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 (GBM), which are extremely radio- and chemo-resistant brain tumors. Currently, there are no DNA double-strand break (DSB) repair inhibitors that have been successful in treating GBM. Our lab has previously demonstrated that the dual PI3K/mTOR inhibitor NVP-BEZ235 can potently inhibit the two central DDR kinases, DNA-PKcs and 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 chemo-sensitizer in pre-clinical mouse GBM models.

**Experimental design:** The radiosensitizing effect of NVP-BEZ235 was tested by following tumor growth in subcutaneous and orthotopic GBM 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 (IR) 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 IR-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 to normal brain tissue. NVP-BEZ235 also sensitized a subset of subcutaneous tumors to temozolomide, a drug routinely used concurrently with IR for the treatment of GBM.
Conclusions: These results demonstrate that it may be possible to significantly improve GBM therapy by combining IR with potent and bioavailable DNA repair inhibitors like NVP-BEZ235.

Translational Relevance

In the past decades, the only significant advance in the treatment of GBMs, 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 GBMs 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 PI3K/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 GBM therapy.
**Introduction**

Glioblastomas (GBM) are deadly brain tumors with very poor prognosis (1). Patients with GBM 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 (TMZ) 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 due to their infiltrative nature and extreme radioresistance (1, 4, 5). As radiation and TMZ remain the mainstay of GBM therapy, novel radio- and chemosensitizing strategies that work with the current therapeutic modality are urgently needed.

Ionizing radiation (IR) 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 non-homologous end joining (NHEJ) pathway or the error-free homologous recombination (HR) pathway, in which the 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 GBM 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 pre-clinical 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 (IC$_{50}$=4-75nM, IC$_{50}$=20nM, respectively) (13); reviewed in (14). We reported earlier that NVP-BEZ235 also potently inhibits both ATM and DNA-PKcs, thereby attenuating both HR and NHEJ and resulting in unprecedented radiosensitization in a panel of GBM cell lines (15). NVP-BEZ235 was also found to inhibit 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 pre-clinical mouse models (17). Thus, this drug provides us with the unique opportunity of carrying out proof-of-principle experiments in pre-clinical mouse models to test the possibility of improving GBM therapy by blocking both DNA-PKcs and ATM.

