Inhibition of DNA Double-Strand Break Repair by the Dual PI3K/mTOR Inhibitor NVP-BEZ235 as a Strategy for Radiosensitization of Glioblastoma

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Abstract

Purpose: Inhibitors of the DNA damage response (DDR) have great potential for radiosensitization of numerous cancers, including glioblastomas, which are extremely radio- and chemoresistant brain tumors. Currently, there are no DNA double-strand break (DSB) repair inhibitors that have been successful in treating glioblastoma. Our laboratory previously demonstrated that the dual phosphoinositide 3-kinase/mTOR inhibitor NVP-BEZ235 can potently inhibit the two central DDR kinases, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia-telangiectasia mutated (ATM), in vitro. Here, we tested whether NVP-BEZ235 could also inhibit ATM and DNA-PKcs in tumors in vivo and assessed its potential as a radio- and chemosensitizer in preclinical mouse glioblastoma models.

Experimental Design: The radiosensitizing effect of NVP-BEZ235 was tested by following tumor growth in subcutaneous and orthotopic glioblastoma models. Tumors were generated using the radioresistant U87-vIII glioma cell line and GBM9 neurospheres in nude mice. These tumors were then treated with ionizing radiation and/or NVP-BEZ235 and analyzed for DNA-PKcs and ATM activation, DSB repair inhibition, and attenuation of growth.

Results: NVP-BEZ235 potently inhibited both DNA-PKcs and ATM kinases and attenuated the repair of ionizing radiation–induced DNA damage in tumors. This resulted in striking tumor radiosensitization, which extended the survival of brain tumor–bearing mice. Notably, tumors displayed a higher DSB-load when compared with normal brain tissue. NVP-BEZ235 also sensitized a subset of subcutaneous tumors to temozolomide, a drug routinely used concurrently with ionizing radiation for the treatment of glioblastoma.

Conclusions: These results demonstrate that it may be possible to significantly improve glioblastoma therapy by combining ionizing radiation with potent and bioavailable DNA repair inhibitors such as NVP-BEZ235.

Introduction

Glioblastomas are deadly brain tumors with very poor prognosis (1). Patients with glioblastoma exhibit median survival times of only about 15 months and a 5-year survival of less than 10% even with aggressive treatment regimens (2). Currently, the standard care consists of surgical resection followed by radiation therapy. Concomitant and adjuvant administration of the chemotherapeutic agent temozolomide was recently added to the standard-of-care and is the only regimen that has improved survival, albeit minimally (2, 3). Despite aggressive treatment, these tumors always recur because of their infiltrative nature and extreme radioresistance (1, 4, 5). As radiation and temozolomide remain the mainstay of glioblastoma therapy, novel radio- and chemo-sensitizing strategies that work with the current therapeutic modality are urgently needed.

Ionizing radiation induces DNA double-strand breaks (DSB), the most deleterious of all types of DNA lesions, that can lead to cell death if left unrepaired (6). Abrogating the DNA damage response (DDR) to these breaks is, in principle, an attractive strategy to sensitize cancers to radiation and chemotherapy (7–9). DSBs can be repaired either through the error-prone nonhomologous end joining (NHEJ) pathway or the error-free homologous...
Translational Relevance

In the past decades, the only significant advance in the treatment of glioblastomas, which are highly aggressive and fatal brain tumors, has been the addition of temozolomide to radiotherapy regimens. However, this treatment modality increases survival only minimally. Therefore, new treatment modalities that improve survival are urgently needed. Targeting the DNA damage response (DDR) has been proposed as a strategy to sensitize glioblastomas to radiation. However, compounds with the necessary specificity, potency, bioavailability, and ability to cross the blood–brain barrier have not yet reached the clinical trial stage. Here, we show that NVP-BEZ235, a phosphoinositide 3-kinase/mTOR inhibitor already in clinical trials, can potently inhibit the DNA repair enzymes, DNA-PKcs and ATM, in vivo, resulting in abrogation of DNA repair and striking radiosensitization of subcutaneous and orthotopic brain tumors. Our results demonstrate that blocking the DDR using potent DNA-PKcs/ATM inhibitors is a viable option for improving glioblastoma therapy.

recombination pathway, in which the phosphoinositide 3-kinase (PI3K)-like kinases, DNA-PKcs and ATM, respectively, are centrally involved (10, 11). Therefore, these two apical kinases are very attractive targets for radiosensitization of glioblastoma and other tumors. Research over the past decade has led to the development of specific DNA-PKcs and ATM inhibitors, some of which are quite potent (12). Unfortunately, potent ATM or DNA-PKcs inhibitors that have good bioavailability for preclinical tumor studies and that can also cross the blood–brain barrier (BBB) have not yet been successfully developed (12).

