






ATR inhibition augments the efficacy of lurbinectedin in small-cell lung cancer

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Abstract

Small-cell lung cancer (SCLC) is the most lethal type of lung cancer. Specifically, MYC-driven non-neuroendocrine SCLC is particularly resistant to standard therapies. Lurbinectedin was recently approved for the treatment of relapsed SCLC, but combinatorial approaches are needed to increase the depth and duration of responses to lurbinectedin. Using high-throughput screens, we found inhibitors of ataxia telangiectasia mutated and rad3 related (ATR) as the most effective agents for augmenting lurbinectedin efficacy. First-in-class ATR inhibitor berzosertib synergized with lurbinectedin in multiple SCLC cell lines, organoid, and *in vivo* models. Mechanistically, ATR inhibition abrogated S-phase arrest induced by lurbinectedin and forced cell cycle progression causing mitotic catastrophe and cell death. High *CDKN1A/p21* expression was associated with decreased synergy due to G1 arrest, while increased levels of *ERCC5/XPG* were predictive of increased combination efficacy. Importantly, MYC-driven non-neuroendocrine tumors which are resistant to first-line therapies show reduced *CDKN1A/p21* expression and increased *ERCC5/XPG* indicating they are primed for response to lurbinectedin–berzosertib combination. The combination is being assessed in a clinical trial NCT04802174.

Keywords ATR inhibitor; biomarker; neuroendocrine; SCLC; synergy

Subject Categories Cancer; Respiratory System

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Introduction

Despite major advances in targeting oncogenes and the immune inhibitory checkpoints, most cancer patients die from chemotherapy-resistant disease. Multiple resistance mechanisms to chemotherapy have been described. For DNA-damaging chemotherapies, reduced intracellular drug intake, intracellular inactivation of the agent, increased DNA repair, activation of alternative DNA repair pathways, and impaired apoptotic signaling are among the most common resistance mechanisms (Housman *et al*, 2014).

Targeting DNA repair pathways using combination strategies represents a rational approach to overcome chemotherapy resistance. The ataxia telangiectasia–mutated and rad3-related (ATR) kinase is a master regulator of DNA damage response that plays a key role in stabilizing the genome when DNA replication is compromised (Ciccia & Elledge, 2010). ATR is activated by regions of single-stranded DNA (ssDNA), commonly generated as a result of DNA replication stress produced pharmacologically or by oncogene activation (Saldivar *et al*, 2018). Once activated, ATR functions to safeguard genomic integrity and safeguard replication by slowing the progression of replication forks, inhibiting distal replication origin firing, ensuring sufficient supply of deoxynucleotides, and promoting cell cycle arrest primarily by activation of intra-S and -G2/M cell cycle checkpoints (Saldivar *et al*, 2018). Accordingly, ATR inhibition leads to the loss of the S and G2/M checkpoints, allowing cells with damaged DNA to progress prematurely into M-phase, leading to mitotic catastrophe and cell death (Saldivar *et al*, 2018; Jo *et al*, 2021b). As such, multiple potent and selective ATR inhibitors are in preclinical and clinical development for

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cancer therapy (Thomas *et al*, 2018, 2021; Yap *et al*, 2020; Kim *et al*, 2021; Jo *et al*, 2021b).

SCLC is a neuroendocrine (NE) tumor characterized by near-universal bi-allelic loss of tumor suppressors *TP53* and *RB1*. *MYC* family genes are amplified, often on extrachromosomal DNA (ecDNA), in ~20% of SCLC and are overexpressed in ~50% of SCLC (George *et al*, 2015; Balanis *et al*, 2019; Pongor *et al*, 2023). Most patients are diagnosed with widely metastatic disease and are treated with a combination regimen of platinum, etoposide, and immunotherapy. Despite initial responses, risk of relapse is high with > 90% of patients progressing within 2 years (Paz-Ares *et al*, 2019). Second-line treatment options include topotecan and lurbinectedin, but the depth and duration of responses are modest and most relapsed tumors do not respond to additional chemotherapy. Furthermore, SCLCs have few targetable alterations (George *et al*, 2015) and tend not to respond to therapies targeted at somatic mutations (Lopez-Chavez *et al*, 2015). Recent studies have revealed heterogeneity of the SCLC neuroendocrine cell state, with tumors consisting of cells with NE and non-neuroendocrine (non-NE) features (Zhang *et al*, 2018). SCLC heterogeneity increases over the course of treatment, with an increase in chemoresistant non-NE cells evolving over time (Wagner *et al*, 2018; Ireland *et al*, 2020). Importantly, NE differentiation is emerging as a potential predictor of response to therapy (Rudin *et al*, 2019; Gay *et al*, 2021; Roper *et al*, 2021; Thomas *et al*, 2021).

Lurbinectedin is a synthetic alkylating derivative of trabectedin that was recently approved for SCLC patients with disease progression on or after platinum-based chemotherapy (Trigo *et al*, 2020). Lurbinectedin covalently binds to DNA-forming adducts that irreversibly stall elongating RNA polymerase II (Pol II) on the DNA template, generating DNA double-strand breaks (DSBs; Takebayashi *et al*, 2001; Santamaria Nunez *et al*, 2016). Here, we report an exquisite, NE differentiation-dependent synergistic interaction between the ATR inhibitor berzosertib and lurbinectedin. Mechanistically, lurbinectedin induces DSBs and activates ATR causing cell cycle arrest, allowing for repair and resistance, a process that is inhibited with berzosertib cotreatment. The combination produced greater synergy in *MYC*-high, chemoresistant, non-NE SCLC. Notably, these findings form the basis for a clinically actionable combination of a DNA repair inhibitor and a DNA-damaging agent to overcome SCLC chemoresistance.

Results

Drug screen identifies synergy of ATR inhibitors with lurbinectedin

To identify agents synergistically cytotoxic with lurbinectedin, we leveraged a previously reported drug screen in the SCLC cell line NCI-H446 wherein lurbinectedin was combined with 43 FDA-approved drugs or agents in late stages of clinical development (Thomas *et al*, 2021). The compound library included DNA-damaging agents and drugs that target mechanistically diverse pathways including DNA synthesis/metabolism, cell cycle or DNA damage repair, apoptosis, and chromatin remodeling (Fig 1A). Synergy was assessed using the highest single-agent (HSA) model (Berenbaum, 1989); positive values denote synergy and negative values antagonism. Lurbinectedin was most synergistic with DNA-damaging agents, drugs targeting cell cycle/DNA damage repair, and chromatin remodeling agents. In contrast, combinations with inhibitors of DNA synthesis and DNA metabolism were antagonistic, with the least synergy observed with the dihydrofolate reductase inhibitor pralatrexate (HSA -524.6) and the DNA polymerase inhibitor cytarabine (HSA -235.7; Table EV1).

Maximal cytotoxic synergy with lurbinectedin was observed for elimusertib (BAY1895344, HSA 881.2) and berzosertib (M6620, VX-970, VE-822, HSA 881.4) inhibitors of ATR, the main transducer of replication stress signaling and regulator of the cell cycle in response to DNA damage (Fig 1B and Table EV1). Both ATR inhibitors displayed greater synergy with lurbinectedin as compared to the topoisomerase II inhibitor doxorubicin (224.4 HSA, Table EV1). Doxorubicin was previously reported to be synergistic with lurbinectedin in preclinical models, however, this combination failed to improve survival compared with standard of care in patients with relapsed SCLC (Helwik, 2021). In a reciprocal screen, lurbinectedin was among the top four agents that showed maximal synergy with berzosertib (Fig 1C and Table EV2). The other top hits were inhibitors of key proteins involved in maintaining genomic stability including ataxia telangiectasia mutated (*ATM*, AZD-0156; Riches *et al*, 2020), *WEE1* (MK-1775; Rajeshkumar *et al*, 2011), and topoisomerase I (*TOP1*; topotecan; Table EV2). The combination of topotecan and berzosertib is being examined in clinical trials (Thomas *et al*, 2018, 2021; NCT04768296, NCT03896503). Notably,

Figure 1. Lurbinectedin and berzosertib synergize in SCLC.

- A A synergy screen was previously performed with 43 agents targeting multiple pathways in combination with each other. NCI-H446 SCLC cells were treated with drugs in a 10 × 10 matrix format, viability was assessed using Cell Titer Glo, and synergy was assessed using highest single-agent (HSA).
- B Lurbinectedin synergy was ranked based on HSA synergy values. The highest synergy was observed with two ATR inhibitors, berzosertib and elimusertib, both of which displayed more synergy than doxorubicin.
- C Berzosertib synergy with therapeutics ranked based on synergy. Topotecan and lurbinectedin both strongly synergized with berzosertib.
- D, E Lurbinectedin and berzosertib synergized in SCLC cell lines NCI-H524 and DMS 114. Synergy of lurbinectedin and berzosertib was assessed by treating these drugs in a 10 × 10 matrix format for 72 h in NCI-H524 (D) (HSA 410.7) and DMS 114 cells (E) (HSA 286.3). Synergy was calculated by adding HSA across all combinations in the matrix (100 combinations, replicates = 3, *n* = 1). Synergy is denoted by blue and antagonism in red.
- F Multiple DNA damage response pathways were activated by lurbinectedin. Treatment with the ATR inhibitor berzosertib specifically inhibited the activation of ATR and its downstream target *CHK1* (both targets indicated by red box). DMS 114 cells were treated with lurbinectedin (1 nM) ± berzosertib (1 μM) for 6 or 24 h, and targets were assessed by immunoblotting.
- G Lurbinectedin activated all three key DNA damage response proteins, DNA-PK, ATR, and *ATM*. Berzosertib is effective at inhibiting the activation of ATR and downstream ATR target *CHK1* (indicated by red box), with less notable effects on other DNA damage repair pathways.

Source data are available online for this figure.

