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PARP1 modulates the lethality of CHK1 inhibitors in mammary tumors.

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Abbreviations: ERK: extracellular regulated kinase; MEK: mitogen activated extracellular regulated kinase; ca: constitutively active; dn: dominant negative; EGFR: epidermal growth factor receptor; CMV: empty vector plasmid or virus; si: small interfering; SCR: scrambled; IP: immunoprecipitation; Ad: adenovirus; TUNEL: Terminal deoxynucleotidyltransferased UTP nick end labeling; ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia and Rad3-related; Chk1 and Chk2: checkpoint kinases 1 and 2; VEH: vehicle; SSBs: single strand DNA breaks; DSBs: double strand DNA breaks; CDKs: cyclin-dependent kinases; UCN-01: 7-hydroxystaurosporine; PARP: Poly(ADP-ribose) polymerase; BER: base excision repair; BRCA1: Breast cancer type 1 susceptibility protein; FA: Fanconi anemia; XRCC3: X-ray repair cross complementing protein 3.

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Abstract.

The present studies sought to define whether CHK1 inhibitors and PARP1 inhibitors interact in vitro and in vivo to kill breast cancer cells. PARP1 and CHK1 inhibitors interacted to kill ER+; ER+ fulvestrant resistant; HER2+; or triple negative mammary carcinoma cells in a manner that was not apparently impacted by PTEN functional status. Expression of dominant negative CHK1 enhanced, and over-expression of wild type CHK1 suppressed, the toxicity of PARP1 inhibitors in a dose dependent fashion. Knock down of PARP1 enhanced the lethality of CHK1 inhibitors in a dose dependent fashion. PARP1 and CHK1 inhibitors interacted in vivo both to suppress the growth of large established tumors and to suppress the growth of smaller developing tumors; the combination enhanced animal survival. PARP1 and CHK1 inhibitors profoundly radiosensitized cells in vitro and in vivo. In conclusion, our data demonstrates that the combination of PARP1 and CHK1 inhibitors has anti-tumor activity in vivo against multiple mammary tumor types and that translation of this approach could prove a useful anti-cancer therapeutic approach.

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Introduction.

DNA damage leads to the activation of checkpoint responses that result in cell cycle arrest or apoptosis. DNA damage checkpoints are a mechanism that retards cell cycle progress and ensures that false genetic information does not pass to daughter cells before the damage is completely repaired. The checkpoint responses are orchestrated by signal transduction cascades, primarily the ATR–CHK1 and ATM–CHK2 pathways (Lee and Paull, 2007; Fernandez-Capetillo et al, 2004; Lukas et al, 2003). Upon phosphorylation and activation by ATR, CHK1 phosphorylates downstream targets that regulate DNA repair and cell cycle progression, such as the protein phosphatase CDC25A. Phosphorylation of CDC25A and CDC25C by CHK1 can result in their degradation and therefore prevent them from dephosphorylating and activating the CDKs that drive cell cycle progression (Sancar et al, 2004; Eymin et al, 2006). Of note, CHK1 and CHK2 share several downstream substrates such as CDC25A/C and p53 for cell cycle control and apoptosis regulation, which potentially suggests their redundant roles in damage response (Bartek and Lukas, 2003). However, several lines of evidence also argue that CHK1 instead of CHK2 plays an essential role in regulating S- and G2-checkpoints in response to double-strand DNA breaks and CHK1 presents as a promising anti-cancer therapeutic target (Carrassa et al, 2004; Cho et al, 2005; Morgan et al, 2006; Carlessi et al, 2007; Zhao et al, 2002).

Multiple CHK1 inhibitors are currently being evaluated as anti-neoplastic agents in clinical trials, both alone and in combination with radiotherapy and chemotherapeutic agents that induce DNA damage (Morgan et al, 2007; Mow et al, 2001; Prudhomme, 2006). These agents were proposed to enhance the toxicity of chemotherapeutic drugs by inhibition of CHK1 with subsequent inappropriate cell cycle progression after DNA damage (Graves et al, 2000). Inhibition of CHK1 may directly promote activation of the protein phosphatase CDC25C and can also interfere with CDC25C elimination by blocking its binding to 14-3-3 proteins and subsequent degradation (Graves et al, 2000; Peng et al, 1997). The CHK1 inhibitor UCN-01 is known to have many additional intracellular kinase targets including the downstream effector of PI3 kinase, PDK-1, as well as “classic” PKC isoforms (Komander et al, 2003).

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We have noted in a variety of tumor cell types that the CHK1 inhibitor UCN-01, and more recently the CHK1 inhibitor AZD7762, activates the ERK1/2 pathway, and that pharmacologic or genetic inhibition of the ERK1/2 pathway potentiates apoptosis and suppresses tumor growth in vivo (Dai et al, 2001; Dai et al, 2002; Hamed et al, 2008; Dai et al, 2008; Mitchell et al, 2010). We also noted that UCN-01, in addition to activating ERK1/2, promotes increased phosphorylation of histone H2AX, indicative that DNA damage was occurring due to the inhibition of CHK1 function, and that inhibition of ERK1/2 further enhanced histone H2AX phosphorylation prior to induction of apoptosis (Dai et al, 2008). Thus CHK1 dependent regulation of ERK1/2 may play an important role DNA damage sensing and repair in multiple human cancer cells.

One central protein in the regulation of multiple forms of DNA repair processes is poly (ADP-ribose) polymerase (PARP), which is essential for repairing DNA damage through the base excision repair pathway (Rouleau et al, 2010). Among the PARP family, only PARP1 and PARP2 have been shown to be activated in response to DNA damage, with PARP-1 accounting for about 85-90% of the activity. PARP1 binds to damaged DNA where its catalytic activity is stimulated and that catalyzes the synthesis of branched, protein-conjugated poly ADP-ribose to itself and other acceptor proteins involved in base excision repair and those in modulating chromatin structure (Ame et al, 1999; Schreiber et al, 2002). Multiple PARP1 inhibitors have been developed including GPI15427, CEP6800, ABT-888, NU1025 and AZD2281 (Graziani and Szabo, 2005). Inhibitors that block PARP1 -mediated ADP ribosylation synergize with conventional genotoxic chemotherapies, including topoisomerase I inhibitors, ionizing radiation (IR), and DNA alkylating agents (Bowman et al, 2001; Ben-Hur et al, 1985; Arundel-Suto et al, 1991; Weltin et al, 1997; Boulton et al, 1995; Bowman et al, 1998; Delaney et al, 2000; Tentori et al, 2002). PARP inhibitors have also shown single-agent activity against tumors deficient in homologous recombination repair, such as BRCA1/2-mutant cells (Bryant et al, 2005; Farmer et al, 2005; Martin et al, 2008; Tutt et al, 2008).

The present studies extended our analyses and determined whether this drug combination has anti-tumor effects in vivo. Our findings demonstrate that the combination of PARP1 and CHK1 inhibitors has anti-tumor activity in vivo against multiple mammary tumor types and can enhance tumor control following radiotherapy.

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Materials and Methods.

Materials.

Human breast cancer cell lines, BT474 (PTEN wt), BT549 (PTEN mut), HCC38 (PTEN mut), HCC1187 (PTEN wt), HCC1954 (PTEN, wt) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were not further validated. Parental and Fulvestrant resistant MCF7 cells were a kind gift from Dr. K. Nephew (Univ. Indiana, Bloomington) (see Fan et al, 2006). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Antibiotics-Antimycotics (100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin B) and Trypsin-EDTA were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). TUNEL kits were purchased from NEN Life Science Products (NEN Life Science Products, Boston, MA). All the primary antibodies used in the present study were purchase from Cell Signaling Technologies (Worcester, MA). The validated siRNA molecules used to knock down PARP1 were from Ambion (Austin, TX): ref# S1098 (4390824); S1099 (4390825); Silencer Negative Control #1 (4390843) Silencer Negative Control #2 (4390846). siPORT™ NeoFX™ transfection agent was purchased from Ambion, Inc (Austin, TX). Lipofectamine 2000 transfection reagent was purchased Invitrogen Life Technologies, Inc. (Carlsbad, CA). The CHK1 inhibitor AZD7762 and the PARP1 inhibitors AZD2281 and ABT-888 were purchased from Axon Medchem (Groningen, Netherlands). The CHK1 inhibitor UCN-01 was purchased from Sigma (St. Louis MO).

Methods.

Culture and in vitro exposure of cells to drugs.

All breast cancer cells were maintained in a RPMI medium supplemented with 10% FBS and 1% antibiotic-antimycotic in a humidified incubator under an atmosphere containing 5% CO2 at 37 °C. In vitro Vehicle / UCN-01 / AZD7762 / AZD2281 treatment was from a 10 mM stock solution of each drug and the maximal concentration of Vehicle (DMSO) in media was 0.02% (v/v).