NVP-BEZ235 is a small molecule inhibitor that was originally identified as a dual PI3K/mTOR inhibitor (IC50 = 4–75 and 20 mmol/L, respectively; ref. 13; reviewed in ref. 14). We reported earlier that NVP-BEZ235 also potently inhibits both ATM and DNA-PKcs, thereby attenuating both homologous recombination and NHEJ and resulting in unprecedented radiosensitization in a panel of glioblastoma cell lines (15). NVP-BEZ235 was also found to inhibit ATM and Rad3-related (ATR), another member of the PI3K-like family (16). Unlike specific inhibitors of PI3K-like kinases (12), NVP-BEZ235 is currently in phase I/II clinical trials as an mTOR inhibitor and has shown great promise in controlling solid tumors in preclinical mouse models (17). Thus, this drug provides us with the unique opportunity of carrying out proof-of-principle experiments in preclinical mouse models to test the possibility of improving glioblastoma therapy by blocking both DNA-PKcs and ATM and abrogating the repair of ionizing radiation–induced DSBs in tumors in vivo, thereby controlling brain tumor growth and significantly prolonging survival of brain tumor–bearing mice. Our results indicate that glioblastoma therapy could possibly be improved by using a combination of ionizing radiation and specific inhibitors of DNA-PKcs and/or ATM that are potent and bioavailable.

Materials and Methods

Cell culture and drug treatment

U87MG cells ectopically expressing EGFRvIII (U87-vIII) have been described before (18) and were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS in a humidified 37°C incubator with 5% CO2. U87-vIII cells were treated with ionizing radiation and specific inhibitors of DNA-PKcs and ATM using luciferase were generated by infection with a lentivirus carrying firefly luciferase under the control of the PKG promoter. Lentivirus was generated by packaging pLenti PGK V5-LUC Puro (19) in 293FT cells using the ViraPower Lentiviral Packaging System (Invitrogen). pLenti PGK V5-LUC Puro was a gift from Dr. Kaufman (Addgene; plasmid #19166). Luciferase-expressing cells were selected with 1 μg/ml Puromycin (GIBCO) and were maintained in medium containing Puromycin. GBM9 neurosphere culture has been described before (20). Briefly, these cells were maintained in DMEM/F12 1:1 media (Life Technologies) supplemented with B27 without Vitamin A (Life Technologies), 10 ng/mL EGF, and 10 ng/mL basic fibroblast growth factor (Peprotech). Once spheres reached a size of ~100 μm, they were dissociated by triturating in Accutase (Sigma-Aldrich) and subcultured at a 1:3 dilution. All cells were Mycoplasma free. NVP-BEZ235 (Selleck Chemicals) and temozolomide (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (Sigma-Aldrich), and 10 and 100 mmol/L stocks, respectively, were stored at −20°C. Cells were treated with drugs for 1 hour before irradiation. Cells were irradiated with γ rays from a 137Cs (21) source (JL Shepherd and Associates) at the indicated doses.

Mouse tumor studies

The Nestin-GFP transgenic mouse (21) was obtained from the Mouse Cancer Models Consortium. Nu/Nu nude mice were obtained commercially (Charles River, Stock#88). Subcutaneous tumors were generated by injecting 3.00 × 106 U87-vIII or 1.00 × 106 GBM9 cells subcutaneously in 50 μL PBS/Matrigel into 6-week-old Nu/Nu mice. Tumors were measured at the indicated times with digital calipers (Fisher Scientific), and tumor volumes were calculated (length × width × height × 0.5). For intracranial stereotactic injections, 5.00 × 105 U87-vIII cells were suspended in PBS (7.5 μL) and delivered into the left corpus striatum of the brains of 6-week-old Nu/Nu nude mice as described before (18). Tumor development was monitored by bioluminescence imaging (BLI). Treatment was initiated when the subcutaneous tumors reached an average size of 150 to 200 mm3, and when the intracranial tumors reached a signal of 0.5 to 1.0 × 109 photons/s. Mice were treated with NVP-BEZ235, temozolomide, or both by oral gavage or with vehicle (N-methyl-2-pyrrolidone/polyethylene...
glycol 300; 1:9, v/v; Sigma-Aldrich) as control; radiation was administered 2 hours after treatment. The treatment regimen consisted of a total of 12 doses of drug and/or ionizing radiation given every other day. Mice bearing subcutaneous tumors were treated with 50 mg/kg NVP-BEZ235 and/or 20 mg/kg temozolomide. Mice bearing intracranial tumors were treated with 75 mg/kg NVP-BEZ235. Subcutaneous as well as intracranial tumors were irradiated with an X-ray device (X-RAD 320, Precision X-ray; 250 kV, 15 mA, 0.2 minutes, 1.65 mm Al filter, at 5 cm) fitted with a specifically designed collimator providing a 10.08-mm diameter field size iso-dose exposure for a total dose of 2 Gy per treatment. Subcutaneous tumors were excised after they reached a group mean volume of 1,000 mm³. Mice were perfused with 1× PBS followed by 4% paraformaldehyde (PFA; Sigma-Aldrich). Excised tumors were postfixied by immersion in 4% PFA and either embedded in paraffin or processed for cryosectioning. Mice bearing intracranial tumors were sacrificed when they became moribund. Symptomatic mice were perfused with PBS followed by 4% PFA. Brains were dissected out, postfixied by immersion in 4% PFA, embedded in paraffin, and sectioned coronally. Mice were weighed 3 times per week during the drug-treatment period and afterward to ensure that weight loss because of drug treatment was less than 20% and that mice regained weight once treatment was stopped. All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center.