In this study, we examined whether NVP-BEZ235 could act as a DDR inhibitor in pre-clinical mouse models and, if so, whether combining this drug with IR could be a viable strategy for improving GBM therapy. We find that NVP-BEZ235 can block both DNA-PKcs and ATM and abrogate the repair of IR-induced DSBs in tumors in vivo, thereby controlling brain tumor growth and significantly prolonging survival of brain tumor-bearing mice. Our results indicate that GBM therapy could possibly be improved by using a combination of IR 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 Modification of Eagle Medium (DMEM) with 10% fetal bovine serum in a humidified 37°C incubator with 5% CO$_2$. 
U87-vIII cells expressing luciferase were generated by infection with a lentivirus carrying firefly
luciferase under the control of the PGK 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
bFGF (Peprotech). Once spheres reached a size of ~100 μm, they were dissociated by triturating
in Accutase (Sigma-Aldrich) and sub-cultured at a 1:3 dilution. All cells were Mycoplasma free.
NVP-BEZ235 (Selleck Chemicals) and TMZ (Sigma-Aldrich) were dissolved in dimethyl
sulfoxide (DMSO; Sigma-Aldrich), and 10 mM and 100 mM stocks, respectively, were stored at
-20°C. Cells were treated with drugs for 1 hour before irradiation. Cells were irradiated with
gamma rays from a $^{137}$Cs 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 3.00x10$^6$ U87-vIII or 1.00x10$^6$
GBM9 cells subcutaneously in 50 μL PBS/Matrigel into 6 weeks-old Nu/Nu mice. Tumors were
measured at the indicated times with digital calipers (Fisher Scientific), and tumor volumes were
calculated (length x width x height x 0.5). For intra-cranial stereotactic injections, 5.00x10$^5$ U87-
vIII cells were suspended in PBS (7.5μl) and delivered into the left corpus striatum of the brains
of 6 weeks-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 150-200 mm$^3$, and when the intra-cranial tumors reached a signal of 0.5-1.0x10$^9$ photons/s. Mice were treated with NVP-BEZ235, TMZ, or both by oral gavage or with vehicle (N-methyl-2-pyrrolidone/polyethylene glycol 300; 1:9 vol/vol; 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 IR given every other day. Mice bearing subcutaneous tumors were treated with 50 mg/kg NVP-BEZ235 and/or 20 mg/kg TMZ. Mice bearing intra-cranial tumors were treated with 75 mg/kg NVP-BEZ235. Subcutaneous as well as intra-cranial tumors were irradiated with an X-ray device (X-RAD 320, Precision X-ray; 250 kV, 15 mA, 0.2 min, 1.65 mm Al filter, at 5cm) 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 1000 mm$^3$. Mice were perfused with 1X phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich). Excised tumors were post-fixed by immersion in 4% PFA and either embedded in paraffin or processed for cryosectioning. Mice bearing intra-cranial tumors were sacrificed when they became moribund. Symptomatic mice were perfused with PBS followed by 4% PFA. Brains were dissected out, post-fixed by immersion in 4% PFA, embedded in paraffin, and sectioned coronally. Mice were weighed three times per week during the drug-treatment period and afterwards to ensure that weight loss due to 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.
Non-invasive intra-cranial bioluminescence imaging. 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 anaesthetized with isoflurane (Baxter International Inc.), and a solution of D-luciferin (180 mg/kg in PBS; total volume: 80 μL; Gold Biotechnology) 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 (ROI) 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 and treated with NVP-BEZ235 or vehicle and irradiated 2 hours later at the indicated doses. Tumors were excised and snap frozen in liquid nitrogen 30 minutes after irradiation. Tumors were homogenized in hypotonic lysis buffer, nuclear extracts were obtained, and Western blot analysis was carried out as described before (22). Antibodies used were as follows: anti-phospho-Akt(S473), anti-Akt, and anti-phospho-p53(S15) (Cell Signaling); anti-ATM (Sigma-Aldrich); anti-p53, (Santa Cruz Biotechnology); anti-phospho-KAP-1(S824), anti-KAP-1 (Bethyl); anti-phospho-ATM(S1981) (GenScript); anti-phospho-DNA-PKcs(S2056) (Abcam); anti-DNA-PKcs (Thermo Fisher); anti-Ku80 (kind gift from Dr. B. Chen); HRP-conjugated secondary antibodies (Biorad); Alexa488/568-conjugated secondary antibodies (Molecular Probes).
Immunofluorescence staining. Subcutaneous tumors and tumor-bearing brains were cut into 20 μm cryosections or 5-10 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-S6(S235/236) (Cell Signaling). A Leica DM5500 microscope was used for imaging. Ki67 and pS6 images were taken at 10X magnification and 53BP1 foci were imaged at 40X magnification.

H&E and TUNEL staining. 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 (1000 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 intra-cranial tumors reached a BLI intensity of 0.5-1 x 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).

**Statistical analysis.** DNA repair and cell proliferation data were analyzed by unpaired, two tailed t-tests with Welch's correction using the Graphpad Prism software package. Mouse survival data were plotted using the Kaplan-Meier method and compared using the logrank test. Tumor growth profiles between different groups were compared by the Mixed Model method. An AR(1) covariance structure for repeated tumor volume measures was used in the model. SAS 9.3 for Windows was used for analysis.

**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 pre-clinical 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 GBM 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 intra-cranial tumors that are very suitable for radiosensitization studies (15). As a prelude to intra-cranial GBM 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 post-IR. We then examined IR-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 IR-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 (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.** In order 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 twelve doses, given every other day, of 50 mg/kg of NVP-BEZ235 given by oral gavage, followed 2 hr later by 2 Gy of IR localized to the tumor. Six mice were randomly allocated for each treatment group: 1) mock-irradiation and vehicle only (Vehicle), 2) irradiation and vehicle only (IR), 3) mock-irradiation and NVP-BEZ235 (BEZ), and 4) irradiation and NVP-BEZ235 (IR+BEZ) (Fig. 2A). Mice treated with IR alone exhibited tumor growth profiles that were only slightly delayed compared to 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 which was not unexpected as adaptive resistance to NVP-BEZ235 has been reported before (35). In striking contrast, combinatorial treatment with IR 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, B; Supplementary Fig. S2A).