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Cell treatments, SDS-PAGE and Western blot analysis.

For in vitro analyses of short-term apoptosis effects, cells were treated with Vehicle / drugs or their combination for the indicated times. Cells were isolated at the indicated times in the Figure by trypsinization. Cell viability was measured with Vi-CELL™ Series Cell Viability Analyzers (Beckman Coulter, Inc.), which is based on the traditional cell viability method of trypan blue exclusion. Cells for colony formation assays were plated at 250-4000 cells per well in sextupilcate and for *in vitro* assays 14 hours after plating were treated with the individual or the drug combination(s), for the indicated time followed by drug removal. Ten-14 days after exposure or tumor isolation, plates were washed in PBS, fixed with methanol and stained with a filtered solution of crystal violet (5% w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount™ plate reader (Oxford Optronics, Oxford, England). Data presented is the arithmetic mean (± SEM) from both counting methods from multiple studies. Colony formation was defined as a colony of 50 cells or greater.

For SDS PAGE and immunoblotting, cells were plated at 5 x 10^5 cells/cm and treated with therapeutic drugs at the indicated concentrations and after the indicated time of treatment, lysed with whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue) in the presence of a protease inhibitor cocktail (Sigma, S8830), and the samples were sonicated and boiled for 5 min. The boiled samples were loaded onto 10–14% SDS-PAGE and were fractionated by SDS-PAGE gels in a Bio-Rad Protean II system. After transferring proteins to the Immobilon-FL PVDF membrane, the membrane was blocked with Odyssey Blocking Buffer from LI-COR Biosciences for 60 min at room temperature and incubated with the primary antibody at appropriate dilutions in Odyssey Blocking Buffer at 4°C overnight. After overnight incubation with appropriate primary antibodies, the membrane was washed (3x) with TBS-T for a total of 15 min, probed with fluorescently-labeled secondary antibody (1:5000) for 80 min at room temperature and washed (3x) with TBS-T for a total of 15 min. The immunoblots were visualized by an Odyssey Infrared Imaging System (LI-COR Biosciences).

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siRNA and plasmid transfection in vitro.

siRNA transfection was performed with siPORT™ NeoFX™ Transfection Agent following the manufacture procedures. In brief, 10 nM of pre-validated siRNA was diluted into 50 μl serum free media. Based on the Manufacturer's instructions, an appropriate amount of iPORT™ NeoFX™ Transfection Agent was diluted into a separate vial containing serum free media. The two solutions were incubated separately at room temperature for 15 min, mixed together by pipetting up and down several times, and the mixture was added drop-wise to the target cells. 24 h after transfection, the transfection medium was replaced with complete medium and 12 hours later the cells are subjected to treatments. Plasmid transfection used similar procedures to those for siRNA but instead used Lipofectamine 2000 transfection reagent.

In vivo Exposure of Mammary Carcinoma Tumors to Drugs.

4- to 6-week-old athymic female NCr-nu/nu mice were obtained from The Jackson Laboratory. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care. For each mouse, a total of 5×10^6 BT474 or BT549 cells were injected s.c. into the 4^{th} mammary fat pad each mouse. For animal administration, AZD7762 and AZD2281 were first dissolved in DMSO and an equal volume of Cremophor (Sigma) was added. After mixing, a 1:10 dilution was made with sterile PBS. Animals were i.p. injected with AZD7762 (50 mg/kg or 12.5 mg/kg body mass), AZD2281 (50 mg/kg or 12.5 mg/kg body mass), or AZD7762+ AZD2281. Each animal in control group was given an i.p. injection of diluent alone in a volume equal to the amount given with the drug. The resulting palpable tumors were measured using a vernier caliper, and tumor volume was calculated using the formula: $(\text{Width})^2 \times \text{Length} \times 0.5$. Tumor growth was expressed as relative fold change in tumor volume, (Tx/ T0), where T is the mean tumor volume of all tumors at a particular time in days x and T0 was the mean tumor volume at day 0. At the end of experiments, animals were euthanized using CO_2. In our studies drug treatment(s) did not negatively impact upon animal body mass.

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Immunohistochemistry and Staining of Fixed Tumor Sections.

Tumors were removed using small scissors, forceps and a disposable scalpel. The collected tumor was placed in 5 mL Streck Tissue Fixative (Fisher Scientific) in a 50 mL conical tube for fixation. Fixed tumors were embedded in paraffin wax and 10 μm slices were obtained using a microtone. Tumor sections were deparafinized and rehydrated and antigen retrieval was done in a 10 mmol/L (w/v) sodium citrate/citric acid buffer (pH 6.7). Prepared sections were then blocked and subjected to immunohistochemistry as per the instructions of the manufacturer for each primary antibody (Ki67; CD31; cleaved caspase-3). The tissue sections were dehydrated, cleared, and mounted with cover slips using Permount.

Data analysis. Comparison of the effects between various in vitro drug treatments was performed following ANOVA using the Student's t test. Differences with a *p*-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points from multiple studies (± SEM). Statistical examination of in vivo animal survival data utilized log-rank statistical analyses between the different treatment groups. Experiments shown are the means of multiple individual points from multiple experiments (± SEM). Median dose-effect isobologram colony-formation analyses to determine synergism of drug interaction were performed according to the methods of Chou and Talalay using the CalcuSyn program for Windows (Biosoft, Cambridge, UK). Cells were treated with agents at an escalating fixed concentration drug dose. A combination index of <1.00 indicates synergy of interaction between the two drugs; a combination index of ~ 1.00 indicates an additive interaction; a combination index (CI) value of >1.00 indicates antagonism of action between the agents.

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Results.

Previously we have published that MEK1/2 inhibitors interact with CHK1 inhibitors in a synergistic manner to kill mammary tumor cells in vitro (Hamed et al, 2008; Dai et al, 2008; Mitchell et al, 2010). In prior studies we had also determined in ER+ and in HER2+ breast cancer cells that PARP1 and CHK1 inhibitors interacted to synergistically cause tumor cell death (Mitchell et al, 2010a). Multiple novel therapeutic options for patients whose tumors are either ER+ or HER2+ have been developed in the last 25 years, whereas patients with so called "triple negative" mammary tumors (lacking ER, PR and HER2) have yet to fully benefit from the more recent application of cell signaling based therapeutic options.

Treatment of triple negative mammary carcinoma cells with PARP1 inhibitors (AZD2281, ABT-888) and CHK1 inhibitors (UCN-01, AZD7762) caused a greater than additive induction of tumor cell killing (Figures 1A-1C) (Zabludoff et al, 2008; Donawho et al, 2007; Khan et al, 2011; Fong et al, 2009; Riches et al, 2008). Of particular note was that the cell death response of tumor cells lacking PTEN function (BT549, HCC38) was not significantly different to cells expressing wild type PTEN (HCC1187, HCC1954). CHK1 and PARP1 inhibitors synergized to kill BT549 cells as judged by combination index values of less than 1.00 (Table 1). Similar data were also observed in HCC1957 cells (data not shown). In estrogen-dependent mammary tumors one therapeutic option is to treat patients with the anti-estrogen Faslodex (Fulvestrant, ICI-182780); however, over time such cells become resistant to anti-estrogen therapy. PARP1 and CHK1 inhibitors were competent to kill both estrogen dependent and fulvestrant resistant MCF7 breast cancer cells with again, very little difference in killing between the cell types (Figure 1D). We next determined the mechanism(s) by which PARP1 and CHK1 inhibitors killed mammary tumor cells. Expression of dominant negative caspase 9 or BCL-XL, but not c-FLIP-s suppressed drug combination lethality, implying that death proceeded via the intrinsic apoptosis pathway rather than the extrinsic pathway (Figure 1E). Treatment of cells with CHK1 and PARP1 inhibitors activated JNK1/2 (Figure 1F). Incubation of cells with the JNK inhibitory peptide blocked JNK activation and suppressed drug combination toxicity.

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We next sought to further define the "on- and off-target effects" of the drugs in our system. BT474 (HER2+) and HCC38 (triple negative) cells were transfected with a vector control plasmid that expresses GFP or plasmids to express dominant negative CHK1-GFP or wild type CHK1-GFP. Treatment of vector control transfected BT474 or HCC38 cells with increasing concentrations of the PARP1 inhibitor AZD2281 resulted in a dose-dependent increase in cell death (Figures 2A and 2B). Treatment of dominant negative CHK1-GFP transfected BT474 or HCC38 cells with increasing concentrations of a PARP1 inhibitor caused a significantly greater amount of cell death than observed in parallel treatments in vector control cells. Expression of wild type CHK1-GFP suppressed PARP1 inhibitor lethality in BT474 and to a lesser extent in HCC38 cells. Transfection of dominant negative GFP-CHK1 blocked radiation (6 Gy) induced phosphorylation of CDC25C S216 (data not shown). In parallel to our CHK1 expression studies with PARP inhibitor treatment, we next assessed the impact of knocking down PARP1 expression on CHK1 inhibitor lethality. Knock down of PARP1 enhanced CHK1 inhibitor lethality in BT474 and in HCC38 cells (Figures 3A and 3B). Similar data for PARP1 knockdown were observed using a different siRNA molecule (Supplemental Figure 1).