Noninvasive intracranial BLI

Serial bioluminescence images of tumor-bearing mice were obtained using the IVIS Lumina System (Xenogen Corp.) coupled to Living Image data acquisition software (Xenogen Corp.). During imaging, mice were anesthetized with isoflurane (Baxter International Inc.), and a solution of d-luciferin (Baxter International Inc.), and a solution of d-luciferin (Baxter International Inc.) was administered subcutaneously in the neck region. Images were acquired between 10 and 20 minutes post-luciferin administration and peak luminescence signals were recorded. The BLI signals emanating from the tumors were quantified by measuring photon flux within the region of interest using the Living Image software package.

Western blot analyses

Nuclear extracts from irradiated cells were prepared and Western blotted as described before (22). For analysis of DNA-PKcs and ATM activation in vivo, tumors were allowed to grow to 100 mm³ or the intracranial tumors reached a BLI intensity of 0.5 to 1 × 10⁹ photons/sec. Tumors were collected at the indicated times and processed for immunofluorescence staining as described above. For quantifying 53BP1 foci, a stack of images along the Z-axis (at 0.5 μm intervals throughout the depth of the tissue) was obtained with a Leica DM5500 microscope and Leica Application Suite software. The Z-stacks were combined (Projection Type: Maximum Intensity) using Image J software and all visible foci were counted manually by the same scorer. Nuclei overlapping with other nuclei were excluded. The number of 53BP1 foci per nucleus (average of >50 nuclei per tumor) was determined for each time point or treatment and plotted after subtracting background (average number of foci per nucleus of vehicle treated, mock-irradiated mice).

Immunofluorescence staining

Subcutaneous tumors and tumor-bearing brains were cut into 20-μm cryosections or 5 to 10 μm paraffin sections. Tissue sections were stained according to immunofluorescence protocols as described (18, 23). Antibodies used for immunofluorescence staining were as follows: anti-53BP1 (Santa Cruz), anti-Ki67 (Abcam), anti-phospho-pS6(S235/236) (Cell Signaling). A Leica DM5500 microscope was used for imaging. Ki67 and pS6 images were taken at ×10 magnification and 53BP1 foci were imaged at ×40 magnification.

Hematoxylin and eosin and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed on paraffin sections using the FragEL DNA Fragmentation Detection Kit (Merck KGaA). Hematoxylin and eosin (H&E) staining was done by standard techniques.

Colony formation assays

Cells were plated in triplicate onto 60-mm dishes (1,000 cells per dish), treated with the indicated drugs, and irradiated 4 hours later with graded doses of radiation. At 16 hours after irradiation, drug-containing medium was replaced with drug-free medium. Surviving colonies were stained with crystal violet approximately 10 to 14 days later as described (18).

DSB repair assays

For monitoring DSB repair in vivo, mice were treated with NVP-BEZ235 (150 mg/kg) or vehicle and irradiated 2 hours later at the indicated doses. Mice were treated when the subcutaneous tumors reached a volume of 100 mm³ or the intracranial tumors reached a BLI intensity of 0.5 to 1 × 10⁹ photons/sec. Tumors were collected at the indicated times and processed for immunofluorescence staining as described above. For quantifying 53BP1 foci, a stack of images along the Z-axis (at 0.5 μm intervals throughout the depth of the tissue) was obtained with a Leica DM5500 microscope and Leica Application Suite software. The Z-stacks were combined (Projection Type: Maximum Intensity) using Image J software and all visible foci were counted manually by the same scorer. Nuclei overlapping with other nuclei were excluded. The number of 53BP1 foci per nucleus (average of >50 nuclei per tumor) was determined for each time point or treatment and plotted after subtracting background (average number of foci per nucleus of vehicle treated, mock-irradiated mice).
DSB repair seen the pronounced inhibition of these PI3K-like kinases and surrogate marker for DSBs (Fig. 1B). Thus, it is clear that tumor sections were immunostained for 53BP1 foci, aminated tumors as seen in a time course experiment in which such as KAP-1 and p53 phosphorylation (ref. 10; Fig. 1A).