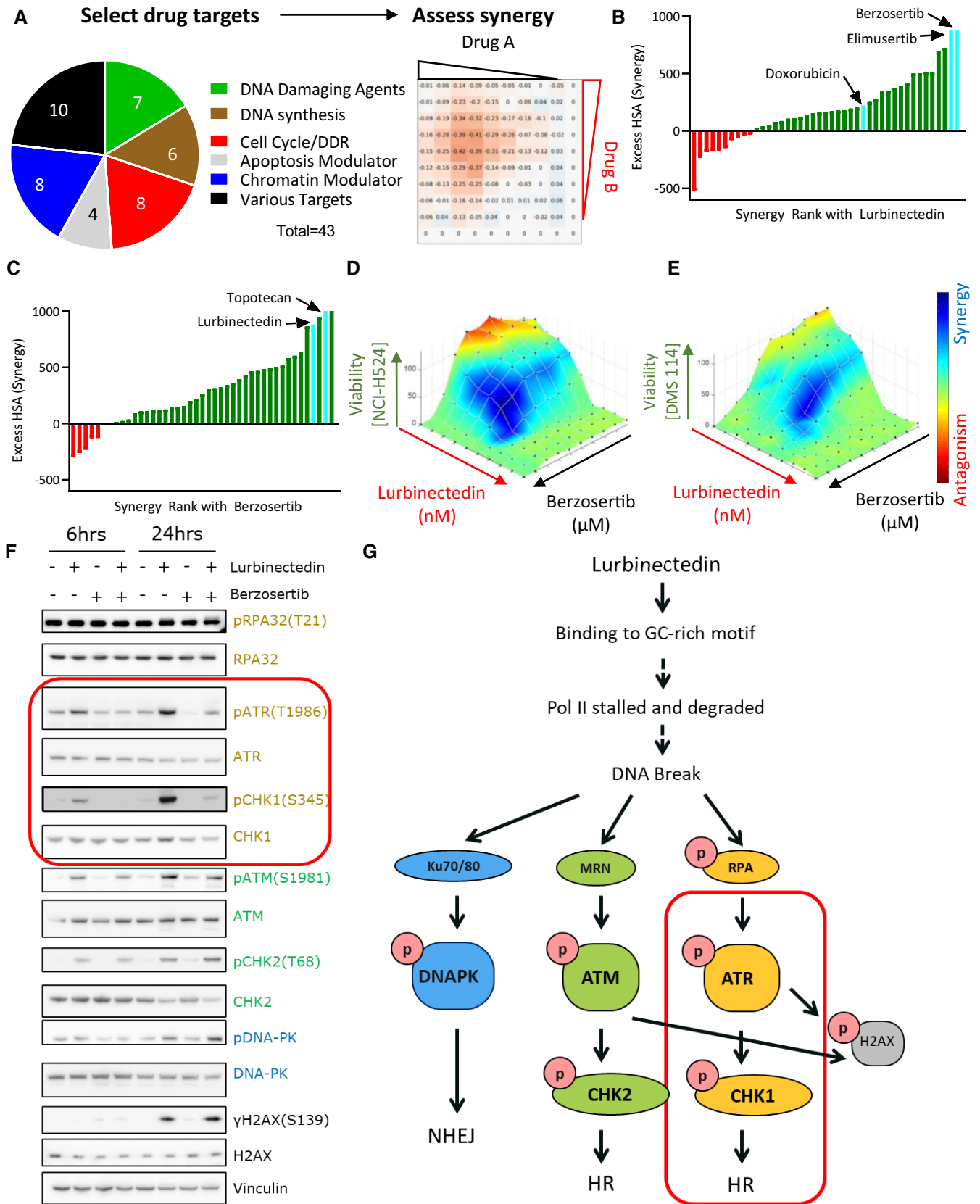


Figure 1.

lurbinectedin was more potent than topotecan in seven of nine SCLC cell lines tested and thus may have improved efficacy (Fig EV1A). The synergy of lurbinectedin and berzosertib was also confirmed in additional SCLC cell lines NCI-H524 (HSA 410.7) and DMS 114 (HSA 286.3; Fig 1D and E).

The combination of lurbinectedin and berzosertib causes mitotic catastrophe

Lurbinectedin treatment induced activation of ATR, ATM- and DNA-dependent protein kinase (DNA-PK), primary kinases that regulate DNA repair, and γ H2AX, a marker of DNA DSBs (Blackford & Jackson, 2017; Fig EV1B). The addition of berzosertib reduced the activation of ATR and its downstream target CHK1, with less notable impact on ATM/CHK2 or DNA-PK (Fig 1F). These results confirm that lurbinectedin-induced DNA damage activates multiple DNA damage sensing and repair pathways, but berzosertib specifically reduces the activation of the ATR-CHK1 axis (Fig 1G).

Given the critical role of ATR and its downstream target CHK1 in initiating cell cycle arrest in response to DNA damage, we investigated whether the synergy of lurbinectedin and berzosertib was cell cycle dependent. Lurbinectedin-induced DNA damage as measured by γ H2AX occurred predominantly in the S-phase of cycling cells (Figs 2A and EV1C). Berzosertib alone or in combination with lurbinectedin reduced γ H2AX in the majority of cells, but in a small fraction (~5%) of the population caused a drastic increase in γ H2AX. Therefore, when assessed using flow cytometry, cotreatment with berzosertib reduced lurbinectedin-dependent S-phase γ H2AX induction in the majority of cells leading to a decrease in median γ H2AX signal in S-phase cells, but consistent with our immunoblotting (Fig 1F) caused an overall increase in average γ H2AX signal in DMS

114 cells (Figs 2B and EV1D–F). We assessed γ H2AX induction using immunostaining and immunoblotting in additional cell lines and found berzosertib overall inhibited lurbinectedin-dependent γ H2AX induction (Fig 2C and Appendix Fig S1A–D). Together, the immunoblotting, immunostaining, and flow cytometry analyses indicated that berzosertib cotreatment reduces lurbinectedin-induced γ H2AX formation. Reduced γ H2AX signal in the majority of cells was not due to a decrease in DNA damage as the combination of lurbinectedin and berzosertib induced more DNA breaks as assessed by alkaline comet assay than either agent alone (Fig 2D and E). Treatment with lurbinectedin reduced DNA replication as indicated by a decrease in 5-ethynyl-2'-deoxyuridine (EdU) incorporation, an effect which was largely rescued by treatment with berzosertib (Figs 2F and EV1G). Lurbinectedin-induced increase in γ H2AX and decrease in EdU incorporation were inhibited by siRNA against ATR, suggesting that these responses are indeed ATR dependent (Fig EV1H and I).

Next, we used molecular combing (DNA fiber assays) to visualize single replicons and measure the impact of lurbinectedin and ATR inhibition on replication dynamics. Control DMS 114 cells displayed roughly equal distribution of initiation (27%), termination (39%), and unidirectional (34%) forks. Lurbinectedin treatment suppressed replication fork initiation by 3-fold while slightly increasing termination and unidirectional forks (1.2-fold and 1.3-fold, respectively). These results were in agreement with our previous data indicating lurbinectedin treatment inhibited DNA synthesis. Berzosertib treatment increased initiation forks by 1.8-fold compared with control, consistent with previous work demonstrating that ATR inhibition causes the unscheduled firing of dormant origins (Jo *et al*, 2021b) with a minimal impact on termination and unidirectional forks. In combination, berzosertib treatment rescued

Figure 2. Berzosertib causes continued cell cycle progression and induces mitotic catastrophe in the presence of lurbinectedin.

- A Lurbinectedin induced changes to γ H2AX across different phases of the cell cycle, with the greatest degree of activation in S-phase. DMS 114 cells were treated with 1 nM lurbinectedin for 1 to 6 h and γ H2AX induction was assessed using flow cytometry; four replicates of 10,000 cells for each timepoint were assessed, and error bars represent SEM, $n = 2$. Cell cycle was assessed using propidium iodide.
- B Lurbinectedin treatment increased γ H2AX induction, while berzosertib treatment caused a small portion of cells to display increased γ H2AX signal. Berzosertib cotreatment with lurbinectedin caused a decrease in γ H2AX accumulation for the majority of cells leading to a decreased median accumulation of γ H2AX, however, an increase in γ H2AX in a smaller portion of cells led to an increased average accumulation (averages and medians are shown in Fig EV1D–F). DMS 114 cells were treated with ± 1 nM lurbinectedin ± 2 μ M berzosertib for 6 h γ H2AX induction was assessed using flow cytometry. 10 mM EdU was added for the last hour prior to collection (assessed Fig 2F). Numbers on the graph indicate the average percent of cells across four replicates of 10,000 cells with low, medium, or high γ H2AX, $n = 3$.
- C Lurbinectedin treatment increased γ H2AX induction, which was reduced with berzosertib treatment. γ H2AX induction was assessed using immunofluorescence in DMS 114 cells were treated with ± 1 nM lurbinectedin ± 2 μ M berzosertib for 6 h. Quantification is from 100 to 150 cells per treatment, with error bars indicating SEM, comparisons were made using an unpaired two-tailed Student's *t*-test in PRISM, $n = 3$. Representative images for this experiment are in Appendix Fig S2A.
- D, E Combination of lurbinectedin and berzosertib caused DNA damage. DMS 114 cells were treated with ± 1 nM lurbinectedin ± 2 μ M berzosertib for 6 h and total DNA damage was assessed using an alkaline comet assay. These data represent average tail length for each group with 150–250 cells measured per group, $n = 3$ error bars represent SEM, *P*-values represent unpaired two-tailed Student's *t*-tests performed in PRISM.
- F Lurbinectedin treatment caused a decrease in DNA replication as assessed by EdU incorporation while berzosertib cotreatment largely rescued this phenotype. Numbers represent the average percent of cells across four replicates with high EdU incorporation indicating functional DNA replication, $n = 3$. These cells are the same as assessed in Fig 2B.
- G Lurbinectedin treatment inhibited fork initiation, an effect that was rescued by the addition of berzosertib. DMS 114 cells were treated with ± 1 nM lurbinectedin ± 2 μ M berzosertib for 6 h, and using DNA combing, we assessed motifs of incorporation of IdU and CldU indicating initiation, termination, or unidirectional DNA forks. Forks were assessed and verified individually to avoid algorithmic assessment, with 100–200 forks quantified per group $n = 2$.
- H, I Berzosertib treatment alone caused a small portion of cells to undergo mitotic catastrophe, however, cotreatment of lurbinectedin and berzosertib caused a large portion of cells to undergo mitotic catastrophe. DMS 114 cells were treated with ± 1 nM lurbinectedin ± 2 μ M berzosertib for 6 h with the final 3 h being in the presence of nocodazole followed by release from all drugs and 45 min of continued growth. Cells that had undergone mitotic catastrophe were assessed as those which were multinucleated. Representative images (H) and quantification of percent of cells that underwent mitotic catastrophe (I). Graph represents average of three separate experiments with statistics determined from assessing 100 nuclei per group, error bars represent SEM, and *P*-values are indicative of unpaired two-tailed Student's *t*-tests performed in PRISM.

Source data are available online for this figure.