A key question remained with respect to any new drug combination that kills tumor cells is whether the approach will translate from an in vitro cell culture setting into animal models of the disease. Multiple PARP1 and CHK1 inhibitors have / are being developed by drug companies. We established large (~250 mm^3) BT549 tumors in the 4^{th} mammary fat pad; this would represent the equivalent of a ~ 1 kg tumor in a patient. Tumors were permitted to form for 10 days and then animals were treated with vehicle, AZD2281, AZD7762 or the drug combination for an additional 5 days (Figure 4A). Tumors treated with either AZD2281 or AZD7762 exhibited modest declines in tumor growth rate compared to the vehicle control value whereas tumors exposed to both drugs showed a significant decline in their growth (Figure 4A). This occurred with no apparent loss in animal body mass (data not shown). Based on IACUC regulations, animals with tumor volumes above 1,500 mm^3 must be humanely sacrificed and for animals treated with vehicle control this resulted in a rapid decline in animal survival (Figure 4B). Animals treated with AZD2281 or AZD7762 exhibited a trend showing some increase in survival compared to vehicle control animals, however, animals treated with both AZD2281 and AZD7762 showed a significant

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increase in survival compared to any other group that was associated with the stabilization of tumor mass (Figures 4A and 4B). Similar tumor growth data to that in BT549 cells were obtained using smaller established (~75 mm^3) BT474 HER2+ tumors (Figure 4C). These effects correlated with disruption of tumor cyto-architecture and increased levels of apoptosis within the tumor as judged by elevated TUNEL+ levels and reduced Ki67 / DAPI staining (Figure 4D).

Radiotherapy is a standard of care treatment for primary diagnoses of breast cancer as well as palliative treatment of metastatic disease. CHK1 and PARP1 inhibitor treatment profoundly radiosensitized cells (Figures 5A and 5B). Expression of BCL-XL or dominant negative caspase 9, but not c-FLIP-s, blunted the radiosensitization effect (Figure 5A). We then determined the impact that expression of wild type and dominant negative CHK1 had on tumor cell radiosensitivity. Over-expression of wild type CHK1-GFP was radio-protective compared to GFP control and expression of dominant negative CHK1-GFP was radio-sensitizing compared to GFP control.

Finally, we determined in vivo whether [CHK1 inhibitor + PARP1 inhibitor] treatment, *using lower dose levels* of the CHK1 and PARP inhibitory drugs, radiosensitized tumors. Treatment of established BT549 tumors with lower doses of [CHK1 inhibitor + PARP1 inhibitor] modestly suppressed tumor growth (Figure 5B). Radiation exposure as a single agent also suppressed growth. The combination of [CHK1 inhibitor + PARP1 inhibitor] plus radiation almost abolished tumor growth and prolonged animal survival (Figures 5C and 5D, data not shown). Collectively our findings indicate that [CHK1 inhibitor + PARP1 inhibitor] treatment is effective at suppressing mammary tumor growth and at radiosensitizing mammary tumors.

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Discussion

Previous studies by this group have demonstrated that MEK1/2 inhibitors as well as PARP1 inhibitors interact with CHK1 inhibitors to promote tumor cell specific killing in a wide variety of malignancies including breast, prostate and multiple hematological cell types (Mitchell et al, 2010; Mitchell et al, 2010a). The output of the protective RAS - MEK1/2 - ERK1/2 pathway has previously been shown to be a critical determinant of tumor cell survival in many cell types (Riches et al, 2008). Activation of this cascade has been observed as a compensatory response of tumor cells to various environmental stresses, including cytotoxic drugs and ionizing radiation. The present studies were initiated to determine in further detail the molecular mechanisms by which PARP1 inhibitors interact with CHK1 inhibitors to promote breast cancer cell killing and to determine whether this drug combination can prove effective at controlling mammary tumor growth in vivo.

PARP1 and CHK1 inhibitors interacted in a synergistic fashion to kill mammary tumor cells. Expression of proteins that block the actions of the intrinsic apoptosis pathway (BCL-XL; dominant negative caspase 9) inhibited drug combination lethality. In contrast, expression of the caspase 8 inhibitor c-FLIP-s had not diminutive effect on cell killing arguing that the extrinsic pathway was not involved in drug combination lethality. Similar findings were made examining the ability of [PARP1 and CHK1 inhibitors] to radiosensitize cells; inhibition of the intrinsic pathway protected cells. Prior studies by our group noted in fibroblasts that loss of BAX and BAK also suppressed PARP and CHK inhibitor lethality. Collectively, these findings argue that our drug combination targets mitochondria for its generation of an apoptotic response.

Cells have a variety of conserved pathways to sense and overcome DNA damage and therefore preserve genomic integrity. Certain types of chemo-radiotherapy lead to DNA lesions and trigger checkpoint activation and consequent cell cycle arrest that permit DNA repair or apoptosis. An additional hallmark of the cellular DNA damage response is activation of PARP1 (Rodon et al, 2009). PARP1 activation results in ADP ribosylation of multiple DNA repair complex proteins, transcription factors as well as PARP1 itself. As a result of this effect on multiple repair proteins, loss of PARP1 function promotes genomic instability and leads to hyper-activation of

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CHK1 with increased cell numbers in G2 phase (Lu et al, 2006). This is also of interest because other groups have postulated the chemotherapy sensitizing effect of CHK1 inhibitors is due to abrogation of the G2 checkpoint (Prudhomme, 2006).

In our studies two chemically distinct CHK1 inhibitors (AZD7762 and UCN-01) rapidly promoted CHK1 and ERK1/2 phosphorylation. UCN-01 has undergone Phase I and II evaluation with poor PK/PD issues preventing full exploitation of the drug and AZD7762 was removed from clinical testing due to cardiac toxicity (Dent et al, 2011; http://www.clinicaltrials.gov/ct2/results?term=AZD7762). In prior studies we have noted that inhibition of CHK1 inhibitor-induced H2AX and ERK1/2 phosphorylation by PARP inhibition is likely explained by the requirement of ATM for PARP1 function, and vice versa (Golding et al, 2007). Previously we presented evidence that inhibition of CHK1–induced ERK1/2 activation further enhanced H2AX phosphorylation, indicative that loss of ERK1/2 signaling increased the amount of DNA damage being induced by the CHK1 inhibitor. This correlated with a subsequent profound induction of apoptosis.

In addition to interacting in a greater than additive fashion to kill breast cancer cells in vitro, the PARP1 inhibitor (AZD2281) and the CHK1 inhibitor (AZD7762) drug combination significantly inhibited the growth of tumors derived from either “triple negative” BT549 or “HER2+” BT474 cells in vivo. Of note BT549 and HCC38 cells also lack expression of PTEN, suggesting that loss of this tumor suppressor gene does not significantly impact on drug combination lethality. AZD2281 and AZD7762 drug combination treatment increased the survival of animals carrying BT549 derived tumors (as defined by a tumor mass of 1.5 cm^3 requiring animal sacrifice). The inhibition of tumor growth in mice treated with AZD2281 and AZD7762 was in parallel associated with reduced levels of the growth marker Ki67 and with elevated apoptosis as evidenced by increased TUNEL+ staining.

Radiotherapy is a mainstay of breast cancer therapy, and particularly of triple negative disease. Our present studies demonstrated that concomitant irradiation following drug treatment radiosensitized tumor cells in vitro. Expression of wild type CHK1 was protective compared to control vector whereas expression of dominant

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negative CHK1 caused further radio-sensitization. In vivo [AZD7762 + AZD2281] also promoted radiation toxicity using the PTEN null BT549 cell line. This data argues that the drug combination could be combined with established breast cancer therapeutic modalities in some of the most therapeutically resistant tumor types.

In conclusion, we have demonstrated that multiple PARP1 and CHK1 inhibitors interact to kill a diverse range of breast cancer cell types in vitro and in vivo. Further studies will be required to more fully define the pathway by which PARP1 and CHK1 inhibitors interact to cause tumor cell death.

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Authorship Contributions.

Participated in research design: TY, AP, YD, SG, PD

Conducted experiments: TY, HAH

Contributed new reagents or analytic tools: SG

Performed data analysis: TY, PD

Wrote or contributed to the writing of the manuscript: TY, PD

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Footnotes

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Figure Legends.