Results
NVP-BEZ235 is a potent inhibitor of DNA-PKcs and ATM in tumors in vivo
NVP-BEZ235 has exhibited good bioavailability as well as potent inhibition of its canonical targets, mTOR and PI3K, in preclinical mouse models (24–34), and is currently in clinical trials for the treatment of solid tumors. We have previously reported that NVP-BEZ235 can also block the PI3K-like kinases, DNA-PKcs and ATM, in vitro (15). This provides us, for the first time, with a potent DSB repair inhibitor that is bioavailable and that can be used to evaluate whether clinical radiosensitization of glioblastoma may be a viable option for improving therapy. For our study, we chose U87MG human glioma cells expressing the constitutively active form of EGFR, EGFRvIII (henceforth called U87-vIII). We have previously shown that these cells form radioresistant intracranial tumors that are very suitable for radiosensitization studies (18). As a prelude to intracranial glioblastoma studies, we wanted to determine whether this drug could inhibit DNA-PKcs and ATM in subcutaneous tumors in vivo. We first confirmed that NVP-BEZ235 could block DNA-PKcs and ATM in U87-vIII cells in culture leading to acute radiosensitization (Supplementary Fig. S1) similar to that reported by us previously for a panel of glioma lines (15). It was previously reported that NVP-BEZ235 levels peak in tumors approximately 2 hours after drug administration (13). Therefore, we treated mice bearing U87-vIII subcutaneous tumors with increasing doses of NVP-BEZ235, focally irradiated the tumors 2 hours later, and harvested them at 0.5 hours after ionizing radiation. We then examined ionizing radiation–induced DNA-PKcs and ATM activation by Western blotting tumor extracts with phospho-specific antibodies that recognize auto-phosphorylated DNA-PKcs or ATM (10, 11). We found that NVP-BEZ235 could inhibit ionizing radiation–induced activation of both ATM and DNA-PKcs in tumors (Fig. 1A). As also seen in vitro (Supplementary Fig. S1A), phosphorylation of DNA-PKcs in tumors was impaired to a greater extent than that of ATM. Nevertheless, the partial inhibition of ATM in tumors was sufficient to block downstream signaling events such as Kap-1 and p53 phosphorylation (ref. 10; Fig. 1A). Consequently, DSB repair was severely impaired in irradiated tumors as seen in a time course experiment in which tumor sections were immunostained for 53BP1 foci, a surrogate marker for DSBs (Fig. 1B). Thus, it is clear that the pronounced inhibition of these PI3K-like kinases and DSB repair seen in vitro can be recapitulated in vivo.

DSB repair inhibition by NVP-BEZ235 results in accumulation of DSBs in tumors and striking radiosensitization
To determine if inhibition of DSB repair by NVP-BEZ235 would result in radiosensitization and tumor regression, we generated subcutaneous tumors using U87-vIII cells, and allowed the tumors to grow to a volume of 200 mm³ before initiating treatment. The treatment regimen consisted of 12 doses, given every other day, of 50 mg/kg of NVP-BEZ235 given by oral gavage, followed 2 hours later by 2 Gy of ionizing radiation localized to the tumor. Six mice were randomly allocated for each treatment group: (i) mock-irradiation and vehicle only (Vehicle), (ii) irradiation and vehicle only (ionizing radiation), (iii) mock-irradiation and NVP-BEZ235 (BEZ), and (iv) irradiation and NVP-BEZ235 (ionizing radiation + BEZ; Fig. 2A). Mice treated with ionizing radiation alone exhibited tumor growth profiles that were only slightly delayed compared with untreated animals, demonstrating the highly radioresistant nature of these tumors. The BEZ arm also showed a minor initial response, but the tumors resumed rapid growth that was not unexpected as adaptive resistance to NVP-BEZ235 has been reported before (35). In striking contrast, combinatorial treatment with ionizing radiation and NVP-BEZ235 resulted in tremendous radiosensitization as evidenced by inhibition of tumor growth throughout the treatment period and for approximately 60 additional days after treatment was stopped (Fig. 2A and B; Supplementary Fig. S2A).

To determine if the regressing tumors (ionizing radiation + BEZ arm) carried a greater burden of DSBs compared with the nonresponding tumors (the other 3 arms), 3 replicate sets of tumor-bearing mice were treated as before, and tumors were harvested at mid-treatment, that is, 24 hours after dose 7. We detected significant accumulation of 53BP1 foci in tumors treated with both ionizing radiation and NVP-BEZ235 compared with those treated with either modality alone (Fig. 2C), which correlated with the tumor response (Fig. 2A). The ionizing radiation + BEZ235 tumors also showed a striking reduction in proliferation assessed by Ki67 staining in accord with the greater burden of DSBs in these tumors (Fig. 2D). The tumors from the different groups did not exhibit significant levels of apoptosis as assayed by TUNEL staining (Supplementary Fig. S2B). However, NVP-BEZ235 has been reported to trigger necrosis (36) as well as autophagy (37) in irradiated cells in vitro, and these processes could also modulate tumor responses in vivo. Taken together, our observations indicate that DNA repair inhibition in irradiated tumors results in a high burden of DSBs and inhibition of tumor growth.