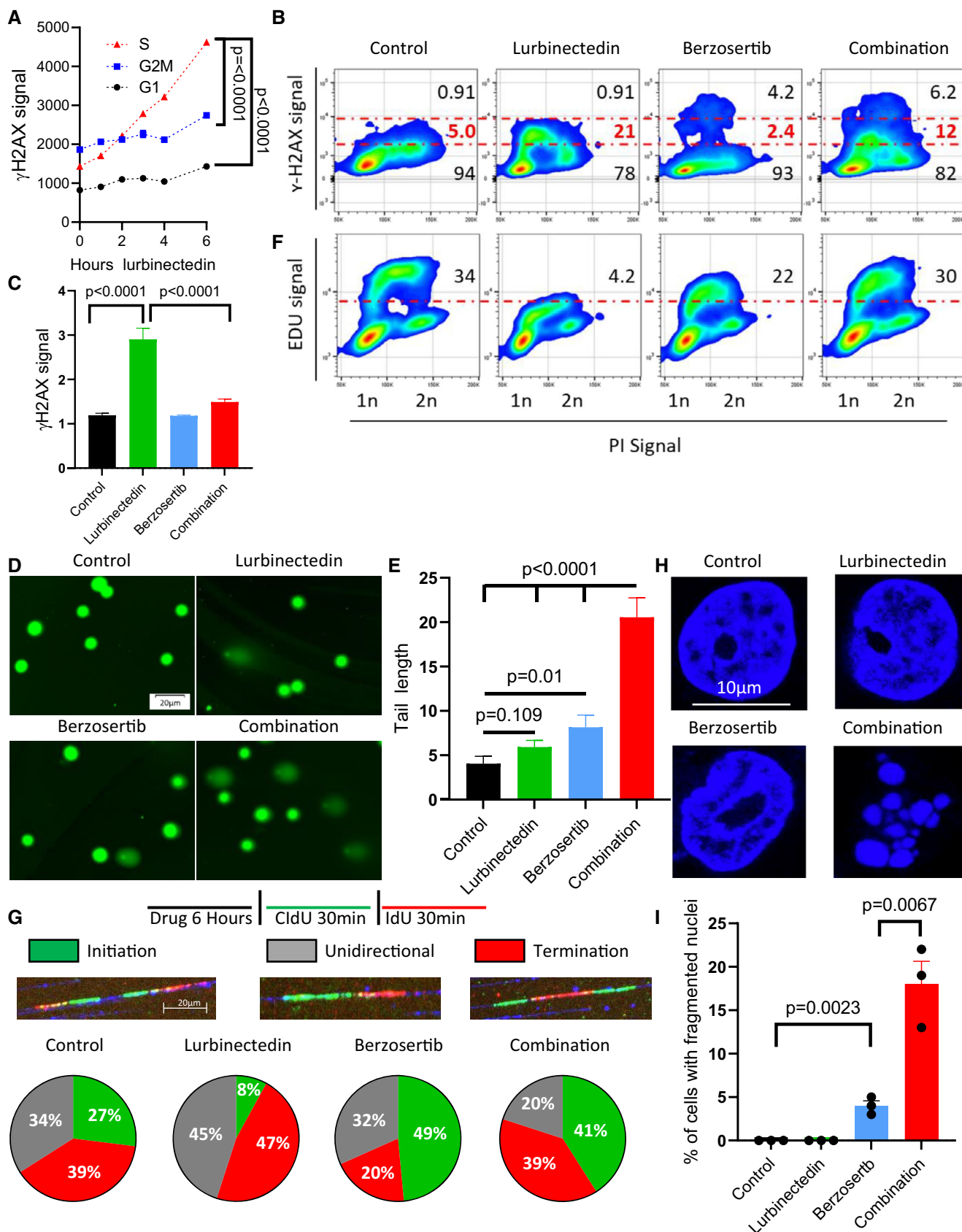


Figure 2.

the suppression of replication fork initiation caused by lurbinectedin, with a 5-fold increase in initiation forks as compared to lurbinectedin treatment, also reducing termination and unidirectional forks to a lesser extent (0.83-fold and 0.44-fold, respectively; Fig 2G; Iyer & Rhind, 2017). These results demonstrated that berzosertib leads to unscheduled replication origin firing and continued DNA replication even in the presence of lurbinectedin-induced DNA damage.

In addition to its crucial role in DNA damage and replication stress response, ATR also promotes accurate chromosome segregation during mitosis (Blackford & Jackson, 2017). We assessed metaphase spreads to determine drug treatment-induced changes to chromosomal integrity during mitosis. Berzosertib monotherapy led to abnormal metaphase spreads, with individual chromosomes failing to segregate appropriately (Appendix Fig S1E and F). This phenotype was recapitulated on treatment with barasertib, an inhibitor of Aurora Kinase B, a downstream target of ATR critical for accurate chromosomal segregation (Appendix Fig S1G). Lurbinectedin alone had little effect on chromosomal integrity at metaphase. Lurbinectedin–berzosertib combination treatment did not impact the frequency of segregation defects caused by berzosertib treatment, but the combination significantly increased the percentage of mitotic cells with multiple breaks in chromosomes (Appendix Fig S1E). We assessed postmitotic viability in order to determine if the chromosome breaks induced by cotreatment affected mitotic competence. Both lurbinectedin and berzosertib had little effect on postmitotic viability by themselves. The combination, however, significantly increased postmitotic death as indicated by multinucleation (Fig 2H and I). Together, these results demonstrate that berzosertib augments DNA damage caused by lurbinectedin and allows cells to progress to mitosis with unrepaired DNA damage, ultimately leading to mitotic catastrophe and cell death.

ERCC5/XPG and SLFN11 are critical determinants of response to lurbinectedin

Similar to the structurally related trabectedin, adducts generated by binding lurbinectedin to the DNA minor groove are recognized by the nucleotide excision repair (NER) pathway. Lurbinectedin then binds NER complex member *ERCC5/XPG*, trapping the complex on the DNA, ultimately leading to the formation of irreversible single-strand breaks, and consequently cell death (Takebayashi *et al*, 2001; Romano *et al*, 2013). Homologous recombination (HR) is critical for the repair of lurbinectedin-induced DNA damage, and loss of the HR pathway increases sensitivity to lurbinectedin (Romano *et al*, 2013). Isogenic models using the chicken B cell line DT40 cells have previously been utilized to demonstrate the importance of NER and HR pathways for lurbinectedin efficacy (Romano *et al*, 2013). We examined the contribution of these DNA repair mechanisms to the efficacy of lurbinectedin, berzosertib, and the combination utilizing isogenic DT40 models. Consistent with previous findings, DT40 cells with knockout (KO) of the NER pathway mediators *ERCC5/XPG* and *XPA* were approximately 10-fold more resistant to lurbinectedin. Alternatively, *BRCA2*-KO DT40 cells were approximately 6-fold more sensitive to lurbinectedin (Fig EV2A). Berzosertib efficacy was not significantly altered in the knockouts (Fig EV2B).

ATR signaling and ATR-induced cell cycle arrest are critical for efficient HR repair (Blackford & Jackson, 2017). To confirm that

berzosertib was inhibiting HR competency, we utilized U2OS cells with a stably integrated GFP HR reporter (Weinstock *et al*, 2006). We confirmed that berzosertib inhibited HR competency at 7-fold lower concentrations than that required for inhibiting viability (Fig EV2C and D). If lurbinectedin–berzosertib synergy was due to reduced HR competency from berzosertib, synergy would be decreased in the *BRCA2*-KO. Supporting this hypothesis, *BRCA2*-KO cells displayed slightly decreased synergy. Consistent with the importance of the NER pathway for lurbinectedin efficacy, synergy was even more markedly reduced with *XPG*-KO and *XPA*-KO cells (Fig EV2E). To standardize the assessment of overall combination efficacy, we defined lurbinectedin IC_{50} in the presence of 500 nM of berzosertib (a concentration where lurbinectedin IC_{50} s are reduced in all synergistic models tested and HR is inhibited) as representative of overall combination efficacy. Lurbinectedin–berzosertib combination displayed similar combination efficacy in the *BRCA2* KO cell line as compared to control. However, the *XPG*-KO and *XPA*-KO cell lines maintained resistance even in the presence of 500 nM berzosertib leading to reduced combination efficacy (Fig EV2F). These results show that lurbinectedin–berzosertib synergy is only partially mediated by berzosertib-inhibiting HR competency, and that the efficacy of lurbinectedin alone and combination efficacy are both dependent on NER competency.

A recent report has indicated that cells with high expression of *SLFN11*, another important mediator of DNA damage response, are significantly more sensitive to lurbinectedin (Kundu *et al*, 2021). *SLFN11* destabilizes paused replication forks following DNA damage causing cell death. Consequently, loss of *SLFN11* leads to broad resistance to a variety of DNA-damaging therapeutics (Jo *et al*, 2021a). We found that *SLFN11*-KO DMS 114 cells were approximately 4-fold more resistant to lurbinectedin than parental cells, while there was little difference in sensitivity to berzosertib (Fig EV2G and H). Consistent with previous findings demonstrating the ability of ATR inhibition to re-sensitize *SLFN11*-KO cells to DNA-damaging agents (Jo *et al*, 2021a), synergy was increased in *SLFN11*-KO cells, and *SLFN11*-KO cells displayed similar combination efficacy as the control cells (Fig EV2I and J). As berzosertib rescued lurbinectedin efficacy in *SLFN11*-KO cells and not in *XPG*-KO cells, we conclude that *ERCC5/XPG* is mechanistically required for lurbinectedin activity even in the presence of an ATR inhibitor, whereas *SLFN11* is not. These results are consistent with *SLFN11* inducing lethal replication fork instability in response to DNA-damaging agents independently of ATR and acting in parallel with the ATR-mediated S-phase checkpoint (Murai *et al*, 2016).

Next, we assessed the efficacy of lurbinectedin, berzosertib, and the combination of both agents in a 10 × 10 matrix format (100 conditions) across a panel of nine SCLC cell lines spanning the spectrum of NE differentiation and lineage transcription factors (Table 1; Rudin *et al*, 2019). Lurbinectedin was highly potent across SCLC cell lines with IC_{50} s ranging from 0.01 to 0.38 nM, while berzosertib IC_{50} s ranged from 0.34 to 5.04 μM (Table 1). Consistent with findings in isogenic models described above, we observed trends toward increased sensitivity to lurbinectedin in cells with high *SLFN11* or *ERCC5/XPG* expression (Fig EV3A). We assessed the IC_{50} of lurbinectedin in the presence of 500 nM berzosertib, a concentration that effectively inhibits HR, and has little effect on viability by itself in most models. Increased *ERCC5/XPG* expression significantly correlated with improved combination efficacy, while higher *SLFN11*

Table 1. Critical variables for lurbinectedin and berzosertib efficacy and synergy in SCLC cell lines.

