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Inhibition of Autophagy as a Strategy to Augment Radiosensitization by the Dual PI3K/mTOR Inhibitor NVP-BEZ235

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Running title: Inhibiting autophagy to radiosensitize cells with NVP-BEZ235

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List of non-standard abbreviations

FRAP1	**FK506 binding protein 12-rapamycin associated protein 1**
PIK3CA	**phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha**
ATG5	**autophagy related 5**
DAPI	**4', 6- diamidino-2-phenylindole**
siRNA	**small inhibitory RNA**
PTEN	**phosphatase and tensin homolog**
mTOR	**mechanistic target of rapamycin (serine/threonine kinase)**
MEFs	**mouse embryonic fibroblasts**
DNA-PKcs	**DNA-dependent protein kinase, catalytic subunit**
DER	**Dose Enhancement Ratio**

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Abstract

We investigated the effect of NVP-BEZ235 (Novartis), a dual PI3K/mTOR inhibitor currently being tested in phase I clinical trials, in radiosensitization. BEZ235 radiosensitized a variety of cancer cell lines including SQ20B head and neck carcinoma cells and U251 glioblastoma cells. BEZ235 also increased *in vivo* radiation response in SQ20B xenografts. Knockdown of Akt1, p110α, or mTOR resulted in radiosensitization, but not to the same degree as with BEZ235. BEZ235 interfered with DNA damage repair following radiation as measured by the Comet assay and resolution of γH2AX foci. BEZ235 abrogated the radiation-induced phosphorylation of both DNA-PKcs and ATM. Knockdown of either p110α or mTOR failed to decrease phosphorylation of DNA-PKcs, suggesting that the effect of the drug was direct rather than mediated via p110α or mTOR. Treatment of cells with BEZ235 also promoted autophagy. To assess the importance of this process in radiosensitization, we used the autophagy inhibitors 3-methyladenine and chloroquine and found that either drug increased cell killing after BEZ235 treatment and radiation. Knocking down the essential autophagy proteins ATG5 and Beclin1 increased BEZ235-mediated radiosensitization. Furthermore, BEZ235 radiosensitized autophagy deficient $ATG5^{-/-}$ fibroblasts to a greater extent than $ATG5^{+/+}$ cells. We conclude that BEZ235 radiosensitizes cell and induce autophagy, by apparently distinct mechanisms. Inhibiting autophagy via pharmacologic or genetic means increases radiation killing following BEZ235 treatment; hence, autophagy appears to be cytoprotective in this situation. Our data offer a rationale for combining BEZ235 along with an autophagy inhibitor (i.e. chloroquine) and radiation in future clinical trials.

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Introduction

Class I PI3 kinases are a family of protein/lipid kinases that regulate many cellular processes through the activation of downstream targets such as Akt and mTOR (Engelman et al., 2006). The PI3K/Akt/mTOR pathway is activated in a large percentage of human cancers through a variety of mechanisms including Ras mutation, loss of *PTEN*, activation of growth factor receptors such as EGFR, and mutations in *PIK3A* (Yuan and Cantley, 2008). Hence, PI3K is an attractive target for anti-cancer drug therapy (Wong et al., 2010). Two of the more commonly used inhibitors of the PI3K pathway are wortmannin (Powis et al., 1994) and LY294002 (Vlahos et al., 1994). However, due to their unfavorable pharmacologic properties, toxicity and non-specific inhibition of other kinases, neither of these drugs is suitable for clinical use (Bain et al., 2007, Gharbi et al., 2007, Norman et al., 1996). For this reason, other more specific and less toxic agents that can inhibit this pathway have been developed. One such drug is the Novartis compound NVP-BEZ235, a dual pan-class I PI3K and mTOR inhibitor that binds the ATP-binding clefts of PI3K and mTOR (Stauffer et al., 2008). BEZ235 inhibits the α, γ, and δ isoforms of the p110 subunits with an IC50 ranging from 4-7 nM and the β isoform with an IC50 of 75 nM (Maira et al., 2008). The IC50 for mTOR kinase is 20 nM; however, the IC50 for kinases such as VEGFR1, HER1, cMet, and Akt1 is orders of magnitude higher (>10,000 nM).

The PI3K/Akt/mTOR pathway, which regulates diverse cellular processes including cell growth, proliferation, survival, metabolism and autophagy (Manning and Cantley, 2007), has also been implicated in radiation resistance. Activation of the PI3K/Akt/mTOR pathway through genetic changes such as Ras mutation, EGFR overexpression/mutation and loss of PTEN have been shown to lead to increased survival after radiation (Bernhard et al., 2000, Grana et al., 2002, Jiang et al., 2007, Mukherjee et al., 2009). Conversely inhibitors of this pathway including EGFR

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inhibitors (Harari and Huang, 2001), LY294002 (Gupta et al., 2001, Kao et al., 2007, Kim et al., 2005, Nakamura et al., 2005), PI103 (Prevo et al., 2008) and BEZ235 (Fokas et al., 2012, Fokas et al., 2012, Konstantinidou et al., 2009, Mukherjee et al., 2012) have been shown to increase sensitivity of cells to radiation. Inhibition of mTOR by rapamycin or related compounds (Crazzolara et al., 2009, Kamada et al., 2000) but also BEZ235 (Fan et al., 2010, Liu et al., 2009) promotes autophagy. We were interested in understanding the mechanism of radiosensitization by BEZ235, in particular the role that autophagy might play in this. We found that this agent is a highly potent radiosensitizer, but its effect cannot be explained solely on its inhibition of the PI3K/Akt/mTOR pathway and is also likely related to inhibition of DNA damage repair through inhibition of other PI3K-like family members such as ATM and DNA-PKcs. BEZ235 does induce autophagy, but this appears to be a protective response following radiation as inhibiting it leads to increased cell killing. Our findings provide insight into how BEZ235 radiosensitizes and how to further improve its efficacy by inhibiting autophagy.

Materials and Methods

Chemicals--NVP-BEZ235 was provided by Novartis (Novartis International Basel Switzerland) and used at a concentration of 50 nM for all *in vitro* experiments. The following chemicals were purchased from Sigma-Aldrich (Saint Louis, MO) and used at the following final concentrations: rapamycin 10 nM, 3-MA 5 uM, staurosporine 1 µM, E64d 10 µg/ml, Pepstain A 10 µg/ml.

Cell growth–SQ20B head and neck squamous cell carcinoma cells, U251MG glioblastoma cells, ATG5 wild-type ($ATG5^{+/+}$) and ATG5 knockout ($ATG5^{-/-}$) mouse embryonic fibroblasts (MEFs) were cultured in DMEM (4,500 mg/L glucose; Invitrogen, NY, USA) containing 10% fetal bovine serum (Atlanta Biologicals; NY, USA), penicillin (100 units/ml), and

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streptomycin (100 mg/ml; Life Technologies, Inc., Gaithersburg, MD) at 37°C in humidified 5%CO_2-95% air. $ATG5^{+/+}$ and $ATG5^{-/-}$ MEFs were generated from ATG5 heterozygous mice generously provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Bunkyo-Ku, Tokyo) (Kuma et al., 2004).

Mouse studies–Pathogen free female Ncr-nu/nu mice were obtained from National Cancer Institute (stock # 01B74) and housed in the vivarium of the University of Pennsylvania Laboratory Animal Resources. All the experiments were carried out in accordance with the protocols approved by University Institutional Animal Care and Use Committee guidelines.

Transfection–Cells were transfected with ON-TARGET plus SMART pool siRNA for AKT-1, FRAP1, PIK3CA, DNA-PKcs, ATG5 and Beclin-1. Cells were harvested and plated at a density of 200,000 cells per well in a six well plate and allowed to attach over night. The next day media was removed and cells were washed twice with PBS and re-fed with 1 ml of OPTI-MEM from Gibco. The six well plate was returned to the incubator for 1 hour before they were transfected. SiRNA was mixed with oligofectamine reagent from Invitrogen for 20 minutes before being added to the dishes.

Radiation survival assays–Cultures in log growth were counted and plated in 60-mm dishes containing 4 ml of medium. Drugs were added to cultures at least 1 h prior to radiation. Cells were irradiated with a Mark I cesium irradiator (J. L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min. Treatment was continued for 8h after irradiation, at which time drug-free medium was added. Colonies were stained and counted 10–14 days after irradiation. The surviving fraction was calculated as follows: (number of colonies formed)/(number of cells plated x plating efficiency). Each point on the survival curve represents the mean surviving fraction from

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at least six dishes. A dose enhancement ratio (DER) was calculated as a ratio of the 10% percent survival rate between cells treated with irradiation alone and those which were treated with irradiation and drug.

Assays for γ-H2AX Activation–Following irradiation, cells were assessed via immunofluorescence for unrepaired DNA damage via phosphorylation of H2AX (γ-H2AX), a standard marker of unrepaired double strand DNA damage. For these experiments, cells were grown on coverslips. All groups of cells were fixed in 4% paraformaldehyde with 0.1% Triton-X100 and probed with anti-γ-H2AX antibody (Upstate Biological, Inc., Lake Placid, NY), followed by secondary antibody (anti-mouse Alexa Fluor 594 (Molecular Probes. subsidiary of Invitrogen). After the staining with specific antibody, the coverslips were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) to mark the nuclei. All treatment groups were then assessed for γ-H2AX foci via sequential imaging through each nucleus. A minimum of 300 cells in each treatment group were counted.