NVP-BEZ235 does not interfere with temozolomide, an agent coadministered with radiation for glioblastoma treatment
The S(N1)-type DNA alkylating drug temozolomide is the only chemotherapeutic agent that offers survival benefit when administered with ionizing radiation for glioblastoma.
therapy (2, 3). Thus, any new radiosensitizing approach for glioblastoma needs to be compatible with temozolomide. We and others have shown that lethality from S

1-type alkylating agents is because of secondary DSBs that are generated during DNA replication (38–40). The antiproliferative activity of NVP-BEZ235 could attenuate induction of such secondary DSBs and thereby antagonize the effects of temozolomide. To rule out this possibility, we generated U87-vIII tumors and allowed them to reach a size of 200 mm

3. The entreated the mice every other day (total of 12 treatments over 24 days; 6 mice per treatment group) with temozolomide only (temozolomide) or a combination of both temozolomide and NVP-BEZ235 (20 and 50 mg/kg, respectively) given at the same time by gavage (temozolomide + BEZ; Fig. 3A). We found that although these tumors regressed upon treatment with temozolomide, they always recurred and grew rapidly thereafter. Upon addition of NVP-BEZ235 to the temozolomide treatment regimen, 50% of temozolomide + NVP-BEZ235–treated tumors showed no major differences in tumor growth, whereas the remaining 50% exhibited significantly longer tumor remission compared with temozolomide alone (Supplementary Fig. S3). This experiment was repeated, and the same split response was observed again. Pooled data from both experiments are shown (Fig. 3A). It is clear from these data that NVP-BEZ235 does not interfere with the antitumor effects of temozolomide, and in a subset of the tumors, dual treatment actually shows an advantage in tumor growth control. A similar synergy between temozolomide and NVP-BEZ235 has also been reported before with a different dosing schedule, although the underlying mechanism was not elucidated (13).

Next, 3 replicate sets of tumor-bearing mice were treated as before with NVP-BEZ235 and/or temozolomide and tumors in the middle of the treatment regimen (i.e., 24 hours after dose 7) from each arm were harvested and immunostained for 53BP1 and Ki67. We found that the temozolomide
treated tumors show an increased number of 53BP1 foci compared with the control tumors (Fig. 3B), which correlates with a decrease in proliferation (Fig. 3C). These effects of temozolomide are clearly not attenuated by the addition of NVP-BEZ235 to the treatment regimen (Fig. 3B and C). Taken together, these data indicate that NVP-BEZ235 does not antagonize the antitumor effects of temozolomide, and in a subset of tumors, actually augments these effects.

Figure 2. Administration of NVP-BEZ235 with radiation results in accumulation of DSBs in tumors and striking radiosensitization. A, subcutaneous tumors generated from U87-vIII cells were allowed to reach a volume of 200 mm³, after which mice were treated every other day with vehicle, NVP-BEZ235, ionizing radiation, or NVP-BEZ235 with ionizing radiation (2 Gy, given 2 hours after drug administration) for a total of 12 doses (shaded area). Tumor growth was monitored until tumors reached 1,000 mm³. n = 6 for each treatment group. Error bars, SEM. *P < 0.0001 (ionizing radiation vs. ionizing radiation + BEZ). B, a set of tumors excised at end of treatment period illustrate the marked reduction in tumor size with combinatorial treatment. Bar, 10 mm. C, a set of tumors was harvested 24 hours after dose 7, and cryosections were immunofluorescence stained for 53BP1 foci (green) and imaged at ×40 magnification to obtain a snapshot of the DNA damage load in tumors undergoing treatment. Nuclei are stained with DAPI (blue). 53BP1 foci in 50 nuclei per tumor were counted and plotted. n = 3 tumors per group. Error bars, SEM. ****, P < 0.0001. D, cryosections were immunofluorescence stained for Ki67 (green) and imaged at ×10 magnification. More than 5,000 nuclei per tumor were scored using Cell Profiler software and the percentages of Ki67-positive cells were plotted. n = 3 tumors per group. Error bars, SEM. ***, P = 0.0018.
NVP-BEZ235 radiosensitizes tumors derived from glioblastoma neurospheres

Next, we wanted to examine if radiosensitization with NVP-BEZ235 could be recapitulated in early-passage, primary glioblastoma cells grown as neurospheres. We chose the previously described GBM9 cells for this purpose as these cells retain high levels of amplified EGFRvIII and are thus complementary to the U87-vIII cells used in this study (20). We found that NVP-BEZ235 could block ionizing radiation–induced activation of DNA-PKcs and ATM in GBM9 cells in culture leading to acute radiosensitization, similar to that seen with the U87-vIII cells (Supplementary Fig. S4). We then generated subcutaneous tumors in Nu/Nu mice using GBM9 cells and found that NVP-BEZ235 could inhibit ionizing radiation–induced activation of both ATM and DNA-PKcs and phosphorylation of downstream substrates in these tumors (Fig. 4A). As a result, DSB repair was compromised in irradiated tumors as seen by immunostaining tumor sections for 53BP1 foci (Fig. 4B).