Cell line	p53 mutation status	RB mutation status	SCLC subtype	NE score	Excess HSA (Synergy)	CDKN1A (RNA)	MYC (RNA)	ERCC5 (RNA)	SLFN11 (RNA)	BRZ IC50 (μM)	Lurb IC50 (nM)	Lurb + 500 nM BRZ IC50 (nM)
NCI-H211	Mutated	Wild-type	POU2F3	-0.699	474.0	7.6	10.8	7.1	6.116	1.001	0.099	0.039
NCI-H524	Mutated	Mutated	NEUROD1	0.264	410.7	7.1	11.2	7.1	4.427	1.270	0.215	0.059
DMS 114	Mutated	Wild-type	YAP1	-0.587	286.3	7.5	9.3	8.1	7.810	0.338	0.157	0.058
NCI-H841	Mutated	Wild-type	YAP1	-0.638	257.1	8.0	9.5	6.0	4.937	5.036	0.383	0.172
NCI-H1048	Mutated	Mutated	POU2F3	-0.699	250.1	8.4	9.0	7.5	8.929	0.434	0.010	0.005
NCI-H1341	Wild-type	Wild-type	YAP1	-0.333	180.6	9.0	10.7	6.9	7.839	1.053	0.123	0.063
NCI-H446	Mutated	Mutated	NEUROD1	0.000	-11.9	9.1	10.4	6.8	4.692	0.507	0.089	0.059
NCI-H146	Wild-type	Mutated	ASCL1	0.395	-128.3	8.5	9.5	7.7	4.625	3.213	0.143	0.078
NCI-H889	Mutated	Wild-type	ASCL1	0.479	-171.3	9.3	5.8	6.0	4.679	2.208	0.108	0.179

Pertinent values from the nine SCLC cell lines utilized in this work. All RNA values are given as log₂ values and these along with NE scores, and RB (RB1 and RB2 both assessed, all mutations in RB1) and p53 mutation status are publicly available at <https://discover.nci.nih.gov/rsconnect/cellminercdb>.

expression trended toward increased combination efficacy (Figs 3A and EV3A, Table 1 and Table EV3). Overall, studies in isogenic systems and human SCLC cell lines demonstrate that SLFN11, HR, and ERCC5/XPG are determinants for the response to lurbinectedin monotherapy, with ERCC5/XPG being a critical determinant of response to the lurbinectedin–berzosertib combination, that is, combination efficacy (Table EV3 and Fig EV3B).

The G1/S checkpoint is a critical determinant of lurbinectedin–berzosertib synergy; consequently, CDKN1A/p21 is a determinant of reduced synergy

The lurbinectedin–berzosertib combination was synergistically cytotoxic in six of nine SCLC cell lines assessed (HSA 180.5 to 474.0) and additive (HSA -11.9 where 0 is additive) or antagonistic (HSA -128.3 to -171.3) in the others (Table 1). Synergy, that is, HSA or the difference between lurbinectedin IC₅₀s +/- berzosertib, did not correlate with sensitivity to either drug or to the expression of SLFN11 or ERCC5/XPG (Fig EV3A). As lurbinectedin and berzosertib greatly affected DNA replication and mitotic division, we assessed whether synergy was dependent on cell cycle dynamics. Cell lines with higher synergy (NCI-H841 and NCI-H1341) upon exposure to lurbinectedin had significantly greater S-phase arrest which was ameliorated with cotreatment with berzosertib as compared to less synergistic cell lines (NCI-H146 and NCI-H889; Figs 3B and C, and EV3C). Therefore, we hypothesized that cells deficient in the G1/S checkpoint could be uniquely sensitive to the combination of lurbinectedin–berzosertib.

SCLC is characterized by loss of RB1 (George et al, 2015), the predominant regulator of G1/S transition leading to increased reliance on cyclin-dependent kinase inhibitor p21 (CDKN1A) for control of the G1/S checkpoint (Hauge et al, 2019). High expression of CDKN1A RNA was associated with reduced lurbinectedin–berzosertib synergy (Fig 3D, and Table 1 and Table EV3). Furthermore, p21 protein expression, which was highly correlated with CDKN1A RNA expression (Fig EV3D), was also associated with decreased synergy (Fig 3E and F). Supporting our hypothesis that p21-mediated G1 arrest could lead to reduced sensitivity to

lurbinectedin–berzosertib combination, thymidine-enforced G1 cell cycle arrest led to a significant reduction in efficacy of the combination (Fig EV3E). Small interfering RNA (siRNA) knockdown of p21 in the least synergistic cell line NCI-H889 resulted in a significant increase in synergy (Fig 3G), while overexpression of p21 in the synergistic DMS 114 cell line led to a decrease in synergy (Fig EV3F). These data are consistent with previous work in which p21 levels predicted reduced sensitivity to agents targeting downstream targets of ATR, CHK1, and Wee1 (Hauge et al, 2019).

ATM-dependent activation of p53 followed by p53-dependent p21 upregulation is a canonical pathway that regulates p21 expression in response to DNA damage (Smith et al, 2020). TP53/p53 is mutated in most SCLC tumors (George et al, 2015), suggesting frequent abrogation of the ATM-p53-p21 axis in SCLC. To determine the impact of the ATM-p53-p21 pathway on lurbinectedin–berzosertib synergy, we utilized two cell lines that exhibited low synergy and high expression of CDKN1A which were TP53 mutated (NCI-H889) or TP53 intact (NCI-H146). Upon lurbinectedin treatment, p21 protein levels were reduced in NCI-H889 cells, whereas NCI-H146 cells displayed an increase in p21 (Fig EV4A). P21 decrease in NCI-H889 cells is expected as lurbinectedin inhibits RNA Pol-II and, due to the rapid turnover rate of p21 protein and RNA, inhibition of RNA Pol-II causes rapid decreases in p21 (Al-Haj et al, 2012). Increased p21 in NCI-H146 cells is consistent with the ability of both DNA-damaging agents and transcription inhibitors to increase p21 expression in an ATM-p53-p21 axis-dependent manner (Smith et al, 2020). Consistent with the importance of ATM in the TP53-intact setting, addition of the ATM inhibitor KU60019 (Golding et al, 2009) significantly increased the synergy of lurbinectedin–berzosertib in NCI-H146 cells. Conversely, in NCI-H889 cells, ATM inhibition was less effective at increasing synergy (Fig EV4B and C).

Together, our *in vitro* data show that high CDKN1A/p21 expression irrespective of TP53 status predicts decreased synergy of lurbinectedin–berzosertib, that is, less impact on lurbinectedin IC₅₀s with the addition of berzosertib, while higher ERCC5/XPG expression predicts greater sensitivity, that is, decreased lurbinectedin IC₅₀s in the presence of berzosertib. We confirmed these results in two small-cell organoid models (Fig EV4D–G). We propose

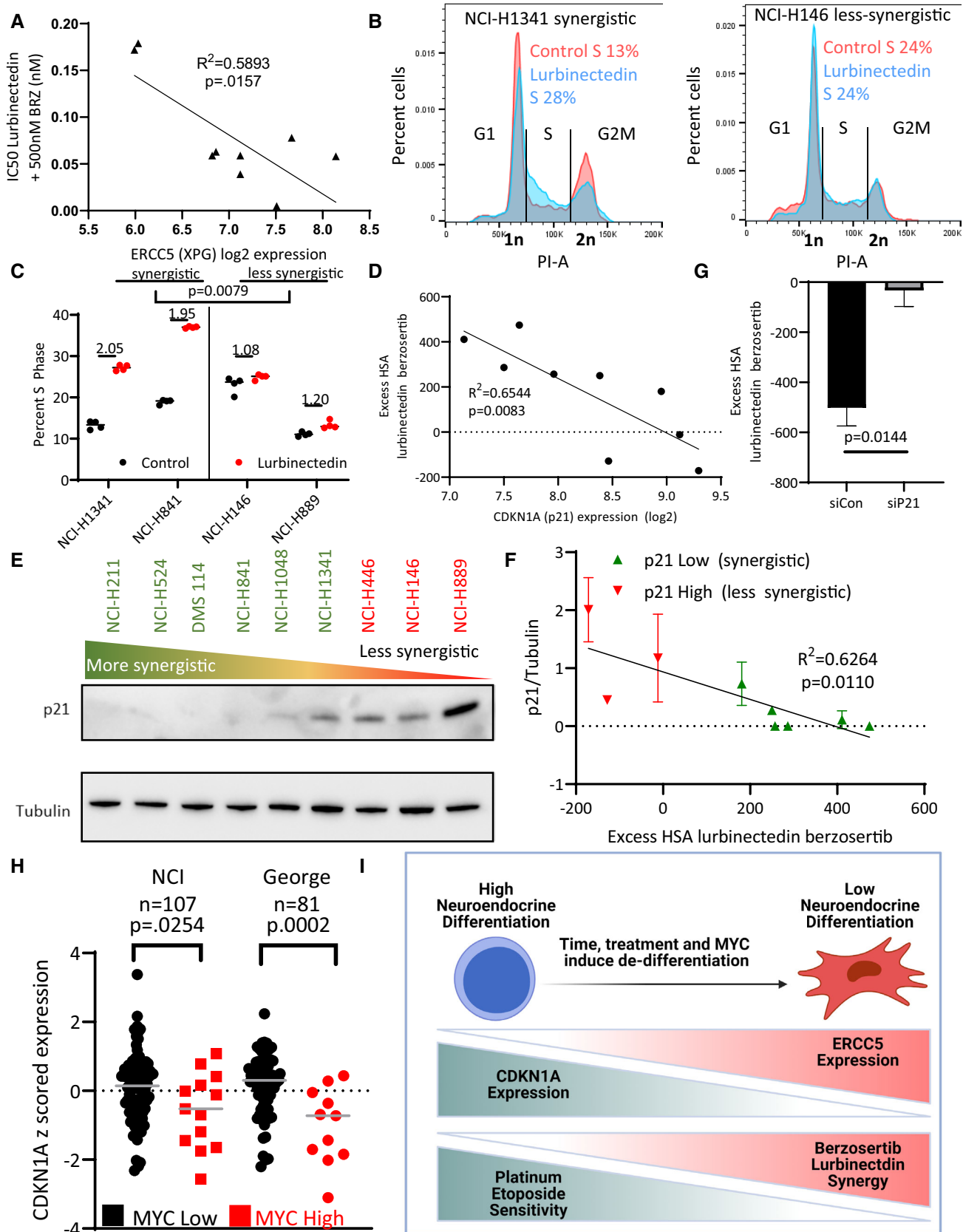


Figure 3.

