

RESEARCH PAPER

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## Ganetespib radiosensitization for liver cancer therapy

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### ABSTRACT

Therapies for liver cancer particularly those including radiation are still inadequate. Inhibiting the stress response machinery is an appealing anti-cancer and radiosensitizing therapeutic strategy. Heat-shock-protein-90 (HSP90) is a molecular chaperone that is a prominent effector of the stress response machinery and is overexpressed in liver cancer cells. HSP90 client proteins include critical components of pathways implicated in liver cancer cell survival and radioresistance. The effects of a novel non-geldanamycin HSP90 inhibitor, ganetespib, combined with radiation were examined on 3 liver cancer cell lines, Hep3b, HepG2 and HUH7, using *in vitro* assays for clonogenic survival, apoptosis, cell cycle distribution,  $\gamma$ H2AX foci kinetics and client protein expression in pathways important for liver cancer survival and radioresistance. We then evaluated tumor growth delay and effects of the combined ganetespib-radiation treatment on tumor cell proliferation in a HepG2 hind-flank tumor graft model. Nanomolar levels of ganetespib alone exhibited liver cancer cell anti-cancer activity *in vitro* as shown by decreased clonogenic survival that was associated with increased apoptotic cell death, prominent G2-M arrest and marked changes in PI3K/AKT/mTOR and RAS/MAPK client protein activity. Ganetespib caused a supra-additive radiosensitization in all liver cancer cell lines at low nanomolar doses with enhancement ratios between 1.33–1.78. These results were confirmed *in vivo*, where the ganetespib-radiation combination therapy produced supra-additive tumor growth delay compared with either therapy by itself in HepG2 tumor grafts. Our data suggest that combined ganetespib-radiation therapy exhibits promising activity against liver cancer cells, which should be investigated in clinical studies.

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## Introduction


Hepatocellular carcinoma (HCC) is a major global health concern. Although more than 80% of HCC cases are found in Africa and East Asia, there is a rising incidence of cases in the western world due to the contraction of hepatitis C in the 1960s and 1970s.<sup>1–4</sup> The low 5-year survival rate of 10–15% can be attributed to the discovery of HCC in its advanced stages in the majority of cases and lack of effective therapies for advanced disease.<sup>5,6</sup>

Surgical tumor resection, with 5 y survival rates of 60%–70%, or transplantation, with 4 y survival rates of 70%–80%, are the preferred methods for HCC treatment, however only a minority of patients are candidates for surgery and recurrence is common.<sup>7</sup> Non-surgical liver-directed therapies such as

ablation, embolization, or radiation are used commonly, but many patients develop recurrent disease or present with an advanced stage of the disease which cannot be cured by these methods.<sup>6,8</sup> Furthermore, HCC is relatively resistant to conventionally fractionated radiation<sup>9</sup> and physical radiation dose escalation is greatly limited by normal liver tissue toxicity. Methods to dose escalate to liver cancer cells selectively would be advantageous as local control in the setting of compromised normal liver parenchyma is commonly a critical issue in many HCC patients.

Targeting the non-oncogene addiction or stress response machinery is gaining recognition as a potent cancer therapeutic strategy. In the high stress environment of cancers, tumor cells are subject to harsh conditions including hypoxia,

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inflammation, DNA damage and alterations in metabolic behavior.<sup>10</sup> Cancer cell survival is maintained through the help of cellular stress response machinery such as heat shock proteins. Heat shock protein 90 (HSP90) is a molecular chaperone that assists in the post-translational folding and stabilization of many proteins, including various growth factor receptors and oncoproteins required for tumor maintenance. HSP90 has been reported to be overexpressed in HCC cell lines and HCC patient tumors when compared to the normal liver.<sup>8</sup> Pre-clinical studies have provided a rationale for inhibition of HSP90 as an anti-cancer target for HCC, but combined effects of HSP90 inhibition with radiation are unknown. We have recently demonstrated targeting HSP90 with a second generation inhibitor potentially radiosensitizes prostate cancer cells and delays prostate tumor growth in immunocompetent mice.<sup>11</sup>

The previous geldanamycin-analog generation of HSP90 inhibitors have shown modest results in several clinical trials, but limitations such as poor solubility, inconsistent pharmacokinetics, and hepatotoxicity, among other factors, have led to the development of newer and more potent class of HSP90 inhibitors.<sup>8,11</sup> Ganetespib is a non-geldanamycin triazolone HSP90 inhibitor with single agent anti-cancer activity which has been shown to have superior pharmacodynamics compared to other HSP90 inhibitors.<sup>12</sup> The drug has shown promise as an antitumor agent in phase II trials of patients with non-small cell lung cancer and metastatic breast cancer.<sup>13,14</sup> For advanced HCC, a phase I pharmacokinetic study of ganetespib showed a very manageable safety profile, and established a recommended phase II dose,<sup>8</sup> but combined effects of ganetespib with radiation are unknown. Herein we report potent *in vitro* and *in vivo* radiosensitizing effects of ganetespib on HCC cells and potential mechanisms of radiosensitization.

## Results

### **Ganetespib (STA-9090) treatment induces radiosensitization of HCC cells *in vitro***

Hep3b, HepG2 and HUH7 cells were treated with increasing concentrations of ganetespib (0, 10, 50 and 100 nM) and all lines showed a dose dependent increase of HSP72 levels by western blotting (Fig. 1A), correlating with degree of HSP90 inhibition. HCC cell lines rely on several cellular pathways for proliferation and survival, the components of which are HSP90 client proteins, such as the RAS/MAPK and PI3K/AKT/mTOR pathways.<sup>15-17</sup> Ganetespib caused down regulation of these signaling pathways in Hep3b, HepG2 and HUH7 cells in a dose dependent fashion (ganetespib 0, 10, 50 and 100 nM). In all 3 HCC cell lines treated with  $\geq 50$  nM ganetespib, pERK1/2 expression was either noticeably reduced or completely lost (Fig. 1A). Phospho-S6 was also seen to be down-regulated in the presence of  $\geq 50$  nM ganetespib in all 3 HCC cell lines. These results suggest reduced activity of both the RAS/MAPK and PI3K/AKT/mTOR pathways in HCC cell lines treated with ganetespib.