In order to determine if the regressing tumors (IR+BEZ arm) carried a greater burden of DSBs compared to the non-responding tumors (the other three arms), three replicate sets of tumor-bearing mice were treated as before, and tumors were harvested at mid-treatment, i.e., 24 hours after dose 7. We detected significant accumulation of 53BP1 foci in tumors treated with both IR and NVP-BEZ235 compared to those treated with either modality alone (Fig. 2C) which correlated with the tumor response (Fig. 2A). The IR+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 TMZ, an agent co-administered with radiation for GBM treatment. The S\textsubscript{N}1-type DNA alkylating drug temozolomide (TMZ) is the only chemotherapeutic agent that offers survival benefit when administered with IR for GBM therapy (2, 3). Thus, any new radiosensitizing approach for GBM needs to be compatible with TMZ. We and others have shown that lethality from S\textsubscript{N}1-type alkylating agents is due to secondary DSBs that are generated during DNA replication (38-40). The anti-proliferative activity of NVP-BEZ235 could attenuate induction of such secondary DSBs and thereby antagonize the effects of TMZ. To rule out this possibility, we generated U87-vIII tumors and allowed them to reach a size of 200mm\textsuperscript{3}. We then treated the mice every other day (total of 12 treatments over 24 days; 6 mice per treatment group) with TMZ only (TMZ) or a combination of both TMZ and NVP-BEZ235 (20 mg/kg and 50 mg/kg, respectively) given at the same time by gavage (TMZ+BEZ) (Fig. 3A). We found that while these tumors regressed upon treatment with TMZ, they always recurred and grew rapidly thereafter. Upon addition of NVP-BEZ235 to the TMZ-treatment regimen, 50% of TMZ+NVP-BEZ235-treated tumors showed no major differences in tumor growth, while the remaining 50% exhibited significantly longer tumor remission compared to TMZ alone (Supplemental Fig. S3). This experiment was repeated, and the same split response
was observed again. Pooled data from both experiments is shown (Fig. 3A). It is clear from these data that NVP-BEZ235 does not interfere with the antitumor effects of TMZ, and in a subset of the tumors, dual treatment actually shows an advantage in tumor growth control. A similar synergy between TMZ and NVP-BEZ235 has also been reported before with a different dosing schedule though the underlying mechanism was not elucidated (13).

Next, three replicate sets of tumor-bearing mice were treated as before with NVP-BEZ235 and/or TMZ 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 for Ki67. We found that the TMZ-treated tumors show an increased number of 53BP1 foci compared to the control tumors (Fig. 3B), which correlates with a decrease in proliferation (Fig. 3C). These effects of TMZ are clearly not attenuated by the addition of NVP-BEZ235 to the treatment regimen (Fig. 3B, C). Taken together, these data indicate that NVP-BEZ235 does not antagonize the anti-tumor effect of TMZ, and in a subset of tumors, actually augments these effects.

**NVP-BEZ235 radiosensitizes tumors derived from GBM neurospheres.** Next, we wanted to examine if radiosensitization with NVP-BEZ235 could be recapitulated in early-passage, primary GBM 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 IR-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 IR-induced activation of both ATM and DNA-PKcs and phosphorylation in the tumors.
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).

In order 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 twelve doses, given every other day, of 50mg/kg of NVP-BEZ235 given by gavage, followed 2 hours later by 2 Gy of IR localized to the tumor. Six mice were randomly allocated to each treatment group. While mice treated with IR or NVP-BEZ235 alone exhibited some delay in tumor growth, combinatorial treatment with both IR 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 staining a set of tumors at the mid-treatment point for 53BP1 foci (compare IR+BEZ arm with the other three arms) (Fig. 4D).

Our results, showing that NVP-BEZ235 can radiosensitize tumors generated from both U87-vIII cells and GBM9 neurospheres, indicate that DSB-repair inhibitors could have broad utility in GBM therapy.

**NVP-BEZ235 crosses the blood-brain-barrier (BBB) and inhibits DSB repair and tumor growth in orthotopic GBM 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 post-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 post-treatment in most regions of the mouse brain, including the cortex, cerebellum, and the two 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 GBM.

