1521-0111/12/8206-1230–1240$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 82:1230–1240, 2012

Vol. 82, No. 6
80408/3807568

Inhibition of Autophagy as a Strategy to Augment Radiosensitization by the Dual Phosphatidylinositol 3-Kinase/ Mammalian Target of Rapamycin Inhibitor NVP-BEZ235[S]

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Received June 7, 2012; accepted September 18, 2012

ABSTRACT

We investigated the effect of 2-methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1*H*-imidazo[4,5-c]quinolin-1-yl]phenyl} propanenitrile (NVP-BEZ235) (Novartis, Basel Switzerland), a dual phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor currently being tested in phase I clinical trials, in radiosensitization. NVP-BEZ235 radiosensitized a variety of cancer cell lines, including SQ20B head and neck carcinoma cells and U251 glioblastoma cells. NVP-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 NVP-BEZ235. NVP-BEZ235 interfered with DNA damage repair after radiation as measured by the CometAssay and resolution of phosphorylated H2A histone family member X foci. NVP-BEZ235 abrogated the radiation-induced phosphorylation of both DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia telangiectasia mutated. Knockdown of either p110α or mTOR failed to decrease the phosphorylation of DNA-PKcs, suggesting that the effect of the drug was direct rather than mediated via p110α or mTOR. The treatment of cells with NVP-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 NVP-BEZ235 treatment and radiation. Knocking down the essential autophagy proteins autophagy related 5 (ATG5) and beclin1 increased NVP-BEZ235-mediated radiosensitization. Furthermore, NVP-BEZ235 radiosensitized autophagy-deficient ATG5(−/−) fibroblasts to a greater extent than ATG5(+/+) cells. We conclude that NVP-BEZ235 radiosensitizes cells and induces autophagy by apparently distinct mechanisms. Inhibiting autophagy via pharmacologic or genetic means increases radiation killing after NVP-BEZ235 treatment; hence, autophagy seems to be cytoprotective in this situation. Our data offer a rationale for combining NVP-BEZ235 along with an autophagy inhibitor (i.e., chloroquine) and radiation in future clinical trials.

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*, activa-

This work was supported by the National Institutes of Health National Institute of Cancer [Grants R01-CA093638, R01-CA133470].

G.J.C. and J.K. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
http://dx.doi.org/10.1124/mol.112.080408.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: PI3K, phosphatidylinositol 3-kinase; NVP-BEZ235, 2-methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]quinolin-1-yl]phenyl}propanenitrile; ATG5, autophagy related 5; ATM, ataxia telangiectasia mutated; DER, dose enhancement ratio; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; 4E-BP1, 4E binding protein 1; E64d, 2*S*,3*S*-*trans*-(ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methylbutyl) amide; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; Gy, gray; HR, homologous recombination; H2AX, H2A histone family member X; γ-H2AX, phosphorylated H2AX; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; 3-MA, 3-methyladenosine; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; NHEJR, nonhomologous end joining repair; P-, phosphorylated-; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PI-103, 3–4-(4-morpholinylpyrido[3′,2′:4,5]furo[3,2-*d*]pyrimidin-2-yl-phenol; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; PTEN, phosphatase and tensin homolog; RAD001, dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.04,9]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone; siRNA, small inhibitory RNA.

tion of growth factor receptors such as EGFR, and mutations in *PIK3A* (Yuan and Cantley, 2008). Hence, PI3K is an attractive target for anticancer drug therapy (Wong et al., 2010). Two of the more commonly used inhibitors of the PI3K pathway are wortmannin (Powis et al., 1994) and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (Vlahos et al., 1994). However, because of their unfavorable pharmacologic properties, toxicity and nonspecific inhibition of other kinases, neither of these drugs is suitable for clinical use (Norman et al., 1996; Bain et al., 2007; Gharbi et al., 2007). 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 2-methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]quinolin-1-yl]phenyl}propanenitrile (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). NVP-BEZ235 inhibits the α, γ, and δ isoforms of the p110 subunits with an IC_{50} ranging from 4 to 7 nM and inhibits the β isoform with an IC_{50} of 75 nM (Maira et al., 2008). IC_{50} for mTOR kinase is 20 nM; however, the IC_{50} for kinases such as vascular EGFR1, human epidermal receptor 1, 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 has 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 inhibitors (Harari and Huang, 2001), LY294002 (Gupta et al., 2001; Kim et al., 2005; Nakamura et al., 2005; Kao et al., 2007), 3–4-(4-morpholinylpyrido[3′,2′:4,5]furo[3,2-*d*]pyrimidin-2-yl-phenol; (Pl-103) (Prevo et al., 2008), and NVP-BEZ235 (Konstantinidou et al., 2009; Fokas et al., 2012a,b; Mukherjee et al., 2012), have been shown to increase the sensitivity of cells to radiation. Inhibition of mTOR by rapamycin or related compounds (Kamada et al., 2000; Crazzolara et al., 2009), as well as NVP-BEZ235 (Liu et al., 2009; Fan et al., 2010), promotes autophagy. We were interested in understanding the mechanism of radiosensitization by NVP-BEZ235, in particular the role that autophagy might play. We found that this agent is a highly potent radiosensitizer, but its effect cannot be explained solely by its inhibition of the PI3K/Akt/mTOR pathway, and it is also probably related to the inhibition of DNA damage repair through the inhibition of other PI3K-like family members such as ATM and DNA-PKcs. NVP-BEZ235 does induce autophagy, but it seems to be a protective response after radiation because inhibiting it leads to increased cell killing. Our findings provide insight into how NVP-BEZ235 radiosensitizes and how to further improve its efficacy by inhibiting autophagy.

Materials and Methods

Chemicals. NVP-BEZ235 was provided by Novartis (Basel, Switzerland) and used at a concentration of 50 nM for all in vitro experiments. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following final concentrations: rapamycin, 10 nM; 3-methyladenosine (3-MA), 5 μM; staurosporine, 1 μM; 2*S*,3*S*-*trans*-(ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methylbutyl) amide (E64d), 10 μg/ml; and pepstain A, 10 μg/ml.

Cell Growth. SQ20B head and neck squamous cell carcinoma cells, U251MG glioblastoma cells, and ATG5 wild-type [ATG5(**+/+**)] and ATG5 knockout [ATG5(**−/−**)] mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (4500 mg/l glucose; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), penicillin (100 units/ml), and streptomycin (100 mg/ml; Life Technologies, Inc., Gaithersburg, MD) at 37°C in humidified 5% CO_2 and 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, Japan) (Kuma et al., 2004).