To assess the efficacy of ganetespib as a potential monotherapy and/or as a radiosensitizer for liver cancer we assayed the effect of ganetespib treatment on clonogenic potential of HCC

cell lines. Clonogenic colony formation assays with Hep3b, HepG2 and HUH7 cell lines treated with ganetespib alone (5 nM) showed a reduction of 53.31%, 56.44% and 34.60% in survival fraction of Hep3b (Fig. 1B,  $p = 0.0032$ ), HepG2 (Fig. 1C,  $p = 0.003$ ) and HUH7 (Fig. 1D,  $p = 0.0172$ ) cell lines, respectively.

Additionally, a greater decrease in survival fraction was found when the HCC cells treated with ganetespib were also subjected to ionizing radiation. The ganetespib enhancement ratios were 1.78 for Hep3b cells (Fig. 1E), 1.33 for HepG2 cells (Fig. 1F) and 1.47 for HUH7 cells (Fig. 1G). These data indicated that ganetespib is a potent inhibitor of clonogenic survival in HCC cells both as a single agent and also as a radiosensitizer.

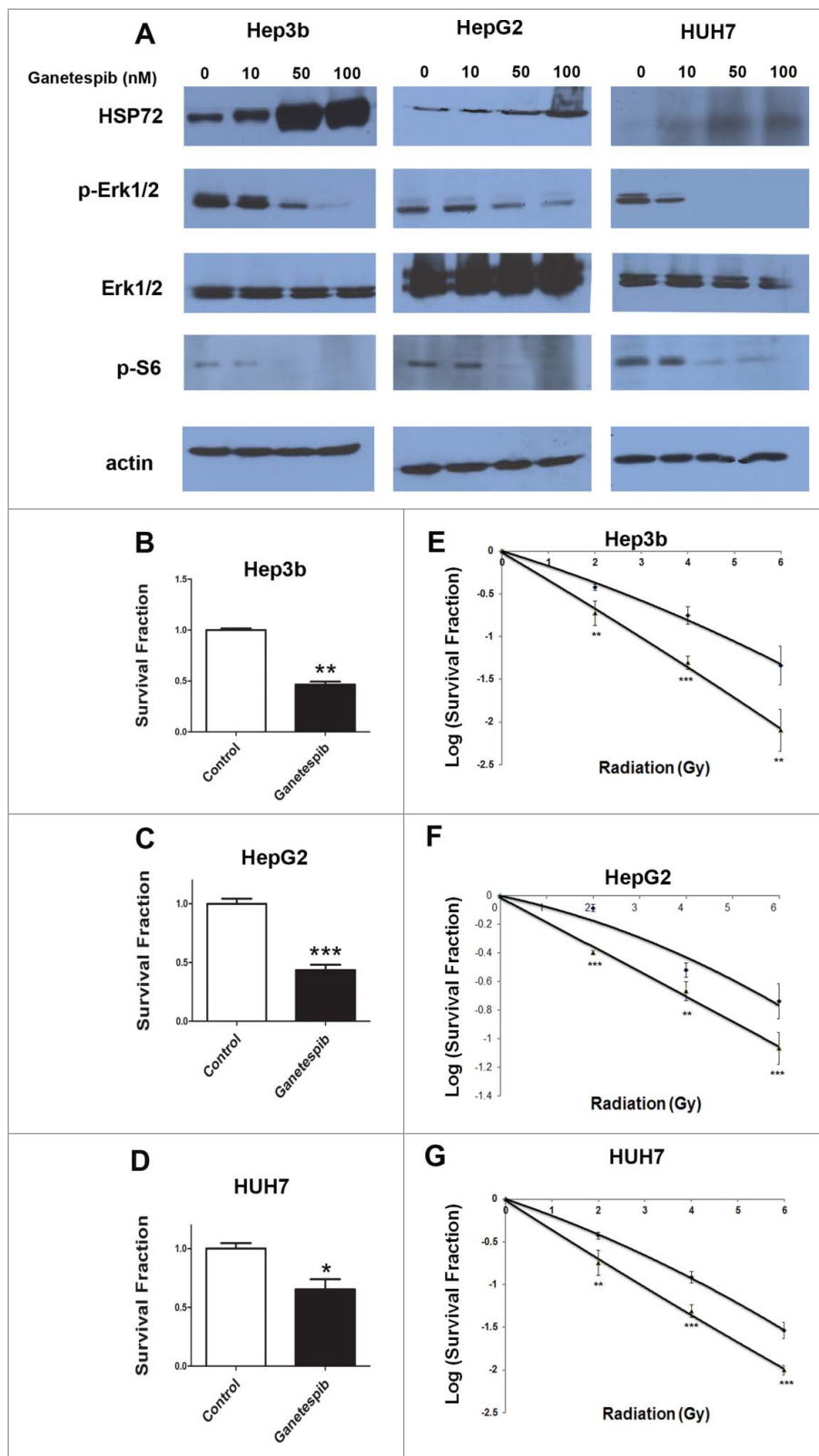
### **Ganetespib causes G2-M arrest and apoptosis induction in HCC cell lines**

We synchronized HCC cells by serum starvation and treatment with aphidicolin and then subsequently treated them with ganetespib for 24 hours and analyzed for nuclear content by flow cytometry. Hep3b cells when treated with vehicle control (DMSO) did not show significant change in the cell cycle profile (Fig. 2A and Fig. S1) after 24 and 48 hours post treatment. However, ganetespib treated Hep3b cells show a marked increase in G2-M cells with 46.633% (SD 0.379) cells at 24 hours and 48.8% (SD 0.866) at 48 hours corresponding to a 11.373% and 13.4% increase over vehicle control at 24 and 48 hours, respectively (Fig. 2A). HepG2 cells showed the most relative arrest in G2-M in presence of ganetespib (Fig. 2B) with a 33.96% and 23.73% increase at 24 and 48 hours, respectively. A qualitatively similar pattern was observed for HUH7 cells (Fig. 2C). Taken altogether, ganetespib treatment caused HCC cells to undergo cell cycle arrest in G2-M, the most radiosensitive phase of the cell cycle.