Protein Extraction and Western Blot Analysis–Protein isolation and quantitation and Western blotting were performed as described previously (Pore et al., 2006). The following antibodies were procured from Cell Signaling Technology (Danvers, MA, USA): anti–phospho-Akt antibody (Ser473 and Thr308), anti-phospho-4E-BP1 (Ser 65), anti-phospho-S6, anti-mTOR, anti-Akt1, anti-PI3K p110α, LC3B, p62, cleaved-PARP. Other antibodies were those directed against DNA-PKcs (Biolegend #612901), DNA-PKcs (G4) (Santa Cruz # 5282); P-H2A.X (Millipore # 05-636); and β-actin antibody (Sigma-Aldrich; St. Louis, MO, USA). The secondary antibody used for these blots was a goat anti-mouse and goat anti-rabbit antibody (Thermo Scientific; Rockford, IL, USA). Antibody binding was detected by chemiluminescence using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

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Split-dose experiments–Cells were seeded into 60 mm dishes and allowed to attach before BEZ235 (50nM) was added to dishes 1 hr. before irradiation. A total dose of 6 Gy was given in two fractions of 3 Gy with an interval of 1, 2, 4 or 6 hours between the first and last dose of irradiation.

Comet assay–Cells were seeded into 60 mm dishes 24 hours prior to drug and irradiation treatment. Cells were treated with drug 1 hour prior to irradiation with 4 Gy. Thirty minutes following irradiation cells were trypsinized and suspended to a final density of 1 X 10^5/ml in molten low melting agarose at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto microscope slides. Samples were then processed following the alkaline comet assay protocol from Trevigen (# 4250-050-K).

Electron microscopy–SQ20B cells were treated with BEZ235 for 1 hr and irradiated and 24 hr later cells were fixed and given to the Electron Microscopy Resource facility for further processing.

GFP-LC3 immunofluorescence microscopy–SQ20B and U251 cells were transfected with pCMV-GFP-LC3 plasmid and placed under G418 selection to obtain stably transfected colonies. The clones were treated with BEZ235 for 24 hr. Following treatment, cells were fixed and subjected to immunofluorescence analysis.

Senescence β-galactosidase cell staining protocol–Cells were seeded into 6 well plates at a density of 1 X 10^5 cells per well. Cells were allowed to attach overnight before treatment with BEZ235 for one hour prior to being irradiated with 4 Gy. 48 hours following irradiation cells were stained following the Senescence *β-galactosidase* staining kit protocol (Cell Signaling # 9860)

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Statistical analysis–A two tailed t-test was used to compare the mean values of the control and treated samples.

Results

BEZ235 inhibits PI3K/mTOR pathway and increases radiation response

Cells were treated with the drug for differing lengths of time, cells harvested, and then Western blotting performed. One hour of drug treatment, with either 25 or 50 nM, down regulated P-AktS473 in SQ20B (**Figure 1A; left panel**) and U251 cells (**Supplemental Figure 1A**). BEZ235 also effectively decreased phosphorylation of 4E-BP1, a downstream marker of mTOR activation. In contrast, even 100 nM of the mTOR inhibitor rapamycin, was less effective in decreasing 4E-BP1 phosphorylation and had no effect on Akt phosphorylation.

After 1 hr. incubation with the drug, cells were irradiated, and then clonogenic survival assays performed. BEZ235 radiosensitized both SQ20B (**Figure 1A right panel**) and U251 cells (**Supplemental Figure 1B**) with a dose enhancement ratio (DER) ranging from 1.35 – 1.63. In contrast, rapamycin (10 or 100 nM) showed minimal effect on increasing radiation-induced cell killing in either SQ20B or U251 cells. BEZ235 also radiosensitized other cancer cell lines with various genetic backgrounds including colon cancer (HT29 with mutant p53; HCT116 with *PIK3CA* mutation) and lung cancer cells (A549 with K-*ras* mutation; H460 with *PIK3CA* mutation) (**Supplemental Figure 2**).

Effect of BEZ235 on non-transformed cells

We also used two non-transformed lines derived from mice, WTFB (wildtype fibroblasts) and C10, an immortalized bronchial epithelial cell line kindly provided by Dr. Malkinson

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(University of Colorado, Denver) (Malkinson et al., 1997). Both cell lines expressed little P-Akt compared to as SQ20B cells; therefore, BEZ235 had little appreciable effect on P-Akt downregulation (**Figure 1B;** compare lanes 1 and either 2 or 3). Of note, both cell lines contained a similar level of P-S6 compared with SQ20B, and this was effectively decreased by BEZ235. WTFB did not show radiosensitization by BEZ235 (**Figure 1D**), and C10 cells only showed radiosensitization at the relatively high dose of 6 Gy (**Figure 1C**).

Downregulation of PI3K/mTOR pathways by siRNA leads to radiosensitization

To investigate the contribution of the PI3K pathway to radiosensitization we used siRNA directed against p110α, Akt1, or mTOR. We chose to examine p110α because the IC50 for inhibition of this isoform by BEZ235 was 5-7 nM. Both p110 γ and p110 δ are also inhibited by these low concentrations of BEZ235; however, SQ20B cells have almost no expression of either of these isoforms compared with P4936, a control lymphoma line (**Supplemental Figure 3).** This is consistent with the literature, which indicates that the γ and δ isoforms are expressed only in selected cell types, such as white blood cells, but not in most epithelial cancer lines (Guillermet-Guibert et al., 2008, Vanhaesebroeck et al., 1997).

Knockdown of the p110α isoform has previously been shown to radiosensitize SQ20B cells (Kim et al., 2005). The same study showed that these cells are radiosensitized by knockdown of Akt1 but not Akt 2 or 3. These siRNAs functioned as expected to knock down levels of the respective proteins in SQ20B and U251 cells (**Figure 2A, 2B**). p110α or mTOR knockdown in SQ20B cells led to a decrease in survival after radiation with a DER of 1.19 – 1.24 (**Figure 2C**). However, the degree of radiosensitization was not as great as that seen with BEZ235 (DER = 1.48), and the combination of siRNA against p110α and mTOR had no greater effect than either

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alone. In U251 cells, knockdown of Akt1, mTOR, or p110α all led to a similar level of radiosensitization with a DER of 1.17 - 1.25 but not as high as seen with BEZ235 (**Figure 2D**; DER of 1.53).

Effects of BEZ235 on DNA damage response following radiation

Previous work from our lab had indicated that inhibition of the PI3K pathway might be related to delayed repair of DNA damage (Kao et al., 2007). To determine whether BEZ235 altered DNA damage repair following radiation, we performed split course experiments in which cells were irradiated either with a fixed dose of radiation or given half the dose, allowed to recover and then given the same dose of radiation hours later. Typically, cells exposed to split course radiation show greater survival than cells exposed to the total dose given at one time because cells have an inherent ability to repair DNA damage. As the time between the 2 doses increased from zero to one to two hours, the survival fraction increased from 1 to 1.6 to 2.4 (**Figure 3A).** Pre-treatment with BEZ235 essentially abolished this effect, suggesting that the drug interfered with DNA damage repair. Similarly, the survival fraction for U251 cells increased from 1 to 1.9 when doses were split by 6 hours, but no such increase was seen when cells were pre-treated with BEZ235 (**Supplemental Figure 4A**).

To assess the possibility of altered DNA damage repair, we assayed H2AX phosphorylation (γH2AX). **Figure 3B** and **C** shows that γH2AX foci were induced to a similar extent in both BEZ235-pretreated and control SQ20B cells at 1 hour, but by 24 hours, there were more foci remaining in the drug-treated cells. We made similar findings in U251 cells (**Supplemental Figure 4B, C**). We also used the Comet assay, which measures unrepaired DNA by assessing the tail moment. We found similar levels of DNA damage by the Comet assay at 1

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hour, but persistently elevated levels by 24 hours in the drug-treated SQ20B cells (**Figure 3D**). We made the same qualitative observations using the Comet assay in U251 cells (**Supplemental Figure 4D**).

To further investigate BEZ235 and DNA damage repair, we examined proteins involved in DNA damage repair. One of the key proteins involved in DNA damage recognition is DNA-PK, which consists of a regulatory subunit, Ku, and a catalytic subunit, DNA-PKcs, which is readily phosphorylated in response to radiation. DNA-PKcs has been reported to be inhibited by the BEZ235 compound (Kong et al., 2009, Toledo et al., 2011). Therefore, we exposed cells to drug, irradiated them and then collected samples, which we analyzed by Western blotting. **Figure 4A** shows that DNA-PKcs was phosphorylated at Ser2056 even at 2 Gy and increased in a dose-dependent manner. However, pre-treatment with BEZ235 attenuated this phosphorylation. Of note, phosphorylation of ATM at Ser1981 was also blunted following radiation. Similar attenuation of the phosphorylation of DNA-PKcs in response to radiation was seen in U251 cells (**Supplemental Figure 5A).**

The effect of BEZ235 on the phosphorylation of various proteins could be indirect through its effects on the PI3K/Akt pathway. To assess this we transfected cells with siRNA directed against p110α or mTOR, irradiated them, then performed Western blotting. **Figure 4B** shows that, in contrast to treatment with BEZ235, knockdown of either p110α or mTOR failed to decrease phosphorylation of DNA-PKcs, which argues against the idea that effect of the drug was mediated via either p110α or mTOR. **Supplemental Figure 5B** shows similar results with U251 cells.

Given that we found a change in DNA-PKcs phosphorylation, we wanted to determine the effect of direct targeting of the protein. We used siRNA against DNA-PKcs to decrease the level

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of the protein (**Figure 4C right panel**) and found that this led to a substantial decrease in clonogenic cell survival, comparable to that seen with BEZ235 (**Figure 4C left panel**). Surprisingly when we combined siRNA against DNA-PK with BEZ235, we observed a highly synergistic effect far greater than that seen with either agent alone.

Effect of BEZ235 on senescence

One publication has suggested that NVP-BEZ235 increased senescence following radiation through its effects on DNA-PKcs (Azad et al., 2011). To investigate this, we performed *β-galactosidase* staining on SQ20B cells 48 hours after irradiation with 4 Gy. 4 Gy resulted in greater *β-galactosidase* staining than no irradiation (**Supplemental Figures 6A**, **B**). However, pre-treatment with BEZ235 followed by 4 Gy did not increase staining compared with 4 Gy alone, suggesting that the drug had no effect on increasing senescence after radiation. Likewise we found no increase in senescence after BEZ235 pre-treatment followed by irradiation in A549 and H226 lung cancer cells, U251 glioblastoma cells and PC3 prostate cancer cells.

