKT (Hafsi et al, 2012). PI3K pathway signaling has been linked to anti-apoptotic effects both at the level of death receptors and at the mitochondrion (Sancho-Martinez and Martin-Villalba, 2009; Fulda, 2012). PI3K signaling, through 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 biomarker several attempts have been made by pharmaceutical companies to inhibit pathway members, most notably the catalytic p110 subunit of PI3K and the PH / allosteric domain of AKT. Examples of the former include the p110 inhibitor PX-866 and the latter perifosine or MK2206 (Ihle et al, 2009; Locatelli et al, 2013). These agents are undergoing clinical evaluation in a variety of tumor types (Hong et al, 2012; Cho et al, 2012). PX-866 is a derivative of wortmannin, and acts as a *suicide inhibitor* of p110α/β/δ/γ. Dual inhibition of the PI3K and ERK1/2 pathways has been proposed as one approach to kill tumor cells and there are multiple clinical trials combining PI3K and MEK1/2 inhibitors, however there are no *on-going* studies examining the interaction of PI3K pathway inhibitors with a multi-kinase inhibitor such as sorafenib or regorafenib (see www.clinicaltrials.gov; Rahmani et al, 2009).

Sorafenib is a multi-kinase 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 anti-angiogenic 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 day) C_{max} for sorafenib is ~21 μM in plasma, with > 95% of the drug protein bound based on in vitro human serum binding assays; none the less *patient data*

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would argue that at least ~5-10 μM of the drug *has* to be bioavailable in a tumor based on its single agent effects on decreasing both ERK1/2 phosphorylation and reducing 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 + "drug" combinations that PDGFRβ is a major target of sorafenib for its interactions with other agents e.g. with HDAC inhibitors (Park et al, 2008). A major biological effect of sorafenib at in vitro concentrations in the ~3 μM range, is the induction of an endoplasmic stress (ER) / unfolded protein response (UPR), with reduced expression of proteins that have short half-lives such as MCL-1 and BCL-XL (Rahmani et al, 2005; Rahmani et al, 2007). Reduced MCL-1 levels due to sorafenib exposure have been linked in many tumor types to increased levels of apoptosis (Kim and El-Deiry, 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 LC3-GFP vesicles and elevated expression of Beclin1 and 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 ER 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-RTK pathways is complex. Inhibition of the PI3K and/or the ERK1/2 pathways can lead to activation of the JNK1/2 pathway, which can control the activity of the pro-apoptotic BH3 domain proteins BAX and BAK (Yacoub et al, 2010). Reduced ERK1/2 activity can facilitate increased expression of the pro-apoptotic BH3 domain protein BIM and dephosphorylation and activation of BAD (Rahmani et al, 2013). Expression of the caspase 8 inhibitor c-FLIP-s as well as other caspase inhibitors such as XIAP 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).

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Materials and Methods.

Materials.

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

Methods.

Cell culture and in vitro exposure of cells to drugs. All established cancer lines were cultured at 37 ^{o}C (5% (v/v CO_2) *in vitro* using RPMI supplemented with 10% (v/v) fetal calf serum and 10% (v/v) Non-essential amino acids. All primary human GBM cells were initially cultured at 37 ^{o}C (5% (v/v CO_2) *in vitro* using RPMI supplemented with 2% (v/v) fetal calf serum and 10% (v/v) Non-essential amino acids at 37 ^{o}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) Non-essential amino acids. For short-term cell killing assays and immunoblotting, cells were plated at a density of 3 x 10^3 per cm^2 and 24h 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 (DMSO) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study.

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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 refs. (Cruickshanks et al, 2012; Park et al, 2008; Yacoub et al, 2010; Bareford et al, 2011). 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 min. The boiled samples were loaded onto 10–14% SDS-PAGE and electrophoresis was run overnight (10–100 µg/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. For presentation, immunoblots were digitally assessed using the provided Odyssey Imager software (the data sets presented are the −Fold increase +/− SEM (n = 3) in expression of the indicated protein compared to GAPDH loading control; for phospho-proteins the −Fold increase +/− SEM (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 previously noted recombinant adenoviruses as per refs. (Cruickshanks et al, 2012; Park et al, 2008; Yacoub et al, 2010; Bareford et al, 2011). Cells were infected with these adenoviruses at an approximate m.o.i. as indicated in the Figure / Legend. Cells were incubated for 24 h to ensure adequate expression of transduced gene products prior to drug exposures.

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Detection of cell death by Trypan Blue, Hoechst, TUNEL and flow cytometric assays. Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37 ^{o}C. Cell death assays were performed as described in refs. (Cruickshanks et al, 2012; Park et al, 2008; Yacoub et al, 2010; Bareford et al, 2011). Briefly, for in vitro analyses of short-term cell death effects, cells were treated with Vehicle or drugs for the indicated times 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 (Geimsa) assay kit or Hoechst stain. Alternatively, the cell volume assay using the Scepter system was carried to determine cell viability out as per the manufacturer's instructions (Millipore).

Assessment of autophagy. Cells were transfected with a plasmid to express a green fluorescent protein (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 X40 objective of a Zeiss Axiovert fluorescent microscope (Cruickshanks et al, 2012; Park et al, 2008; Yacoub et al, 2010; Bareford et al, 2011).

Plasmid transfection.

Plasmids: Cells were plated as described above and 24 h after plating, transfected. Plasmids (0.5 μg) expressing a specific mRNA or appropriate vector control plasmid DNA was diluted in 50 μl serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 μl Lipofectamine 2000 (Invitrogen), 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 min. This mixture was added to each well/dish of cells containing 200 μl serum-free and antibiotic-free medium for a total volume of 300 μl and the cells were incubated for 4h at 37^{o}C. An equal volume of 2X medium was then added to

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each well. Cells were incubated for 48h, 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%.

siRNA: Cells were plated in 60 mm dishes from a fresh culture growing in log phase as described above, and 24h after plating transfected. Prior to transfection, the medium was aspirated and 1 ml serum-free medium was added to each plate. For transfection, 10 nM of the annealed siRNA, the positive sense control doubled stranded siRNA targeting GAPDH 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, Valencia, CA; occasional alternate siRNA molecules were purchased from Ambion, Inc., Austin, Texas). Ten nM siRNA (scrambled or experimental) was diluted in serum-free media. Four μl 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 min, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37 ^{o}C for 2h. One ml of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37 ^{o}C for 24-48h before re-plating (50 x 10^3 cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0-48h). Trypan blue exclusion assays and SDS PAGE / immunoblotting analyses were then performed at the indicated time points.