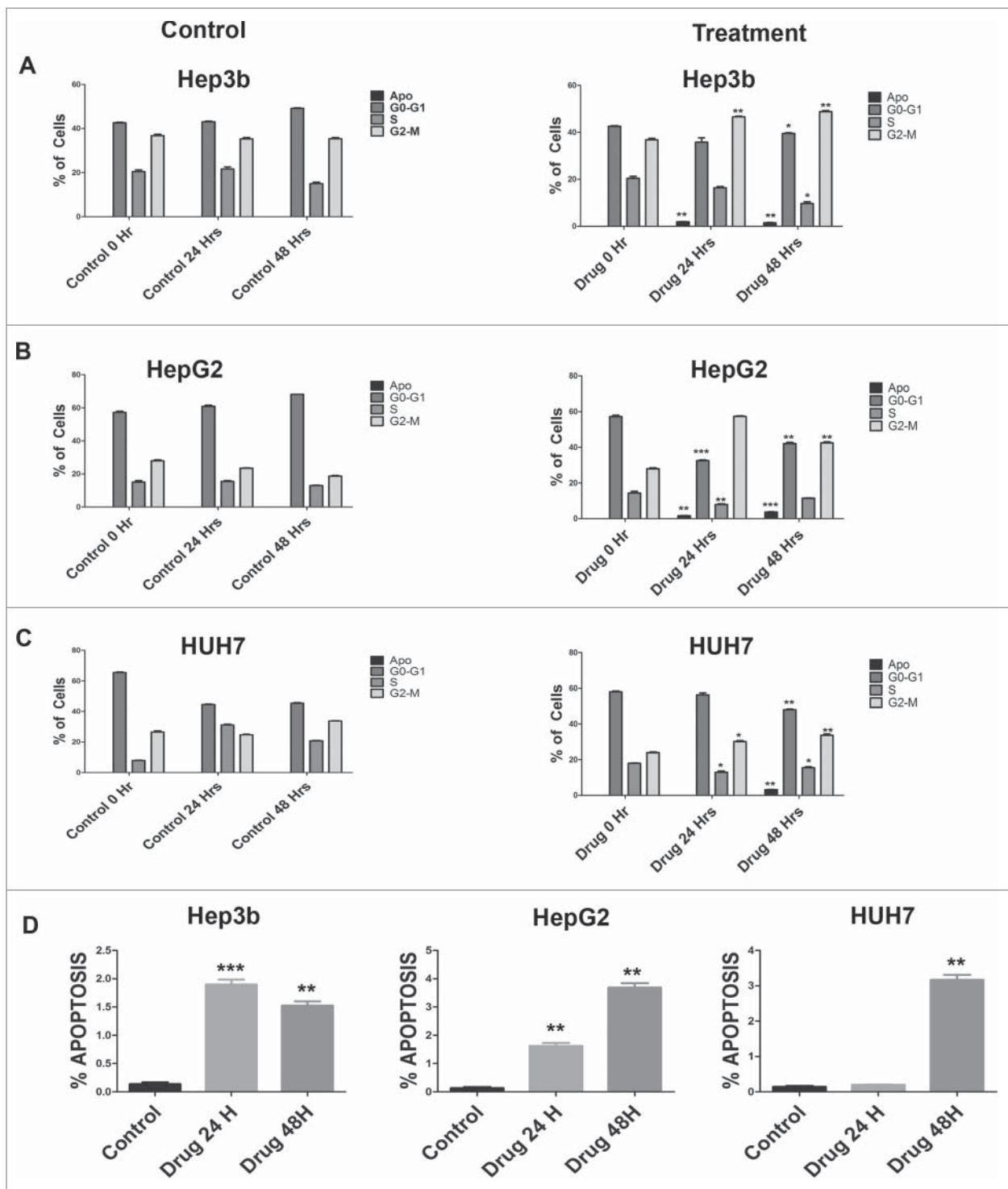
Treatment of HCC cell lines with ganetespib caused an increase of sub-G1 population of cells indicative of apoptosis. Hep3b, HepG2 and HUH7 cells showed a 1.365% (SD 0.035), 3.687% (SD 0.27) and 3.16% (SD 0.252) increase in cells sub-G1 cells by 48 hours, respectively (Fig. 2D). We confirmed the induction of apoptosis by ganetespib as shown by Western blotting showing increased cleaved caspase 3 (Fig. S2). These data suggest that ganetespib anti-cancer effects in HCC cell lines may be partially from induction of apoptosis.