Effect of BEZ235 on autophagy

Because BEZ235 has been reported to induce autophagy (Fan et al., 2010, Liu et al., 2009), we investigated whether this might be involved in radiosensitization with the drug. We performed Western blotting for LC3, a protein that is embedded in autophagosomes that becomes lipidated during active autophagy. Hence, conversion of the LC3-I form to the faster-migrating LC3-II form is a commonly used marker of autophagy. BEZ235 treatment of SQ20B cells led to conversion of the LC3-I to the LC3-II form, consistent with the induction of autophagy (**Figure 5A** compare lanes 6-10 with 1-5). Accumulation of LC3-II can occur without activation of

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autophagy, for example in the case of an agent that decreases autophagic flux by inhibiting autophagy at a later stage. Hence, many have advocated performing autophagic flux measurements to distinguish between the two possibilities (Klionsky et al., 2008). We used the protease inhibitors E64d and Pepstatin A, which blocks autophagic flux by inhibiting the late stage of autophagy. The combination of E64d and Pepstatin A and BEZ235 clearly increased LC3-II levels at hours 8 and 24 compared to BEZ235 by itself (**Figure 5B**; compare lanes 2 vs. 5 and 6 vs. 8). This result strongly indicates that the accumulation of LC3-II with BEZ235 is due to autophagy induction rather than reduced autophagy flux. **Figure 5A** also shows that by 24 hours (lane 10), with BEZ235 treatment there was a drop in the level of p62, a protein that participates in and is degraded by the autophagic process, thus strengthening the idea that BEZ235 induces autophagy.

We repeated this study with 4 Gy of radiation and extended it out to 72 hours. BEZ235 clearly led to LC3-I to LC3-II conversion at 24-72 hours. (**Figure 5C**; lanes 8-10); however, 4 Gy did not (lanes 5-7). The combination of BEZ25 + 4 Gy (lanes 11-13) appeared the same as BEZ235 alone.

To further confirm these effects on autophagy, we transiently transfected SQ20B cells with GFP-labeled LC3. We administered various treatments, and then monitored GFP-LC3 localization using immunofluorescence**.** BEZ235 treatment for 24 hours led to accumulation of punctate foci in the cytoplasm, consistent with localization to the autophagosomes (and induction of autophagy) (**Figure 5D)**. Irradiation with 4 Gy did not appreciably increase punctate GFP-labeled LC3, but these foci were seen in cells receiving BEZ235 and radiation.

Electron microscopy imaging analysis of SQ20B cells treated with BEZ235 for 24 hours. revealed numerous vacuoles in the cytoplasm (**Supplemental Figure 7A and insets with**

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arrows). We speculate that this represents cells at a later stage of autophagy during which the autophagosomes have merged with lysosomal vacuoles to form single-membraned autolysosomes containing degraded cytoplasmic contents as has been described previously (27). These vacuoles were not seen in cells irradiated with 4 Gy but were seen in cells that were treated with BEZ235 + 4 Gy.

We found qualitatively similar results with U251 cells. Western blotting showed no conversion of LC3-I to LC3-II 24-72 hours following 4 Gy (**Supplemental Figure 7B**). BEZ235 resulted in LC3-I to LC3-II conversion at 24 hours, but in contrast to the situation with SQ20B cells (**Figure 5B**), this was prolonged after BEZ235 + 4 Gy, visible even out to 72 hours.

Effect of autophagy inhibitors on radiosensitization with BEZ235

To determine whether inhibiting the transit of cells through autophagy would influence radiosensitivity we first used the drug 3-methyladenosine (3-MA), which blocks autophagy at an early stage by inhibiting the autophagy protein Vps34 (Castino et al., 2010). **Figure 6A** shows that pre-treatment of SQ20B cells with 3-MA prior to BEZ235 treatment led to a decrease in the LC3-II band compared with BEZ235 treatment alone (compare lanes 8 and 9), consistent with inhibition of autophagy. Of note, 3-MA by itself and in combination with BEZ235 led to an increase in PARP cleavage. Using the combination of 3-MA and BEZ235 we found an increase in the cell killing after radiation with a DER of 2.40 (**Figure 6B**).

We also used chloroquine, which inhibits autophagy at a later stage by blocking lysosomal activity. As in the case with 3-MA, treatment of SQ20B cells with chloroquine and BEZ235 led to decreased clonogenic survival, with a DER of 2.63 (**Figure 6C**). Therefore, both of these drugs that inhibited autophagy potentiated BEZ235-mediated radiosensitization.

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Genetic inhibition of autophagy and its effect on radiosensitization with BEZ235

The experiments described in **Figure 6** used pharmacological agents to inhibit autophagy. The possibility exists that these results were due to off-target effects; hence, we used genetic approaches to confirm these results. First, we used siRNA directed against two key autophagic proteins, Beclin1 and ATG5, which downregulated their intended targets (**Figure 7A**). Knockdown of both proteins significantly increased BEZ235 mediated radiosensitization with a DER of 2.34, whereas knockdown of either one alone had no effect (**Figure 7B**).

We also used cells deficient in the autophagy protein ATG5. BEZ235 treatment (for 24 hours) of the wildtype ($ATG5^{+/+}$) fibroblasts led to a conversion of LC3-I to LC3-II; however, this was completely absent in $ATG5^{-/-}$ cells, consistent with their loss of autophagic capacity (**Figure 7C)**. Also of note, there was little downregulation of P-Akt in either cell line with BEZ235 treatment, reminiscent of what we found with WTB and C10 cells (**Figure 1B**). We performed clonogenic survival studies with these cells following radiation (Figure 7D). First, we noted little difference in the radiation survival curves between $ATG5^{-/-}$ and $ATG5^{+/+}$ cells, consistent with the idea that autophagy does not generally play much role in survival after radiation. Secondly, BEZ235 treatment of $ATG5^{+/+}$ cells, resulted in a very modest degree of radiosensitization (DER of 1.11), similar to what we had saw in WTB and C10, two other non-transformed lines. Lastly, BEZ235 treatment of $ATG5^{+/+}$ cells, which cannot undergo autophagy, resulted in a greater degree of radiosensitization than that seen in $ATG5^{+/+}$ cells (DER 1.36).

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Discussion

NVP-BEZ235 is a recently developed combined PI3K/mTOR inhibitor being used clinically that has been shown to be a radiosensitizer (Fokas et al., 2012, Konstantinidou et al., 2009, Mukherjee et al., 2012). We found that the compound radiosensitizes a variety of cancer cell lines with varying genetic changes including EGFR amplification (SQ20B), p53 mutation (HT29), and PTEN deletion (U251), and K-*ras* mutation (A549). We tested the drug in 3 different non-cancer cell lines (WTFB, C10 and wildtype MEFs) and found that there was minimal radiosensitization.

The primary purpose of our study was to understand mechanistically how BEZ235 radiosensitizes cancer cells. Our findings indicate that the effect of the drug on the PI3K/mTOR pathway does not completely explain its radiosensitizing effect. In both SQ20B and U251 cells, knockdown of p110α, an isoform associated with radiation resistance (Kim et al., 2005), or of mTOR, or of the two together led to modest radiosensitization, but not as great as that seen with BEZ235. Of interest, although we found some radiosensitization using mTOR siRNA, we found none with rapamycin. Likewise, other groups have not found rapamycin to radiosensitize cancer cells (Gupta et al., 2001, Nakamura et al., 2005). In contrast, Paglin *et al.* found that rapamycin in combination with radiation led to increased cell death (Paglin et al., 2005). Albert *et al*. also showed that the rapamycin derivative RAD001 radiosensitized breast cancer cell lines (Albert et al., 2006). The same group found that RAD001 radiosensitized prostate cancer cells lines but to a very modest extent (calculated DER from their survival curves for PC3 ~1.20 and for DU145 ~1.02) (Cao et al., 2006). In another study by this group, neither rapamycin nor RAD001 radiosensitized GL261 glioma cells to any appreciable extent (Shinohara et al., 2005). We speculate that the reason for these conflicting results in the literature is that treating cells with rapamycin inhibits the mTOR/raptor (TORC1) complex but not the mTOR/rictor (TORC2)

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complex, which can then lead to compensatory increase in Akt activation, perhaps via insulin receptor substrate-1 (IRS-1) (Guertin and Sabatini, 2007, Tamburini et al., 2008). The extent to which this happens is cell line dependent. On the other hand, we found that mTOR siRNA, but not rapamycin, has some effect on radiosensitization, perhaps because this siRNA inhibits both complexes containing mTOR.

As inhibition of PI3K and mTOR did not seem to explain radiosensization by BEZ235 completely, we explored its effects on DNA damage repair. Based on split course radiation experiments and analysis of the kinetics of γ-H2AX foci resolution and the Comet assay, we found that the drug impairs the repair of DNA damage. Furthermore, we found that it abrogates the radiation-induced phosphorylation of DNA-PKcs and ATM, key proteins involved DNA damage repair. Akt has been implicated in regulating DNA-PKcs phosphorylation (Toulany et al., 2008). However, since knockdown of p110α or mTOR did not alter DNA-PKcs phosphorylation, it is much more likely that the effect of BEZ235 on ATM and DNA-PKcs is a direct one, given that they are both members of the PI3K-like family.

A recent paper showed that BEZ235 decreased both HR and NHEJR following radiation (Mukherjee et al., 2012). However, our experiments using the combination of BEZ235 and DNA-PKcs knockdown show a surprisingly robust increase in cell killing, far greater than that seen with either alone. This finding is suggestive of synthetic lethality with a possible explanation that BEZ235 only partially inhibits HR and NHEJR but combining it with DNA-PKcs leads to complete inhibition of NHEJR with minimal compensatory upregulation of HR.