Mouse Studies. Pathogen-free female *Ncr-nu/nu* mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in the vivarium of the University of Pennsylvania Laboratory Animal Resources. All 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 (Dharmacon RNA Technologies, Lafayette, CO) for AKT-1, FK506 binding protein 12-rapamycin-associated protein 1, 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 overnight. The next day, media were removed, and cells were washed twice with phosphate-buffered saline and refed with 1 ml of OPTI-MEM (Invitrogen). The six-well plate was returned to the incubator for 1 h before being transfected. siRNA was mixed with Oligofectamine reagent (Invitrogen) for 20 min 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 before 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 8 h after irradiation, at which time drug-free medium was added. Colonies were stained and counted 10 to 14 days after irradiation. The surviving fraction was calculated as follows: (number of colonies formed)/(number of cells plated × plating efficiency). Each point on the survival curve represented the mean surviving fraction from at least six dishes. A dose enhancement ratio (DER) was calculated as a ratio of the 10% survival rate between cells treated with irradiation alone and those treated with irradiation and drug.

Assays for γ-H2AX Activation. After irradiation, cells were assessed via immunofluorescence for unrepaired DNA damage via the phosphorylation of H2AX (γ-H2AX), a standard marker of unrepaired double-stranded DNA damage. For these experiments, cells were grown on coverslips. All groups of cells were fixed in 4% paraformaldehyde with 0.1% Triton X-100 and probed with anti-γ-H2AX antibody (Upstate Biological, Inc., Lake Placid, NY), followed by secondary antibody (anti-mouse Alexa Fluor 594; Molecular Probes, Carlsbad, CA). After staining with the specific antibody, the coverslips were counterstained with 4′,6-diamidino-2-phenylindole 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 was 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): antiphospho-Akt antibody (Ser473 and Thr308), antiphospho-4E-BP1 (Ser 65), antiphospho-S6, anti-mTOR, anti-Akt1, anti-PI3K p110α, LC3B, p62, and cleaved-PARP. Other antibodies were those directed against DNA-PKcs (BioLegend, San Diego, CA), DNA-PKcs (G4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), P-H2A.X (Millipore Corporation), and β-actin antibody (Sigma-Aldrich). The sec-

ondary antibody used for these blots was a goat anti-mouse and goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA). Antibody binding was detected by chemiluminescence using an enhanced chemiluminescence kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

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

CometAssay. Cells were seeded into 60-mm dishes 24 h before drug and irradiation treatment. Cells were treated with drug 1 h before 4-Gy irradiation. Thirty minutes after irradiation cells were trypsinized and suspended to a final density of 1×10^5/ml in molten low-melting agarose at a ratio of 1:10 (v/v), and 50 μl was immediately pipetted onto microscope slides. Samples were then processed by following the alkaline CometAssay protocol from Trevigen (Gaithersburg, MD).

Electron Microscopy. SQ20B cells were treated with NVP-BEZ235 for 1 h and irradiated, and 24 h later cells were fixed and given to the Electron Microscopy Resource facility at the University of Pennsylvania 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 NVP-BEZ235 for 24 h. After treatment, cells were fixed and subjected to immunofluorescence analysis.

Senescence β-Galactosidase Cell Staining Protocol. Cells were seeded into six-well plates at a density of 1×10^5 cells per well. Cells were allowed to attach overnight before treatment with NVP-BEZ235 for 1 h before being irradiated with 4 Gy of irradiation. Forty eight hours after irradiation cells were stained following the Senescence β-galactosidase staining kit protocol (Cell Signaling Technology).

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

Results

NVP-BEZ235 Inhibits PI3K/mTOR Pathway and Increases Radiation Response. Cells were treated with the drug for differing lengths of time, cells were harvested, and then Western blotting was performed. One hour of drug treatment, at either 25 or 50 nM, down-regulated P-AktS473 in SQ20B (Fig. 1A, left) and U251 cells (Supplemental Fig. 1A). NVP-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