Figure 1. PARP1 and CHK1 inhibitors interact in a greater than additive fashion in killing triple negative and fulvestrant resistant mammary carcinoma cells. (A) BT549 and HCC1187 cells were treated with CHK1 inhibitor, UCN-01 (50 nM), PARP1 inhibitor, AZD2281 (1 μM) or UCN-01+AZD2281 for 24 or 48 hours. Floating and attached cells were isolated after drug exposure and cell viability was measured by trypan blue exclusion (±SEM, n = 3). **(B)** BT549 and HCC1187 cells were treated with CHK1 inhibitor, AZD7762 (50 nM), PARP1 inhibitor, ABT888 (1.0 μM), PARP1 inhibitor, AZD2281 (1 μM), AZD7762+AZD2281, or ABT888+AZD7762 for 48 hours. Floating and attached cells were isolated after drug exposure and cell viability was measured by trypan blue exclusion (±SEM, n = 3). **(C)** HCC38 and HCC1954 cells were treated with AZD7762 (50 nM), ABT888 (1.0 μM), AZD2281 (1 μM), AZD7762+AZD2281, or ABT888+AZD7762 for 48 hours. Floating and attached cells were isolated after drug exposure and cell viability was measured by trypan blue exclusion (±SEM, n = 3). **(D)** MCF7 and fulvestrant resistant MCF7 (MCF7F) cells were treated with AZD7762 (50 nM), AZD2281 (1 μM), AZD7762+AZD2281 for 48 h. Floating and attached cells were isolated after drug exposure and viability was measured by trypan blue exclusion (±SEM, n = 3). **(E)** MCF7 cells were infected with empty vector virus (CMV) or with viruses to express dominant negative caspase 9, BCL-XL or c-FLIP-s. Twenty four h after infection cells were treated with vehicle (DMSO) or with AZD7762 (50 nM) +AZD2281 (1 μM). Cells were fixed 48h later and viability determined using terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (TUNEL) assay (±SEM, n = 3). *Upper inset blot:* Cells were isolated 24h after drug exposure and immunoblotting performed to detect the levels of cleaved caspase 3 (n=2). **(F)** MCF7 cells were pre-treated for 30 min with vehicle (DMSO) or the JNK inhibitory peptide (10 μM, JNK-IP). After 30 min cells were treated with vehicle (DMSO) or with AZD7762 (50 nM) +AZD2281 (1 μM). Cells were fixed 48h later and viability determined by trypan blue exclusion (±SEM, n = 3). Upper inset blot: Cells were isolated 24h after drug exposure and immunoblotting performed to detect the levels of JNK1/2 phosphorylation (n=2).

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Figure 2. Dominant negative Chk1 increased the sensitivity of the cells toward PARP inhibitor treatment. (A) BT474 or **(B)** HCC38 cells were transfected with WT CHK-GFP, DN CHK1-GFP or GFP control vector. Twenty four hours after transfection, the cells were treated with the PARP1 inhibitor AZD2281 (0.3, 1, 3 μM) for 48 hours. Floating and attached cells were isolated after drug exposure and cell viability was measured by trypan blue exclusion (±SEM, n = 3) * $p < 0.05$ greater than corresponding vehicle control; # $p < 0.05$ less than corresponding value in GFP; $ $p < 0.05$ greater than corresponding value in GFP.

Figure 3. Knockdown of PARP1 sensitized breast cancer cells toward CHK1 inhibitors. (A) BT474 or **(B)** HCC38 cells were infected with siRNA control (siSCR) or a siRNA to knock down PARP1 (siPARP1). Thirty six hours after transfection, the cells were treated with vehicle (VEH, DMSO), UCN-01 (10, 30, 50 nM) or AZD7762 (10, 30, 50 nM) for 48 hours. Floating and attached cells were isolated after drug exposure and cell viability was measured by trypan blue exclusion (±SEM, n = 3) * $p < 0.05$ greater than corresponding value in siSCR cells. **Top inserts:** the knockdown of PARP1 was validated by western blot in BT474 and HCC38 cells.

Figure 4. PARP1 inhibitor interacted with CHK1 inhibitor in a greater than additive fashion in inhibiting the tumor grow and in enhancing the animal survival in vivo. (A) BT549 cells (5×10^6) were injected into the 4th mammary fat pad. Tumors were permitted to form to ~250 mm^3. Initial volumes of the tumor groups were: Vehicle 256 mm^3; AZD7762 241 mm^3; AZD2281 281 mm^3; AZD7762+AZD2281 245 mm^3. Animals were injected with vehicle, AZD7762 (50 mg/kg), AZD2281 (50 mg/kg), or AZD7762+ AZD2281 for 5 days. Tumors were calipered to determine tumor volume as described in the Methods. The mean ± SEM tumor volume for all animals in each treatment condition was plotted (n = 7 animals per group, 2 separate studies). *, $p < 0.05$, less than vehicle control value. **Upper panel**: tumors were isolated 7 days after the start of treatment were fixed and stained with TUNEL staining to examine tumor cell death. **(B)** For animals carrying BT549 tumors in *(A)* after receiving the indicated drug treatment, animals were monitored daily and when tumor volumes were > 1.5 cm^3 animals

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were sacrificed and survival of animals is plotted as a percentage of animals alive on any given day. **(C)** BT474 cells (8 x 10^6) were injected into the 4th mammary fat pad. Tumors permitted to form to ~75mm^3. Initial volumes of the tumor groups were: Vehicle 81 mm^3; AZD7762 70 mm^3; AZD2281 78 mm^3; AZD7762+AZD2281 68 mm^3. Animals were injected with vehicle, AZD7762 (50 mg/kg body mass), AZD2281 (50 mg/kg body mass), or AZD7762+ AZD2281 for 5 days. Tumors were calipered to determine the tumor volume as described in Methods. The mean ± SEM tumor volume for all animals in each treatment condition was plotted (n = 10 animals per group). *, $p < 0.05$, less than vehicle control value. Representative of two independent studies. **(D)** The tissues collected from BT474 derived tumors (day 15) were fixed and stained with TUNEL staining to examine tumor cell morphology and tumor cell death, and with an anti-Ki67 antibody to measure proliferative index.

Figure 5. PARP and CHK inhibitors radiosensitize tumor cells. BT474 cells were infected with empty vector virus (CMV) or with viruses to express dominant negative caspase 9, BCL-XL or c-FLIP-s. Twenty four h after infection cells were treated with vehicle (DMSO) or with AZD7762 (50 nM) +AZD2281 (1 μM). Cells were irradiated (4 Gy) 30 min after drug treatment. Cells were fixed 24h later and viability determined using TUNEL assay (±SEM, n = 3). **(B)** BT474 cells were transfected with WT CHK-GFP, DN CHK1-GFP or GFP control vector. Twelve h after transfection single cells were re-plated in sextuplicate. Twelve h after plating cells were treated with vehicle (VEH, DMSO), AZD2281 (1 μM), AZD7762 (50 nM) or AZD7762 + AZD2281. Cells were irradiated or mock exposed 30 min after initiation of drug treatment (2 Gy). Colonies were permitted to form over the following 10-14 days. # $p < 0.05$ greater than corresponding value in GFP transfected cells; * $p < 0.05$ less than corresponding value in GFP transfected cells. **(C)** BT549 cells (5 x 10^6) were injected into the 4th mammary fat pad. Tumors were permitted to form to ~75 mm^3. Initial volumes of the tumor groups were: Vehicle 84 mm^3; AZD7762 73 mm^3; AZD2281 66 mm^3; AZD7762+AZD2281 76 mm^3. Animals were injected with vehicle, AZD7762 (12.5 mg/kg), AZD2281 (12.5 mg/kg), or AZD7762+ AZD2281 for 5 days. Tumors were irradiated on Day2 and Day 4 (4 Gy). Tumors were calipered to determine tumor volume as described in the Methods. The

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mean ± SEM tumor volume for all animals in each treatment condition was plotted (n = 8 animals per group, 2 separate studies). **(D)** For animals carrying BT549 tumors in *(B)* after receiving the indicated drug treatment, animals were monitored daily and when tumor volumes were > 1.5 cm^3 animals were sacrificed and survival of animals is plotted as a percentage of animals alive on any given day.

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Table 1. PARP1 and CHK1 inhibitors synergize to kill mammary carcinoma cells. BT549 cells were plated as single cells in sextuplicate. Twelve h after plating cells were treated with AZD7762 (25-75 nM), UCN-01 (20-60 nM) or AZD2281 (500-1500 nM) as indicated in the Table. Forty eight h after treatment the media was removed and replaced with drug free media. Colonies were permitted to form for 10 days after which colonies were fixed and stained. A group of > 50 cells was defined as a colony. An assessment of drug interaction was made using the Calcusyn for Windows program. A combination index (CI) less than 1.00 was indicative of a synergistic drug interaction (Fa: Fraction affected).