Figure 3. p21 inhibits lurbinectedin and berzosertib synergy, with greater synergy expected in recalcitrant MYC-driven non-NE cells.

- A *ERCC5* (XPG) expression inversely correlated with combination efficacy, that is, when the expression of *ERCC5* (XPG) was high, the combination was more effective. The IC₅₀ of lurbinectedin in the presence of 500 nM berzosertib (combination efficacy) was determined in nine cell lines after assessing all nine lines using a 10 × 10 matrix of lurbinectedin and berzosertib combinations (replicates = 3, n = 1); Pearson correlation was assessed in PRISM.
- B, C Lurbinectedin increased S-phase arrest as assessed by propidium iodide staining. Synergistic cell lines had a greater increase in S-phase than less synergistic lines. Synergistic (NCI-H841 and NCI-H1341) and less synergistic (NCI-H146 and NCI-H889) cell lines were treated with ±1 nM lurbinectedin ±2 μM berzosertib. Quantification in (C) is representative of four technical replicates of 10,000 cells per group biological replicates, n = 3, with fold change in S-phase cells being compared between synergistic and less synergistic cell lines using an unpaired two-tailed Student's *t*-test in PRISM. These data are also described in Appendix Fig S4C.
- D *CDKN1A* (p21) RNA expression inversely correlated with synergy of lurbinectedin and berzosertib, indicating that *CDKN1A* (p21) could potentially inhibit synergy. Pearson correlation was assessed in PRISM.
- E, F Synergy was lower in cells that displayed higher expression of p21 protein. (E) Cell lines were ordered by HSA synergy score (most-to-least synergy, left to right), and p21 protein expression was assessed by immunoblotting. (F) Quantitation of p21 protein compared to control tubulin across two biological replicates, error bars represent SD; Pearson correlation was assessed in PRISM.
- G Knockdown of *CDKN1A* (p21) in NCI-H889 cells led to increased synergy. NCI-H889 cells were treated with siRNA against control or *CDKN1A* (p21), followed by dosing in a 10 × 10 matrix format in triplicate with lurbinectedin and berzosertib and collection after 72 h. HSA synergy was determined and summed across the 10 × 10 matrix, graph represents the average of three independent experiments of 10 × 10 matrixes in triplicate (technical replicates), error bars = SD, and unpaired two-tailed Student's *t*-test performed in PRISM.
- H High MYC family member patient samples had lower *CDKN1A* (p21) expression consistent with high MYC family member expression causing decrease in *CDKN1A* (p21). In two independent SCLC datasets, *MYC*, *MYCL*, and *MYCN* expressions were z-scored (within each database) and the max MYC family member z-score expression was determined for each sample. Those samples which were greater than 1 SD above average were considered to be high MYC family member expressing. *P*-values are indicative of unpaired two-tailed Student's *t*-test assessed in PRISM.
- I As SCLC progresses, cancer cells progress from a NE-differentiated state to a non-NE state. These non-NE cells have a lower expression of *CDKN1A* (p21) and a higher expression of *ERCC5* (XPG). These are characteristics that make them less responsive to the standard first-line platinum/etoposide regimen, however, this should make them more sensitive to the combination of lurbinectedin and berzosertib.

Source data are available online for this figure.

CDKN1A/P21 as the primary negative determinant of synergy for lurbinectedin–berzosertib while *ERCC5*/XPG expression is required for lurbinectedin-induced DNA damage and determines combination efficacy (Table EV3).

MYC expression and non-NE differentiation are associated with lurbinectedin–berzosertib synergy

SCLC is characterized by NE differentiation which decreases as tumors progress and following chemotherapy (Wagner *et al*, 2018; Ireland *et al*, 2020). NE differentiation as characterized by a previously validated 50-gene signature (Zhang *et al*, 2018) negatively associated with lurbinectedin–berzosertib synergy, with the highest synergy observed in non-NE SCLC cells (Fig EV4H). MYC, as a driver of both non-NE differentiation and decreased *CDKN1A* expression (Fiorentino *et al*, 2016; Ireland *et al*, 2020), could potentially cause the increased synergy of lurbinectedin–berzosertib in non-NE cells. In metastatic small-cell patient tumors, *MYC* expression was negatively correlated with NE differentiation (Appendix Fig S2A). In our metastatic dataset along with additional primary SCLC patient tumor, circulating tumor cell-derived xenograft, and patient-derived xenograft (PDX) datasets, samples overexpressing *MYC* paralogs (*MYC*, *MYCL*, or *MYCN*) consistently displayed significantly lower *CDKN1A* expression (Fig 3H and Appendix Fig S2B). Similar results were observed in SCLC cell lines where *MYC* expression negatively correlated with *CDKN1A* expression and NE differentiation (Appendix Fig S2C and D).

Confirming the recalcitrance of high MYC non-NE SCLC tumors (Alves Rde *et al*, 2014), of 50 hallmark gene sets, we found that the 2 gene sets predictive of MYC activity, MYC-targets-V1 and MYC-targets-V2, were, respectively, the first and third most highly associated with platinum resistance in SCLC patients (Horita *et al*, 2015; Liberzon *et al*, 2015; Appendix Fig S2E). MYC-targets-V1

significantly differentiated platinum/etoposide sensitivity (Appendix Fig S2F and G), with MYC-low patients displaying hazard ratios of 0.2130 and 0.1830 at the median and quartile levels, respectively, for platinum resistance (response duration of < 90 days to carboplatin etoposide therapy). These data indicate that MYC-low SCLCs were ~5-fold less likely to be resistant to carboplatin/etoposide therapy as compared to MYC-high tumors. Interestingly, SCLC cell lines with enrichment of MYC-targets-V2 were associated with increased lurbinectedin–berzosertib combination efficacy (Appendix Fig S2H). NE differentiation negatively correlated with *ERCC5*/XPG expression in multiple clinical datasets, with non-NE tumors displaying higher *ERCC5*/XPG expression (Appendix Fig S2I and J). Together, these data indicate that MYC-driven, non-NE tumors are resistant to platinum-based chemotherapy, but they may be uniquely sensitive to lurbinectedin–berzosertib combination due to high *ERCC5*/XPG and low *CDKN1A*/p21 expression (Fig 3I and Table EV3).

In vivo synergy and schedule dependence of lurbinectedin–berzosertib combination

In our previous work and ongoing clinical trials, we have observed efficacy using a dosing regimen in which topotecan is treated daily for 5 days while berzosertib is dosed on days 2 and 5 of 7-day cycles (Thomas *et al*, 2021). Adjusting this model for the standard dosing of lurbinectedin, we assessed the efficacy of lurbinectedin and berzosertib in mouse models of SCLC dosing lurbinectedin on day 1, followed by berzosertib on days 2 and 5 of 7-day cycles. In a patient-derived xenograft (PDX) model of SCLC, PDX-06, lurbinectedin was extremely effective by itself almost completely inhibiting tumor growth. Likely due to the high efficacy of lurbinectedin alone, we observed minimal increased activity with the addition of berzosertib (Fig 4A). We assessed a separate cohort of mice treated in the same manner for target engagement 24 h after dosing and found

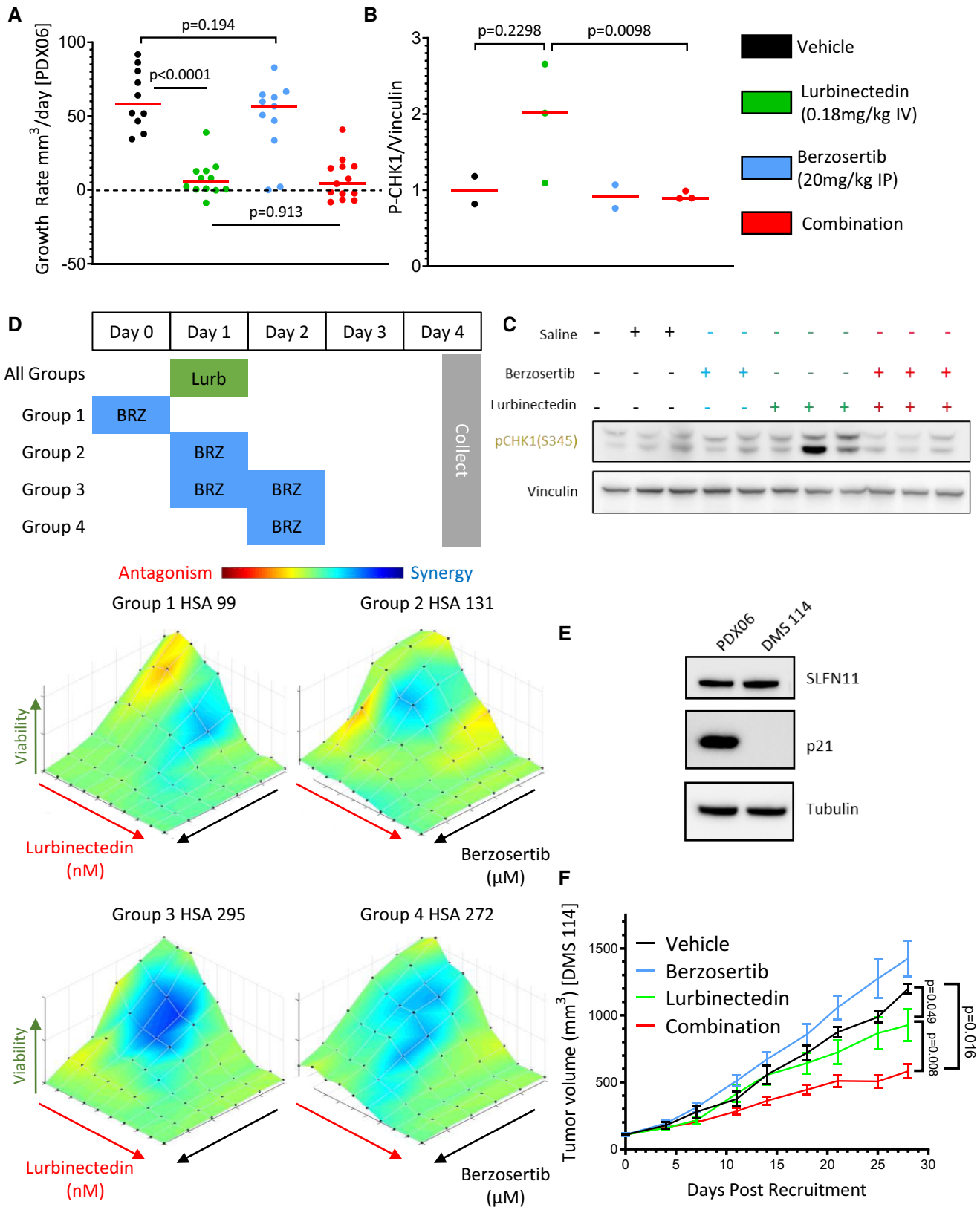


Figure 4.