Animal studies. Athymic female NCr-v/v mice (NCI-Fredrick) weighing ~20 g, were used for this study (Bareford et al, 2011). Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, Washington, DC, 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 4 usable tumors per group) in 10 μl of growth medium. Fourteen days after tumor cell implantation mice were

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mice were PO administered drugs or the drug combination QD for 3 days (see legends). Animals were monitored daily and tumor volume determined every fourth day. When the volume of the tumor reached > 2,000 mm^3, animals were killed.

Immunohistochemistry and staining of fixed tumor sections. Post sacrifice, tumors were fixed in OCT compound (Tissue Tek); 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; anti-cleaved caspase 3; anti-phospho-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 Apototic Detection Kit according to the manufacturer's instructions. Slides were applied to high powered light/confocal microscopes (Zeiss LSM 510 Meta-confocal scanning microscope; Zeiss HBO 100 microscope with Axio Cam MRm camera) at the indicated magnification in the Figures / Figure legends (X40). 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 utilized log rank statistical analyses between the different treatment groups. Experiments shown are the means of multiple individual points from multiple experiments (± SEM).

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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 μM) did not exhibit significant toxicity over 24h in the tumor cells used in our studies (Figure 1). Sorafenib (1.0-3.0 μM) and PX-866 cooperated in a dose-dependent fashion to enhance sorafenib lethality in multiple hepatoma cell lines (Figures 1A-1C). As judged by DNA condensation / Hoechst staining, sorafenib and PX-866 also cooperated to kill cells (Figure 1D). Sorafenib and PX-866 enhanced the radiosensitivity of hepatoma cells (Figures 1E). Similar data to that in hepatoma cells combining sorafenib and PX-866 was obtained in kidney cancer, breast cancer, lung cancer and brain cancer cells, with similar levels of killing observed in cells regardless of PTEN expression (Figures 1F-1I). Notably, the ~IC50 of sorafenib in our system, with cells cultured with 10% FCS, to reduce phosphorylation of wild type VEGFR2 and ERK2 was ~ 2 μM and ~2 μM, respectively. Sorafenib and PX-866 cooperated to suppress colony formation of multiple liver cancer cell lines *regardless of CD95 expression status* (Figure 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 PMCs (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 (Figure 2A). PX-866 enhanced the lethality of the novel fluorinated inhibitor derived from sorafenib, regorafenib, in hepatoma and colon cancer cells (Figures 2B and 2C) (Carr et al, 2013). Perifosine enhanced the lethality of regorafenib in hepatoma cells (Figure 2D). The AKT inhibitor MK2206 enhanced regorafenib toxicity in hepatoma cells (Figure 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

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specifically activates PI3K-AKT were more sensitive to MK2206 (Figure 2F). Of note, HCT116 cells deleted for K-RAS D13 but still expressing a mutant active p110α PI3K were less sensitive to MK2206 (Caron et al, 2005). Expression of dominant negative AKT or combined knock down of p110α/β expression enhanced cell killing by sorafenib (Figures 2G and 2H).

Studies next defined changes in the activity of signal transduction pathways and apoptosis regulator mediators. Treatment of cells with PX-866 abolished AKT phosphorylation (Figure 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 (Figure 3B). This correlated with reduced activation of BAX (Figure 3B, inset). Expression of an activated form of mTOR but not of p70 S6K more modestly suppressed drug combination toxicity (Figures 3C and 3D). Expression of an activated form of MEK1 did not suppress drug combination toxicity (Figure 3E). Activated MEK1 prevented the drug-induced expression of BIM. Expression of dominant negative GSK3 (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 (Figure 3F graph and upper insert 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 a SRC dependent manner (Figure 3F, upper inset right). Knock down of BAX, but not BIM, protected cells from the drug combination (Figure 3G). Over-expression of BCL-XL or dominant negative caspase 9, but not c-FLIP-s, suppressed drug combination toxicity (Figures 3H-3J).

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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 (Figures 4A). Combined treatment of hepatoma cells with sorafenib and PX-866 reduced LC3-GFP vesicle levels to near those observed in cells treated with of sorafenib alone. Expression of ca-mTOR suppressed the reduction in vesicle numbers comparing PX-866 and PX-866 + sorafenib treatments (Figure 4B). Sorafenib treatment appeared to enhance the levels of a faster migrating form of Raptor on SDS PAGE and combined exposure of cells to PX-866 and sorafenib abolished the interaction between Raptor and mTOR (Figure 4B, upper inset blot). Treatment with sorafenib weakly reduced p62 levels whereas PX-866 strongly increased expression of both p62 and LC3 and LC3II (Figure 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 (Figure 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, knock down 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 knock down of ATG5 or Beclin1 reduced but did not abolish sorafenib and PX-866 toxicity in our hepatoma cell lines (Figure 4E).

We determined whether [sorafenib and PX-866] or [regorafenib and MK2206] cooperated in vivo to suppress tumor growth and increase animal survival. In pre-formed HuH7 tumors both sorafenib and PX-866 reduced tumor growth (Figure 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

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staining), decreased phospho-ERK1/2 staining, and increased levels of tumor cell apoptosis (cleaved caspase 3) (Figures 5B and 5C). Normal tissue effects, as judged by altered H&E staining of liver, kidneys and heart were not evident at Day 15 (Figure 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 drug combination abolished growth and tumor mass slightly declined compared to vehicle (Figure 5E).

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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 PH 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 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 PX-866 as a single agent only 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 that by itself did not alter phospho-ERK1/2 levels, further reduced P-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 knock down of BAX suppressed activation of BAX and apoptosis.

In a recent manuscript by Roulin et al the authors argued that very high concentrations of sorafenib cooperated with the PI3K / mTOR inhibitor BEZ235 to kill renal carcinoma cells; by MTT assay no interaction was observed; by DNA fragmentation there was an additive killing effect; by caspase 3 cleavage

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there was a greater than additive killing effect observed (Roulin et al, 2011). 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. 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 knock down of BAX, but not over-expression 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 pro-apoptotic 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 knock down 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

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reduced the levels of LC3-GFP vesicles, p62 and PC3II. As expected from our prior data and those of others, knock down of Beclin1 or ATG5 abolished the induction of autophagy as judged by formation of LC3-GFP vesicles. Knock down of Beclin1 or ATG5 moderately reduced sorafenib and PX-866 toxicity by ~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 anti-tumor effect of these drugs are the inhibition of class III receptor tyrosine kinases e.g. PDGFRβ, 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 to 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 re-activation 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

Molecular Pharmacology Fast Forward. Published on July 22, 2013 as DOI: 10.1124/mol.113.088005
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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 // regorafenib and MK2206 resulted in slower tumor growth and increased levels of cell death within the tumor.