### **Ganetespib delays repair of radiation induced double stranded breaks in HCC cells**

To further explore mechanisms of ganetespib mediated radiosensitization of HCC cells, we examined dynamics of DNA double strand break repair (DSBR) in HCC treated cells. HCC cells were treated with DMSO control or with 20 nM of ganetespib, 24 hours post drug treatment the cells were subjected to 4 Gy of radiation and assessed for  $\gamma$ H2AX foci formation indicative of DNA double strand breaks (DSBs). Hep3b (Figs. 3A and 3B), HepG2 (Fig. 3C and Fig. S3) and HUH7 (Fig. 3D and Figs. S3) control and ganetespib alone treated cells show very low basal levels of DSBs. Radiation alone and radiation-



**Figure 1.** GanetespiB radiosensitizes hepatocellular carcinoma cell lines *in vitro*. (A) Cells were exposed to 24 hours of ganetespiB at the specified concentration before protein extraction. Representative Western blotting showed a down-regulation of phospho-S6 and phospho-Erk1/2, but an upregulation of Hsp72. Total Erk1/2 levels remained constant throughout the treatment dosages. Clonogenic survival assay show the anticancer activity of ganetespiB used as a single agent in (B) Hep3b, (C) HepG2, and (D) HUH7. Radiation clonogenic survival assays for (E) Hep3b, (F) HepG2, and (G) HUH7 demonstrated 5 nM ganetespiB-induced radiosensitization in all 3 cell lines with enhancement ratios of 1.78, 1.33, and 1.47, respectively. All experiments were done in triplicate; error bars indicate SD and repeated at least twice. The \* -  $p < 0.05$ , \*\* -  $p < 0.01$  and \*\*\* -  $p < 0.001$ .

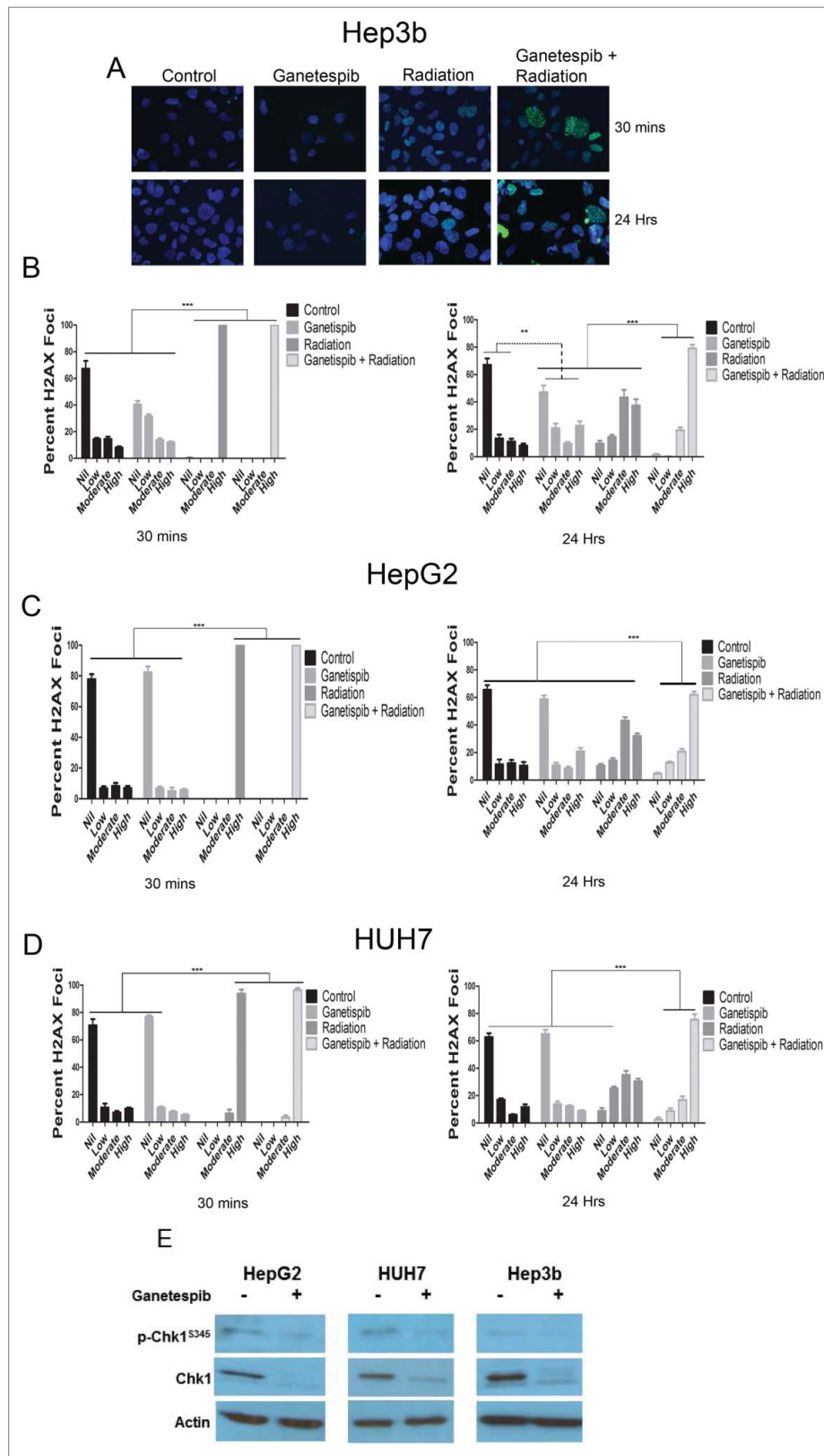


**Figure 2.** Cell cycle perturbations induced by ganetespib on HCC cells *in vitro*. Synchronized cells were exposed to vehicle control or 20 nM ganetespib for 0, 24, or 48 hours and then fixed with ethanol for cell cycle analysis with propidium iodide. Treatment with ganetespib caused a G<sub>2</sub>-M arrest in all cell lines as can be seen by the increase in percentage of cells in the G<sub>2</sub>-M phase at 24 and 48 hours when compared to vehicle controls for (A) Hep3b, (B) HepG2, and (C) HUH7 cell lines. Percent of cells in G<sub>1</sub>, S, G<sub>2</sub> and G<sub>2</sub>-M phases is plotted for the control and ganetespib arms. (D) The sub-G<sub>1</sub> population of cells as shown by flow cytometry of HCC cells treated with ganetespib (20 nM) or vehicle control for Hep3b, HepG2 and HUH7 cell lines. All experiments were done in triplicate and repeated at least twice. Error bars indicate SD. The \* -  $p < 0.05$ , \*\* -  $p < 0.01$  and \*\*\* -  $p < 0.001$ .

ganetespib treated cells as expected showed cells with high numbers of  $\gamma$ H2AX foci at 30 minutes ( $p < 0.001$ , Fisher's exact test). At 24 hours following treatment,  $\gamma$ H2AX foci were significantly reduced in all 3 HCC cell lines in the radiation alone arm (Fig. 3B-D), while cells treated with radiation-

ganetespib still showed cells with high levels of  $\gamma$ H2AX foci ( $p < 0.001$  for Hep3b and HUH7 and  $p = 0.0002$  for HepG2, Fisher's exact test).