Figure 4. Berzosertib improves lurbinectedin efficacy *in vivo*.

- A Lurbinectedin was very efficacious and the addition of berzosertib did not significantly improve the almost complete inhibition of tumor growth caused by lurbinectedin in a PDX mouse model of SCLC (PDX-06), $n = 10\text{--}13$ mice per group, red bar indicates median, and P -values are indicative of unpaired two-tailed Student's t -test calculated in PRISM. Tumor-bearing mice were treated with lurbinectedin (0.18 mg/kg IV) and berzosertib (20 mg/kg IP) in a format mirroring our clinical trial with topotecan/berzosertib in SCLC, lurbinectedin day 1, and berzosertib days 2 and 5 of a 7-day cycle. Tumor growth rates were calculated as the difference between the initial tumor size and the final tumor size after death of the mouse due to toxicity or progression of tumor divided by the total number of days treated.
- B, C We determined that lurbinectedin trended towards increasing p-chk1 while berzosertib cotreatment significantly reduced p-chk1 activation *in vivo* indicating target engagement. Tumors from mice with the same tumor type (PDX-06) as in (A) were collected 24 h after being dosed with the indicated drugs. P -values are indicative of unpaired two-tailed Student's t -test calculated in PRISM; western blot for (C) is also shown in Appendix Fig S2A.
- D Lurbinectedin synergy was maximal in DMS 114 cells when treated on day 1 with lurbinectedin and days 1 and 2 with berzosertib. DMS 114 cells were treated with lurbinectedin and berzosertib in a 10×6 matrix format replicates = 4, $n = 1$. All groups were treated for 24 h with lurbinectedin, while group 1 was pretreated with berzosertib, group 2 was cotreated with berzosertib, group 3 was co- and posttreated with berzosertib, and group 4 was posttreated with berzosertib for 24 h. At the end of 5 days, cells were collected and synergy was assessed across the matrixes.
- E PDX-06 (less synergistic) and DMS 114 (more synergistic) cells had equivalent SLFN11, while DMS 114 had less p21. p21 and SLFN11 protein expression was assessed using immunoblotting.
- F Berzosertib cotreatment improved lurbinectedin efficacy in a DMS 114 xenograft mouse model of SCLC. Lurbinectedin was dosed at 0.18 mg/kg (intravenous) and berzosertib at 50 mg/kg (oral) for four 7-day cycles in dosing regimens consistent with (D) $n = 10$ mice per group. Consistent with our results in (D), the greatest degree of increased and overall efficacy was seen in group 3 (lurbinectedin day 1, berzosertib days 1 and 2), P -values are indicative of paired two-tailed Student's t -test calculated in PRISM, and error bars are representative of SEM. The other groups are displayed in Appendix Fig S7H and I.

Source data are available online for this figure.

that although results were variable, lurbinectedin caused an increase in p-CHK1, a downstream target of ATR, while berzosertib cotreatment reduced p-CHK1 activation (Figs 4B and C, and EV5A). Even though in initial testing, mice treated for a single cycle displayed no toxicity, the combination was toxic with repeated dosing. In particular, significant damage at tail veins of lurbinectedin and combination dosed mice was observed, and the majority of these mice were sacrificed due to weight loss (Appendix Fig S2K).

To better assess combination efficacy, we utilized a more aggressive PDX model PDX-03. PDX-03 displayed reduced *CDKN1A*/p21 and *SLFN11* expression and increased aggressiveness as compared to PDX-06, and thus we expected it to be more resistant to lurbinectedin and to display increased synergy for the combination (Appendix Fig S2L). To reduce toxicity, we doubled the volume of lurbinectedin dosed for tail vein injections (100–200 μ l). We found in this model that lurbinectedin and combination efficacy were significantly reduced, potentially due to increased overall tumor aggressiveness in the PDX-03 model (Fig EV5B and C, and Appendix Fig S2L). Although toxicity was reduced in the PDX-03 model, several mice in lurbinectedin and combination treatment arms were sacrificed due to toxicity (Fig EV5D). While the combination showed little increased efficacy as compared to lurbinectedin alone, we found trends toward decreased marker of proliferation ki-67 and increased cleaved caspase-3 and necrosis in tumors treated with the combination as compared to either lurbinectedin or berzosertib alone (Fig EV5E). The heightened toxicity of lurbinectedin and combination may potentially be due to the strain of mice, as severe combined immunodeficient (SCID) are inherently more sensitive to DNA-damaging agents (Biedermann *et al*, 1991), we thus decided to utilize a nude mouse model which would likely have reduced toxicity.

We assessed in two cell lines (NCI-H446 and DMS 114) whether dosing sequence was critical for lurbinectedin–berzosertib synergy by dosing berzosertib before, after, or at the same time as lurbinectedin (lurbinectedin day 1 and berzosertib day(s) 0/1/2/1 + 2). The greatest synergy was observed when berzosertib was cotreated with lurbinectedin and then maintained after the removal of

lurbinectedin (Figs 4D and EV5F). As DMS 114 cells had a lower NE score than NCI-H446 cells, we chose the DMS 114 cells to represent the recalcitrant non-NE subtype for our mouse model. We were able to derive a cell line from the NE PDX mouse model PDX-06, although unfortunately, PDX-03 cells did not take to culture as well. We determined that PDX-06 cells expressed similar levels of SLFN11, but significantly higher p21 than the non-NE DMS 114 cells (Fig 4E). Consistent with our findings that p21 is a critical determinant of synergy, although DMS 114 cells were more resistant to lurbinectedin alone, they displayed significantly higher synergy for the lurbinectedin–berzosertib combination than PDX-06 cells (Fig EV5F and G). In a xenograft model of DMS 114 cells in nude mice, the combination of berzosertib and lurbinectedin was significantly more effective as compared to either agent alone (Fig 4F). Consistent with our *in vitro* data, the greatest degree of synergy and antitumor activity was observed with lurbinectedin treatment on day 1 and berzosertib dosed on days 1 and 2. This combination was also well tolerated in nude mice as 0/10 mice succumbed to toxicity as compared to 11/13 and 3/11 in the PDX-06 and PDX-03 combination arms of the SCID models, respectively (Figs 4F, and EV5H and I). Of note, berzosertib administered after lurbinectedin was the least synergistic in the DMS 114 xenograft model (Fig EV5H) and suboptimal in both cell lines (Figs 4D and EV5F). As both the PDX-06 and PDX-03 models were treated with lurbinectedin first followed by berzosertib, the low synergy observed in these models could be due to the sequential model of dosing. Notably, plasma drug levels achieved in the xenograft experiments were comparable to those of human patients (Fig EV5J and K; Fudio *et al*, 2021; Thomas *et al*, 2021).

Overall, lurbinectedin causes DNA damage in an *ERCC5*/XPG-dependent manner, at which point cells can either be arrested in a *CDKN1A*/p21 (G1-phase checkpoint) or ATR (S-phase checkpoint)-dependent manner. Cells arrested in S-phase due to ATR activation will either die via SLFN11-dependent lethal replication fork instability or resolve DNA damage through HR and continue through the cell cycle. The addition of berzosertib causes cells that would normally halt cell cycle progression in S-phase to continue through the

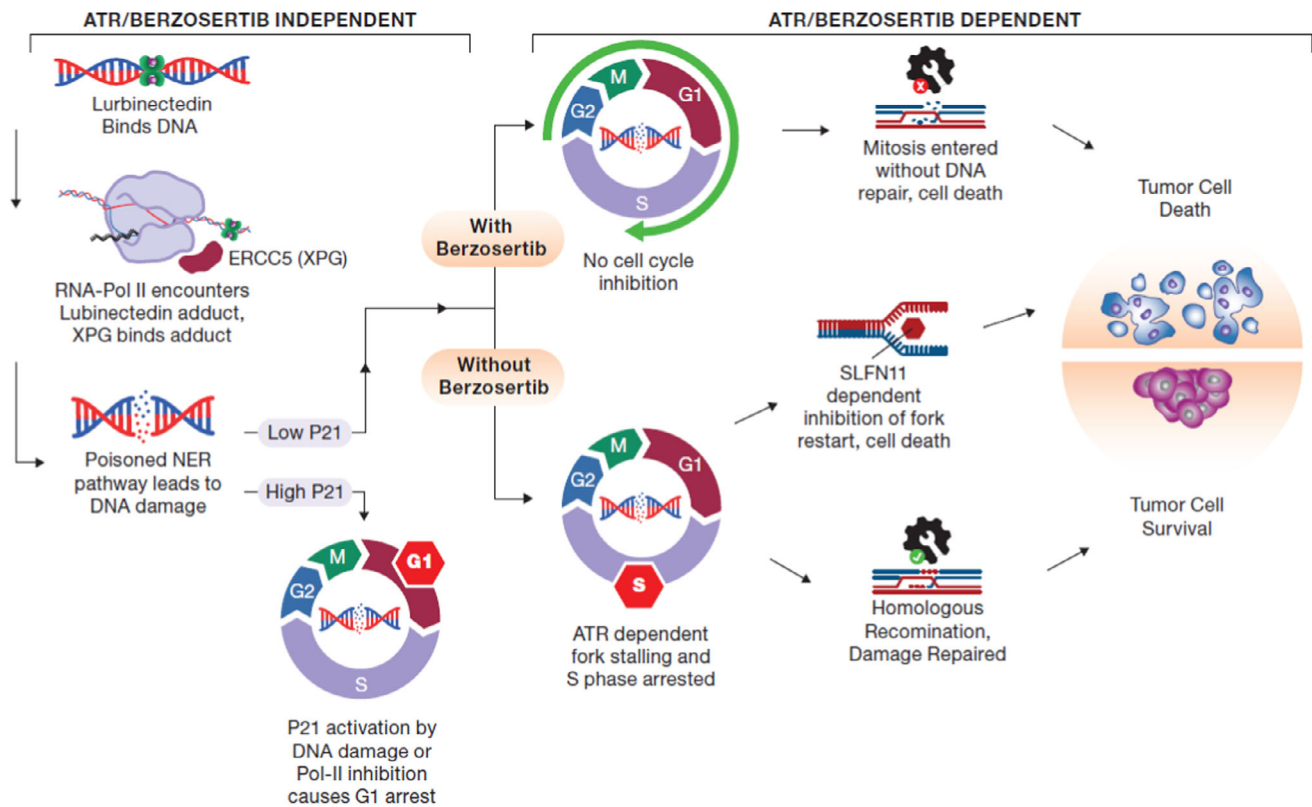


Figure 5. Berzosertib synergizes with lurbinectedin by inhibiting S-phase arrest and inducing mitotic catastrophe.