Molecular Pharmacology Fast Forward. Published on July 22, 2013 as DOI: 10.1124/mol.113.088005
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Authorship Contribution

Participated in research design: Dent, Grant, Poklepovic

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

Contributed new reagents: n/a

Performed data analysis: Dent

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

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Footnotes

Support for the present study was funded from PHS grants from the National Institutes of Health [R01-CA141704, R01-CA150214, R01-DK52825]; the Department of Defense [W81XWH-10-1-0009]. PD is the holder of the Universal Inc. Professorship in Signal Transduction Research. The authors have no conflicts of interest to report.

Molecular Pharmacology Fast Forward. Published on July 22, 2013 as DOI: 10.1124/mol.113.088005
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Figure Legends

Figure 1. Sorafenib and PI3K pathway inhibitors interact to kill multiple tumor cell types. (A) HEPG2 cells were treated with vehicle (DMSO), sorafenib (sor, 1.0-3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(B)** HEP3B cells were treated with vehicle (DMSO), sorafenib (sor, 1.0-3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(C)** HuH7 cells were treated with vehicle (DMSO), sorafenib (sor, 1.0-3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(D)** HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by levels of nuclear DNA condensation (n = 3, +/- SEM) #p < greater than vehicle control. Upper inset panel: 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 μM) and PX-866 (0.5 μM) (PX+SOR). Thirty minutes after drug treatment cells were irradiated (4 Gy, XRT). Cells were isolated 12h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than corresponding unirradiated control. **(F)** A498, UOK121LN and CAKI cells were treated with vehicle (DMSO), sorafenib (sor, 2.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(G)** BT549, BT474 and MCF7 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(H)** GBM5 and GBM14 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(I)** A549, H460 and H1975 cells were treated with vehicle (DMSO), sorafenib (sor,

Molecular Pharmacology Fast Forward. Published on July 22, 2013 as DOI: 10.1124/mol.113.088005
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3.0 μM) and/or PX-866 (0.5 μM) as indicated. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 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 12h treated with drugs. Cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM) and/or PX-866 (PX, 0.5 μM) as indicated for 24h. 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, +/- SEM) *p < less than vehicle control.

Figure 2. Inhibitors of RAF and the PI3K pathway interact to kill hepatoma cells. (A) HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM) and/or perifosine (Peri, 1.0 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 0.05 greater than vehicle control. **(B)** HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), regorafenib (Reg, 0.5 μM) and/or PX-866 (PX, 0.5 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 0.05 greater than vehicle control. **(C)** SW620, SW480, HT29 and HCT116 cells were treated with vehicle (DMSO), regorafenib (Reg, 0.5 μM) and/or PX-866 (PX, 0.5 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 0.05 greater than vehicle control. **(D)** HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), regorafenib (Reg, 0.5 μM) and/or perifosine (peri, 1.0 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 0.05 greater than vehicle control. **(E)** HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), sorafenib (SOR, 2.0 μM) and/or MK2206 (MK, 1.0 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #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); 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 μM) and/or MK2206 (MK, 1.0 μM) as indicated for 24h. Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < 0.05 greater than vehicle control; *p < 0.05 less than corresponding value in WT cells; $ p < 0.05 greater than corresponding value in WT cells. **(G)** HEPG2 cells were infected with empty vector control virus (CMV) or a virus to express dominant negative AKT (dnAKT). Twenty four h after infection cells were treated with vehicle (DMSO) or sorafenib (sor, 3.0 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than vehicle control. **(H)** HEPG2 cells were transfected with scrambled siRNA (siSCR) or with siRNA molecules to knock down p110α and p110β (si-p110). Thirty six h after transfection cells were treated with vehicle (DMSO) or sorafenib (sor, 3.0 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #p < greater than corresponding value in siSCR cells.

Figure 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 μM) and/or PX-866 (PX, 0.5 μM) as indicated for 12h. 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 h after infection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < less than corresponding value in CMV cells. Inset panel: 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 h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and

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viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < 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 h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < 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 (caMEK). Twenty four h after infection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM). Upper inset panel: 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 dominant negative glycogen synthase kinase 3 (GSK3) (dnGSK3) or a plasmid to express dominant negative SRC (dnSRC), as indicated. Twenty four h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < less than corresponding value in CMV cells. Upper inset panels: expression of dnGSK3 suppresses the decrease in MCL-1 expression and activation of BAX; dnSRC prevents tyrosine phosphorylation of GSK3. **(G).** HEPG2, HEP3B and HuH7 cells were transfected with scrambled control siRNA (siSCR) or siRNAs to knock down expression of BIM or of BAX. Thirty six h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < 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 h after infection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) *p < less than corresponding value in CMV cells.

Figure 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 h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were microscopically examined 12h after exposure and the number of GFP punctae determined in at least 40 random cells per condition (n = 3, +/- SEM). # $p < 0.05$ 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 h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were microscopically examined 12h after exposure and the number of GFP punctae determined in at least 40 random cells per condition (n = 3, +/- SEM). Inset Panel: Cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Twelve h after treatment cells were isolated and mTOR immunoprecipitated. The amount of Raptor co-precipitating with mTOR was assessed. The input of mTOR and GAPDH total protein loading are also presented. **(C)** HEPG2, HEP3B and HuH7 cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 3h/6h/9h 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 (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 15h 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 knock down (Knk Dwn) expression of Beclin1 (siB1) or ATG5 (siA5). Thirty six h after transfection cells were treated with vehicle (DMSO), sorafenib (sor, 3.0 μM), and/or PX-866 (PX, 0.5 μM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) #$p <$ 0.05 greater than corresponding value in CMV cells. *$p <$ less than corresponding value in siSCR cells.