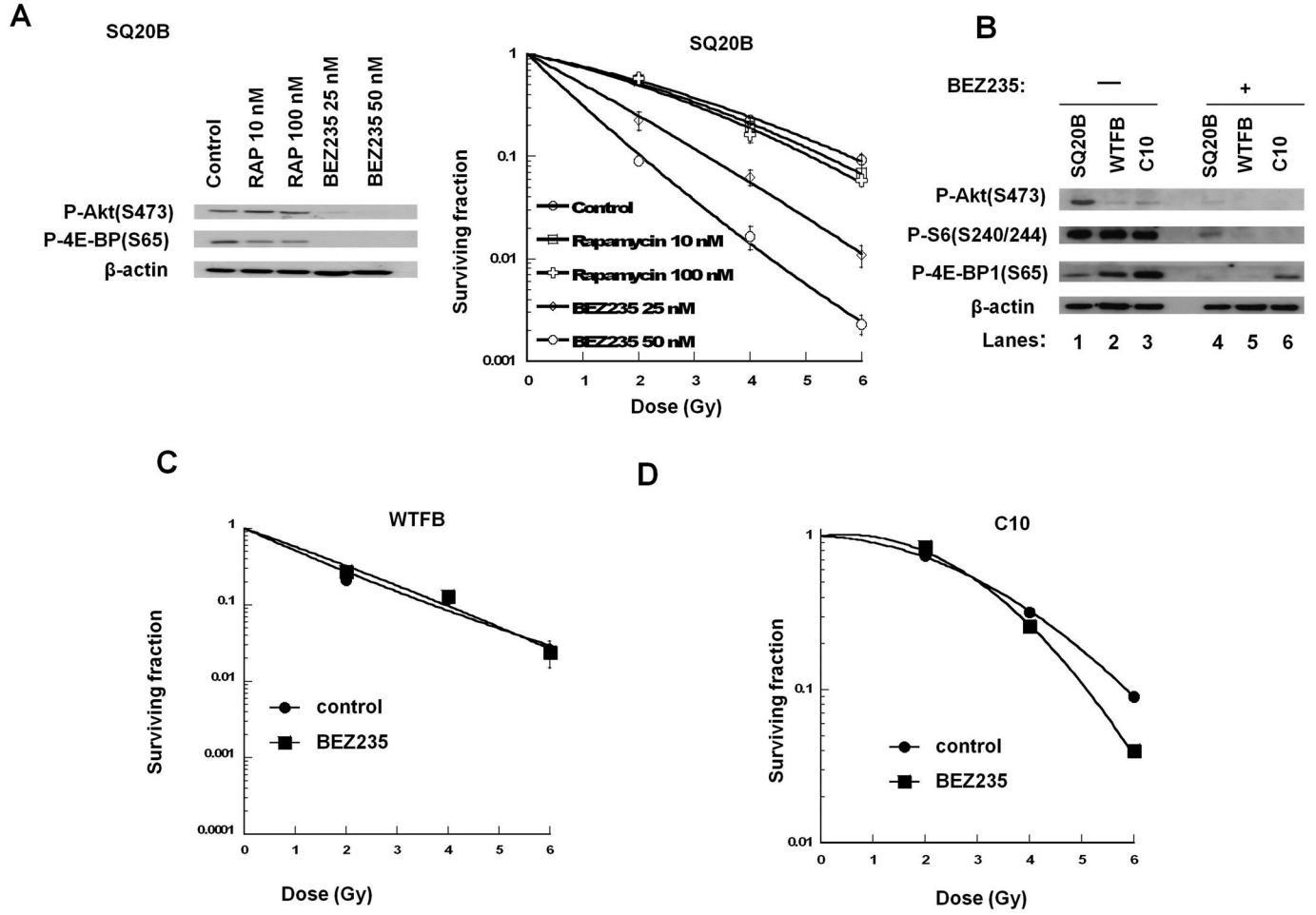

Fig. 1. Effect of NVP-BEZ235 on radiosensitivity of cancer and nontransformed cell lines. A, left, SQ20B cells were treated with drugs as indicated for 1 h, and thereafter protein samples were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Right, SQ20B cells were plated and allowed to attach before drug treatment. Cells were then treated with NVP-BEZ235 or rapamycin for 1 h 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 before 1-h treatment with NVP-BEZ235. After the treatment protein samples were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. C and D, WTFB (C) and C10 (D) cells were plated and allowed to attach before drug treatment. Cells were treated with NVP-BEZ235 for 1 h before irradiation. Ten to 14 days later, dishes were stained, and colonies were counted.

effective in decreasing 4E-BP1 phosphorylation and had no effect on Akt phosphorylation.

After 1-h incubation with the drug, cells were irradiated, and then clonogenic survival assays were performed. NVP-BEZ235 radiosensitized both SQ20B cells (Fig. 1A, right) and U251 cells (Supplemental Fig. 1B) with a DER ranging from 1.35 to 1.63. In contrast, rapamycin (10 or 100 nM) showed minimal effect on increasing radiation-induced cell killing in either SQ20B or U251 cells. NVP-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 (A549 with K-*ras* mutation; H460 with *PIK3CA* mutation) (Supplemental Fig. 2).

Effect of NVP-BEZ235 on Nontransformed Cells. We also used two nontransformed lines derived from mice, WTFB (wild-type fibroblasts) and C10 (an immortalized bronchial epithelial cell line kindly provided by Dr. A. Malkinson, University of Colorado, Denver, CO) (Malkinson et al., 1997). Both cell lines expressed little P-Akt compared with SQ20B cells; therefore, NVP-BEZ235 had little appreciable effect on P-Akt down-regulation (Fig. 1B, compare lanes 1 and either 2 or 3). It is noteworthy that both cell lines contained a similar level of P-S6 compared with SQ20B, and this was effectively decreased by NVP-BEZ235. WTFB cells did not show radiosensitization by NVP-BEZ235 (Fig. 1D), and C10 cells showed radiosensitization only at the relatively high dose of 6 Gy (Fig. 1C).

Down-Regulation 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 IC_{50} for inhibition of this isoform by NVP-BEZ235 was 5 to 7 nM. Both p110γ and p110δ are also inhibited by these low concentrations of NVP-BEZ235; however, SQ20B cells have almost no expression of either of these isoforms compared with P4936, a control lymphoma line (Supplemental Fig. 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 (Vanhaesebroeck et al., 1997; Guillermet-Guibert et al., 2008).

Knockdown of the p110α isoform has been shown previously to radiosensitize SQ20B cells (Kim et al., 2005). The same study showed that these cells are radiosensitized by knockdown of Akt1 but not Akt2 or Akt 3. These siRNAs functioned as expected to knock down levels of the respective proteins in SQ20B and U251 cells (Fig. 2, A and B). p110α or mTOR knockdown in SQ20B cells led to a decrease in survival after radiation with a DER of 1.19 to 1.24 (Fig. 2C). However, the degree of radiosensitization was not as great as that seen with NVP-BEZ235 (DER of 1.48), and the combination of siRNA against p110α and mTOR had no greater effect than either alone. In U251 cells, knockdown of Akt1, mTOR, or p110α led to a similar level of radiosensitization with a DER of 1.17 to 1.25 but not as high as seen with NVP-BEZ235 (Fig. 2D; DER of 1.53).

Effects of NVP-BEZ235 on DNA Damage Response after Radiation. Our previous work had indicated that the inhibition of the PI3K pathway might be related to delayed repair of DNA damage (Kao et al., 2007). To determine whether NVP-BEZ235 altered DNA damage repair after 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-

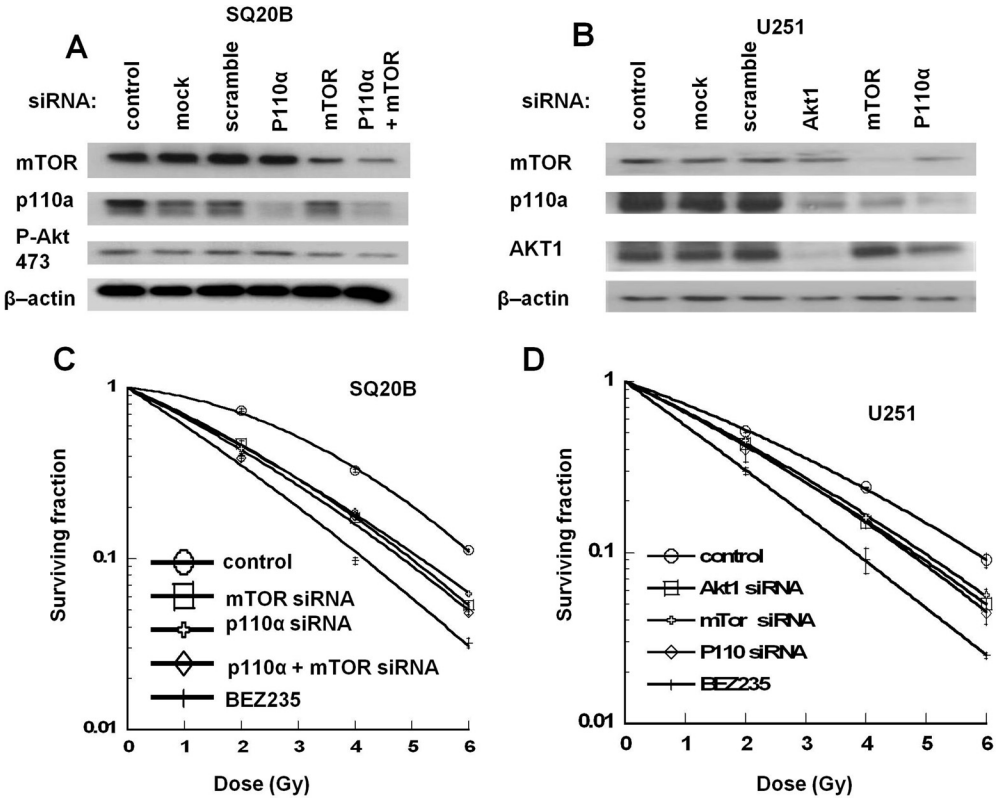

Fig. 2. Comparison of effects on radiosensitization: NVP-BEZ235 versus siRNA inhibition of the PI3K/mTOR pathway. SQ20B and U251 cells were seeded in six-well plates and transfected the next day with 25 nM scrambled (control), mTOR, Akt1, p110α, or combinations of mTOR with p110α siRNA. A and B, 72 h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