To determine if NVP-BEZ235 could radiosensitize tumors generated from GBM9 cells, we allowed the tumors to grow to a volume of approximately 150 mm³ before initiating treatment. As with the U87-vIII tumors, the treatment regimen consisted of 12 doses, given every other day, of 50 mg/kg of NVP-BEZ235 given by gavage, followed 2 hours later by 2 Gy of ionizing radiation localized to the tumor. Six mice were randomly allocated to each treatment group. Although mice treated with ionizing radiation or NVP-BEZ235 alone exhibited some delay in tumor growth, combinatorial treatment with both ionizing radiation and NVP-BEZ235 resulted in striking radiosensitization as evidenced by inhibition of tumor growth throughout the treatment period and for at least 45 additional days after treatment was stopped (Fig. 4C). Tumor regression was clearly accompanied by a greater burden of DSBs in the tumors as seen by...
Figure 4. NVP-BEZ235 radiosensitizes tumors derived from glioblastoma neurospheres. A, subcutaneous tumors generated from GBM9 neurospheres were mock-irradiated or irradiated (ionizing radiation) with a total dose of 10 Gy. Tumor-bearing mice were treated with the indicated doses of NVP-BEZ235 or with vehicle alone 2 hours before irradiation. Tumors were excised 30 minutes after irradiation and tumor extracts were Western blotted with the indicated antibodies. Note attenuation of ionizing radiation–induced autophosphorylation of DNA-PKcs and ATM and reduced phosphorylation of ATM targets, KAP-1 and p53. B, to quantify DSB repair in irradiated tumors, tumor-bearing mice were treated with NVP-BEZ235 and irradiated with 4 Gy of ionizing radiation after 2 hours. Tumors were harvested at 0.5 or 24 hours after irradiation, and cryosections were immunofluorescence stained for 53BP1 (red) and imaged at ×40 magnification. Nuclei are stained with DAPI (blue). 53BP1 foci in 50 nuclei per tumor were counted and plotted for each time point. n = 3 tumors per time point. Error bars, SEM. ****, P < 0.0001. C, tumors were allowed to reach a volume of 150 mm³, after which mice were treated every other day with vehicle, NVP-BEZ235, ionizing radiation, or NVP-BEZ235 with ionizing radiation (2 Gy, given 2 hours after drug administration) for a total of 12 doses (shaded area). Tumor growth was monitored until tumors reached a size of 1,000 mm³. n = 6 mice per treatment group. Error bars, SEM. ****, P < 0.0001. D, a set of tumors was harvested 24 hours after dose 7, and cryosections were immunofluorescence stained for 53BP1 (red) and imaged at ×40 magnification. 53BP1 foci in 50 nuclei per tumor were counted and plotted. n = 3 tumors per group. Error bars, SEM. ****, P < 0.0001.

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Figure 5. NVP-BEZ235 can cross the BBB and inhibit DSB repair in brain tumors. A, Nestin-GFP mice were treated with NVP-BEZ235 and sacrificed at the indicated times after treatment. Cryosections of the mouse brain were immunofluorescence stained for phospho-S6 (red) and imaged at ×10 magnification as a measure of inhibition of the PI3K-Akt pathway. The SVZ, SGZ, cortex, and cerebellum were imaged. Stem cell compartments are labeled with GFP (green) and nuclei are stained with DAPI (blue). Note reduction in phospho-S6 staining between 2 and 8 hours after treatment. B, U87-vIII cells were injected intracranially to generate orthotopic brain tumors. After 7 days, mice were treated with NVP-BEZ235 or vehicle by gavage, intracranially irradiated (2 Gy) after 2 hours, and sacrificed at 0.5 or 12 hours after ionizing radiation. Intracranial tumors were identified in coronal brain sections by H&E staining. C, paraffin sections of tumor-bearing brains were immunofluorescence stained for phospho-S6 (red) and imaged at ×10 magnification as a measure of inhibition of the PI3K-Akt pathway. D, brain tumor sections were also immunofluorescence stained for 53BP1 foci (green) and imaged at ×40 magnification to quantify residual DSBs. n = 2 tumors per time point. Error bars, SEM. **, P = 0.002.
Figure 6. NVP-BEZ235 sensitizes brain tumors to radiation and prolongs survival of tumor-bearing mice. A, U87-vIII cells expressing firefly luciferase were injected intracranially to generate orthotopic brain tumors (6 mice per treatment group) that were monitored by BLI. Once tumors reached a signal of 0.5 to 1.0 \( \times 10^9 \) photons/sec, mice were treated every other day with vehicle alone, NVP-BEZ235, ionizing radiation (2 Gy), or NVP-BEZ235 in combination with ionizing radiation (2 Gy given 2 hours after drug administration) for a total of 12 doses. Top panel shows tumor progression in a representative mouse for each treatment arm. Bottom panel shows quantifications of all measurements. Each line represents an individual brain tumor. (Continued on the following page.)
staining a set of tumors at the mid-treatment point for 53BP1 foci (compare ionizing radiation + BEZ arm with the other 3 arms; Fig. 4D). Our results, showing that NVP-BEZ235 can radiosensitize tumors generated from both U87-vIII cells and GBM9 neurospheres, indicate that DSBR repair inhibitors could have broad utility in glioblastoma therapy.