Figure 5. Sorafenib and PX-866 interact to suppress tumor growth in vivo. (A) HuH7 cells (1 x 10^7) were implanted into the rear flank and tumors permitted to form for fourteen days. The mean initial tumor volume in each group was: vehicle (155 +/- 25 mm^3); sorafenib (60 +/- 18 mm^3); PX-866 (80 +/- 39 mm^3); sorafenib+PX-866 (123 +/- 69 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 (+/- SEM, n = 8, 2 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 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 x 10^7) were implanted into the rear flank and tumors permitted to form for fourteen days. The mean initial tumor volume in each group was: vehicle (133 +/- 21 mm^3); regorafenib (79 +/- 15 mm^3); MK2206 (102 +/- 28 mm^3); regorafenib+MK2206 (109 +/- 37 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 (+/- SEM, n = 6, 2 separate studies) # $p < 0.05$ less than vehicle treated animals; ## $p < 0.05$ less than MK2206 treated animals.

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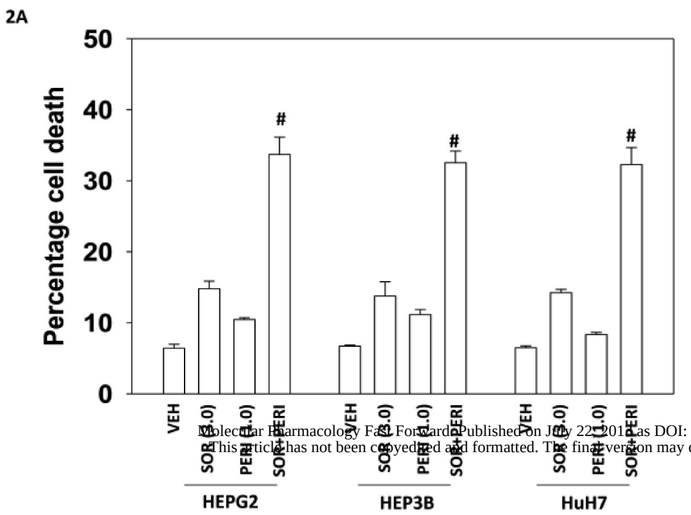
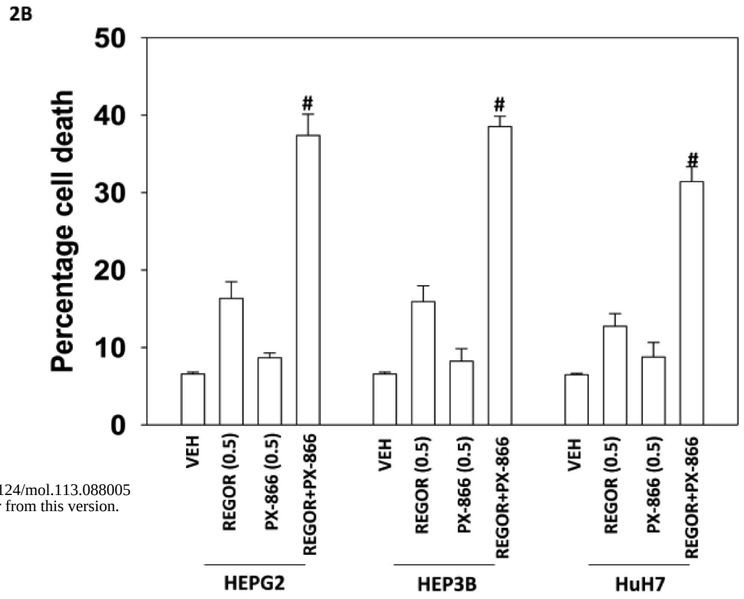
Table 1. Sorafenib/Regorafenib cooperate with PI3K/AKT inhibitors to kill tumors cells. HuH7 and HCT116 cells (250-1500 cells / well) were plated in sextuplicate and allowed to attach for 12h. Cells; HuH7 [sorafenib + P-866]; HCT116 [regorafenib + MK2206], were treated with sorafenib (SOR, 2.0-4.0 μM); regorafenib (REGOR, 0.5-1.0 μM); PX-866 (0.5-1.0 μM); MK2206 (MK, 1.0-2.0 μM) for 24h. Media was removed, plates washed, and cells cultured for the next 10-14 days in media lacking drugs. After ~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 to determine synergy (n = 3). A CI (combination index) of less than 0.70 indicates a strong level of synergy.

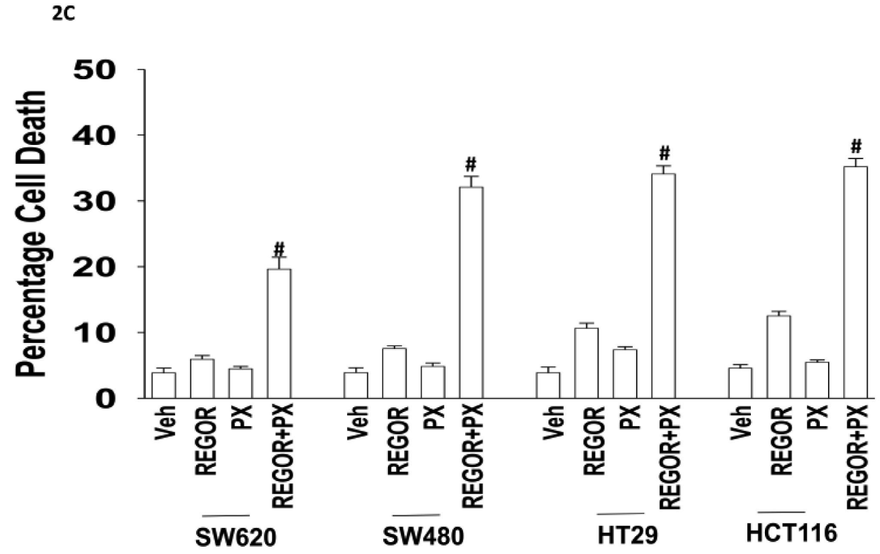
HuH7			**HCT116**		
PX-866, μM	**SOR, μM**	**CI**	**MK2206, μM**	**REGOR, μM**	**CI**
0.50	2.0	0.51	1.0	0.50	0.65
0.75	3.0	0.46	1.5	0.75	0.54
1.00	4.0	0.48	2.0	1.00	0.51