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 two doses increased from 0 to 1 to 2 h, the survival fraction increased from 1 to 1.6 to 2.4 (Fig. 3A). Pretreatment with NVP-BEZ235 essentially abolished this effect, suggesting that the drug interfered with DNA damage repair. Likewise, the survival fraction for U251 cells increased from 1 to 1.9 when doses were split by 6 h, but no such increase was seen when cells were pretreated with NVP-BEZ235 (Supplemental Fig. 4A).

To assess the possibility of altered DNA damage repair, we assayed H2AX phosphorylation (γ-H2AX). Figure 3, B and C shows that γ-H2AX foci were induced to a similar extent in both NVP-BEZ235-pretreated and control SQ20B cells at 1 h, but by 24 h there were more foci remaining in the drug-treated cells. We made similar findings in U251 cells (Supplemental Fig. 4, B and C). We also used the CometAssay, which measures unrepaired DNA by assessing the tail moment. We found similar levels of DNA damage by the CometAssay at 1 h, but persistently elevated levels by 24 h in the drug-treated SQ20B cells (Fig. 3D). We made the same qualitative observations using the CometAssay in U251 cells (Supplemental Fig. 4D).

To further investigate NVP-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 NVP-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, pretreatment with NVP-BEZ235 attenuated this phosphorylation. It is notewor-

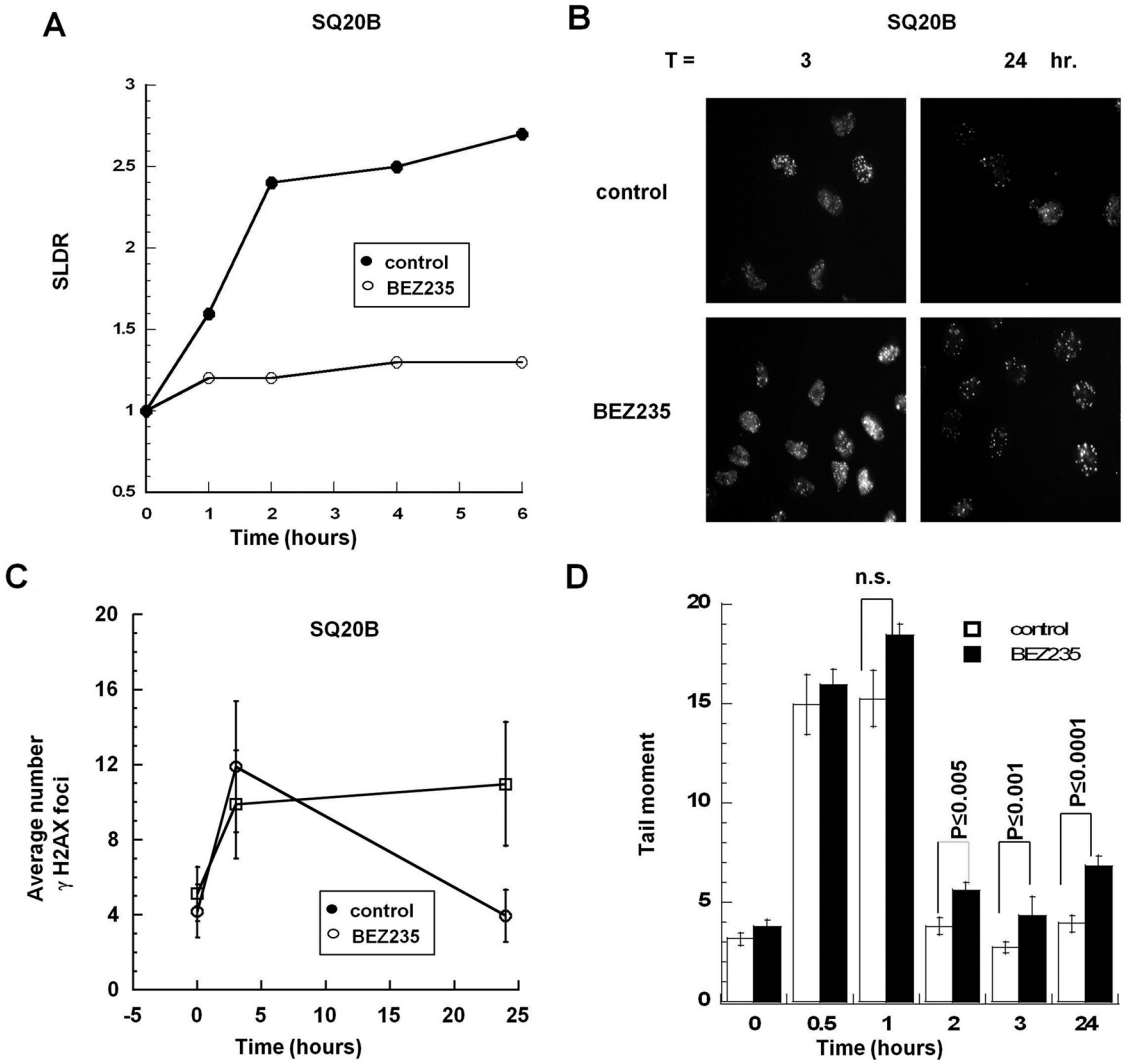

Fig. 3. Effect of NVP-BEZ235 on DNA damage repair. A, split-dose experiments. Changes in the surviving fraction of SQ20B cells after a total dose of 6 Gy are shown as a function of the interval (hour) between two doses of 3 Gy. Cells were seeded into dishes and allowed to attach before 50 nM NVP-BEZ235 was added to dishes 1 h 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 h between the first and last doses of irradiation. Ten to 14 days later, dishes were stained, and colonies were counted. SLDR, sublethal damage repair. B, SQ20B cells were seeded and allowed to attach before treatment for 1 h with NVP-BEZ235. Cells were irradiated and stained for H2AX foci at the time points shown. C, the graph represents the average number of foci per 100 cells counted for each time point. D, SQ20B cells were treated with NVP-BEZ235 1 h before irradiation with 4 Gy, and samples were taken at 0.5, 1, 2, 3, and 24 h after irradiation. Samples were then processed following the alkaline CometAssay protocol. n.s., not significant.