**NVP-BEZ235 crosses the BBB and inhibits DSBR repair and tumor growth in orthotopic glioblastoma models**

A concern with new compounds for the treatment of diseases localized in the central nervous system is whether they will be able to cross the BBB. To see if NVP-BEZ235 can cross the BBB, we treated Nestin-GFP mice (21) with NVP-BEZ235 and sacrificed mice at time points ranging from 0.5 to 16 hours after treatment. Inhibition of the PI3K-Akt-mTOR pathway by NVP-BEZ235 was assessed by staining for phosphorylation of the ribosomal protein S6 (29). We detected a significant decrease in phospho-S6 staining between 2 to 8 hours after treatment in most regions of the mouse brain, including the cortex, cerebellum, and the 2 neurogenic niches, the subventricular zone (SVZ) and the subgranular zone (SGZ), both marked by nestin promoter-driven GFP expression. (Fig. 5A). These data clearly indicate that NVP-BEZ235 can cross the BBB making it a very attractive candidate for radiosensitization of glioblastoma.

To test the efficacy of NVP-BEZ235 in blocking DSBR repair in an orthotopic glioblastoma model, we stereotactically injected U87-vIII cells intracranially into the left corpus striatum of Nu/Nu mice as described before (15) and allowed approximately 7 days for tumor development (Fig. 5B). We treated tumor-bearing mice with vehicle or NVP-BEZ235 and irradiated them intracranially 2 hours later with 2 Gy of ionizing radiation. Irradiated mice were sacrificed at different times after ionizing radiation, and tumor sections were immunofluorescence stained for phospho-S6 (Ser236) and for 53BP1 foci. We found ablation of phospho-S6 staining upon treatment with NVP-BEZ235 confirming delivery of the drug to these intracranial tumors (Fig. 5C). 53BP1 staining indicated that the irradiated tumors harbored extensive DNA DSBs at 0.5 hours after ionizing radiation. These breaks were almost completely repaired by 12 hours in the vehicle-treated arm, but a significant portion remained unrepaired in the NVP-BEZ235-treated arm (Fig. 5D). This clearly indicates that NVP-BEZ235 can cross the BBB and block DSBR repair in intracranial tumors.

To examine if combinatorial treatment with NVP-BEZ235 and ionizing radiation could control brain tumor growth, intracranial tumors were generated in Nu/Nu mice using U87-vIII cells expressing luciferase to allow monitoring of tumor growth by bioluminescent imaging (BLI), as described before (18). Treatment was initiated when tumors reached BLI intensities in the 0.5 to 1.0 × 10^5 photons/sec range and were presumably of similar size at time of treatment. As with the subcutaneous tumors, we followed a 12-dose regimen with treatments given every other day. Each dose consisted of vehicle or NVP-BEZ235 (75 mg/kg) followed after 2 hours by mock-irradiation or 2 Gy of ionizing radiation delivered intracranially. BLI radiance was recorded and plotted over time, and mice were sacrificed once they became moribund because of the brain tumor burden. Intracranial irradiation (ionizing radiation) or NVP-BEZ235 (BEZ) alone had little effect on tumor growth rates compared with nontreated tumors (Vehicle; Fig. 6A). Strikingly, combinatorial treatment with both modalities (ionizing radiation + BEZ) resulted in reduced tumor growth rates, and in the case of one mouse (out of a cohort of 6), complete ablation of BLI signal was observed around day 30 after treatment with no recurrence for at least 12 months after treatment. There was very little improvement in overall survival (Kaplan–Meier plots) when the mice were treated with either modality alone (Fig. 6B). In contrast, combinatorial treatment significantly prolonged survival of tumor bearing mice: treatment with ionizing radiation or NVP-BEZ235 alone resulted in an increase of only 5.5 and 5 days in median survival, respectively, whereas combined treatment extended median survival by 35.5 days. Thus, as with the subcutaneous tumor model, we see striking synergy between NVP-BEZ235 and ionizing radiation in brain tumor growth control, as well as in the median survival of tumor-bearing mice.

To examine if this synergy correlated with a greater burden of DSBs in the tumors receiving combinatorial treatment, additional sets of orthotopic tumors were treated as before, and tumors were harvested at mid-treatment, that is, 24 hours after dose 7. Upon staining for 53BP1 foci, we observed that tumors treated with both ionizing radiation and NVP-BEZ235 harbored higher numbers of DSBs than tumors treated with either modality alone (Fig. 6C). Importantly, the DSB load was much greater in tumors compared with the normal areas of the irradiated mouse brain. This indicates that there might exist a therapeutic window in which combinatorial treatment could result in greater damage to the tumor relative to the normal brain thereby minimizing collateral damage because of radiosensitization. Taken together, our preclinical results clearly indicate that significant improvement of glioblastoma radiotherapy may become possible in the clinic with potent and bioavailable DDR inhibitors such as NVP-BEZ235.

**Discussion**

Glioblastomas remain one of the most lethal of all tumors for which no effective treatment exists despite...
decades of research (1). Thus, there is an urgent need for the development of new therapeutic modalities for glioblastoma. The standard-of-care for glioblastoma is surgical resection followed by ionizing radiation and concomitant and adjuvant temozolomide (3). As both ionizing radiation and temozolomide induce DSBs (6, 38), blocking the DDR to these breaks is, in principle, a rational approach for sensitizing glioblastomas and other cancers (7–9). More than 10 years of research efforts focused on the development of potent DNA-PKcs and ATM inhibitors have yielded specific compounds, some of which are very useful in the laboratory for cell-based studies (12). Unfortunately, no single compound shows the necessary potency, bioavailability, and ability to cross the BBB that would allow their evaluation in preclinical mouse glioblastoma models. Against this backdrop, our results, demonstrating that NVP-BEZ235 potently inhibits both ATM and DNA-PKcs in tumors, are exciting as they open up new therapeutic possibilities for glioblastoma.