AZD2281 (nM)	AZD7762 (nM)	Fa	CI
500	25	0.09	0.49
1000	50	0.22	0.37
1500	75	0.37	0.28
AZD2281 (nM)	UCN-01 (nM)	Fa	CI
500	20	0.16	0.38
1000	40	0.29	0.25
1500	60	0.38	0.24

Figure 1

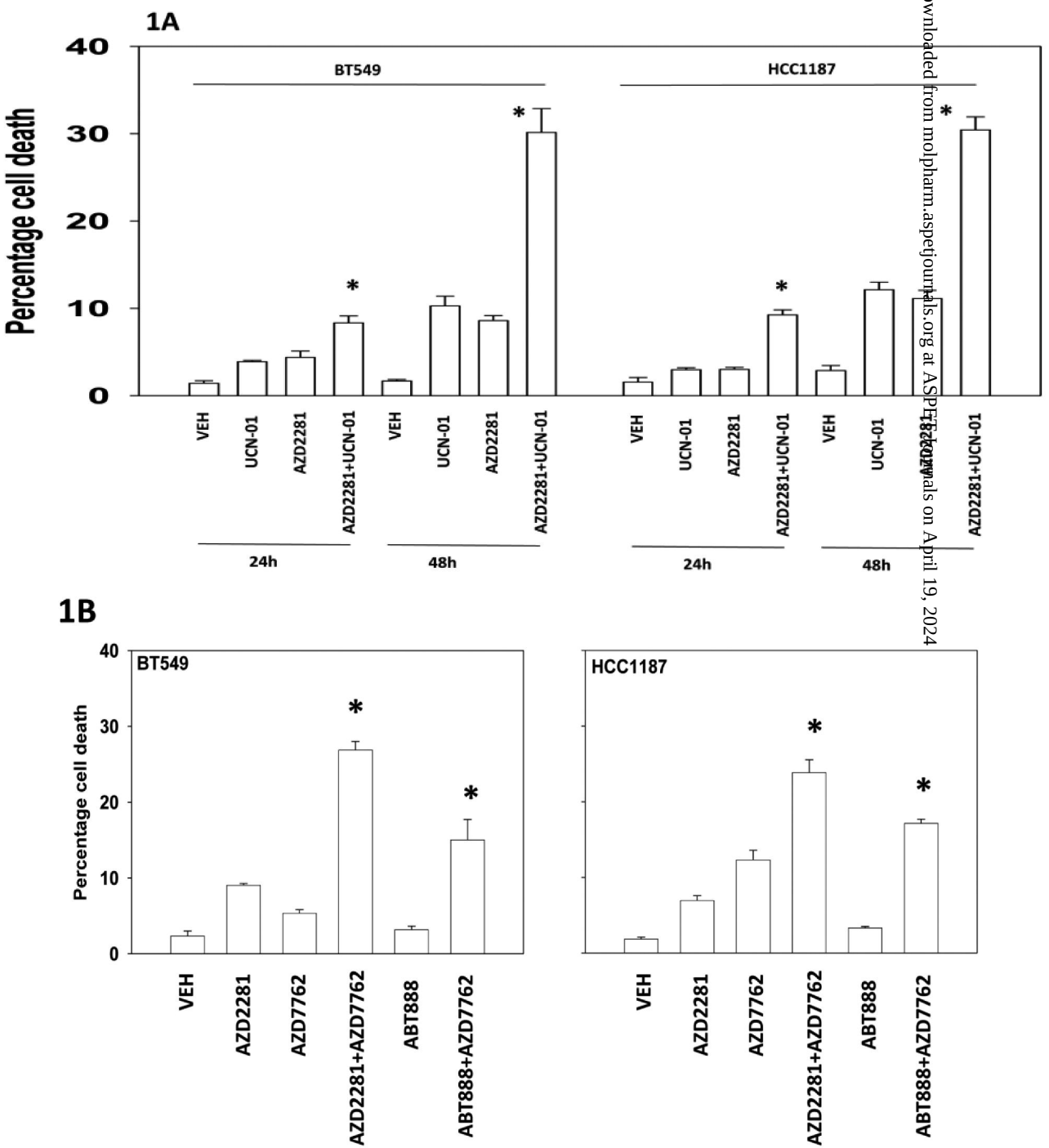


Figure 1

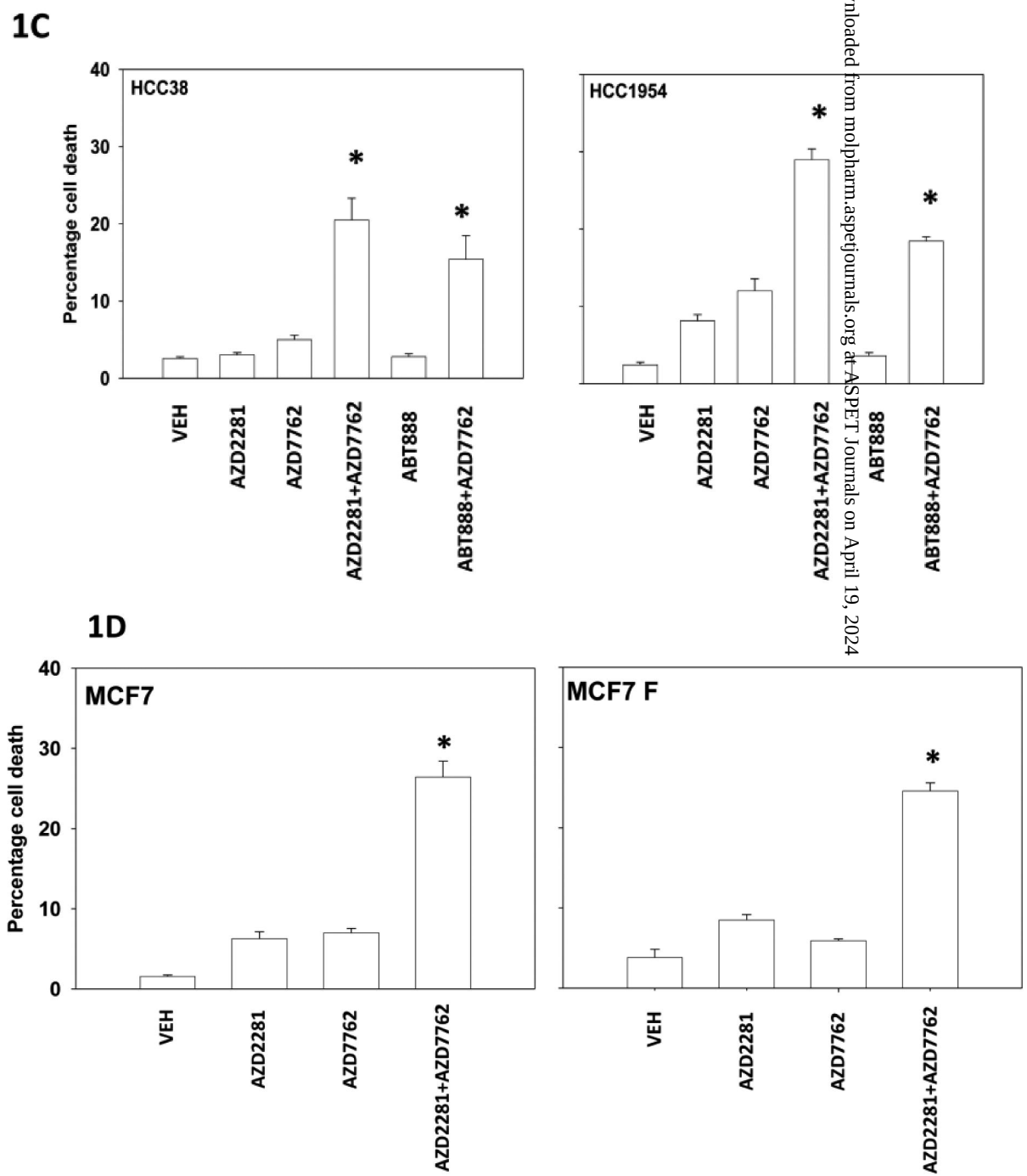
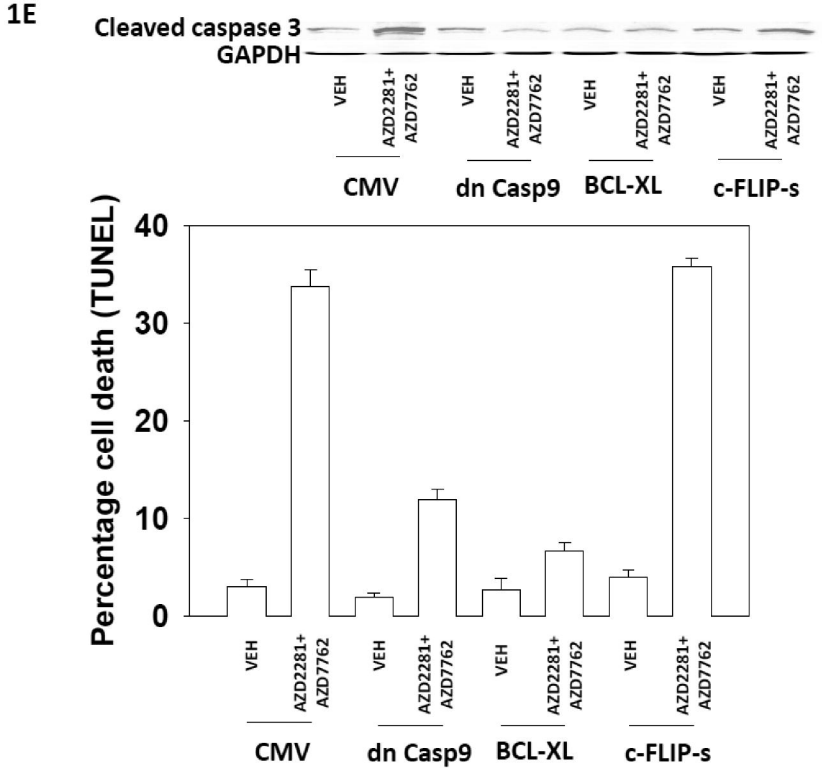