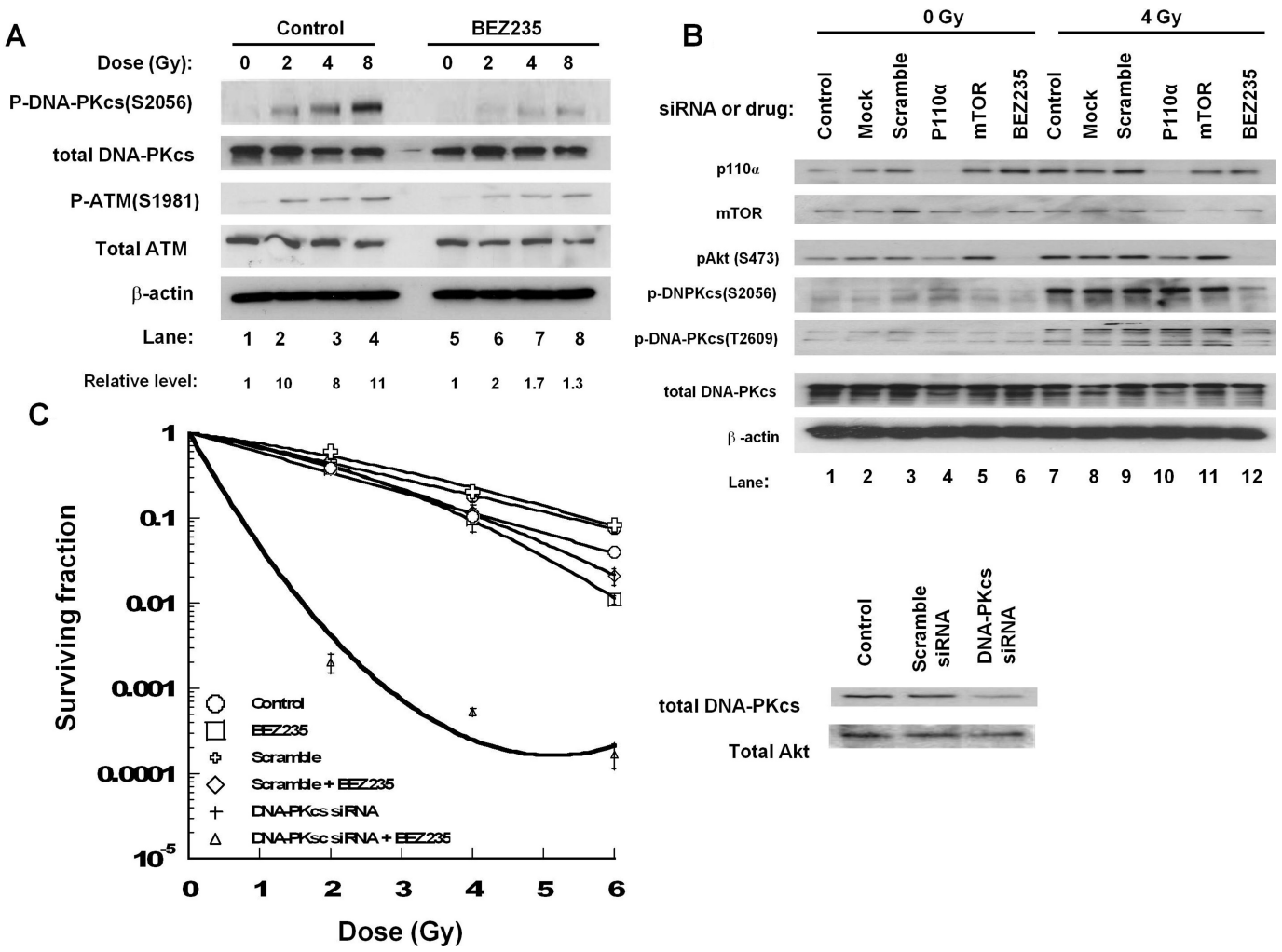

Fig. 4. Effect of NVP-BEZ235 **on** DNA-PKcs phosphorylation. A, SQ20B cells were plated and allowed to attach before 1-h treatment with NVP-BEZ235. Immediately after drug treatment cells were treated with 0, 2, 4, or 8 Gy of irradiation. Two hours after irradiation protein samples were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. The relative integrated densitometry values for P-ATM were quantified and normalized by that of total ATM signal using AlphaEaseFC software (Alpha Innotech, San Leandro, CA). Values are expressed as a ratio of those obtained in the control group (dose = 0 Gy), which were assigned a value of 1.0. B, SQ20B cells were seeded in six-well plates and transfected the next day with 25 nM scrambled (control), mTOR, Akt1, p110α, or combinations of mTOR with Akt1 or p110α siRNA. Seventy two hours after transfection, cells were irradiated and harvested for protein. C, SQ20B cells were seeded in six-well plates and transfected the next day with 50 nM scrambled (control) or DNA-PKcs siRNA. Cells were harvested 72 h after transfection and plated for survival analysis. The next day, drug was added 1 h before irradiation, and protein was harvested for immunoblotting.

thy that phosphorylation of ATM at Ser1981 was also blunted after radiation. Similar attenuation of the phosphorylation of DNA-PKcs in response to radiation was seen in U251 cells (Supplemental Fig. 5A).

The effect of NVP-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, and then performed Western blotting. Figure 4B shows that, in contrast to treatment with NVP-BEZ235, knockdown of either p110α or mTOR failed to decrease the phosphorylation of DNA-PKcs, which argues against the idea that the 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 of the protein (Fig. 4C, right) and found that it led to a substantial decrease in clonogenic cell survival, comparable with that seen with NVP-BEZ235 (Fig. 4C, left). We were surprised to find that when we combined siRNA against DNA-PK with NVP-BEZ235 we observed a highly synergistic effect far greater than that seen with either agent alone.