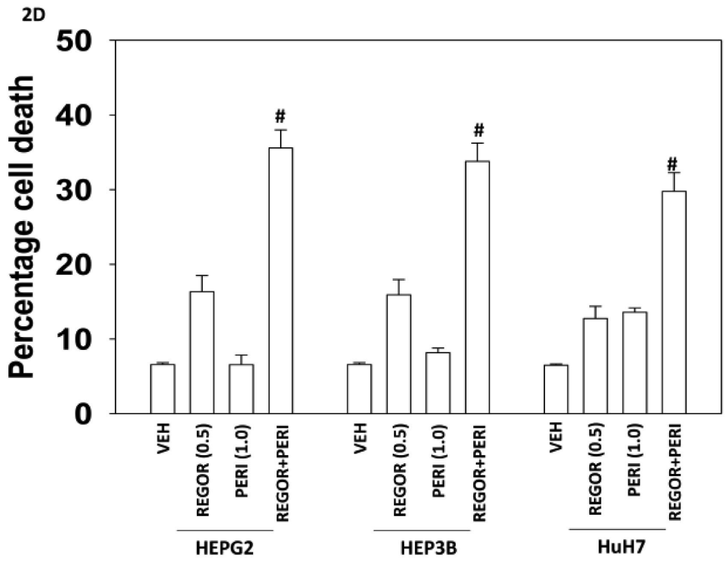
Figure 1

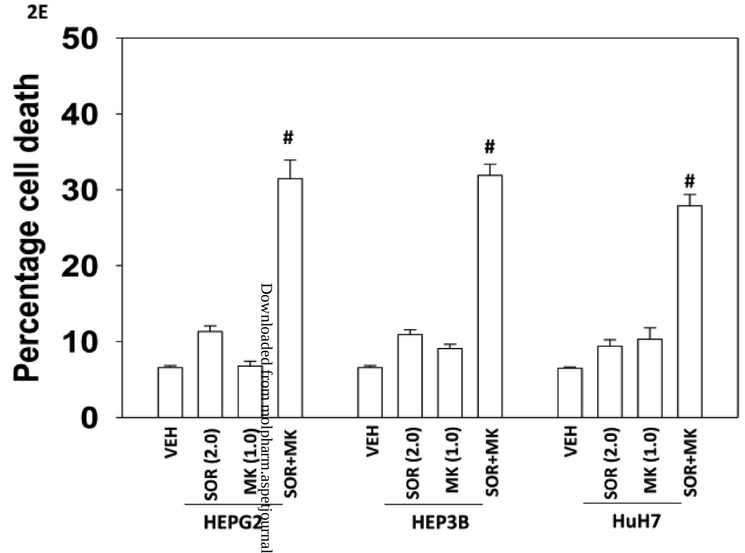
Figure 2

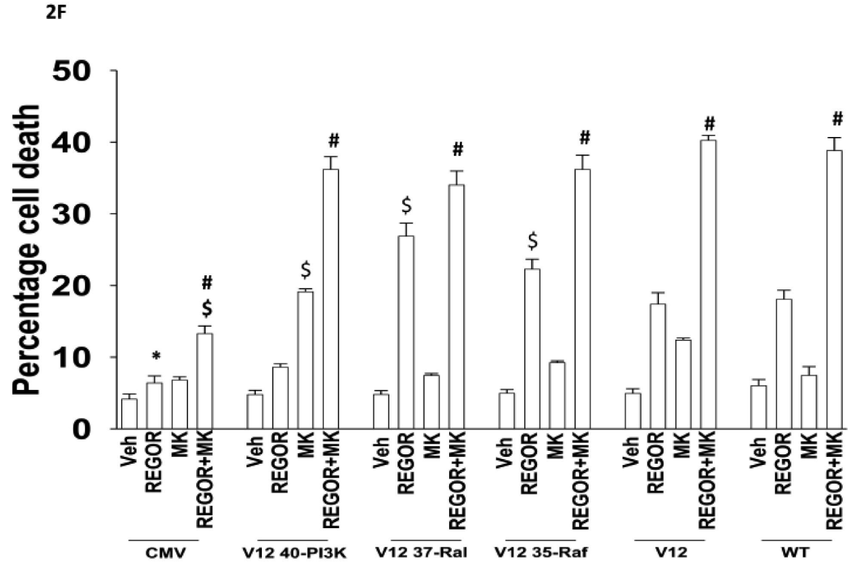


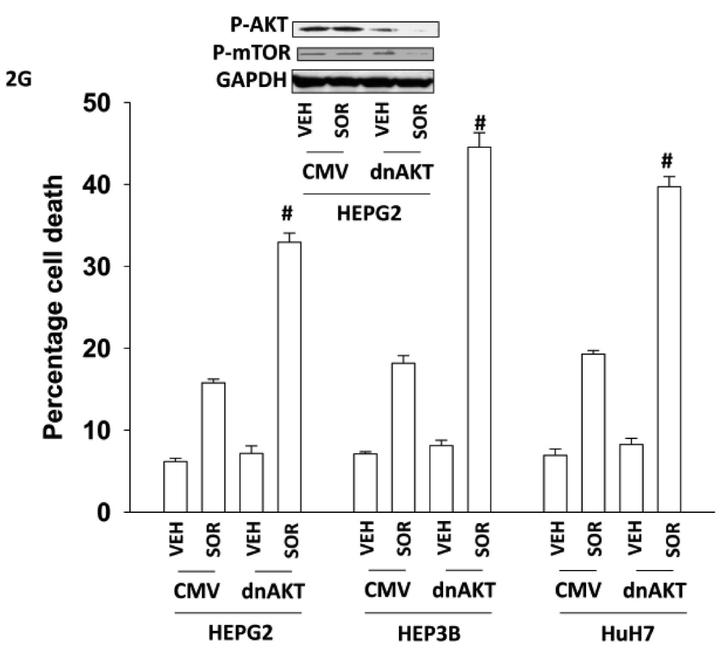