Lurbinectedin binds to DNA and then induces DNA damage through nucleotide excision repair poisoning in an XPG (*ERCC5*)-dependent manner. P21 (*CDKN1A*) activation can lead to G1 arrest in cells, however, those cells which have low *CDKN1A* expression are halted in S-phase in an ATR-dependent manner. Cells paused in S-phase either die through inability to restart fork progression in a SLFN11-dependent manner, or the DNA damage is repaired through homologous recombination. ATR inhibition with berzosertib treatment leads to a loss of the intra-S-phase checkpoint and thus cells enter mitosis with unrepaired DNA damage and ultimately undergo mitotic catastrophe. XPG and p21 are determinants of response even in the presence of berzosertib as they are required for the initial DNA damage or G1 arrest, respectively, processes which are upstream of ATR-dependent S-phase arrest.

cell cycle despite DNA damage and undergo mitotic catastrophe. XPG-dependent induction of DNA damage or p21-dependent G1 arrest occur upstream of ATR activation and thus are unaffected by berzosertib treatment. As such, *ERCC5/XPG* and *CDKN1A/p21* are both critical determinants of lurbinectedin–berzosertib efficacy (Fig 5 and Table 1 and Table EV3).

Discussion

SCLC is a recalcitrant disease, and the majority of patients die of chemotherapy-resistant disease. We determined that the ATR inhibitor berzosertib strongly synergized with approved second-line chemotherapeutic lurbinectedin in SCLC, particularly in MYC-high, chemoresistant, non-NE models. Combination synergy is dependent on the ability of lurbinectedin to induce DNA damage, and berzosertib to augment this damage by inhibiting cell cycle checkpoints leading to mitotic catastrophe. We identified two main factors which determine response to the combination. First, the ability of lurbinectedin to cause DNA damage is reliant upon NER, and in particular, the expression of *ERCC5/XPG*. Second, the expression of *CDKN1A/p21*

is predictive of synergy, with higher *CDKN1A/p21* expression leading to decreased synergy. Importantly, both factors are associated with NE differentiation and MYC overexpression, with recalcitrant high-MYC, non-NE tumors displaying increased *ERCC5/XPG* and decreased *CDKN1A/p21* leading to increased synergy of the combination.

SCLC may be particularly responsive to this combination due to the frequent mutations of *TP53* and thus decreased ability of cells to activate *CDKN1A/p21* in response to DNA damage. Previous work has indicated that ATM inhibitors were required for ATR inhibitors to synergize effectively with lurbinectedin (Lima *et al*, 2016). We found that in the majority of SCLC cell lines, ATR and lurbinectedin were synergistic in the absence of ATM inhibitors, potentially due to the high frequency of loss-of-function *TP53* mutations. Furthermore, ATM inhibition was more impactful in the rare *TP53*-proficient cells as compared to *TP53*-mutant cells.

HR deficiency led to an increase in lurbinectedin efficacy. This observation is consistent with previous findings in breast cancer where HR-deficient breast cancers, particularly *BRCA2*-mutant tumors, were more likely to respond to lurbinectedin than *BRCA1/2* wild-type tumors (Cruz *et al*, 2018). Classical HR deficiency due to

HR pathway member mutation (i.e., *BRCA1/2*) is rare in SCLC (Sato *et al*, 2013; George *et al*, 2015; Farago *et al*, 2019; Simpson *et al*, 2020). However, based on our results in the *BRCA2*-KO DT40 cells where synergy was reduced but not abolished, we believe that the addition of berzosertib would still be meaningful in an HR-deficient setting. This is supported by our results in the SCLC cell line NCI-H1048, in which lurbinectedin and berzosertib were synergistic. This is noteworthy as NCI-H1048 cells were ~10-fold more sensitive to DNA-damaging agents (lurbinectedin and topotecan) as compared to other SCLC cell lines tested and displayed low *BRCA2* expression indicating this line may be HR deficient or have reduced HR competency.

Consistent with recently published work (Kundu *et al*, 2021), *SLFN11* expression trended toward predicting lurbinectedin efficacy, and *SLFN11*-KO reduced lurbinectedin efficacy and increased lurbinectedin–berzosertib synergy. However, we found that *SLFN11* expression across cell lines did not correlate with synergy and that there was still significant synergy *in vitro* and *in vivo* even in SCLC models with high expression of *SLFN11*. While cell lines/tumors with high *SLFN11* are more sensitive to lurbinectedin alone, thus reducing the impact of adding an ATR inhibitor, lurbinectedin–berzosertib synergy is not mechanistically dependent on *SLFN11* expression levels. This is supported by *ERCC5/XPG*-knockout cells which are also resistant to lurbinectedin, but unlike *SLFN11*-knockout cells, they display decreased synergy and lurbinectedin efficacy cannot be rescued with the addition of berzosertib. Based on these data and the high degree of heterogeneity in second-line SCLC, we are not currently stratifying patients based on *SLFN11* or HR status although we will continue to observe these parameters.

In a previous clinical trial, lurbinectedin in combination with the topoisomerase II inhibitor doxorubicin failed to improve survival as compared to standard of care (Helwik, 2021). This may be due to similar mechanisms of action for both agents as both lurbinectedin and doxorubicin cause DNA damage through poisoning DNA–protein interactions (lurbinectedin binds DNA and XPG while doxorubicin binds DNA and topoisomerase II), likely leading to similar resistance and repair mechanisms (i.e., HR and *SLFN11*). We have demonstrated that lurbinectedin has much greater synergistic potential with ATR inhibitors, in particular berzosertib, than with doxorubicin as part of our initial screen. In light of the improved synergy of lurbinectedin and berzosertib as compared to doxorubicin, and the diverse mechanisms of action, we believe lurbinectedin and berzosertib may have improved efficacy in SCLC patients as compared to lurbinectedin and doxorubicin.

Berzosertib is currently being assessed in combination with topotecan for the treatment of SCLC and has so far shown promise leading to durable responses in several patients (Thomas *et al*, 2018, 2021). Both topotecan and lurbinectedin synergized highly with berzosertib based on our work, and although lurbinectedin was more potent in 7/9 SCLC cell lines tested, due to different dosing schemes and bioavailability whether this will impact efficacy in patients is unclear. The combination of berzosertib and topotecan has, however, shown the greatest response in patients with high-NE differentiation (Thomas *et al*, 2021), whereas our work has shown that the lurbinectedin–berzosertib combination is likely to have the greatest degree of improvement in non-NE patients. In particular, the increased *ERCC5/XPG* in non-NE tumors should only effect lurbinectedin–berzosertib combination efficacy, with little to no

effect on topotecan–berzosertib combination efficacy. With continued assessment of biomarkers for both combinations, in future, second-line patients with NE SCLC could be treated with topotecan–berzosertib while patients with non-NE SCLC may be treated with lurbinectedin–berzosertib.

Based on these results, a phase I/II clinical trial with a combination of lurbinectedin and berzosertib was launched (NCT04802174) to determine antitumor efficacy in SCLC patients. In light of our findings described here, it will be important to assess whether NE differentiation along with *ERCC5/XPG*, *CDKN1A/p21*, and *MYC* family member expression are predictors for the efficacy of the combination treatment in patients.

Materials and Methods

Cell lines

NCI-H211, NCI-H524, DMS-114, NCI-H841, NCI-H1048, NCI-H1341, NCI-H446, NCI-H146, NCI-H889, and U2OS DRGFP (Nakanishi *et al*, 2011) cell lines were purchased from ATCC. NCI-H211, NCI-H889, NCI-H1048, NCI-H1341, and U2OS DRGFP cell lines are female and the rest are male, additional information for cell lines can be observed in Table 1. Cell lines were authenticated using short tandem repeat analysis, and were monthly tested for mycoplasma contamination. PDX-03 and PDX-06 were derived from a male and a female SCLC patient, respectively. Cell medium was RPMI-1640 supplemented with 10% FBS for all lines to maintain consistency. DT40 (chicken cell lines) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS 10% and chicken serum 5%. Cells were grown at 37°C and 5% CO₂.

Organoids

Human small-cell autopsies/biopsy specimens were obtained from the CCR-NCI biobank following patient consent and NIH institutional review board (IRB) and ethical approval. The pathological specimens were immediately stored in storage media (1× DMEM/F12, 1× Glutamax, and 10 mM HEPES buffer) on ice. The tissues were immediately subjected to enzymatic disassociation. Human small-cell organoids were cultured in minimal basal media (MBM) as described previously (Kim *et al*, 2019; Sedlack *et al*, 2022). Briefly, PDOs were cultured in drop of growth factor-reduced basement membrane extract (BME; Corning), and medium was refreshed every 4 days. The culture media contain DMEM/F12 (Gibco) with 50 ng/ml EGF (StemCell Technologies), 100 nM IGF-1 (StemCell Technologies), 1× N2 supplement (Thermo Fisher Scientific), 1× B27 (Thermo Fisher Scientific), and 10 μM Y-27632 (StemCell Technologies). The organoids were passage through shear stress with cold 1 U/ml dispase/DMEM/F12 solution (StemCell Technologies) followed by trypsin–EDTA (Invitrogen). Organoids were grown at 37°C and 5% CO₂. PDX-456648 was derived from a male with small-cell cancer of the bladder and PDX-592484 was from a male with small-cell lung cancer.

Mouse tumor models

Eight-week-old male or female NSG mice (NOD.Cg-Prkdc scid Il2rg tm1Wjl/SzJ; # 005557, The Jackson Laboratory, Bar Harbor, ME)

were implanted subcutaneously with fresh patient-needle biopsy supported with Matrigel (Corning) to generate our PDX model. Mice were treated weekly with lurbinectedin at 0.18 mg/kg intravenously on day 1, and berzosertib at 20 mg/kg intraperitoneally on day 1/5. For our pharmacodynamic study, mice were harvested 24 h after the second dose of lurbinectedin and berzosertib. The Animal Study Protocol was approved and followed the Frederick National Laboratory Animal Care and Use Committee guidelines.