Probing for DNA damage response (DDR) machinery in HCC cell lines by western blotting showed that

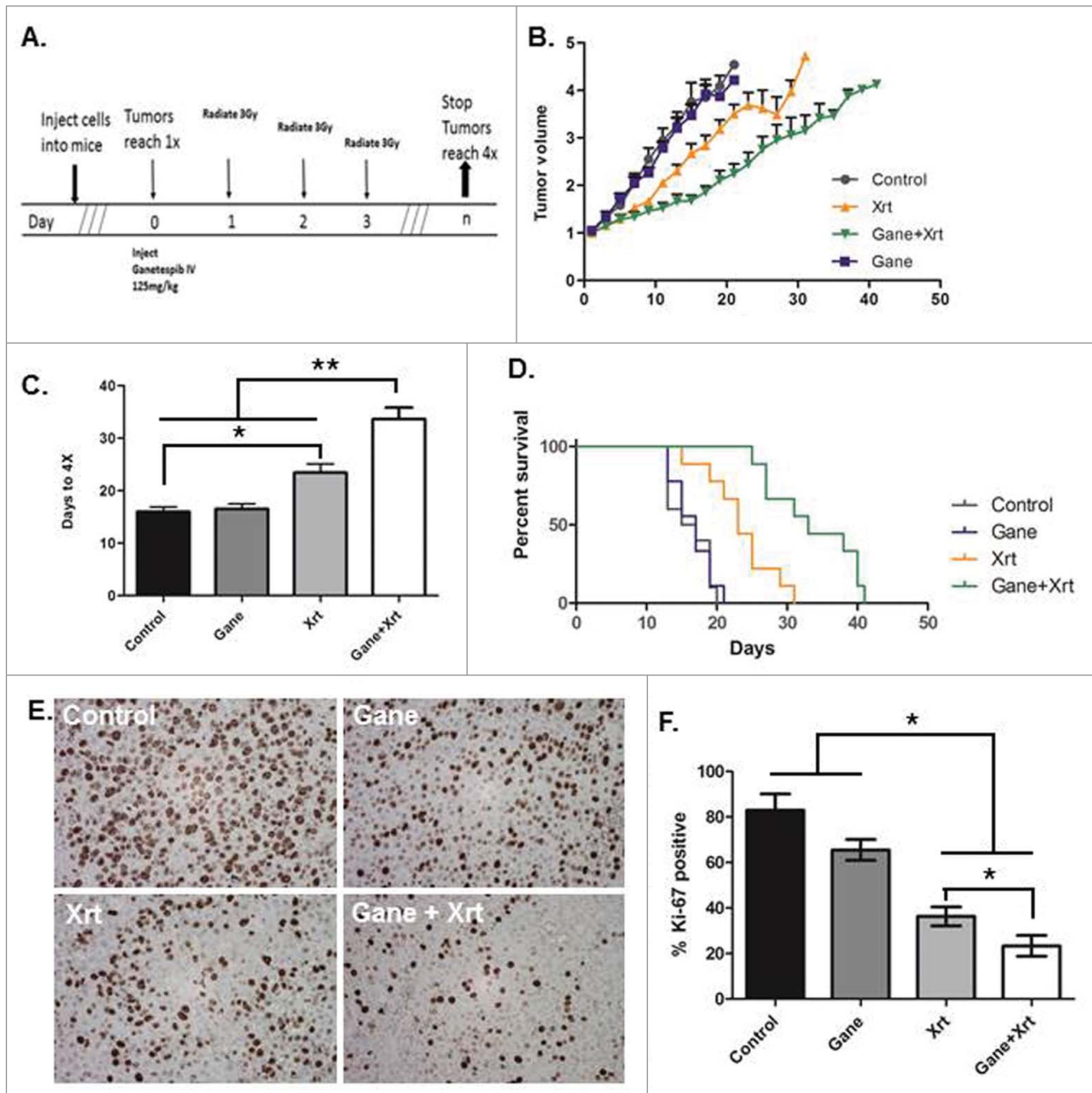


**Figure 3.** Ganetespib delays the repair of radiation-induced double strand breaks and downregulates the double strand break repair protein Chk1 in HCC cells. Immunofluorescence (IF) for  $\gamma$ H2AX foci counterstained with DAPI and images captured using a fluorescent microscope. (A) Representative images are shown for Hep3B cell line at 30 minutes and 24 hours for each of the treatment arms; 20 nM ganetespib and/or 4 Gy of radiation when used. (B-D) Quantification of the percent of nuclei demonstrating no, low (<10 foci), moderate (10–25 foci) or high (>25)  $\gamma$ -H2AX foci per nuclei was quantified for all cell lines for each of the treatment arms and depicted graphically with standard error of the mean (SEM). For all cell lines, radiation alone and ganetespib-radiation resulted in a greater percentage of nuclei with a high number of  $\gamma$ -H2AX foci at 30 minutes when compared to any other treatment arm ( $p < 0.001$ , Fisher’s exact test). At 24 hours, the ganetespib-radiation arm maintained a larger percentage of high  $\gamma$ -H2AX foci when compared to radiation alone ( $p < 0.001$  for Hep3b and HUH7 and  $p = 0.0002$  for HepG2, Fischer’s exact test). (E) Cells were exposed to 24 hours of 50 nM ganetespib followed by an additional 24 hours prior to protein extraction and Western blotting for DNA damage response regulator p-Chk1-Ser345 and total Chk1 in all 3 HCC cell lines.

components and/or activity levels of the DDR, ATM, Chk1 and Wee1, were downregulated by ganetespi treatment. Phospho-Chk1-Ser345 and total Chk1 levels were downregulated in the presence of 50 nM ganetespi in all 3 HCC cell lines (Fig. 3E). In addition we observed total ATM and total Wee1 which are upstream and downstream DDR components, respectively, from Chk1 were similarly downregulated with 50 nM ganetespi treatment (Fig. S4). These results indicate that ganetespi radiosensitization of HCC cells may be explained by the prevention of repair of radiation-induced DSBs by inhibition of DDR client machinery.