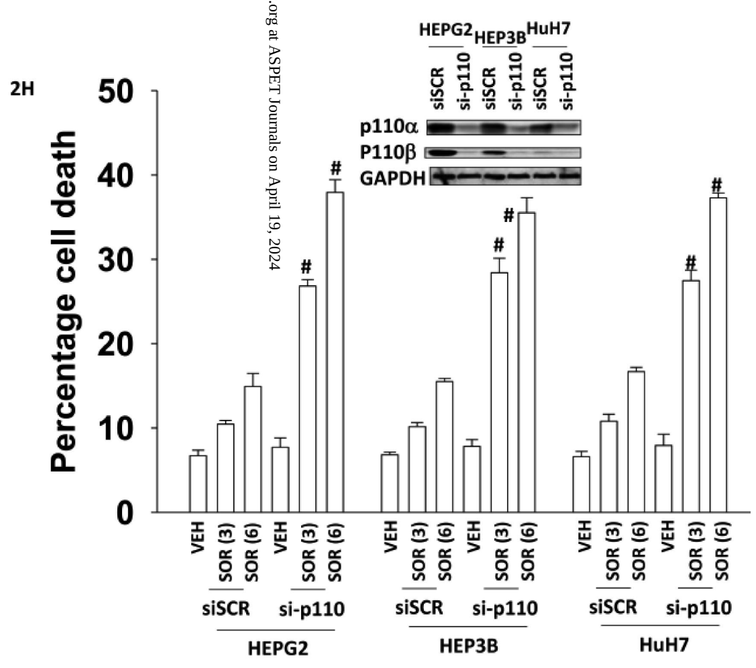
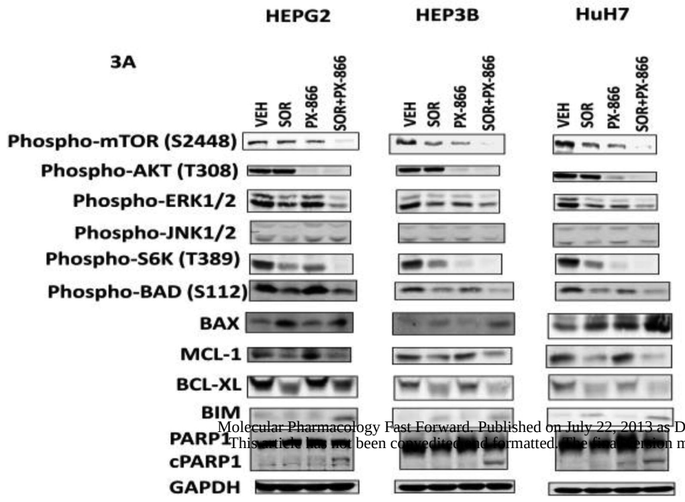
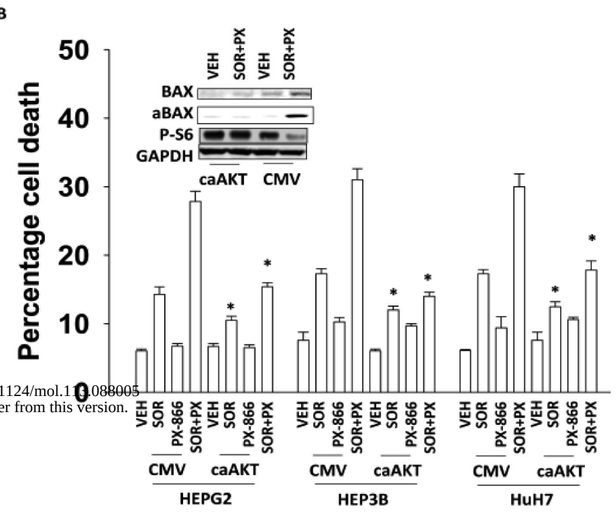
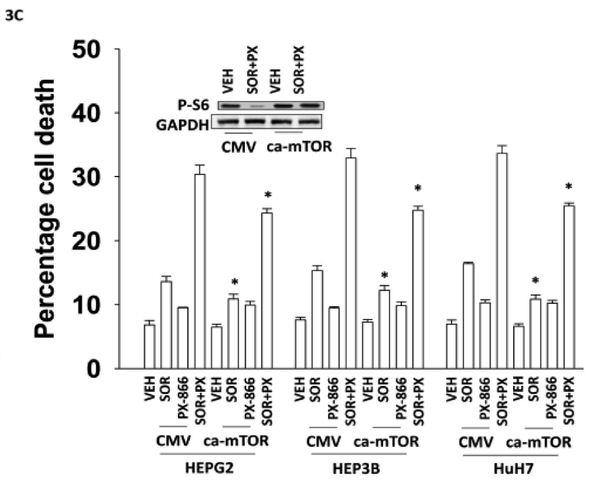
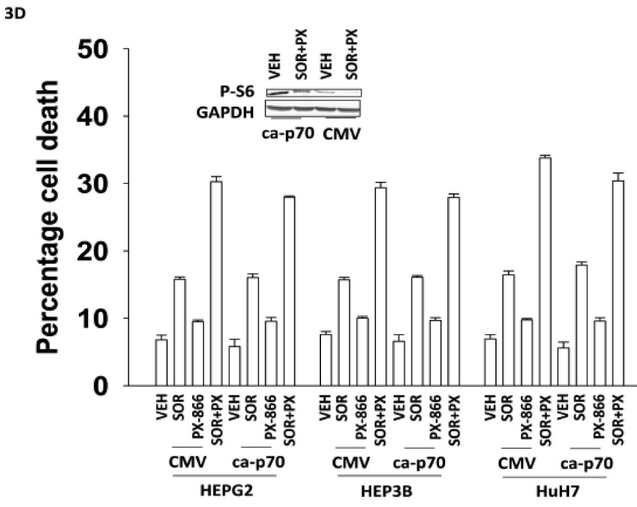