In contrast to a published paper (Azad et al., 2011), we found no evidence that BEZ235 leads to increased senescence following radiation using a variety of cell lines. However we did find that the drug led to increased autophagy. Autophagy is a process by which cells

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"cannibalize" themselves in an effort to stay alive during stress conditions (mainly nutrient stress) and can potentially be cytoprotective. Our data raise several important points regarding autophagy. Our results do not indicate that autophagy occurs to any appreciable extent following radiation alone (without BEZ235). Paglin *et al*. reported that a novel response of cancer cells to radiation involved autophagy (Paglin et al., 2001). While autophagy may occur in some cells after radiation, the fact that neither SQ20B nor U251 cells show much evidence of this process following radiation alone and knocking out ATG5 in fibroblasts does not alter their radiosensitivity compared with $ATG5^{+/+}$ (wildtype) fibroblasts suggests that this process does not play a dominant role in the survival of cells following radiation. On the other hand, radiation in combination with BEZ235 may modulate the latters' effects on autophagy, at least in some cell lines. In U251, the combination did appear to prolong the duration of autophagy (**Supplemental Figure 7**). When combined with radiation, we believe that the autophagy induced by BEZ235 plays a cytoprotective role. This is supported by the fact that three distinct approaches that inhibit autophagy (chemical inhibitors, siRNA knockdown of ATG5/Beclin1, and cells from ATG5 knockout mice), all lead to increased radiosensitization with BEZ235.

If the induction of autophagy by BEZ235 protects cells from dying after irradiation, how is it that the drug radiosensitizes cells? We hypothesize that BEZ235 has two distinct effects on cells; it induces autophagy by inhibiting mTOR, but it also impairs DNA damage repair by inhibiting the PI3K/mTOR pathway and other PI3K-like family members such as DNA-PKcs. These have two opposing effects on radiosensitivity; the former decreases it whereas the latter increases it. The fact that these two occur together confounds the issue and has led to conflicting reports in the literature. Some groups have suggested that promoting autophagy is the mechanism by which mTOR inhibition sensitizes cells to radiation (Kim et al., 2008, Kuwahara et al., 2011).

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Our results do not support this idea. In fact, there is controversy in the literature as to whether cells can actually die by autophagy (Hippert et al., 2006, Shen et al., 2012). Kroemer and colleagues, who recently performed a screen of ~1400 compounds and used ATG7 knockdown to determine whether this attenuated cell death, found that not a single agent induced death by autophagy (Shen et al., 2011). Our results do however offer a rationale for inhibiting autophagy with an agent such as chloroquine when using BEZ235 to radiosensitize cells. This is particularly relevant since both chloroquine and BEZ235 have been used in patients.

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Authorship Contributions

Participated in research design: Cerniglia, Karar, Tyagi, Christofidou-Solomidou, Koumenis, Maity

Conducted experiments: Cerniglia, Karar, Tyagi

Performed data analysis: Cerniglia, Karar, Tyagi, Christofidou-Solomidou, Koumenis, Maity

Wrote or contributed to writing of manuscript: Cerniglia, Karar, Tyagi, Christofidou-Solomidou, Rengan, Koumenis, Maity

Molecular Pharmacology Fast Forward. Published on September 18, 2012 as DOI: 10.1124/mol.112.080408
This article has not been copyedited and formatted. The final version may differ from this version.

References

Albert JM, Kim KW, Cao C, and Lu B (2006) Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. *Molecular cancer therapeutics* **5**: 1183-1189.

Azad A, Jackson S, Cullinane C, Natoli A, Neilsen PM, Callen DF, Maira SM, Hackl W, McArthur GA, and Solomon B (2011) Inhibition of DNA-dependent protein kinase induces accelerated senescence in irradiated human cancer cells. *Mol Cancer Res* **9**: 1696-1707.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, and Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* **408**: 297-315.

Bernhard EJ, Stanbridge EJ, Gupta S, Gupta AK, Soto D, Bakanauskas VJ, Cerniglia GJ, Muschel RJ, and McKenna WG (2000) Direct evidence for the contribution of activated N-ras and K-ras oncogenes to increased intrinsic radiation resistance in human tumor cell lines. *Cancer Res* **60**: 6597-6600.

Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, and Lu B (2006) Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer Res* **66**: 10040-10047.

Castino R, Bellio N, Follo C, Murphy D, and Isidoro C (2010) Inhibition of PI3k class III-dependent autophagy prevents apoptosis and necrosis by oxidative stress in dopaminergic neuroblastoma cells. *Toxicol Sci* **117**: 152-162.

Crazzolara R, Bradstock KF, and Bendall LJ (2009) RAD001 (Everolimus) induces autophagy in acute lymphoblastic leukemia. *Autophagy* **5**: 727-728.

Engelman JA, Luo J, and Cantley LC (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**: 606-619.

Fan QW, Cheng C, Hackett C, Feldman M, Houseman BT, Nicolaides T, Haas-Kogan D, James CD, Oakes SA, Debnath J, Shokat KM, and Weiss WA (2010) Akt and autophagy cooperate to promote survival of drug-resistant glioma. *Sci Signal* **3**: ra81.

Fokas E, Im JH, Hill S, Yameen S, Stratford M, Beech J, Hackl W, Maira SM, Bernhard EJ, McKenna WG, and Muschel RJ (2012) Dual inhibition of the PI3K/mTOR pathway increases tumor radiosensitivity by normalizing tumor vasculature. *Cancer Res* **72**: 239-248.

Fokas E, Yoshimura M, Prevo R, Higgins G, Hackl W, Maira SM, Bernhard EJ, McKenna WG, and Muschel RJ (2012) NVP-BEZ235 and NVP-BGT226, dual phosphatidylinositol 3-kinase/Mammalian target of rapamycin inhibitors, enhance tumor and endothelial cell radiosensitivity. *Radiat Oncol* **7**: 48.

Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, Timms JF, and Waterfield MD (2007) Exploring the specificity of the PI3K family inhibitor LY294002. *Biochem J* **404**: 15-21.

Grana TM, Rusyn EV, Zhou H, Sartor CI, and Cox AD (2002) Ras mediates radioresistance through both phosphatidylinositol 3-kinase-dependent and Raf-dependent but mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-independent signaling pathways. *Cancer research* **62**: 4142-4150.

Guertin DA, and Sabatini DM (2007) Defining the role of mTOR in cancer. *Cancer Cell* **12**: 9-22.

Guillermet-Guibert J, Bjorklof K, Salpekar A, Gonella C, Ramadani F, Bilancio A, Meek S, Smith AJ, Okkenhaug K, and Vanhaesebroeck B (2008) The p110beta isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110gamma. *Proc Natl Acad Sci U S A* **105**: 8292-8297.

Gupta AK, Bakanauskas VJ, Cerniglia GJ, Cheng Y, Bernhard EJ, Muschel RJ, and McKenna WG (2001) The Ras radiation resistance pathway. *Cancer Res* **61**: 4278-4282.

Harari PM, and Huang SM (2001) Radiation response modification following molecular inhibition of epidermal growth factor receptor signaling. *Semin Radiat Oncol* **11**: 281-289.

Hippert MM, O'Toole PS, and Thorburn A (2006) Autophagy in cancer: good, bad, or both? *Cancer Res* **66**: 9349-9351.

Jiang Z, Pore N, Cerniglia GJ, Mick R, Georgescu MM, Bernhard EJ, Hahn SM, Gupta AK, and Maity A (2007) Phosphatase and tensin homologue deficiency in glioblastoma confers resistance to radiation and temozolomide that is reversed by the protease inhibitor nelfinavir. *Cancer Res* **67**: 4467-4473.

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, and Ohsumi Y (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* **150**: 1507-1513.

Kao GD, Jiang Z, Fernandes AM, Gupta AK, and Maity A (2007) Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. *J Biol Chem* **282**: 21206-21212.

Kim IA, Bae SS, Fernandes A, Wu J, Muschel RJ, McKenna WG, Birnbaum MJ, and Bernhard EJ (2005) Selective inhibition of Ras, phosphoinositide 3 kinase, and Akt isoforms increases the radiosensitivity of human carcinoma cell lines. *Cancer Res* **65**: 7902-7910.

Kim KW, Hwang M, Moretti L, Jaboin JJ, Cha YI, and Lu B (2008) Autophagy upregulation by inhibitors of caspase-3 and mTOR enhances radiotherapy in a mouse model of lung cancer. *Autophagy* **4**: 659-668.

Molecular Pharmacology Fast Forward. Published on September 18, 2012 as DOI: 10.1124/mol.112.080408
This article has not been copyedited and formatted. The final version may differ from this version.

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA, Jr., Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevez C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E, Kondo S, Kovacs AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, Lopez-Otin C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Melendez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B, Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Munz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nurnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A, Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M,

Schneider C, Seglen PO, Seleverstov O, Settleman J, Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE, Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Talloczy Z, Tanaka K, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A, Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcategui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, and Deter RL (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**: 151-175.

Kong D, Yaguchi S, and Yamori T (2009) Effect of ZSTK474, a novel phosphatidylinositol 3-kinase inhibitor, on DNA-dependent protein kinase. *Biol Pharm Bull* **32**: 297-300.

Konstantinidou G, Bey EA, Rabellino A, Schuster K, Maira MS, Gazdar AF, Amici A, Boothman DA, and Scaglioni PP (2009) Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. *Cancer research* **69**: 7644-7652.

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, and Mizushima N (2004) The role of autophagy during the early neonatal starvation period. *Nature* **432**: 1032-1036.

Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, Ohtake Y, Ohkubo Y, Mori S, Uchiyama Y, and Fukumoto M (2011) Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. *Cell Death Dis* **2**: e177.