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

Effect of NVP-BEZ235 on Autophagy. Because NVP-BEZ235 has been reported to induce autophagy (Liu et al., 2009; Fan et al., 2010), 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. NVP-BEZ235 treatment of SQ20B cells led to conversion of the LC3-I to the LC3-II form, consistent with the induction of autophagy (Fig. 5A, compare lanes 6–10 with lanes 1–5). Accumulation of LC3-II can occur without the activation of 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 NVP-BEZ235 clearly increased LC3-II levels at 8 and 24 h compared with NVP-BEZ235 by itself (Fig. 5B, compare lanes 2 versus 5 and 6 versus 8). This result strongly indicates that the accumulation of LC3-II with NVP-BEZ235 is caused by autophagy induction rather than reduced autophagy flux. Figure 5A also shows that by 24 h (lane 10) with NVP-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 NVP-BEZ235 induces autophagy.

We repeated this study with 4 Gy of radiation and extended it to 72 h. NVP-BEZ235 clearly led to LC3-I to LC3-II conversion at 24 to 72 h (Fig. 5C, lanes 8–10); however, 4 Gy of radiation did not (Fig. 5C, lanes 5–7). The combination of BEZ25 + 4 Gy of radiation (Fig. 5C, lanes 11–13) appeared the same as NVP-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 by using immunofluorescence. NVP-BEZ235 treatment for 24 h led to accumulation of punctate foci in

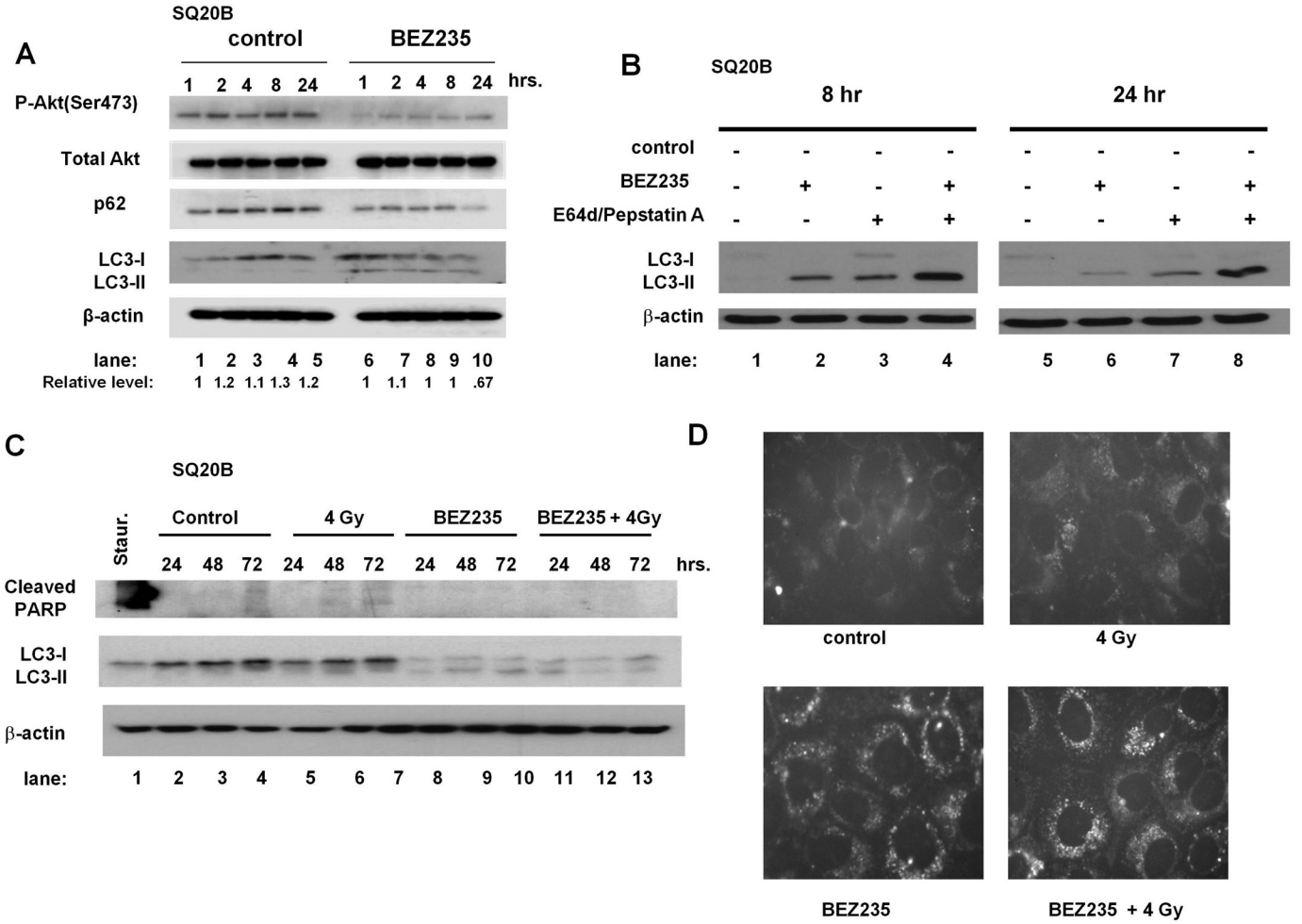

Fig. 5. Effect of NVP-BEZ235 and radiation on autophagy. A, SQ20B cells were treated with NVP-BEZ235 for 0, 1, 2, 4, 8, and 24 h before protein harvesting. Proteins were analyzed by SDS-PAGE and immunoblotting with the antibodies shown including P-Akt (Ser473), p62, and LC3. The relative integrated densitometry values 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 the control group (dose = 0 Gy), which was assigned a value of 1.0. B, SQ20B cells were seeded, then after attachment NVP-BEZ235 (50 nM) was added. Two hours later, E64d and pepstain A were added. Samples were collected either 8 or 24 h after E64d/pepstatin A treatment. C, SQ20B cells were treated with 50 nM NVP-BEZ235 for 1 h before receiving 4 Gy of irradiation. Protein samples were harvested at 24, 48, and 72 h after treatment. Proteins were analyzed by SDS-PAGE and immunoblotting with the antibodies shown including cleaved PARP and LC3. D, SQ20B cells were stably transfected with GFP-LC3 and treated with NVP-BEZ235 for 1 h before receiving 4 Gy of irradiation. Twenty four hours after treatment cells were examined by fluorescence microscopy.

the cytoplasm, which is consistent with localization to the autophagosomes (and induction of autophagy) (Fig. 5D). Irradiation with 4 Gy did not appreciably increase punctate GFP-labeled LC3, but these foci were seen in cells receiving NVP-BEZ235 and radiation.

Electron microscopy imaging analysis of SQ20B cells treated with NVP-BEZ235 for 24 h revealed numerous vacuoles in the cytoplasm (Supplemental Fig. 7A and insets with 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 (Kamada et al., 2000). These vacuoles were not seen in cells irradiated with 4 Gy of radiation but were seen in cells that were treated with NVP-BEZ235 + 4 Gy of radiation.