To test the efficacy of NVP-BEZ235 in blocking DSB repair in an orthotopic GBM model, we stereotactically injected U87-vIII cells intra-cranially 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 intra-cranially 2 hours later with 2 Gy of IR. Irradiated mice were sacrificed at different times post-IR, 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 intra-cranial tumors (Fig. 5C). 53BP1 staining indicated that the irradiated tumors harbored extensive DNA DSBs at 0.5 hours post-IR. 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 DSB repair in intra-cranial tumors.

To examine if combinatorial treatment with NVP-BEZ235 and IR could control brain tumor growth, intra-cranial 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 (15). Treatment was initiated when tumors reached BLI intensities in the 0.5 - 1.0 x 10⁹ 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 IR delivered intra-cranially. BLI radiance was recorded and plotted over time, and mice were sacrificed once they became moribund due to the brain tumor burden. Intracranial irradiation (IR) or NVP-BEZ235 (BEZ) alone had little effect on tumor growth rates compared to non-treated tumors (Vehicle) (Fig. 6A). Strikingly, combinatorial treatment with both modalities (IR+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 post-treatment with no recurrence for at least 12 months post-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 IR 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 IR 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, i.e., 24 hours after dose 7. Upon staining for 53BP1 foci, we observed that tumors treated with both IR 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 to 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 due to radiosensitization. Taken together, our pre-clinical results clearly indicate that
significant improvement of GBM radiotherapy may become possible in the clinic with potent
and bioavailable DDR inhibitors such as NVP-BEZ235.

Discussion

GBMs 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 GBM. The standard-of-care for GBM is surgical resection followed by
IR and concomitant and adjuvant TMZ (3). As both IR and TMZ induce DSBs (6, 38), blocking
the DNA damage response to these breaks is, in principle, a rational approach for sensitizing
GBMs and other cancers (7-9). More than ten 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 pre-clinical mouse GBM 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 GBM.

In our study, using pre-clinical mouse models and a drug that is bioavailable and in
clinical trials, we show that radio- and chemo-sensitization of GBM 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) (15). Here, we have established the in vivo utility of this drug as a radiosensitizer in pre-clinical mouse GBM models. We show that this drug can block the phosphorylation of both ATM and DNA-PKcs in subcutaneous tumors and attenuate the repair of IR-induced DSBs. Importantly, we show that the drug can cross the BBB and inhibit DSB repair in intra-cranial 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 IR. Consequently, the addition of NVP-BEZ235 to the irradiation schedule resulted in a significant increase in survival of mice bearing intra-cranial tumors.

Notably, upon analyzing mouse brains during the middle of the IR+BEZ treatment regimen, we observed that the normal brain tissue harbored a lower burden of DSBs compared to 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 due to 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 (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 to human glioma cell lines (see Supplementary Fig. S1 and S4 and (15)). These differential effects on normal 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 TMZ is integral to the current GBM therapeutic regimen and constitutes the only significant advance in the treatment of GBM in decades, improving median survival by 2 months compared to IR 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 TMZ. We find that NVP-BEZ235 does not interfere with the antitumor effects of TMZ. Rather, NVP-BEZ235 and TMZ seemed to synergize in at least half of the combinatorially-treated tumors. Potentiation of the effects of TMZ could be due to the inhibition of repair of breaks that are secondarily induced by TMZ (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 TMZ treatment has important clinical implications since NVP-BEZ235 could possibly be given in the clinic both concomitantly with IR and TMZ, and as an adjuvant with TMZ.

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 to the specific DNA-PKcs or ATM inhibitors tested thus far (46-50), possibly because it potently
inhibits both DNA-PKcs and ATM. GBM radioresistance is a pressing and intractable problem with no possibility of improvement currently in sight. While we await the development of better clinically-suitable DDR inhibitors, our proof-of-principle experiments indicate that it could be possible to significantly improve GBM therapy in the future by combining radiation with DNA-PKcs and ATM inhibitors.
Acknowledgements

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References

Figure Legends

Figure 1. NVP-BEZ235 blocks both ATM and DNA-PKcs in tumors and inhibits DSB repair. **A.** Subcutaneous tumors generated from U87-vIII cells were mock-irradiated or irradiated (IR) 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 prior to irradiation. Tumors were excised 30 minutes after irradiation and tumor extracts were Western blotted with the indicated antibodies. Note attenuation of IR-induced autophosphorylation of DNA-PKcs and ATM and reduced phosphorylation of ATM targets, KAP-1 and p53. **B.** Tumor-bearing mice were treated with NVP-BEZ235 and irradiated 2 hours later with 4 Gy of IR. Tumors were harvested at 0.5, 2, 4, 8, or 24 hours post-irradiation, and cryosections were immunofluorescence stained for 53BP1 foci (green) and imaged at 40X magnification. Nuclei are stained with DAPI (blue). 53BP1 foci in 50 nuclei per tumor were counted and plotted for each time point. n = 2 tumors per time point. Error bars, S.E.M. Note abrogated DSB repair in NVP-BEZ235-treated tumors.