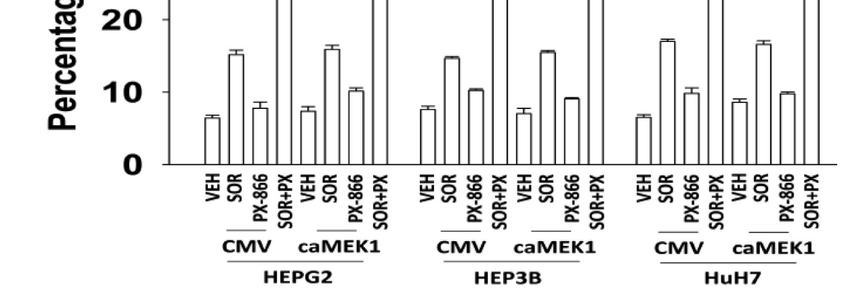
Figure 3

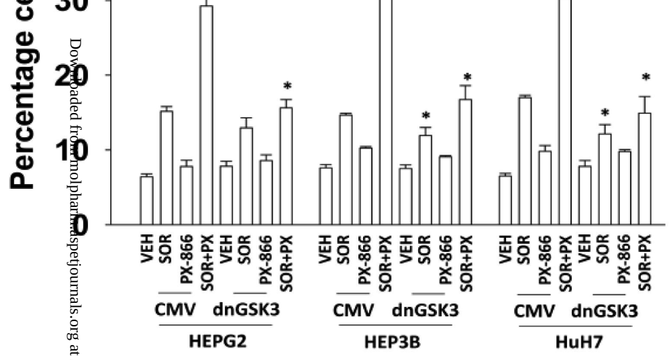


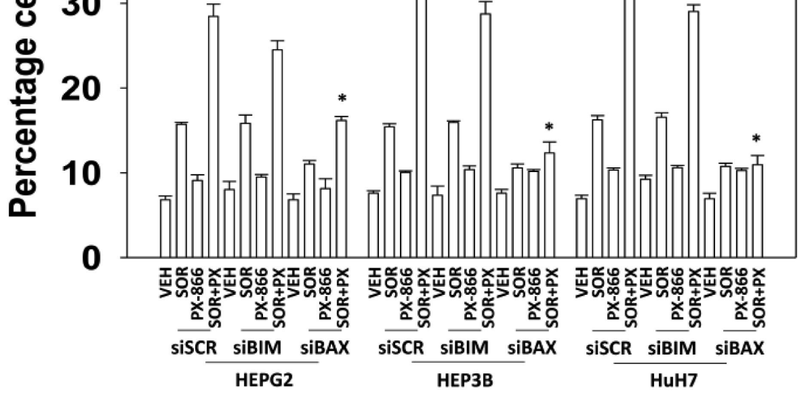


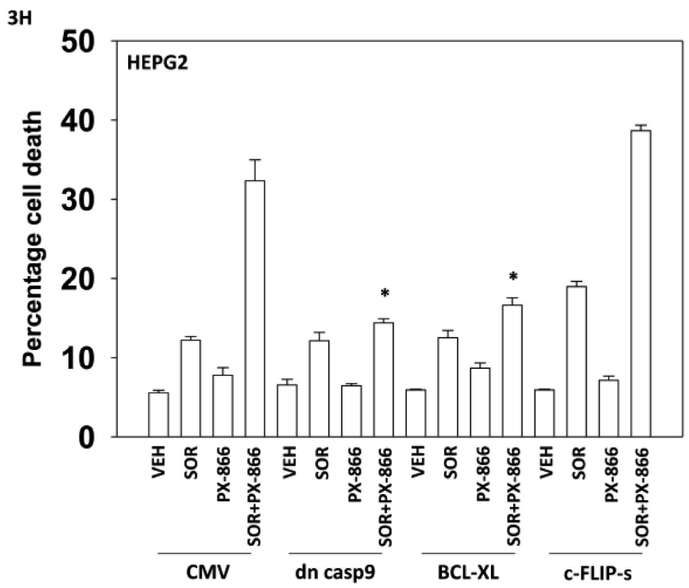


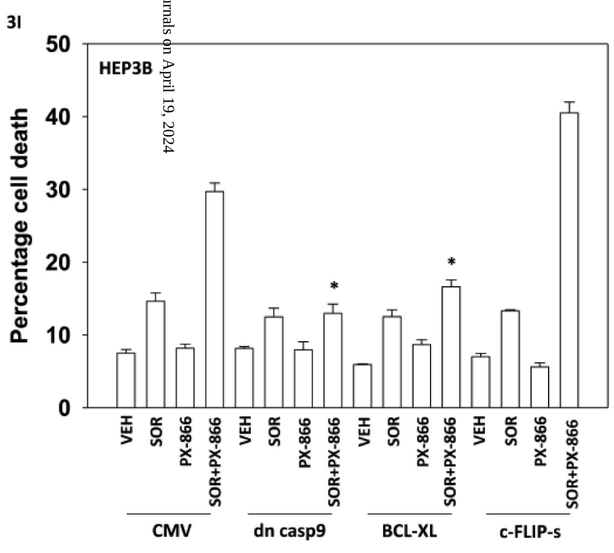












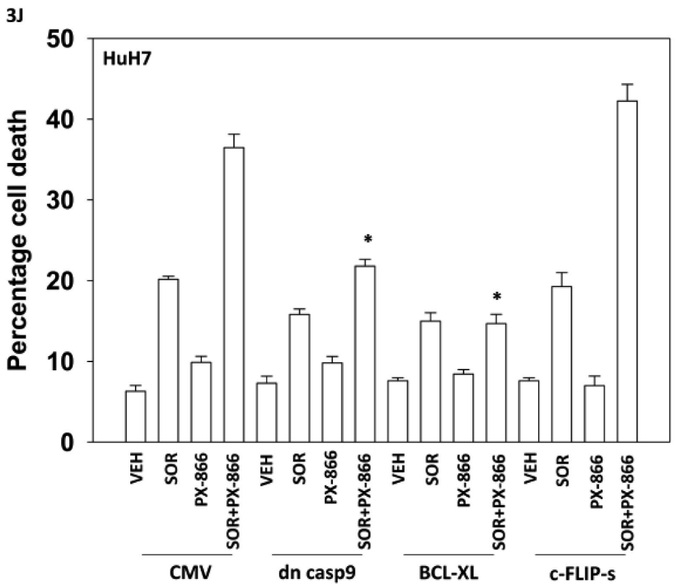