For the DMS 114 xenograft mouse model, 8- to 10-week-old female H2dRag2 mice (C;129P2-H2d-Rag2 < tm1Fwa IL2rgtm1; Taconic, Denmark) were used. Mice were treated with weekly cycles consisting of the following arms: vehicle, lurbinectedin alone, berzosertib alone, lurbinectedin day 1 berzosertib day 1, lurbinectedin day 1 berzosertib day 2, and lurbinectedin day 1 berzosertib days 1 and 2. Lurbinectedin was dosed at 0.18 mg/kg intravenously, and berzosertib at 50 mg/kg orally. The study design and animal usage were approved by local animal welfare authorities (Regierungspräsidium Darmstadt, Hesse, Germany, protocol registration number DA4/Anz.1040). All animal housing and husbandry were performed according to veterinary standards.

Screening and synergy

The screen analyzed in Fig 1A–C, and Tables EV1 and EV2 was performed in NCI-H446 SCLC cells as described previously (Thomas *et al*, 2021); the data for this screen (12831) are available at <https://matrix.ncats.nih.gov/>. For further synergy analysis, cells were seeded at 1,000 cells per well in 384-well plates, and collected using Cell Titer Glo (Promega, Madison, WI, USA) 72 h after drug treatment. Highest single-agent (HSA) synergy was determined by calculating the difference between the most effective single agent and the combination of agents. All matrix formats stated in this work will be presented as lurbinectedin x berzosertib with one control, that is, a 10 × 6 matrix would have 10 concentrations of lurbinectedin with 1 being 0 across 6 concentrations of berzosertib. For thymidine-based experiments, cells were plated at 1,000 cells per well in 384-well plates. On the next day, they were treated with thymidine 2 mM for 18 h followed by a 6–9 h release and then treated with thymidine at 2 mM ± 1 nM lurbinectedin ± 2 μM berzosertib for 72 h.

Comet assay

DMS 114 cells were plated at 500 K cells per well in a six-well plate, and cells were treated with ±1 nM lurbinectedin and ±2 μM berzosertib for 6 h and collected. Comet assays were performed using the Comet Assay Single Cell Gel Electrophoresis Assay (Trevigen, Gaithersburg, MD) according to manufacturer's instructions. Images were captured using BioSpa Live Cell Analysis System (Biotek) and comet tail length was calculated using OpenComet (<https://cometbio.org/>), a plugin for the image processing program ImageJ.

Immunoblotting

For immunoblotting experiments, we utilized the following antibodies: –γH2AX(S139) (Cell Signaling #80312), H2AX (Sigma-Aldrich # 07627), pRPA32(T21) (Abcam #ab109394), RPA32 (Cell Signaling #35869), –pATR(T1986) (Cell Signaling #58014 and #30632), ATR (Cell Signaling #13934), pCHK1(S345) (Cell Signaling

#12302), CHK1 (Cell Signaling #2360), pATM(S1981) (Abcam #ab81292), ATM (Cell Signaling #2873), pCHK2(T68) (Cell Signaling #2197), CHK2 (Cell Signaling #6334), pDNA-PK(S2056) (Abcam# ab18192), DNA-PK (Abcam# ab32566), vinculin (Sigma-Aldrich # V9131), histone H3 (Sigma-Aldrich #07-690), and SLFN11 (Santa-Cruz #sc-374339). All antibodies were diluted at 1:1,000 except SLFN11 1:2,000, tubulin 1:4,000, and vinculin 1:4,000, and all secondary antibodies were diluted at 1:4,000.

Immunofluorescence

Cells were fixed with 2% paraformaldehyde/PBS for 20 min at room temperature, washed three times in PBS, cells were deposited on slide glass by cytopspin, then put in prechilled 70% ethanol for 20 min, and blocked with 5% BSA/PBSTT (PBS-containing 0.5% Tween 20 and 0.1% Triton X-100) for 30 min. Slides were incubated with primary antibodies for 2 h and secondary antibodies for 1 h at room temperature. Images were captured with a Zeiss LSM 780 confocal microscope.

DNA combing

DMS 114 cells were treated with ±1 nM lurbinectedin and ±2 μM berzosertib for 6 h followed by treatment with CIDU for 30 min and then IDU for 30 min and collected. DNA combing assay was performed as described previously (Josse *et al*, 2014; Thomas *et al*, 2021).

Assessment of mitotic catastrophe by fluorescence microscopy

Using fluorescence microscopy, mitotic catastrophe was identified based on the characteristic morphologies of the DAPI-stained nuclei. Cells were seeded in 12-well plates on sterilized coverslips and treated the next day with vehicle, 1 nM lurbinectedin, 2 μM berzosertib, or combination of both for 6 h. Nocodazole was added into media for 3 h during the drug treatment (to enrich mitotic cells), then nocodazole and drugs were removed and fresh media were added for 45 min. After washing cells with cold PBS, fixation was done in 4% paraformaldehyde in PBS for 15 min at RT, followed by permeabilization with 0.2% Triton-X 100/PBS for 15 min. After washing with PBS, cells were mounted with DAPI (VECTASHIELD, Vector Laboratories). Images of nuclei were captured with Zeiss LSM 880 confocal microscope with 63× objective lens.

RNA analysis, NE calculation, and enrichment analysis

RNA data and neuroendocrine score calculation for cell lines are publicly available on CellMiner (Tlemsani *et al*, 2020). Patient tumor and CDX datasets of SCLC were obtained from publicly available datasets (integrated genomic and transcriptomic analysis of small-cell lung cancer reveals inter- and intratumoral heterogeneity and a novel chemotherapy refractory subtype; under revision, data available at dbGaP Study Accession: phs002541.v1.p1; Sato *et al*, 2013; George *et al*, 2015; Farago *et al*, 2019; Simpson *et al*, 2020). High MYC was determined in the patient and CDX datasets through assessing the expression of MYC, L-MYC, and N-MYC, z-scoring them, and selecting the max z-scored expression; high-MYC was defined as 1 SD above mean. ssGSEA hallmark and

The paper explained

Problem

Small-cell lung cancer (SCLC) is a recalcitrant disease with a 9% 5-year survival rate. This recalcitrance is driven in part by a high degree of intratumoral heterogeneity. SCLC tumors present with high neuroendocrine (NE) differentiation and evolve, driven by MYC, to a non-neuroendocrine (non-NE) state. DNA-damaging RNA Pol-II inhibitor lurbinectedin was approved for second-line therapy in 2020, however, only 35% of patients respond and the vast majority of patients still succumb to their disease. Combinatorial approaches are required to improve the efficacy of lurbinectedin.

Results

Here, we find that lurbinectedin shows high synergy with the ataxia telangiectasia–mutated and rad3-related (ATR) inhibitor berzosertib. Efficacy of the combination is dependent on expression of *ERCC5/XPG* and can be inhibited by *CDKN1A/p21*-dependent G1 arrest. Based on the high expression of XPG and decreased *CDKN1A* in MYC-high non-NE SCLC, we propose that non-NE SCLC is likely to be most susceptible to the combination of lurbinectedin and berzosertib.

Impact

ATR inhibition synergizes with lurbinectedin in SCLC models. *CDKN1A/p21* and *ERCC5/XPG* expression are determinants of response. An ongoing clinical trial is examining the combination of lurbinectedin and berzosertib in relapsed SCLC (NCT04802174).

neuroendocrine enrichment scores were computed using the GSVA R/Bioconductor package (Hanzelmann *et al*, 2013; Liberzon *et al*, 2015; Zhang *et al*, 2018).

Metaphase spread

DMS 114 cells were plated at 1 million cells per 10 cm plate, and treated with ± 1 nM lurbinectedin and ± 2 μ M berzosertib for 6 h and collected. Metaphase spread was performed as described https://ccr.cancer.gov/sites/default/files/metaphase_preparation_from_adherent_cells.pdf. Cells were imaged using a Leica Thunder Imager.

Flow cytometry

Cells were treated with ± 1 nM lurbinectedin and ± 2 μ M berzosertib for 6 h, and ethynyl deoxyuridine (EDU) at 100 μ M was added for the last hour prior to collection. EdU was detected using flow cytometry (Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay, Invitrogen), DNA using DAPI, and γ H2AX was assessed by staining cells with JBW301 (Millipore Sigma). Data were acquired using a BD LSRFortessa Flow Cytometer and analyzed using FlowJo.

Statistics

Statistical analysis was performed using Prism 9.3.1 (GraphPad). *P*-values < 0.05 were considered statistically significant. For animal experiments, mice were randomized in an unbiased fashion. Researchers were not blinded during mouse experiments. Samples sizes were selected to, based on estimated efficacy data, give a 90% chance of observing statistically significant deviations at *P* < 0.05 in

efficacy between the combination and either individual treatment arm.

Patient data

NIH IRB, Office of Human Subjects Research Protections at NCI, approved the studies; all patients provided written informed consent for tumor sample sequencing. The experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available [online](#).

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Author contributions

Christopher W Schultz: Conceptualization; data curation; formal analysis; validation; investigation; visualization; methodology; writing – original draft; project administration; writing – review and editing. **Yang Zhang:** Data curation; investigation; methodology; writing – review and editing. **Rajaa Elmeskini:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Astrid Zimmermann:** Formal analysis; investigation; methodology; writing – review and editing. **Haiqing Fu:** Investigation; methodology; writing – review and editing. **Yasuhisa Murai:** Investigation; methodology. **Darawalee Wangsa:** Investigation; methodology. **Suresh Kumar:** Resources; investigation. **Nobuyuki Takahashi:** Conceptualization; investigation; writing – review and editing. **Devon Atkinson:** Investigation; methodology; writing – review and editing. **Liton Kumar Saha:** Investigation; methodology. **Chien-Fei Lee:** Investigation; methodology. **Brian Elenbaas:** Investigation; methodology; writing – review and editing. **Parth Desai:** Investigation; methodology; writing – review and editing. **Robin Sebastian:** Resources; investigation; methodology. **Ajit Kumar Sharma:** Methodology. **Melissa Abel:** Visualization. **Brett Schroeder:** Visualization. **Manan Krishnamurthy:** Validation. **Rajesh Kumar:** Data curation. **Nitin Roper:** Resources. **Mirit Aladjem:** Resources; writing – review and editing. **Frank T Zenke:** Resources; visualization; writing – review and editing. **Zoe Weaver Ohler:** Resources; visualization; writing – review and editing. **Yves Pommier:** Resources; visualization; writing – review and editing. **Anish Thomas:** Conceptualization; resources; supervision; funding acquisition; visualization; methodology; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

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For more information

Screening data for this paper along with other screens is available at <https://matrix.ncats.nih.gov/>. CellminerCDB is a website which allows access to and compiles many different primarily cell-line based datasets, this useful website can be found at <https://discover.nci.nih.gov/rsconnect/cellminerfdb/>.

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