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 mm$^3$ following which mice were treated every other day with vehicle, NVP-BEZ235, IR, or NVP-BEZ235 with IR (2 Gy, given 2 hours after drug administration) for a total of 12 doses (shaded area). Tumor growth was monitored until tumors reached 1000 mm$^3$. n = 6 for each treatment group. Error bars, S.E.M. P < 0.0001 (IR vs. IR+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 40X 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, S.E.M. ****, P < 0.0001. D. Cryosections were immunofluorescence stained for Ki67 (green) and imaged at 10X magnification. More than 5000 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, S.E.M. **, P = 0.0018.

**Figure 3. NVP-BEZ235 does not interfere with the anti-tumor effects of TMZ.**

A. Subcutaneous tumors generated from U87-vIII cells were allowed to reach a volume of 200 mm$^3$ and were treated every other day with TMZ, NVP-BEZ235, or a combination of NVP-BEZ235 and TMZ (given at the same time) for 12 doses (shaded area). Tumor growth was monitored until tumors reached a size of 1000 mm$^3$. Note that the addition of NVP-BEZ235 does not interfere with the effects of TMZ and actually hinders tumor growth further. n = 12 mice per treatment group. Error bars, S.E.M. P = 0.04 (TMZ vs. TMZ+BEZ). Vehicle and BEZ plots from Figure 2A are shown for comparison. B. A set of tumors was harvested 24 hours after dose 7, and cryosections were immunofluorescence stained for 53BP1 foci (green) and imaged at 40X 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, S.E.M. C. Cryosections were immunofluorescence stained for Ki67 (green) and imaged at 10X magnification. More than 5000 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, S.E.M.

**Figure 4. NVP-BEZ235 radiosensitizes tumors derived from GBM neurospheres.**

A. Subcutaneous tumors generated from GBM9 neurospheres were mock-irradiated or irradiated (IR) 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 prior to irradiation. Tumors were excised 30 minutes after irradiation and tumor extracts were Western blotted with the indicated antibodies. Note attenuation of IR-induced autophosphorylation of DNA-PKcs and ATM and reduced phosphorylation of ATM targets, KAP-1 and p53. B. In order to quantify DSB repair in irradiated tumors, tumor-bearing mice were treated with NVP-BEZ235 and irradiated with 4 Gy of IR after 2 hr. Tumors were harvested at 0.5 or 24 hr post-irradiation, and cryosections were immunofluorescence stained for 53BP1 (red) and imaged at 40X 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, S.E.M. ****, P < 0.0001 C. Tumors were allowed to reach a volume of 150 mm$^3$ following which mice were treated every other day with vehicle, NVP-BEZ235, IR, or NVP-BEZ235 with IR (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 1000 mm$^3$. n = 6 mice per treatment group. Error bars, S.E.M. P < 0.0001 (IR vs. IR+BEZ). D. A set of tumors was harvested 24 hours after dose 7, and cryosections were immunofluorescence stained for 53BP1 (red) and imaged at 40X magnification. 53BP1 foci in 50 nuclei per tumor were counted and plotted. n = 3 tumors per group. Error bars, S.E.M. ****, P < 0.0001.
Figure 5. NVP-BEZ235 can cross the blood-brain-barrier and inhibit DSB repair in brain tumors. 

A. Nestin-GFP mice were treated with NVP-BEZ235 and sacrificed at the indicated times post-treatment. Cryosections of the mouse brain were immunofluorescence stained for phospho S6 (red) and imaged at 10X magnification as a measure of inhibition of the PI3K-Akt pathway. The subventricular zone (SVZ), subgranular zone (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 – 8 hours post-treatment. 