Figure 1



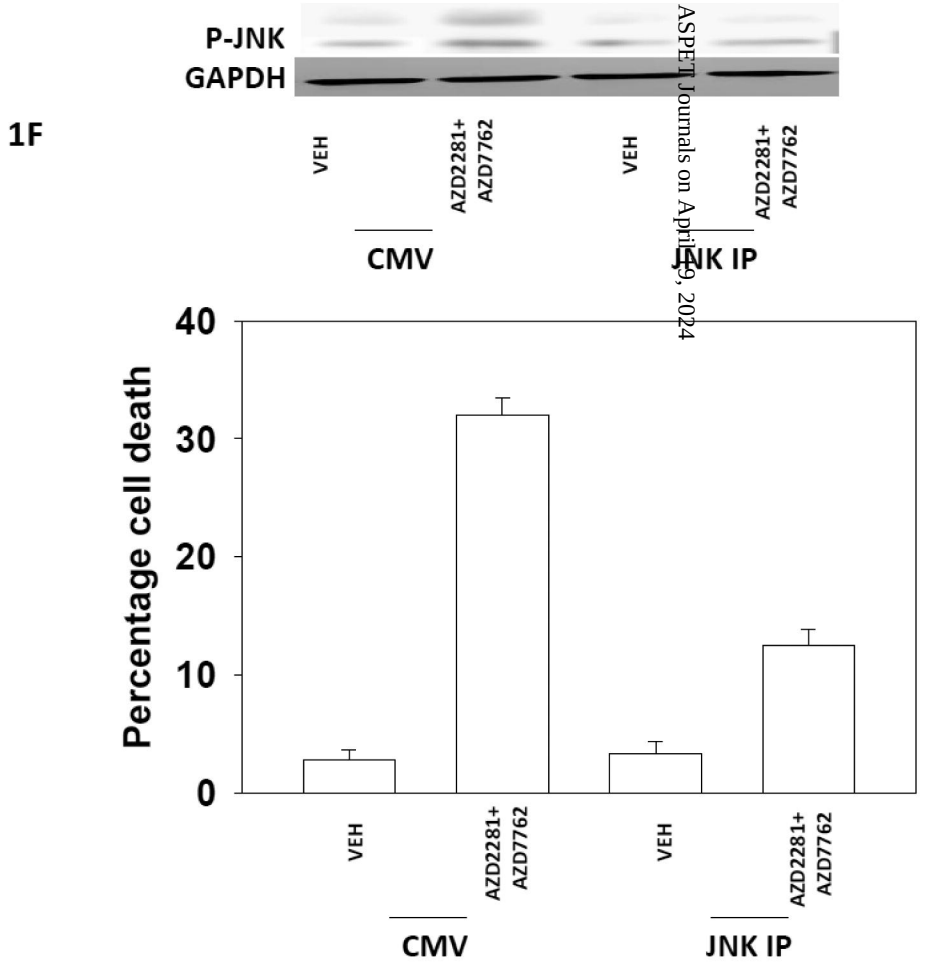


Figure 2

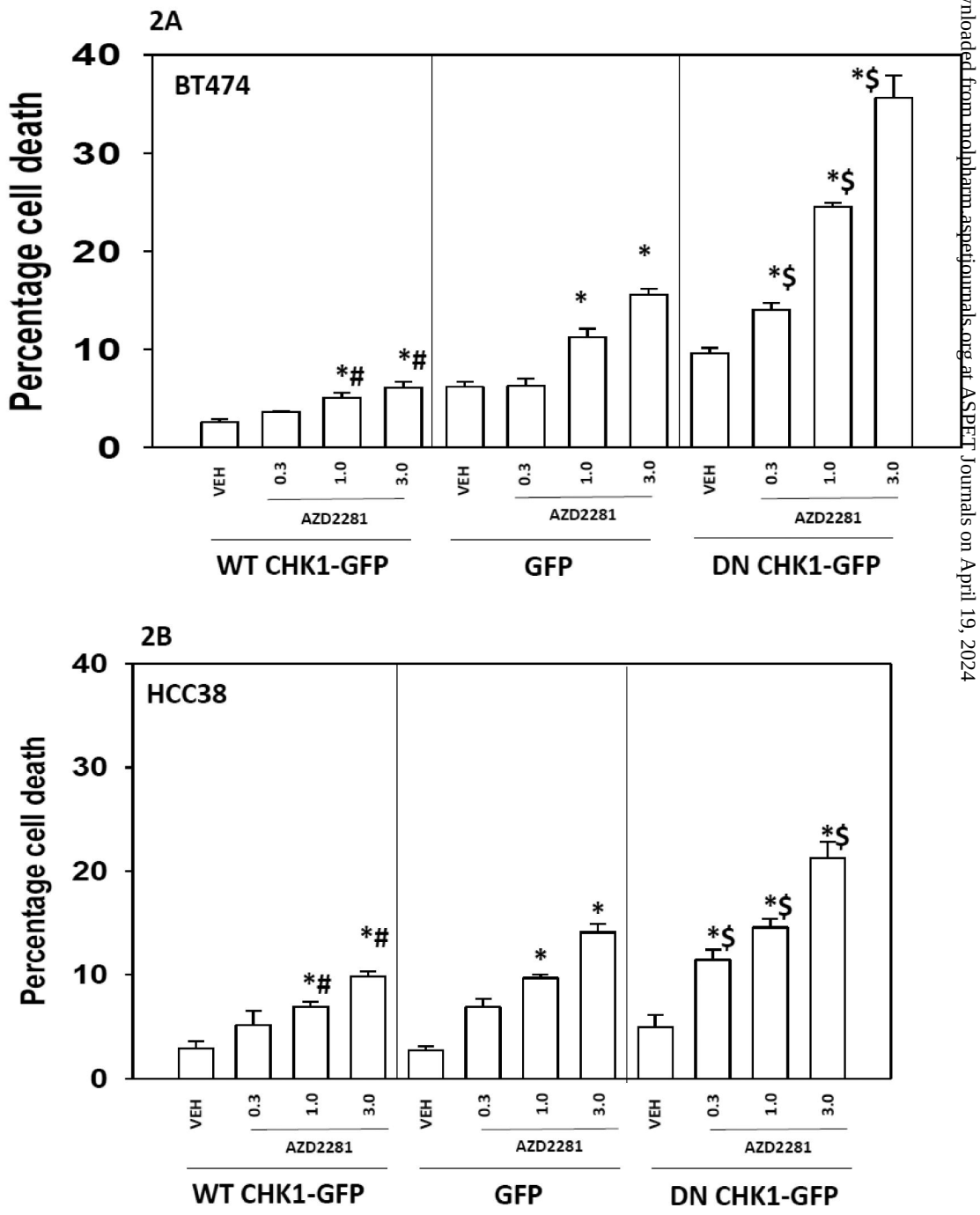


Figure 3

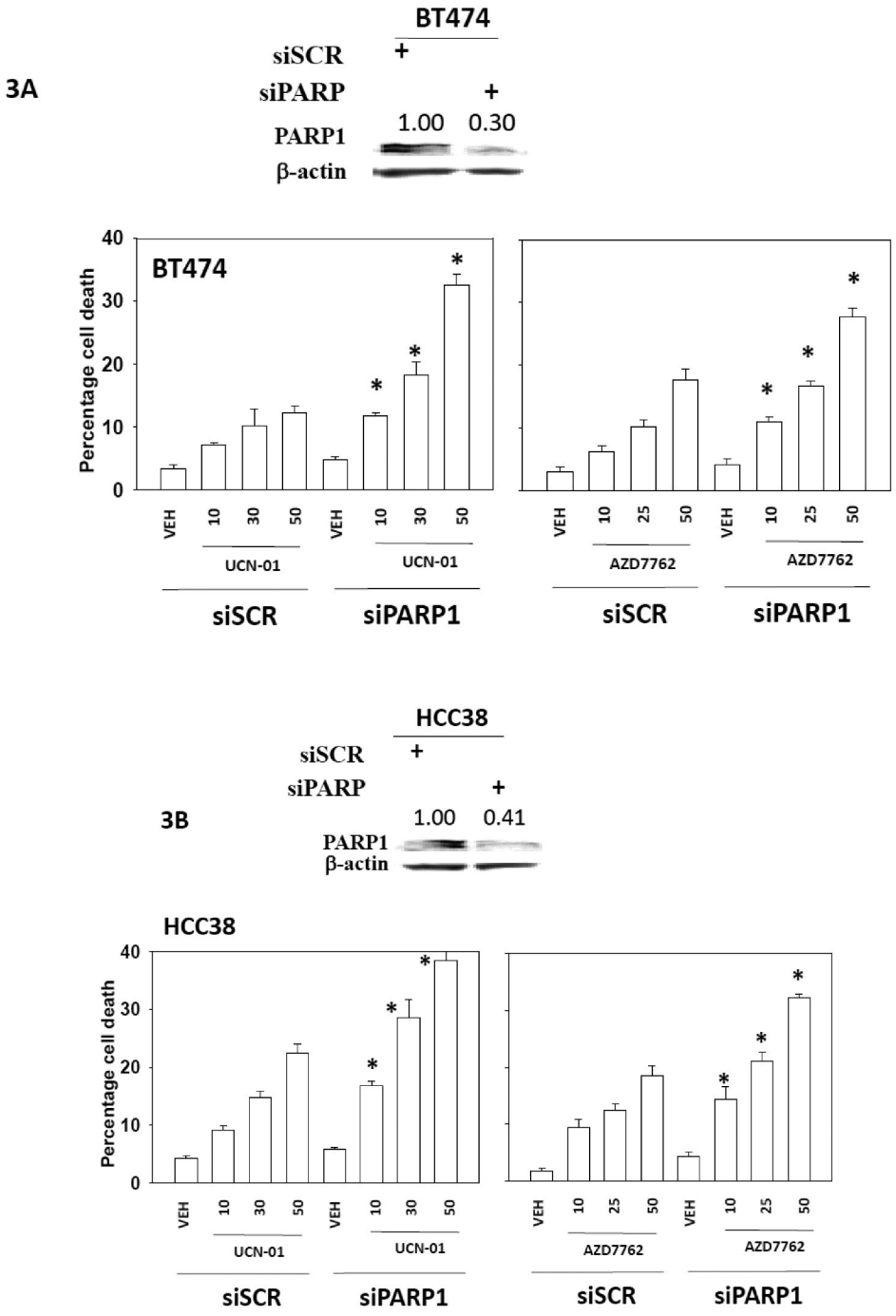