### Combined treatment with ganetespi and radiation delayed tumor growth *in vivo*

To assess the effects of ganetespi and fractionated radiation *in vivo*, HepG2 cells were implanted in the hind flanks of female nude mice with the treatment scheme shown in Fig. 4A. Combined radiation-ganetespi treatment delayed tumor volume growth (Fig. 4B) and quadrupling time compared with the single-treatment and control arms (Fig. 4C). The mean tumor quadrupling times were  $16 \pm 0.98$  d for no treatment,  $16.56 \pm 0.92$  d for ganetespi alone,  $23.44 \pm 1.63$  d for radiation alone and  $33.67 \pm 2.173$  d for the combined radiation-ganetespi



**Figure 4.** Ganetespi radiosensitizes HepG2 HCC cells and delays tumor growth *in vivo* in a supra-additive manner. A HepG2 hind-flank tumor growth delay model was used to assay (1) no treatment (Control), (2) ganetespi only (G or Gane), (3) fractionated radiation 3 Gy x 3 consecutive days (XRT), and (4) ganetespi and radiation (Gane-Xrt). (A) Treatment schema. The results were analyzed using (B) fold tumor volume change over time, (C) time to tumor quadrupling, and (D) Kaplan-Meier survival analysis where the event was considered time to tumor quadrupling. Ganetespi-radiation resulted in greater than additive tumor growth delay: ganetespi-radiation = 17.67 d > ganetespi alone = 0.56 d + radiation alone = 7.44 d. (E) Tumor samples were taken and analyzed by Ki67 IHC with representative images shown. (F) Right panel bar graph shows quantification of the percent Ki67 was significantly reduced in the combined ganetespi-radiation treatment arm compared to the other arms ( $p < 0.05$  by t-test for pairwise comparisons of ganetespi-radiation *versus* all other arms). The \* -  $p < 0.05$ , and \*\* -  $p < 0.005$ .

treatment arm (Fig. 4C,  $p < 0.005$  for radiation-ganetespib vs. any other arm by Mann-Whitney U test). HepG2 tumors treated with radiation-ganetespib required on average 17.67 d more to quadruple compared with untreated tumors. Interestingly, treatment with a single dose of ganetespib alone did not alter tumor progression, however, combined radiation-ganetespib exhibited greater than additive tumor growth delay of HepG2 tumors than seen with radiation alone (radiation-ganetespib 17.67 d > ganetespib 0.56 d + radiation 7.44 days). Similarly, using a Kaplan-Meier analysis with an event defined as time to tumor quadrupling, radiation-ganetespib resulted in significantly longer median time to quadrupling than the radiation alone treatment arm (Fig. 4D,  $p < 0.01$ , log-rank test). No observable differences in normal tissue toxicity, such as weight loss, diarrhea, dermatitis and ulceration, were noted between the combined radiation-ganetespib arm and either of the single-treatment arms (data not shown). Examining the xenograft tumors for proliferation by Ki67 IHC staining (Fig. 4E), the proliferative index was substantially decreased in radiation treated tumors (Fig. 4F, 46.37%,  $p = 0.0026$  by Student's t-test) as compared to the DMSO control treated tumors (83%). Although a single dose of ganetespib treatment alone did not significantly alter the proliferative index of HepG2 cells *in vivo* (Fig. 4F, 65.53%,  $p = 0.09$  by Student's t-test), combined radiation-ganetespib treatment showed the least proliferative index of all treatments (Fig. 4F, 32.34%,  $p < 0.05$  by Student's t-test for all arms). In summary, ganetespib demonstrated potent radiosensitization as measured by tumor growth delay of HCC cells *in vivo*.

## Discussion

Herein we have investigated the anti-tumor and radiosensitizing activity of ganetespib, a second generation HSP90 inhibitor, for liver cancer therapy. Ganetespib was able to reduce activity of multiple client signal transduction proteins believed to be important for liver cancer cell survival and proliferation, ERK1/2 and S6 proteins, in a dose dependent manner. We found that ganetespib treatment alone reduced clonogenic survival in all 3 HCC cell lines tested and further was able to radiosensitize these HCC cells *in vitro*. Treatment with ganetespib also resulted in increased apoptosis in HCC cells. Potential mechanisms for radiosensitization by ganetespib we observed were perturbations of the cell cycle resulting in increased numbers of cells in a G2-M arrest and decreased levels of the DSBR response protein CHK1, both of these findings were correlated with an increased number of unrepaired DSBs following irradiation. Finally, we showed that ganetespib was able to radiosensitize HCC cells to fractionated radiation *in vivo* using a tumor xenograft model.

HCC cells exhibits aberrant over expression and dysregulation of key cellular signaling molecules,<sup>18,19</sup> exemplified by activation of the MAPK pathway<sup>20,21</sup> and PI3K/AKT/mTOR pathway in 30–50% of cases.<sup>22</sup> Sorafenib, a multi-kinase inhibitor is the only current effective systemic treatment for advanced HCC, but only has a modest durable response in most patients.<sup>23–25</sup> HSP90 is known to be overexpressed in HCC cancers<sup>26,27</sup> and thus, inhibition of HSP90 for treatment of HCC is a logical strategy as many oncoproteins important for cancer

cell survival pathways are stabilized by HSP90. In addition, HSP90 inhibitors can target non-oncogene-dependent processes or stress response machinery that are also equally critical to the survival of HCC cells.