In our study, using preclinical mouse models and a drug that is bioavailable and in clinical trials, we show that radio- and chemo-sensitization of glioblastoma may be a viable option for improving therapeutic outcomes. We have previously shown that NVP-BEZ235, a dual PI3K/mTOR inhibitor, can also inhibit both DNA-PKcs and ATM in vitro at low doses (in the nanomolar range; ref. 15). Here, we have established the in vivo utility of this drug as a radiosensitizer in preclinical mouse glioblastoma models. We show that this drug can block the phosphorylation of both ATM and DNA-PKcs in subcutaneous tumors and attenuate the repair of ionizing radiation–induced DSBs. Importantly, we show that the drug can cross the BBB and inhibit DSB repair in intracranial tumors. Therefore, a striking efficacy in controlling tumor growth was observed in both subcutaneous and orthotopic tumor models when the drug was administered in combination with ionizing radiation. Consequently, the addition of NVP-BEZ235 to the irradiation schedule resulted in a significant increase in survival of mice bearing intracranial tumors.

Notably, upon analyzing mouse brains during the middle of the ionizing radiation + BEZ treatment regimen, we observed that the normal brain tissue harbored a lower burden of DSBs compared with the tumor. This hints at the existence of a therapeutic window wherein DNA damage in the tumor may be higher than in the normal brain. This differential inhibition of DSB repair could possibly be attributed to a higher drug intake by the tumor because of a leaky BBB that is usually observed in brain tumors (41). Moreover, aberrant hyperproliferation of tumor cells results in replication stress and DSBs (6, 42), and this could be compounded by the effects of NVP-BEZ235. Heightened activation of DSB repair pathways has been observed in glioma cell lines (18) and human glioma stem cells (4) by us and others; reviewed in ref. 43. Thus, it is possible that glioma cells may be more dependent on these repair pathways, a phenomenon referred to as "non-oncogene addiction" (44). Non-oncogene addiction to DSB repair pathways, in addition to oncogene addiction to the PI3K-Akt-mTOR pathway, both of which would be targeted by NVP-BEZ235, may render tumor cells particularly vulnerable to this drug. Hence, NVP-BEZ235 might have a more drastic effect on tumor cells than on normal brain cells resulting in a greater burden of DSBs in the tumor versus the normal brain during therapy. Such a differential effect was also seen in vitro in preliminary experiments in which we found that normal human astrocytes (45) were radiosensitized to a lesser extent by NVP-BEZ235 (Supplementary Fig. S5) compared with human glioma cell lines (see Supplementary Figs. S1 and S4 and ref. 15). These differential effects on normal cells versus tumor cells, as well as the effects of NVP-BEZ235 on glioma initiating cells and the tumor microenvironment merit further investigation in the future.

Administration of temozolomide is integral to the current glioblastoma therapeutic regimen and constitutes the only significant advance in the treatment of glioblastoma in decades, improving median survival by 2 months compared with ionizing radiation only and increasing the 5-year survival from 2% to 10% (2, 3). Therefore, it is very important that new therapeutic modalities be compatible with temozolomide. We find that NVP-BEZ235 does not interfere with the antitumor effects of temozolomide. Rather, NVP-BEZ235 and temozolomide seemed to synergize in at least half of the combinatorially treated tumors. Potentiation of the effects of temozolomide could be because of the inhibition of repair of breaks that are secondarily induced by temozolomide (38–40). However, further experiments are clearly needed to understand why this synergy was only seen in a subset of tumors. Regardless, the potentiating effect of NVP-BEZ235 on temozolomide treatment has important clinical implications because NVP-BEZ235 could possibly be given in the clinic both concomitantly with ionizing radiation and temozolomide, and as an adjuvant with temozolomide.

A few recent studies have evaluated the radiosensitizing effects of specific DNA-PKcs or ATM inhibitors in vivo (46–50); however, none of these compounds have reached clinical trials. In contrast, NVP-BEZ235 is currently in phase I/II clinical trials for the treatment of advanced solid tumors as a monotherapeutic agent and shows good pharmacokinetics in vivo (13, 14, 17). Moreover, NVP-BEZ235 shows a significantly greater radiosensitizing effect in vivo compared with the specific DNA-PKcs or ATM inhibitors tested thus far (46–50), possibly because it potently inhibits both DNA-PKcs and ATM. Glioblastoma radioresistance is a pressing and intractable problem with no possibility of improvement currently in sight. Although we await the development of better clinically suitable DDR inhibitors, our proof-of-principle experiments indicate that it could be possible to significantly improve glioblastoma therapy in the future by combining radiation with DNA-PKcs and ATM inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Inhibition of DNA Repair in Glioblastoma

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