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

Effect of Autophagy Inhibitors on Radiosensitization with NVP-BEZ235. To determine whether inhibiting the transit of cells through autophagy would influence radiosensitivity we first used the drug 3-MA, which blocks autophagy at an early stage by inhibiting the autophagy protein Vps34 (Castino et al., 2010). Figure 6A shows that pretreatment of SQ20B cells with 3-MA before NVP-BEZ235 treatment led to a decrease in the LC3-II band compared with NVP-BEZ235 treatment alone (compare lanes 8 and 9), which is consistent with the inhibition of autophagy. It is noteworthy that 3-MA by itself and in combination with NVP-BEZ235 led to an increase in PARP cleavage. Using the combination of 3-MA and NVP-BEZ235 we found an increase in cell killing after radiation with a DER of 2.40 (Fig. 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 NVP-BEZ235 led to decreased clonogenic survival with a DER of 2.63 (Fig. 6C). Therefore, both of these drugs that inhibited autophagy potentiated NVP-BEZ235-mediated radiosensitization.

Genetic Inhibition of Autophagy and Its Effect on Radiosensitization with NVP-BEZ235. The experiments shown in Fig. 6 used pharmacological agents to inhibit autophagy. The possibility exists that these results were caused by 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 down-regulated their intended targets (Fig. 7A). Knockdown of both proteins significantly increased NVP-BEZ235-mediated radiosensitization with a DER of 2.34, whereas knockdown of either one alone had no effect (Fig. 7B).

We also used cells deficient in the autophagy protein ATG5. NVP-BEZ235 treatment (for 24 h) of the wild-type [ATG5(+/+)] fibroblasts led to a conversion of LC3-I to LC3-II; however, this was completely absent in ATG5(−/−) cells, which is consistent with their loss of autophagic capacity (Fig. 7C). In addition, it is noteworthy that there was little down-regulation of P-Akt in either cell line with NVP-BEZ235 treatment, which is reminiscent of what we found with WTB and C10 cells (Fig. 1B). We performed clonogenic survival studies with these cells after radiation (Fig. 7D). First, we noted little difference in the radiation survival curves between ATG5(−/−) and ATG5(+/+) cells, which is consistent with the idea that autophagy does not generally play much of a role in survival after radiation. Second, NVP-BEZ235 treatment of ATG5(+/+) cells resulted in a very modest degree of radiosensitization (DER of 1.11), similar to what we had seen in WTB and C10 cells, two other nontransformed lines. Finally, NVP-BEZ235 treatment of ATG5(−/−) cells, which cannot undergo autophagy, resulted in a greater degree of radiosensitization than that seen in ATG5(+/+) cells (DER of 1.36).

Discussion

NVP-BEZ235 is a recently developed combined PI3K/mTOR inhibitor being used clinically that has been shown to be a radiosensitizer (Konstantinidou et al., 2009; Fokas et al., 2012a,b; Mukherjee et al., 2012). We found that the com-

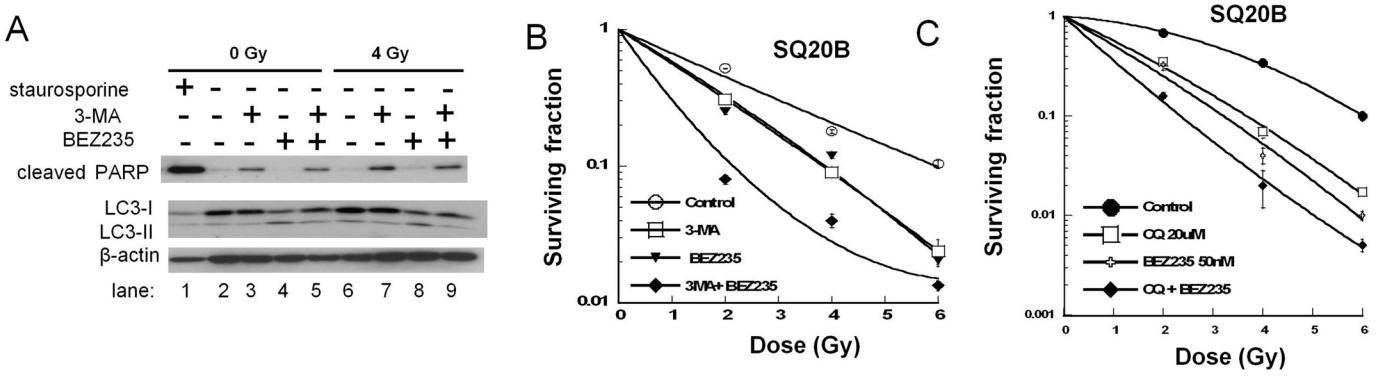

Fig. 6. Effect of chemical inhibitors of autophagy on radiosensitization by NVP-BEZ235. SQ20B cells were allowed to attach before treatment with staurosporine (1 μM), 3-MA (5 mM), chloroquine (20 μM), or NVP-BEZ235 (50 nM) for 1 h. A, SQ20B cells were allowed to attach before treatment with drugs for 1 h. Cells were then irradiated with 4 Gy of irradiation, and protein samples were taken 24 h later. Proteins were analyzed by SDS-PAGE and immunoblotting with the antibodies shown including cleaved PARP and LC3. B, SQ20B cells were plated and allowed to attach before drug treatment. Cells were then treated with NVP-BEZ235, 3-MA, or NVP-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 NVP-BEZ235, chloroquine, or NVP-BEZ235 + chloroquine. One hour later, they were irradiated. Ten to 14 days later, dishes were stained, and colonies were counted.