Ganetespib has already shown single agent pre-clinical activity against other cancers of the lung, prostate, colon and breast, as well as melanoma and leukemia cells *in vitro* and in some cases *in vivo*.<sup>28–30</sup> A number of phase II and phase III clinical trials employing ganetespib are underway currently in a variety of non-HCC tumor histologies showing reasonable response rates.<sup>14</sup> A recent phase I trial of ganetespib in advanced HCC patients showed a manageable toxicity profile even in patients with mild liver dysfunction.<sup>8</sup> The radiosensitizing potential of ganetespib has also been recently demonstrated with lung cancer,<sup>31</sup> stomach cancer<sup>32</sup> and oropharyngeal squamous cell carcinoma.<sup>33</sup>

An important feature of HCC is local control of the tumor that frequently coexists with liver cirrhosis. This need has driven development of non-surgical options such as refinements in radiation therapy for HCC. However, HCC is relatively resistant to conventionally fractionated radiation<sup>9</sup> and is limited by normal liver tissue toxicity. Thus, novel means to dose escalate to liver cancer cells selectively is an important area of research and positions ganetespib as an ideal candidate. First generation HSP90 inhibitors including geldanamycin analogs 17-AAG and 17-DMAG have been shown to radiosensitize a variety of tumor cells, but are plagued with prohibitive pharmacological and toxicity limitations.<sup>34–37</sup> Second generation HSP90 inhibitors overcome these limitations and show similar potent radiosensitizing activity.<sup>11</sup> Proposed mechanisms of HSP90 inhibitor-induced radiosensitization include inhibition of DSBR, as Chk1 and Rad51 are established HSP90 client proteins.<sup>38</sup> Other non-mutually exclusive mechanisms include redistribution of cells into more radiosensitive phases of the cell cycle such as M-phase which appears to be a common effect of second generation HSP90 inhibitors. The effect of cell cycle distribution on radiosensitization is important in liver cancer cells as shown by concurrent *versus* sequential treatment of radiation with sorafenib.<sup>39</sup>

In summary we show that ganetespib inhibits HSP90 in a dose dependent manner leading to anti-cancer activity and potent radiosensitization of HCC cell lines by affecting key cellular signaling pathways, cell cycle arrest into more radiosensitive phases of the cell cycle, and inhibiting DNA repair mechanisms. These findings suggest ganetespib, when combined strategically with radiation, offers promise for liver cancer treatment and should be tested in clinical trials.

## Materials and methods

### Cell lines

Three human HCC cell lines, HepG2 (wild-type p53), HUH7 (Y220C-mutated p53; p21 deficient), and Hep3b (p53 and pRb deficient; hepatitis B virus positive) were used. Cells were grown and maintained in DMEM medium supplemented with 10% FBS

and 1% penicillin-streptomycin. All cells will be incubated at 37°C in humidified 5% CO<sub>2</sub>. Cells were sub-cultured at 70–80% confluence and all experiments were carried out with the cells in an exponential growth phase. All cell lines were checked by short tandem repeat profiling and mycoplasma testing services of the Johns Hopkins Medicine Genetic Resources Core Facility.

### Drug treatment

Ganetespib was obtained from Synta Pharmaceuticals Corp. Ganetespib (STA-9090) was dissolved in DMSO and stored at –20°C in 1 mM aliquots for the *in vitro* studies. For our *in vivo* experiments, the drug (dissolved in DMSO) was formulated in 20% Cremophor RH40 and 80% dextrose (5%) water and injected at 125 mg/kg by tail vein injection.

### Radiation

For *in vitro* experiments, cells were irradiated with 0, 2, 4, or 6 Gy at room temperature using GammaCell irradiator with a <sup>137</sup>Cs source at a dose rate of 50 cGy/min. For *in vivo* experiments, mice were treated using the Small Animal Radiation Research Platform (SARRP).<sup>40</sup> The tumors were irradiated with a square beam of 1-cm × 1-cm with 3 consecutive daily fractions of 3 Gy.

### Clonogenics assay

Cells in exponential growth phase were counted and plated in 10 cm dishes. Depending on the cell type, drug concentration, and radiation dose, 300–15,000 cells were plated. Ganetespib was added to the medium 24 hours after plating and radiation was delivered 24 hours after drug treatment. The drug was removed 24 hours post radiation by adding fresh growth medium. Colonies were stained and counted 10–14 d after irradiation by fixing with 0.1% Gentian Violet dissolved in a mixture of methanol and DI water in a 1:1 ratio. Colonies were counted under an inverted phase contrast microscope (Nikon Instruments Inc., Melville, NY) with a colony defined as comprising of at least 50 cells. Survival fraction was calculated as a function of plating efficiency. All arms were done in triplicate and repeated at least 3 times to ensure reproducibility.

### Cell cycle analysis

For experiments with synchronized cells, 100,000–300,000 cells were seeded per well in 6-well plates allowed to attach in normal growth media for 24 hours, serum starved for 48 hours (0% serum), then grown in the presence of 10% serum and aphidicolin (2 μg/mL) for 24 hours before being released into normal growth medium (10% serum, without aphidicolin) containing ganetespib (20 nM). At various time points after adding ganetespib, cells were detached, washed with phosphate-buffered saline (PBS), and fixed with chilled 70% ethanol. Cells were pelleted and washed in PBS+1% BSA, then treated with 20 μg/mL RNase-A with 10 μg/mL propidium iodide for 2 hours. DNA content was analyzed with FACSCalibur (BD Biosciences, Franklin Lakes, NJ) and FlowJo analysis software (Tree Star, Ashland, OR).