Liu TJ, Koul D, LaFortune T, Tiao N, Shen RJ, Maira SM, Garcia-Echevrria C, and Yung WK (2009) NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. *Mol Cancer Ther* **8**: 2204-2210.

Malkinson AM, Dwyer-Nield LD, Rice PL, and Dinsdale D (1997) Mouse lung epithelial cell lines--tools for the study of differentiation and the neoplastic phenotype. *Toxicology* **123**: 53-100.

Manning BD, and Cantley LC (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**: 1261-1274.

Mukherjee B, McEllin B, Camacho CV, Tomimatsu N, Sirasanagandala S, Nannepaga S, Hatanpaa KJ, Mickey B, Madden C, Maher E, Boothman DA, Furnari F, Cavenee WK, Bachoo RM, and Burma S (2009) EGFRvIII and DNA double-strand break repair: a molecular mechanism for radioresistance in glioblastoma. *Cancer Res* **69**: 4252-4259.

Mukherjee B, Tomimatsu N, Amancherla K, Camacho CV, Pichamoorthy N, and Burma S (2012) The dual PI3K/mTOR inhibitor NVP-BEZ235 is a potent inhibitor of ATM- and DNA-PKCs-mediated DNA damage responses. *Neoplasia* **14**: 34-43.

Nakamura JL, Karlsson A, Arvold ND, Gottschalk AR, Pieper RO, Stokoe D, and Haas-Kogan DA (2005) PKB/Akt mediates radiosensitization by the signaling inhibitor LY294002 in human malignant gliomas. *J Neurooncol* **71**: 215-222.

Norman BH, Shih C, Toth JE, Ray JE, Dodge JA, Johnson DW, Rutherford PG, Schultz RM, Worzalla JF, and Vlahos CJ (1996) Studies on the mechanism of phosphatidylinositol 3-kinase inhibition by wortmannin and related analogs. *J Med Chem* **39**: 1106-1111.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, and Yahalom J (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* **61**: 439-444.

Paglin S, Lee NY, Nakar C, Fitzgerald M, Plotkin J, Deuel B, Hackett N, McMahill M, Sphicas E, Lampen N, and Yahalom J (2005) Rapamycin-sensitive pathway regulates mitochondrial membrane potential, autophagy, and survival in irradiated MCF-7 cells. *Cancer Res* **65**: 11061-11070.

Pore N, Jiang Z, Shu HK, Bernhard E, Kao GD, and Maity A (2006) Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol Cancer Res* **4**: 471-479.

Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, Zalkow L, Matter WF, Dodge J, Grindey G, and et al. (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res* **54**: 2419-2423.

Prevo R, Deutsch E, Sampson O, Diplexcito J, Cengel K, Harper J, O'Neill P, McKenna WG, Patel S, and Bernhard EJ (2008) Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity. *Cancer Res* **68**: 5915-5923.

Shen S, Kepp O, and Kroemer G (2012) The end of autophagic cell death? *Autophagy* **8**: 1-3.

Shen S, Kepp O, Michaud M, Martins I, Minoux H, Metivier D, Maiuri MC, Kroemer RT, and Kroemer G (2011) Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. *Oncogene* **30**: 4544-4556.

Shinohara ET, Cao C, Niermann K, Mu Y, Zeng F, Hallahan DE, and Lu B (2005) Enhanced radiation damage of tumor vasculature by mTOR inhibitors. *Oncogene* **24**: 5414-5422.

Stauffer F, Maira SM, Furet P, and Garcia-Echeverria C (2008) Imidazo[4,5-c]quinolines as inhibitors of the PI3K/PKB-pathway. *Bioorg Med Chem Lett* **18**: 1027-1030.

Tamburini J, Chapuis N, Bardet V, Park S, Sujobert P, Willems L, Ifrah N, Dreyfus F, Mayeux P, Lacombe C, and Bouscary D (2008) Mammalian target of rapamycin (mTOR) inhibition activates phosphatidylinositol 3-kinase/Akt by up-regulating insulin-like growth factor-1 receptor signaling in acute myeloid leukemia: rationale for therapeutic inhibition of both pathways. *Blood* **111**: 379-382.

Toledo LI, Murga M, Zur R, Soria R, Rodriguez A, Martinez S, Oyarzabal J, Pastor J, Bischoff JR, and Fernandez-Capetillo O (2011) A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. *Nat Struct Mol Biol* **18**: 721-727.

Toulany M, Kehlbach R, Florczak U, Sak A, Wang S, Chen J, Lobrich M, and Rodemann HP (2008) Targeting of AKT1 enhances radiation toxicity of human tumor cells by inhibiting DNA-PKcs-dependent DNA double-strand break repair. *Mol Cancer Ther* **7**: 1772-1781.

Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, Higashi K, Volinia S, Downward J, and Waterfield MD (1997) P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A* **94**: 4330-4335.

Vlahos CJ, Matter WF, Hui KY, and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269**: 5241-5248.

Wong KK, Engelman JA, and Cantley LC (2010) Targeting the PI3K signaling pathway in cancer. *Curr Opin Genet Dev* **20**: 87-90.

Yuan TL, and Cantley LC (2008) PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**: 5497-5510.

Footnotes

GJC and JK contributed equally to this work.

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Figure legends

FIGURE 1. **Effect of BEZ235 on radiosensitivity of cancer and non-transformed cell lines.** A) Left panel: SQ20B cells were treated with drugs as indicated for 1 hour, and thereafter protein samples were analyzed by SDS-PAGE and immunoblotting using antibodies as indicated. Right panel: SQ20B cells were plated and allowed to attach before drug treatment. Cells were then treated with BEZ235 or rapamycin for 1 hour and then irradiated. Ten to 14 days later, dishes were stained and colonies were counted. B) C10 and WTFB cells were plated and allowed to attach prior to 1 hour treatment with BEZ235. Cells were then treated with BEZ235 for 1 hour, and thereafter protein samples were analyzed by SDS-PAGE and immunoblotting using antibodies as indicated. C, D) C10 or WTFB cells were plated and allowed to attach before drug treatment. Cells were then treated with BEZ235 for one hour before irradiation. Ten to 14 days later, dishes were stained and colonies were counted

FIGURE 2 Comparison of effects on radiosensitization: BEZ235 versus siRNA inhibition of the PI3K/mTOR pathway. SQ20B and U251 cells were seeded in 6 well plates and transfected next day with 25nM scrambled (control), mTOR, Akt1, p110α or combinations of mTOR with p110α siRNA. (A, B) 72 hours post transfection, cells were harvested for protein and immunoblotting performed using antibodies as indicated. C) and D) 72 hours post transfection, cells were plated and allowed to attach before drug treatment. Cells were then treated with BEZ235 for 1 hour. Following drug treatment cells were irradiated and 10 to 14 days later, dishes were stained and colonies were counted.

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FIGURE 3 Effect of BEZ235 on DNA damage repair. A) Split dose experiments. Changes in the surviving fraction of SQ20B cells after a total dose of 6 Gy as a function of the interval (hour) between two doses of 3 Gy. Cells were seeded into dishes and allowed to attach before 50 nM BEZ235 was added to dishes 1 hour before irradiation. A total dose of 6 Gy was given in two fractions of 3 Gy with an interval of 1, 2, 4 or 6 hours between the first and last dose of irradiation. Ten to 14 days later, dishes were stained and colonies were counted. B) SQ20B cells were seeded and allowed to attach prior to treatment for 1 hour with BEZ235. Cells were irradiated and stained for H2AX foci at the time points shown. C) The graphs represent average number of foci per 100 cells counted for each time point. D) SQ20B cells were treated with BEZ235 1 hour prior to irradiation with 4 Gy and samples were taken at 0.5, 1, 2, 3 and 24 hours post irradiation. Samples were then processed following the alkaline comet assay protocol.

FIGURE 4. **Effect of BEZ235 DNA-PKcs phosphorylation**. A) SQ20B cells were plated and allowed to attach prior to 1 hour treatment with BEZ235. Immediately following drug treatment cells were treated with 0, 2, 4, or 8 Gy. Two hours post irradiation protein samples were analyzed by SDS-PAGE and immunoblotting using antibodies as indicated. The relative integrated densitometry values (IDV) for P-ATM were quantified and normalized by that of total ATM signal using AlphaEaseFC software. Values are expressed as a ratio of those obtained in control group (dose = 0 Gy), which were assigned a value of 1.0. B) SQ20B cells were seeded in 6 well plates and transfected next day with 25nM scrambled (control), mTOR, Akt1, p110α or combinations of mTOR with Akt1 or p110α siRNA. 72 hours post transfection, cells were irradiated and harvested

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for protein. C) SQ20B cells were seeded in 6 well plates and transfected next day with 50 nM scrambled (control) or DNA-PKcs siRNA. Cells were harvested 72 hours post transfection, and plated for survival analysis. The next day drug was added 1 hour before irradiation and protein harvested for immunoblotting.

FIGURE 5. **Effect of BEZ235 and radiation on autophagy.** A) SQ20B cells were treated with BEZ235 for 0, 1, 2, 4, 8 and 24 hours prior to protein harvesting. Proteins were analyzed by SDS-PAGE and immunoblotting with antibodies shown including P-Akt (Ser473), p62, and LC3. The relative integrated densitometry values (IDV) for p62 were quantified and normalized by that of β-actin signal using AlphaEaseFC software. Values are expressed as a ratio of those obtained in control group (dose = 0 Gy), which was assigned a value of 1.0. B) SQ20B cell were seeded, then after attachment BEZ235 (50 nM) was added. Two hours later, E64d and Pepstain A were added. Samples were collected either 8 or 24 hours following E64d/Pepstatin A treatment. C) SQ20B cells were treated with 50 nM BEZ235 for one hour before receiving 4 Gy irradiation. Protein samples were harvested at 24, 48 and 72 hours following treatment. Proteins were analyzed by SDS-PAGE and immunoblotting with antibodies shown including cleaved PARP and LC3. D) SQ20B cells were stably transfected with GFP-LC3 and treated with BEZ235 for 1 h prior to receiving 4 Gy. 24 hours following treatment cells were examined by fluorescence microscopy.