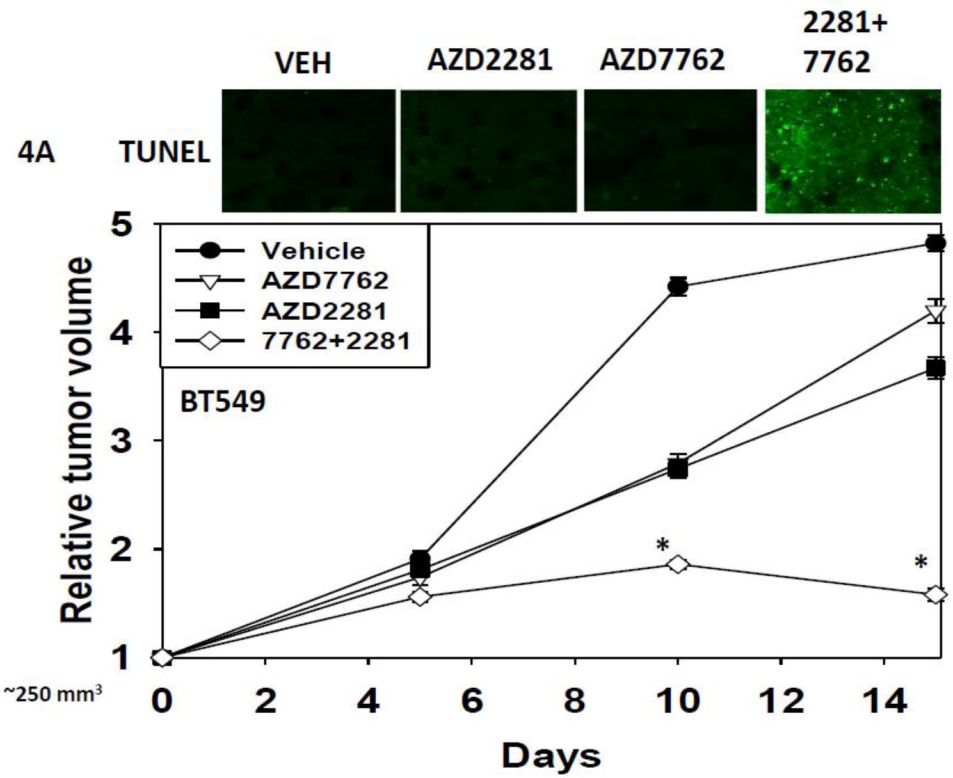


Figure 4



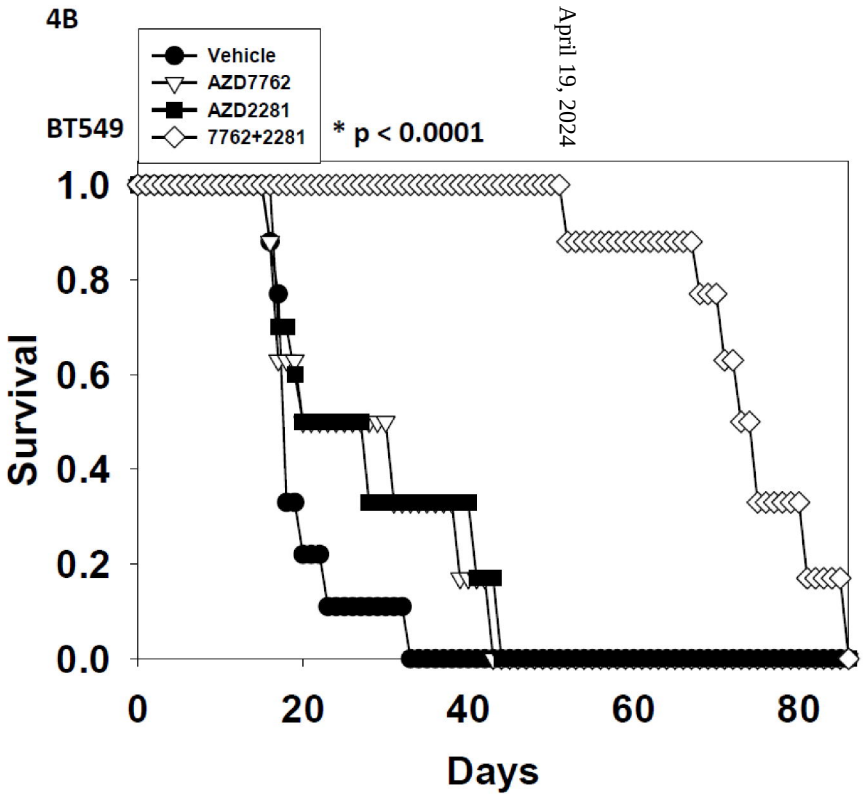
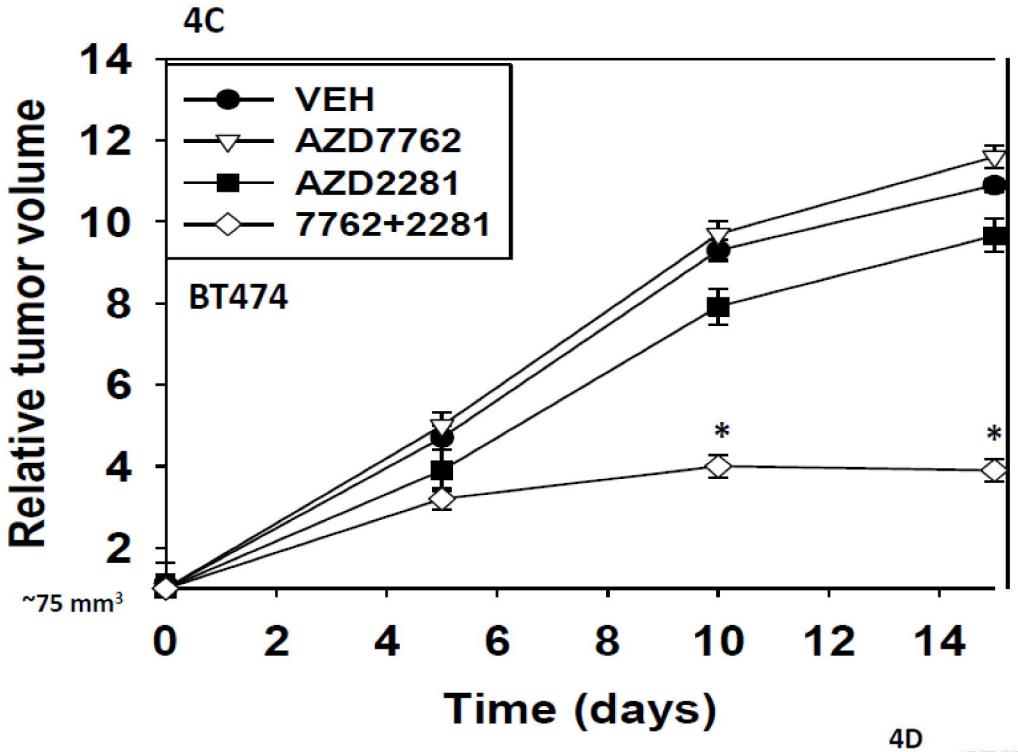