B. U87-vIII cells were injected intra-cranially to generate orthotopic brain tumors. After 7 days, mice were treated with NVP-BEZ235 or vehicle by gavage, intra-cranially irradiated (2 Gy) after 2 hours, and sacrificed at 0.5 or 12 hours post-IR. Intra-cranial 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 10X 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 40X magnification to quantify residual DSBs. n = 2 tumors per time point. Error bars, S.E.M. **, 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 intra-cranially to generate orthotopic brain tumors (6 mice per treatment group) that were monitored by BLI. Once tumors reached a signal of 0.5-1.0X10^9 photons/s, mice were treated every other day with vehicle alone, NVP-BEZ235, IR (2Gy), or NVP-BEZ235 in combination with IR (2 Gy given 2 hours after drug administration) for a total of 12 doses. Upper panel shows tumor progression in a representative mouse for each treatment arm. Lower panel shows quantifications of all
measurements. Each curve represents an individual brain tumor. Scale, 0-2x10^8 protons/s/cm^2/sr.
P < 0.0001 (IR vs. IR+BEZ). Note delay in reappearance of BLI signals in IR+BEZ arm. B. Survival of brain tumor-bearing mice was recorded and represented in a Kaplan-Meier plot. n = 6 mice per group. P = 0.0152 (IR vs. IR+BEZ). Note improved survival of IR+BEZ mice. C. A set of brain tumor-bearing mice was sacrificed at 24 hours after dose 7 and paraffin sections of the whole brain were obtained. Sections were immunofluorescence stained for 53BP1 (green) and imaged at 40X magnification, and 53BP1 foci in 50 nuclei within each tumor were quantified. Nuclei are stained with DAPI (blue). 53BP1 foci were also quantified in the cortex, SVZ, and SGZ and plotted. n = 2 brains per group. Error bars, S.E.M. **, P = 0.003. ##, P = 0.0064. ^^, P = 0.0046.
Figure 1

A

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- pAkt (Ser473)
- Akt
- pDNA-PKcs (S2056)
- DNA-PKcs
- pATM (Ser1981)
- ATM
- pKAP-1 (Ser824)
- KAP-1
- pP53 (Ser15)
- P53
- Ku80

B

Time after IR

Mock IR | 0.5 hr | 2 hr | 24 hr

Vehicle

NVP-BEZ235

53BP1 foci

Time after IR (hours)
Figure 2

A) Time (days)

0 20 40 60 80 100

Vehicle IR BEZ IR+BEZ

B) Treatment period

Vehicle IR BEZ IR+BEZ

C) Vehicle IR BEZ IR+BEZ

D) Vehicle IR BEZ IR+BEZ

**% Ki67-positive cells**

Vehicle IR BEZ IR+BEZ

53BP1 foci

Vehicle IR BEZ IR+BEZ

% Ki67-positive cells

Vehicle IR BEZ IR+BEZ

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Figure 3

A

U87-vIII tumor volume (mm$^3$)

Treatment period  
Time (days)

Vehicle  
BEZ  
TMZ  
TMZ+BEZ

B

Vehicle  
TMZ  
BEZ  
TMZ+BEZ

53BP1 foci

Vehicle  
TMZ  
BEZ  
BEZ+TMZ

C

Vehicle  
TMZ  
BEZ  
TMZ+BEZ

% Ki67-positive cells

Vehicle  
TMZ  
BEZ  
BEZ+TMZ

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Figure 4

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- pAkt (Ser473)
- Akt
- pDNA-PKcs (S2056)
- DNA-PKcs
- pATM (Ser1981)
- ATM
- pKAP-1 (Ser824)
- KAP-1
- pP53 (Ser15)
- P53
- Ku80

B

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C

- GBM9 tumor volume (mm³)
- Time (days)
- Treatment period
- Vehicle
- IR
- BEZ
- IR+BEZ

D

- 53BP1 foci
- Vehicle
- IR
- BEZ
- IR+BEZ
Figure 6

A. Radiance (photons/s/cm²/sr) over time for different treatments.

B. Percent survival over time for different treatments.

C. Quantification of 53BP1 foci in various regions.

Vehicle, IR, BEZ, BEZ+IR comparison showing treatment effects on cellular damage markers.

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Clinical Cancer Research

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

Carlos Gil del Alcazar, Molly Gillam, Bipasha Mukherjee, et al.

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