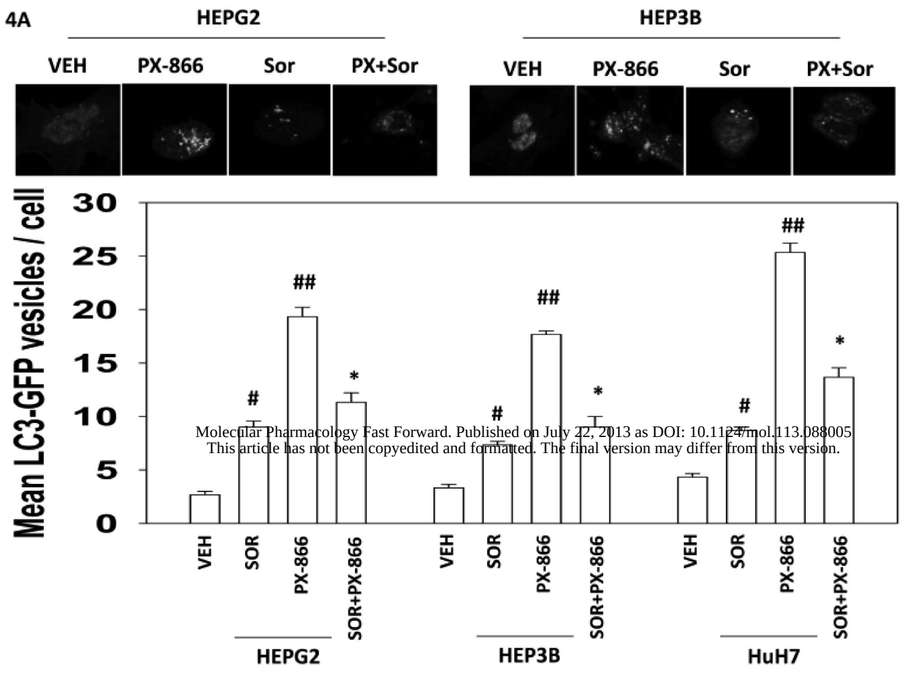
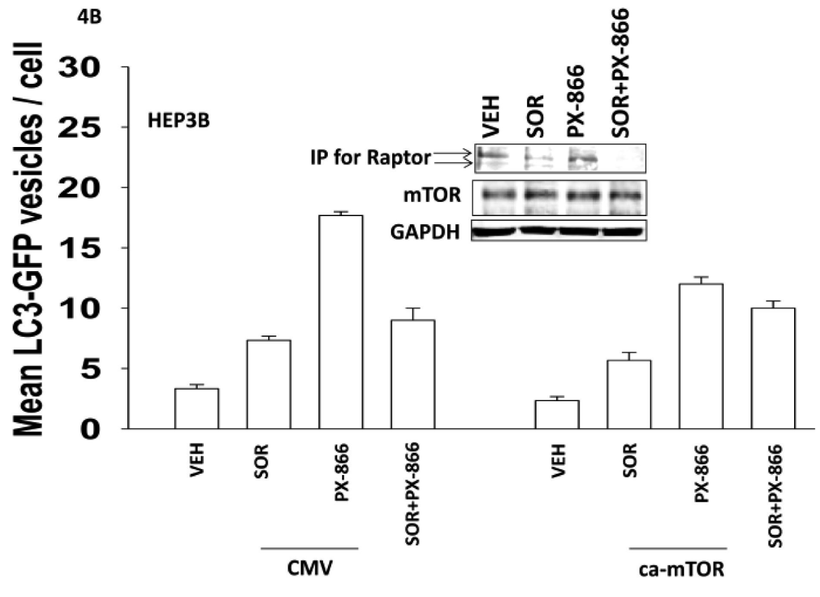
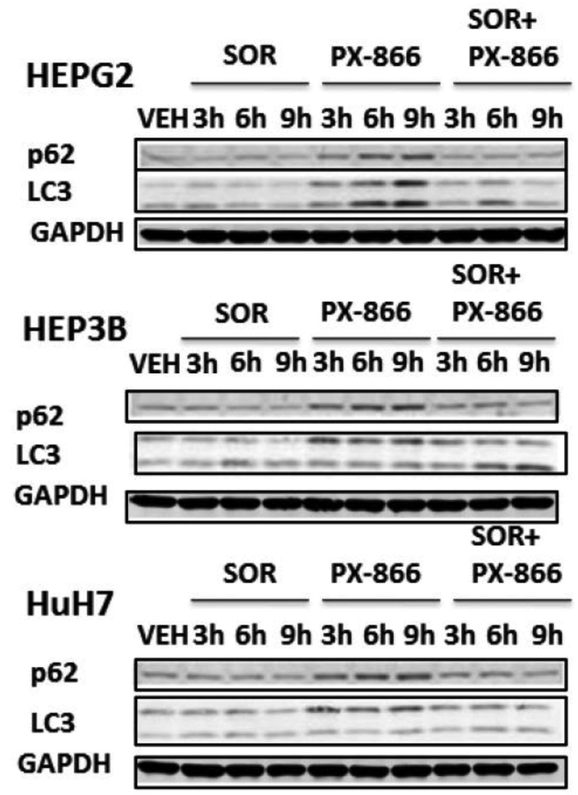
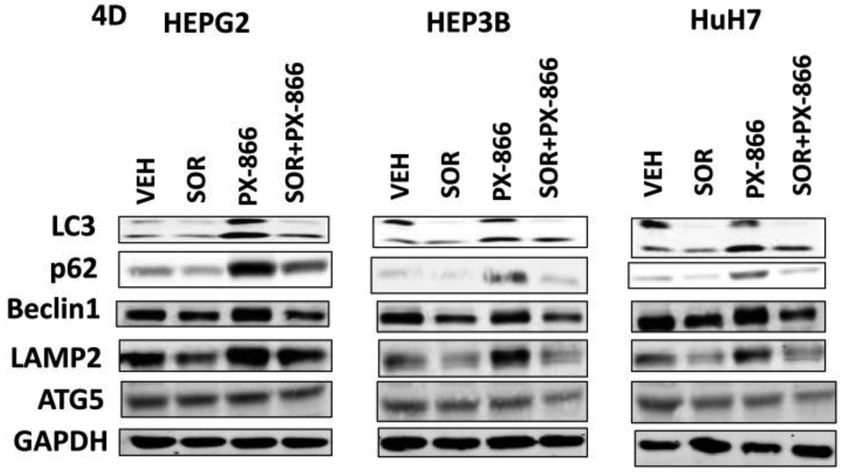


Figure 4









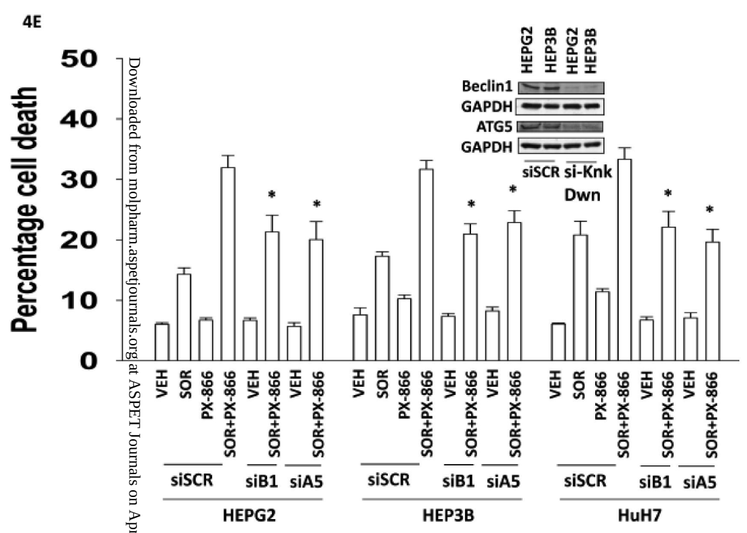


Figure 5