Figure 4



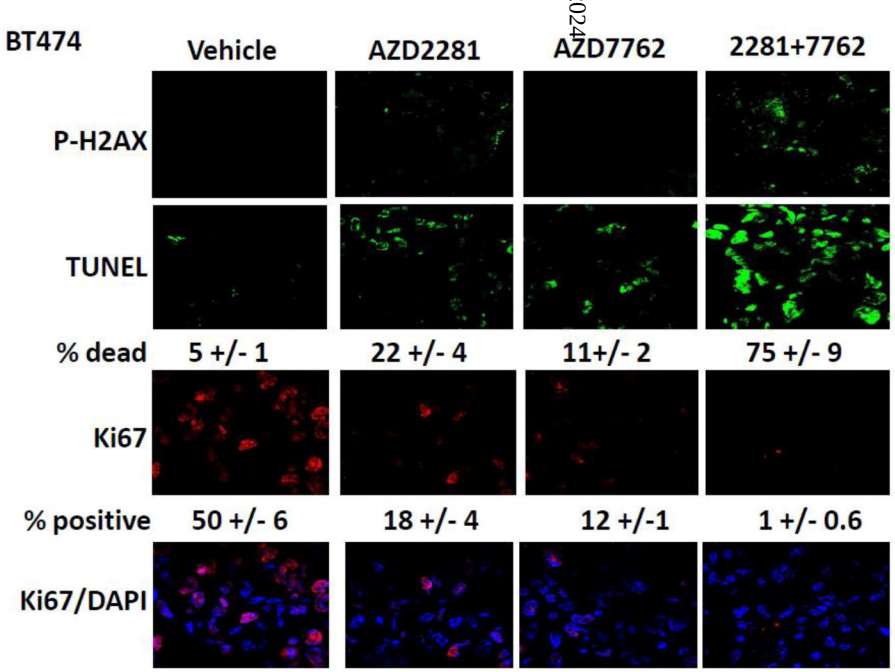
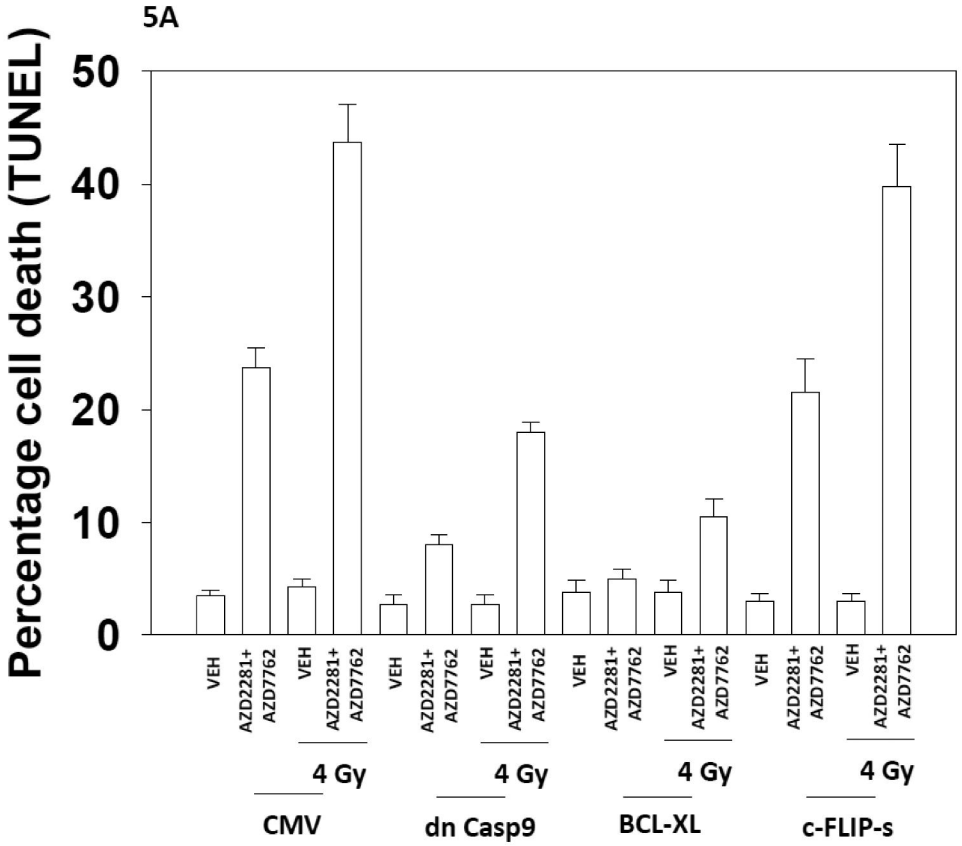


Figure 5



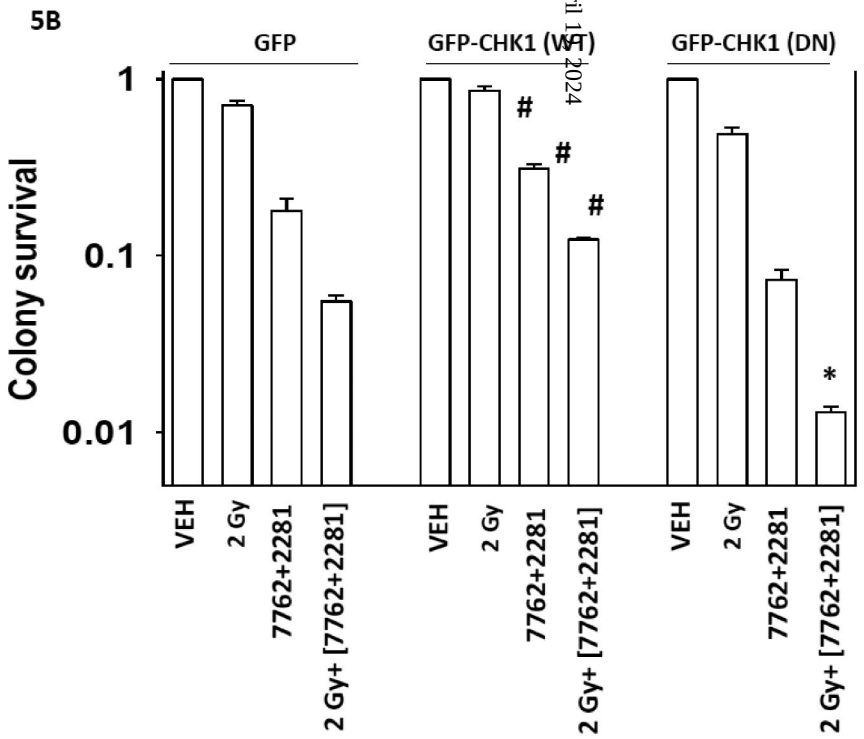


Figure 5