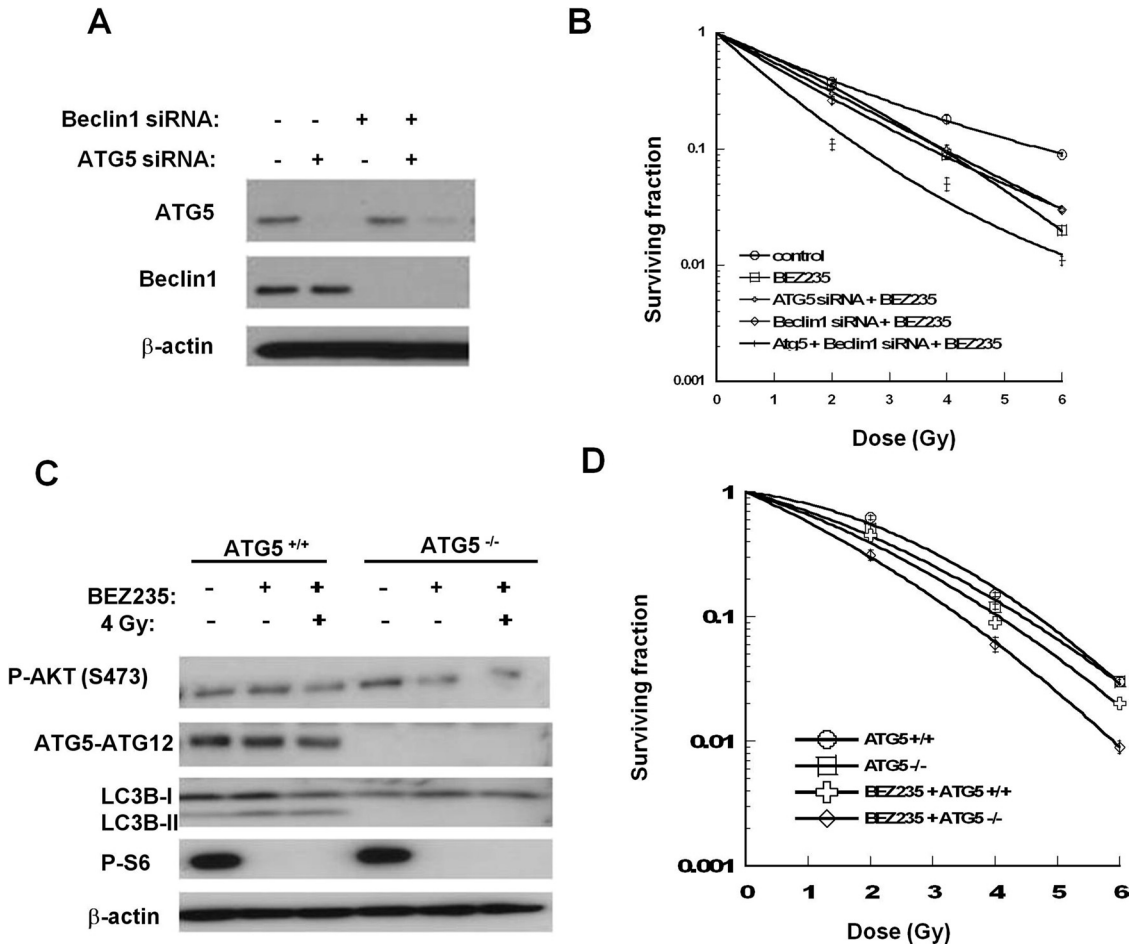

Fig. 7. Effect of knocking down ATG5 and/or beclin1 on radiosensitization by NVP-BEZ235. A and B, SQ20B cells were seeded in six-well plates and transfected the next day with 50 nM beclin1 siRNA, ATG5 siRNA, or a combination of both beclin1 and ATG5 siRNA. Cells were harvested for protein or survival curves 72 h after transfection. A, Western blots. B, clonogenic survival circles. C, ATG5 wild-type [ATG5(+/+)] and knockout [ATG5(−/−)] MEFs were seeded out and allowed to attach before treatment with NVP-BEZ235 for 1 h. After drug treatment, cells were irradiated with 4 Gy, and 24 h after irradiation protein samples were collected and analyzed by SDS-PAGE and immunoblotting with the antibodies shown. D, ATG5 wild-type [ATG5(+/+)] and knockout [ATG5(−/−)] MEFs were seeded out, allowed to attach, and then treated with NVP-BEZ235 for 1 h before being irradiated. Fourteen days later, dishes were stained and colonies were counted.

pound radiosensitizes a variety of cancer cell lines with varying genetic changes, including EGFR amplification (SQ20B), p53 mutation (HT29), PTEN deletion (U251), and K-*ras* mutation (A549). We tested the drug in three different noncancer cell lines (WTFB, C10, and wild-type MEFs) and found that there was minimal radiosensitization.

The primary purpose of our study was to understand mechanistically how NVP-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), mTOR, or the two together led to modest radiosensitization, but not as great as that seen with NVP-BEZ235. It is noteworthy that 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. (2005) found that rapamycin in combination with radiation led to increased cell death. Albert et al. (2006) also showed that the rapamycin derivative dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.04,9]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (RAD001) radiosensitized breast cancer cell lines. 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 that 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 is that treating cells with rapamycin inhibits the mTOR/raptor (TORC1) complex but not the mTOR/rictor (TORC2) complex, which can then lead to compensatory increase in Akt activation, perhaps via insulin receptor substrate-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.

Because inhibition of PI3K and mTOR did not seem to explain radiosensization by NVP-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 CometAssay, 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, which are key proteins involved in DNA damage repair. Akt has been implicated in regulating DNA-PKcs phosphorylation (Toulany et al., 2008). However, because knockdown of p110α or mTOR did not alter DNA-PKcs phosphorylation, it is much more likely that the effect of NVP-BEZ235 on ATM and DNA-PKcs is a direct one, given that they are both members of the PI3K-like family.

A recent article showed that NVP-BEZ235 decreased both HR and NHEJR after radiation (Mukherjee et al., 2012). However, our experiments using the combination of NVP-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 NVP-BEZ235 only partially inhibits HR and NHEJR but combining it with DNA-PKcs leads to complete inhibition of NHEJR with minimal compensatory up-regulation of HR.

In contrast to a published article (Azad et al., 2011), we found no evidence that NVP-BEZ235 leads to increased senescence after radiation in a variety of cell lines. However, we did find that the drug led to increased autophagy. Autophagy is a process by which cells "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 after radiation alone (without NVP-BEZ235). Paglin et al. (2001) reported that a novel response of cancer cells to radiation involved autophagy. Although autophagy may occur in some cells after radiation, the fact that neither SQ20B nor U251 cells show much evidence of this process after radiation alone and knocking out ATG5 in fibroblasts does not alter their radiosensitivity compared with ATG5(+/+) (wild type) fibroblasts suggests that this process does not play a dominant role in the survival of cells after radiation. On the other hand, radiation in combination with NVP-BEZ235 may modulate the latter's effects on autophagy, at least in some cell lines. In U251 cells, the combination did seem to prolong the duration of autophagy (Supplemental Fig. 7). When combined with radiation, we believe that the autophagy induced by NVP-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 NVP-BEZ235.

If the induction of autophagy by NVP-BEZ235 protects cells from dying after irradiation, how is it that the drug radiosensitizes cells? We hypothesize that NVP-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). 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). Shen et al. (2011), 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. Our results do, however, offer a rationale for inhibiting autophagy with an agent such as chloroquine when using NVP-BEZ235 to radiosensitize cells. This is particularly relevant because both chloroquine and NVP-BEZ235 have been used in patients.

Authorship Contributions

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

Conducted experiments: Cerniglia, Karar, and Tyagi.

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

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

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