### Immunoblot analysis

Cells were plated into 10 cm dishes and grown to sub confluence. Ganetespib (1–100 nM) was added to the medium 24 hours after plating. Cells were harvested, homogenized 24 hours later and 50 μg of total protein was loaded into each well of an 8–12% polyacrylamide gel and separated. Protein was transferred onto a polyvinylidene fluoride (BioRad) blotting membrane and blocked for an hour using 5% BSA or milk in TBST (tris-buffered saline supplemented with 0.1% Tween-20). Phospho-antibodies were incubated with 5% BSA in TBST, other antibodies were incubated with 5% milk in TBST. The membranes were probed with antibodies for phospho-S6 (Cell Signaling), ATM (Millipore), Wee1 (Cell Signaling), caspase 3 (Cell Signaling), phospho-Chk1-Ser345 (Santa Cruz), Chk1 (Santa Cruz), Hsp72 (Selleck chemicals), p42/44 (Cell Signaling), phospho-p42/p44 (Cell Signaling), and subsequently with horseradish peroxidase-labeled mouse anti-rabbit secondary antibodies (Sigma-Aldrich). Each antibody incubation step was followed by 3–4 washes with TBST. The secondary antibody was then coupled with GE ECL Plus kit (GE Life Sciences) and protein levels were detected using autoradiography films (Denville Scientific, Inc.). Experiments were done at least twice.

### Immunofluorescence

Cells were plated on poly-L-lysine-coated (13.3 mg/ml) glass chamber slides/cover glass and incubated for 24 hours at 37°C in 5% CO<sub>2</sub> and fixed for 15 minutes with freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS, the cells were permeabilized for 15 minutes with PBST (PBS with 0.1% Triton X-100). The cells were then blocked with 2% FBS, 3% BSA (bovine serum albumin) in PBS for 30 minutes and incubated at room temperature for 1 hour with primary antibody (1:250) diluted in PBS. After washing with PBS, the cells were incubated with an Alexa Flour 488-conjugated secondary antibody (1:300, Molecular Probes, USA) for 1 hour at room temperature. Cells were washed in PBS and coverslips stained with DAPI prior to mounting. Fluorescent images were captured using a fluorescent confocal microscope. The cells were probed with primary antibodies for γH2AX (Cell Signaling).

### Mouse tumor graft models and tumor growth delay experiments

Female athymic nude mice (Harlan, Indianapolis, IN) were maintained under pathogen-free conditions and given food/water *ad libitum* in accordance with Johns Hopkins Animal Care and Use Committee guidelines. Mice were injected subcutaneously in both flanks with 3 × 10<sup>6</sup> HepG2 cells in 100 μL of Hank's balanced salt solution and Matrigel (Invitrogen) mixed 1:1. Once tumors reached 100 mm<sup>3</sup>, 5–6 mice were randomly assigned to each of the 4 treatment arms: (1) no treatment; (2) ganetespib only; (3) radiation only and (4) ganetespib + radiation. Mice in ganetespib treated arms were given a single dose of 125 mg/kg, intravenously through the tail vein on the first day of treatment. The radiation arms were irradiated according to a fractionated scheme wherein, the mice were radiated for 3



consecutive days with a 3 Gy focal beam. Mice allocated to the combination arm were also subjected to the same fractionated radiation scheme as the radiation alone arm, with the first fraction of radiation being delivered 6 hours post drug treatment. The tumors were measured 3 times a week, until the tumors reached 4 times (4x) their pre-treatment volume. Tumor volume was calculated using the formula: length  $\times$  width  $\times$  height  $\times \pi / 6$ . All experiments repeated twice.

### Immunohistochemistry

Mice with established flank tumors from each treatment arm (1)-(4) above were sacrificed; for arms (3) and (4) in which radiation was a component of therapy, mice were sacrificed 1 hour after the first radiation delivery. Tumors were harvested, fixed in 10% formalin for 3 days, then transferred to PBS, fixed in paraffin, and sectioned by the Johns Hopkins Tissue Core Facility. Sections were stained for Ki67 (Abcam) as previously described using immunohistochemistry (IHC).<sup>41</sup> The number of positively staining foci were counted and compared for at least 5 randomly chosen high power fields per tumor.

### Statistics

Error bars included in graphical figures represent standard deviation (SD) unless otherwise specified. Two-tailed unpaired Student's t-test was used to compare cell cycle analysis, and Ki67 immunohistochemistry results between treatment arms. Fisher's exact test was used to compare  $\gamma$ H2AX foci results between treatment arms. Clonogenic survival curves were fitted with a linear quadratic model using Microsoft Excel using a least squares fit, weighted to minimize the relative distances squared, and compared using the extra-sum of squares *F* test. Mean inactivation doses were determined using the method of Fertit<sup>42</sup> and enhancement ratios calculated as the ratio of the mean inactivation dose for control vs. drug-treated arms as described by Morgan.<sup>43</sup> A value significantly  $>1$  indicates radiosensitization. Tumor growth delay assay results were compared by 2 distinct methods: (1) by using the log-rank test to compare median quadrupling times after creating a Kaplan-Meier plot using quadrupling in volume as the event of interest; and (2) by comparing quadrupling times between all tumors in 2 arms using the Mann-Whitney U test. A *p*-value of  $\leq 0.01$  for 2-sided tests was considered significant.

### Disclosure of potential conflicts of interest

David Proia is an employee of Synta Pharmaceuticals Corp makers of ganetespib. The remaining authors declare that they have no competing interests.

### Contributions

SC, RM and AA participated in the design of the study, performed all the experiments, analyzed the data and drafted the manuscript. MB and SM helped with the Western blotting and clonogenic survival experiments. HW, JC and KT participated in the cell cycle and immunofluorescence studies. KM and YK assisted in the xenograft animal experiments. ZB, RA, DP, ML and JH helped draft the manuscript. PT conceived of the study,

participated in its design coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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