FIGURE 6. Effect of chemical inhibitors of autophagy on radiosensitization by BEZ235. SQ20B cells were allowed to attach before treatment with staurosporine (1uM), 3-MA (5mM), chloroquine (20 μM) or BEZ235 (50 nM) for 1 hour. A) SQ20B cells were allowed to attach

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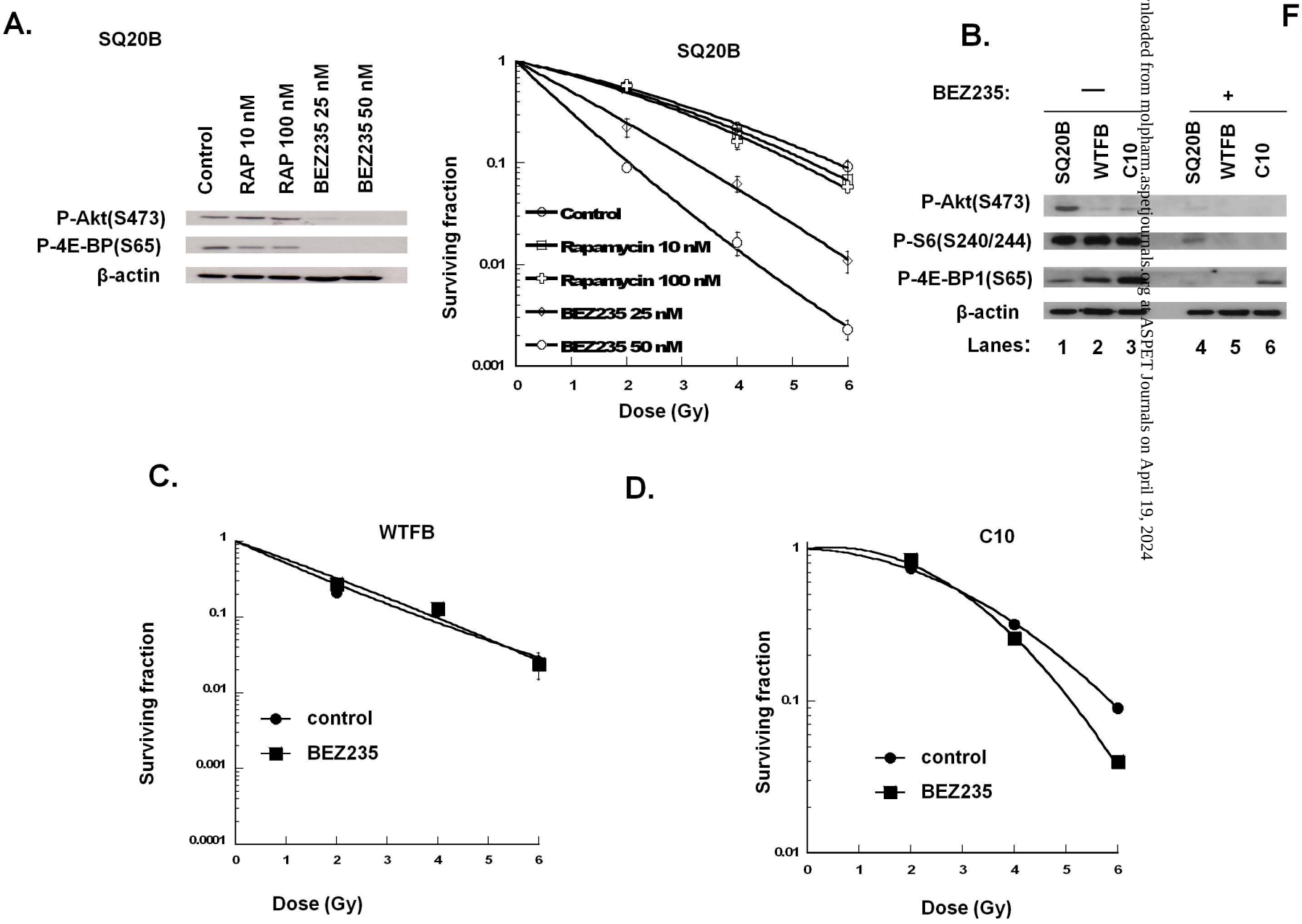
before treatment with drugs for an hour. Cells were then irradiated with 4 Gy and protein samples taken 24 hours later. Proteins were analyzed by SDS-PAGE and immunoblotting with antibodies shown including cleaved PARP and LC3. B) SQ20B cells were plated and allowed to attach before drug treatment. Cells were then treated with BEZ235, 3-MA or BEZ235 + 3-MA. One hour later, they were irradiated. Ten to 14 days later, dishes were stained, and colonies were counted. C) SQ20B cells were plated and allowed to attach before drug treatment. Cells were then treated with BEZ235, chloroquine or BEZ235 + chloroquine. One hour later, they were irradiated. Ten to 14 days later, dishes were stained, and colonies were counted.

FIGURE 7. **Effect of knocking down ATG5 and/or Beclin1 on radiosensitization by BEZ235**. A) and B) SQ20B cells were seeded in 6 well plates and transfected next day with 50nM Beclin1 siRNA, ATG5 siRNA or combination of both Beclin1 and ATG5 siRNA. Cells were harvested for protein or survival curves 72 hours post transfection. C) ATG5 wild-type ($ATG5^{+/+}$) and knockout ($ATG5^{-/-}$) MEFs were seeded out and allowed to attach prior to treatment with BEZ235 for 1 hour. Following drug treatment cells were irradiated with 4 Gy, and 24 hours following irradiation protein samples were collected and analyzed by SDS-PAGE and immunoblotting with antibodies shown. D) ATG5 wild-type ($ATG5^{+/+}$) and knockout ($ATG5^{-/-}$) MEFs cells were seeded out allowed to attach and then treated with BEZ235 for 1 hour prior to being irradiated. 14 days later, dishes were stained and colonies were counted.

Fig. 1



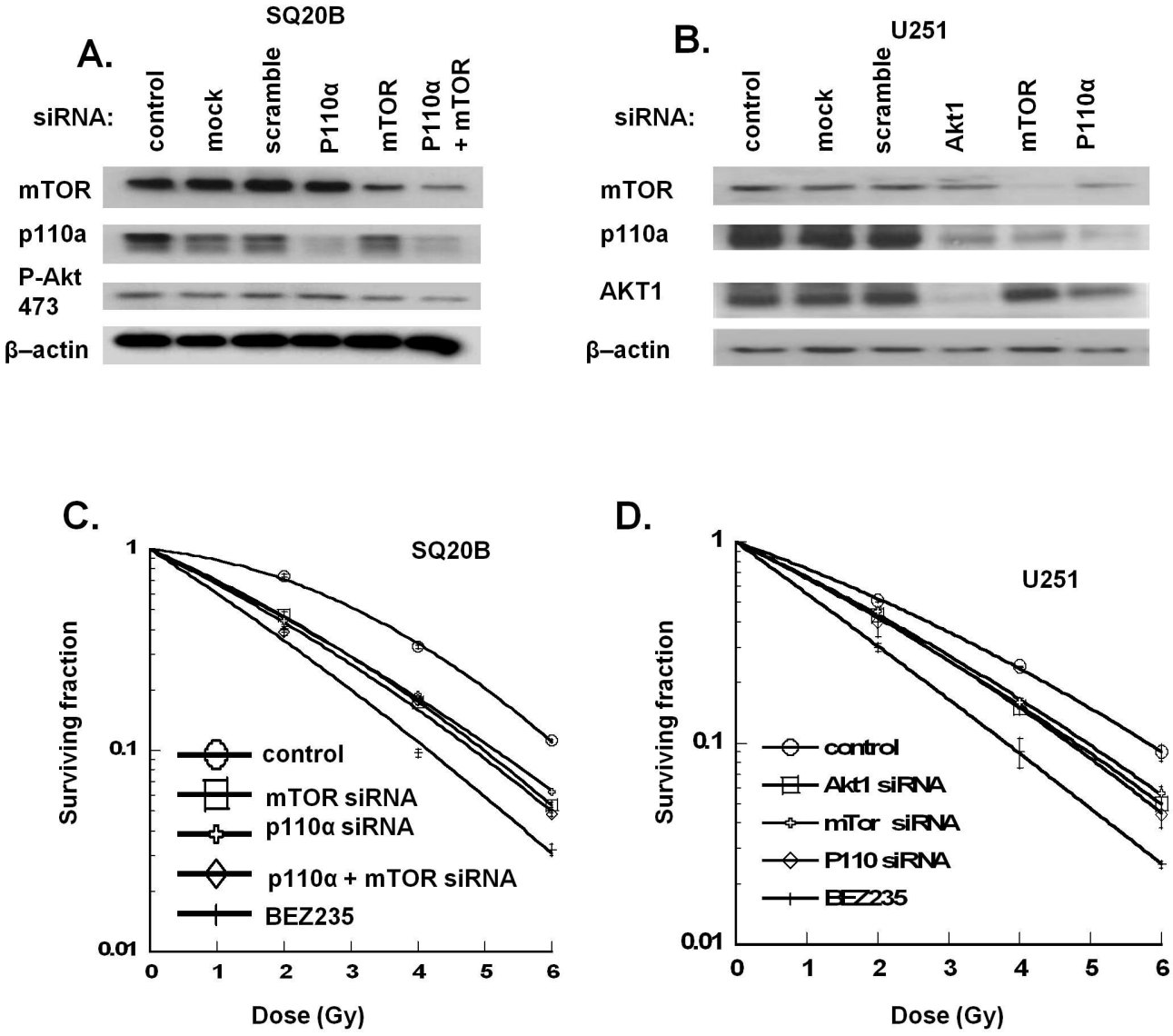


Fig. 2

Fig. 3

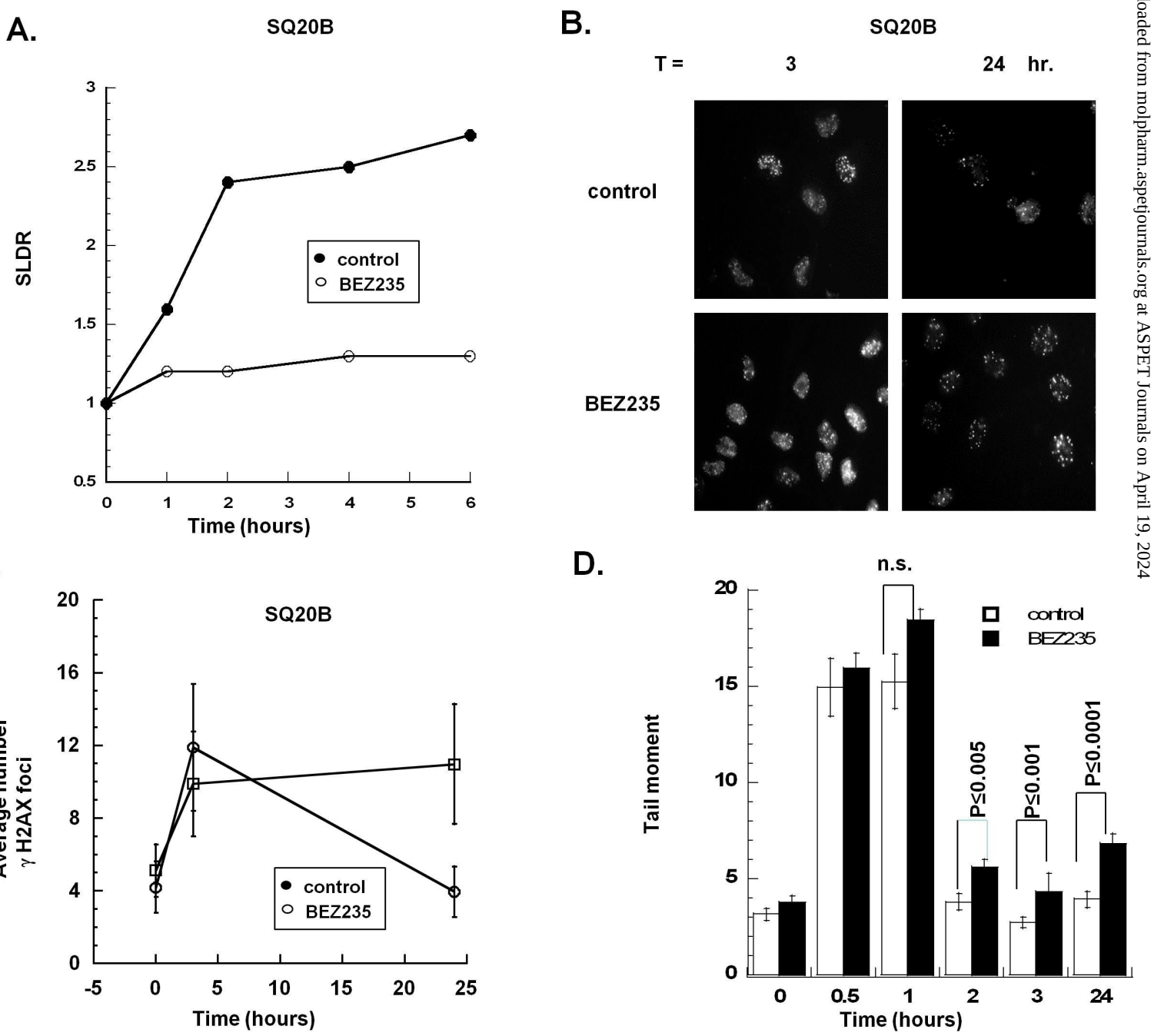
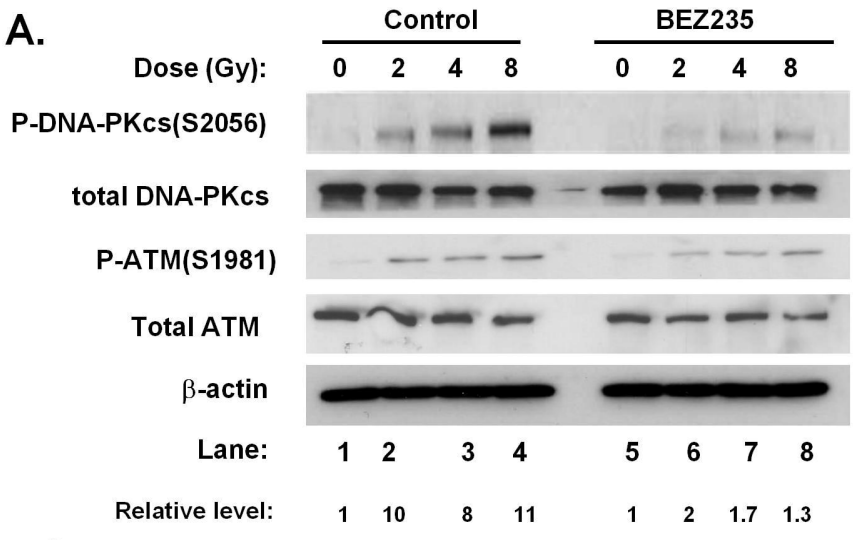
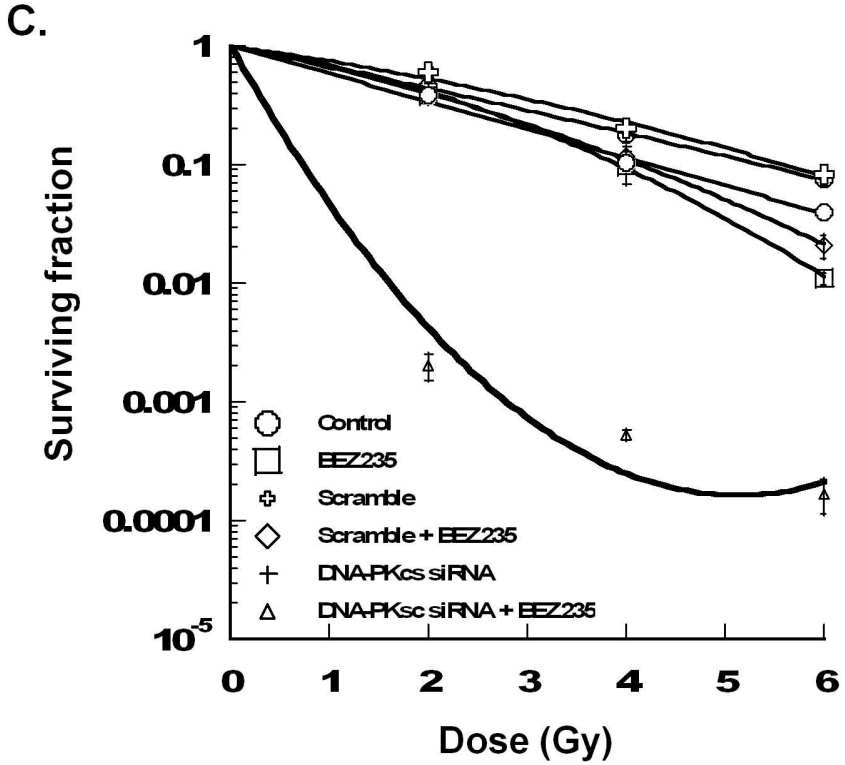
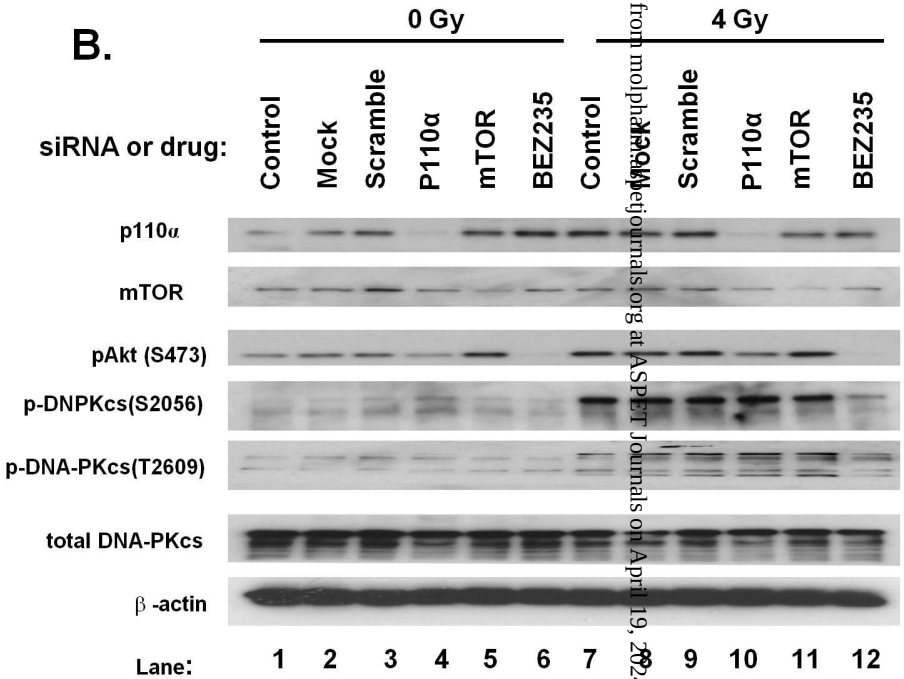


Fig. 4







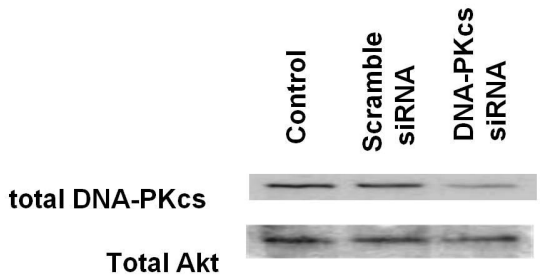
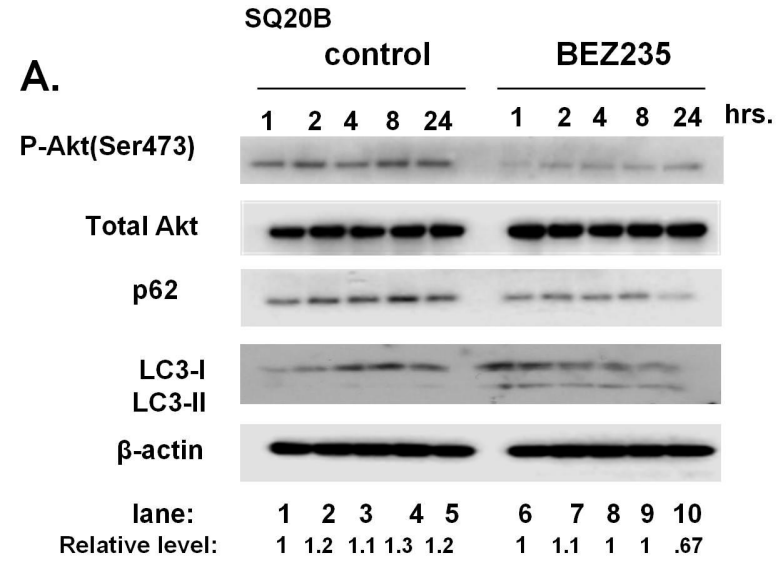
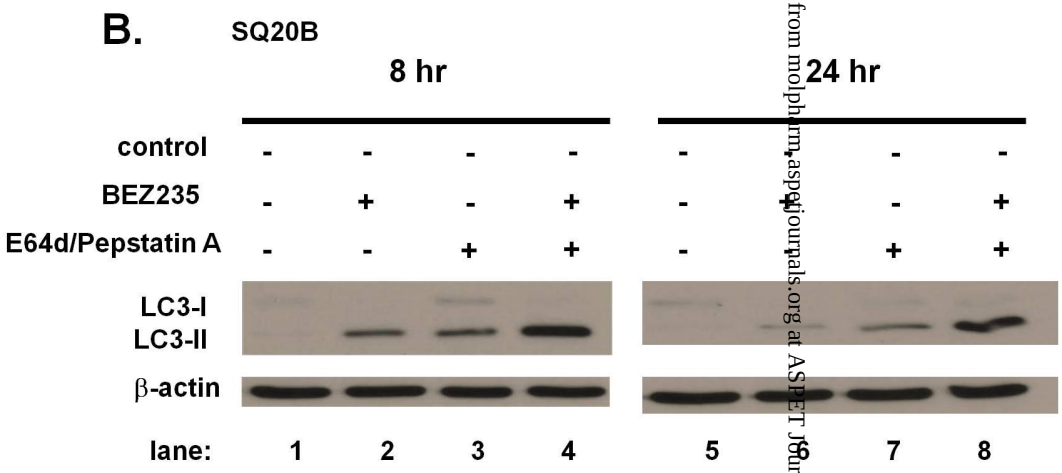
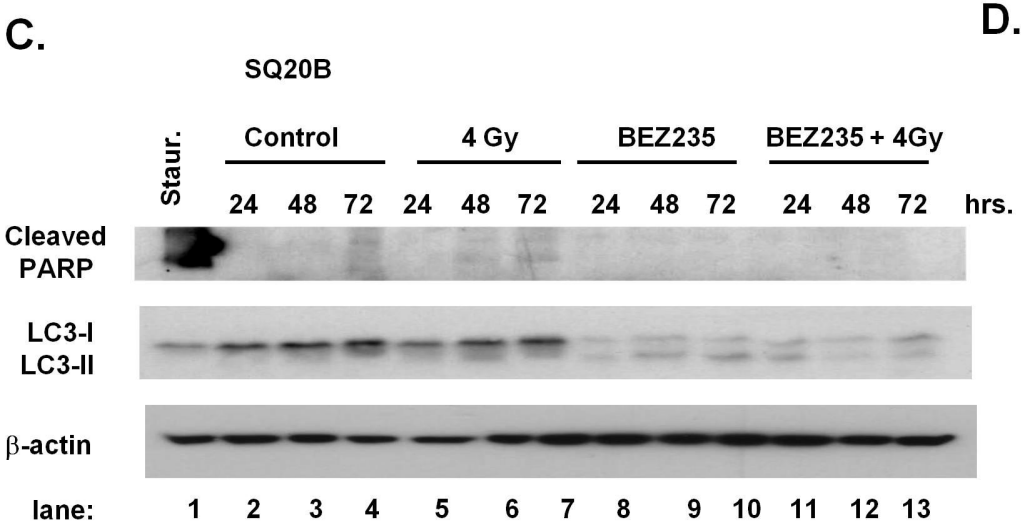


Fig. 5







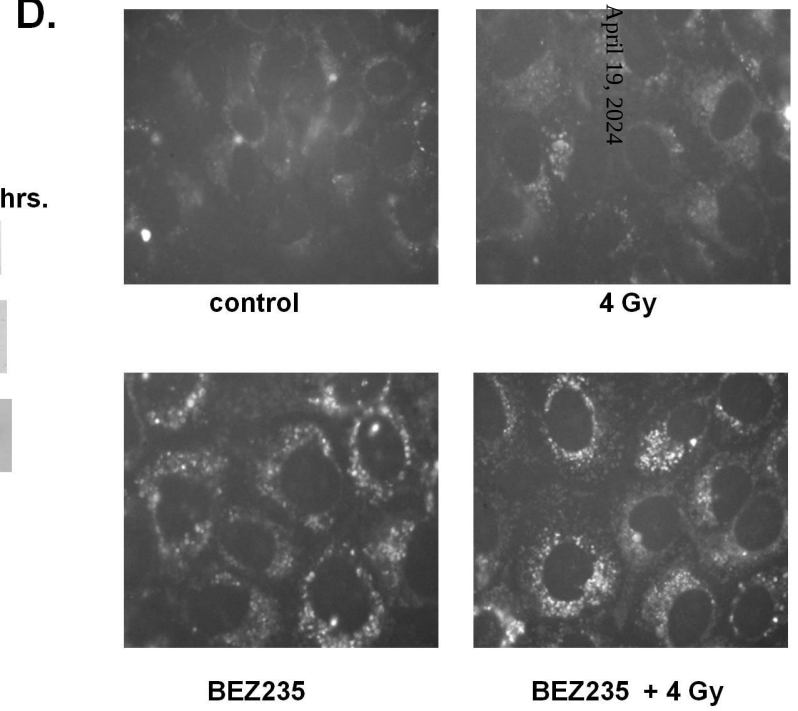
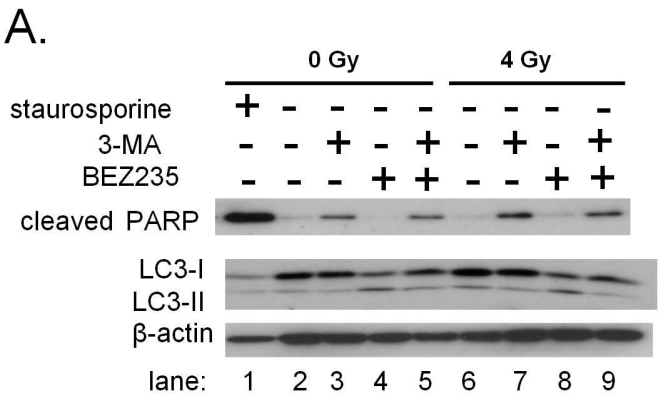
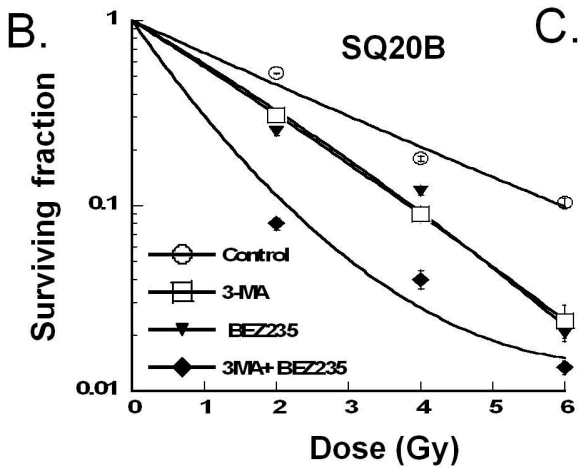


Fig. 6





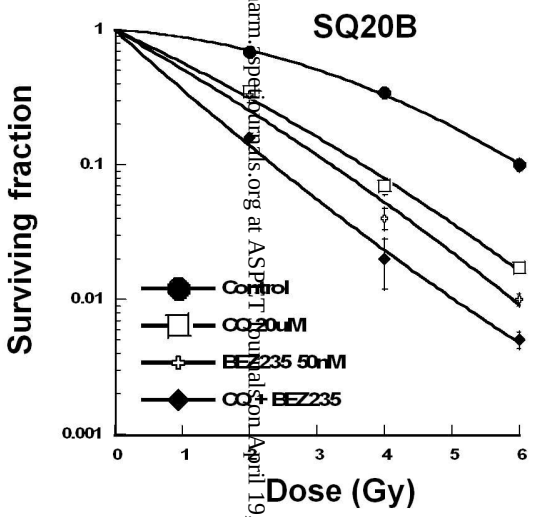


Fig. 7