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Original Article

Preclinical studies with ONC201/TIC10 and lurbinectedin as a novel combination therapy in small cell lung cancer (SCLC)

Nicholas R Liguori[1,2], Ashley Sanchez Sevilla Uruchurtu[2,3], Leiqing Zhang[2,3], Abbas E Abbas[2,3,4,5,7], Young S Lee[2], Lanlan Zhou[2,3,6,7], Christopher G Azzoli[5,6,7], Wafik S El-Deiry[2,3,5,6,7]

[1]Temple University, Lewis Katz School of Medicine, Philadelphia, Pennsylvania 19140, USA; [2]Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Warren Alpert Medical School, Brown University, Providence, Rhode Island 02903, USA; [3]Department of Pathology and Laboratory Medicine, Warren Alpert Medical School, Brown University, Providence, Rhode Island 02903, USA; [4]Department of Surgery, Brown University, Providence, Rhode Island 02912, USA; [5]Hematology/Oncology Division, Department of Medicine, Lifespan Health System and Brown University, Providence, Rhode Island 02903, USA; [6]Joint Program in Cancer Biology, Lifespan Health System and Brown University, Providence, Rhode Island 02903, USA; [7]Legorreta Cancer Center at Brown University, Providence, Rhode Island 02912, USA

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Abstract: The American Cancer Society estimates that ~15% of all lung cancers are categorized as small cell lung cancer (SCLC) with an overall five-year survival rate of less than 7%. Due to disease aggressiveness, more other malignancies, the standard of care is based on clinical efficacy rather than helpful biomarkers. Lurbinectedin is a small molecule RNA polymerase II inhibitor that binds the minor groove of DNA to induce double-strand breaks. Lurbinectedin has efficacy towards SCLC cells at sub-nM concentration and received accelerated FDA approval in 2020 for metastatic SCLC that progressed on platinum-based therapy. ONC201/TIC10 is a TRAIL pathway-inducing compound that with demonstrated clinical efficacy in H3K27M-mutated diffuse midline glioma and neuroendocrine tumors, in early phase clinical trials. We hypothesized that combining ONC201 and lurbinectedin may yield synergistic and targeted killing of SCLC cells. SCLC cell lines H1048, H1105, H1882, and H1417 were treated with ONC201 and lurbinectedin and cell viability was determined using a CellTiter-Glo assay using varying drug concentrations. Synergistic growth inhibition of SCLC cells was noted with combination of ONC201 and lurbinectedin. Induction of the integrated stress response mediator ATF4 and CHOP was observed with ONC201 and lurbinectedin along with induction of PARP cleavage indicative of apoptosis in response to cellular stress. Additionally, SCLC lines treated with the combination therapy displayed increased DNA breakage-related proteins such as phosphorylated Chk-1, Wee1 and γ-H2AX. Combination index revealed the most potent synergy occurred at the concentrations of 0.16 μM ONC201 and 0.05 nM lurbinectedin in the H1048 cell line, demonstrating highly efficient and selective killing of these tumor cells *in vitro*. While these therapies showed potency against the cell lines derived from SCLC patients, it is noteworthy that the combination showed significantly less toxicity to healthy human lung epithelial cells. Future studies could explore the combination of ONC201 and lurbinectedin in SCLC cell lines, SCLC patient-derived organoids, other tumor types, including in vivo studies and clinical translation.

Keywords: SCLC, lurbinectedin, chemotherapy, genomics, ONC201, TIC10

Introduction

Lung cancer is the second most common cancer diagnosed in the United States and is the malignancy with the highest mortality rate. SCLC comprises approximately 15% of all lung cancer diagnoses. Small cell lung cancer can be attributed to the man-made epidemic caused by cigarette smoking. Cigarette smoke contains polycyclic aromatic hydrocarbons, arsenic, and other DNA-damaging carcinogenic agents.

SCLC has a difficult natural history, with the disease tending to grow and spread quickly and aggressively. Doubling times are as short as

25-30 days and SCLC has the unique propensity for hematogenous spread [1]. Survival rates for SCLC patients are poor, and patients often face dismal prognoses upon presentation. Limited stage disease has a median survival time of 15-20 months and the overall five-year survival rate in SCLC is less than 7% [2]. Unfortunately, the survival for extensive disease upon presentation is measured in weeks, and the poor overall survival rate for SCLC patients remains unchanged in the past four decades [3]. Due to its aggressive nature, and the frequency of metastases and progressive disease upon presentation, SCLC does not conform to the clinical or genomic categorization that management of other malignancies may benefit from.

Though there is often significant response to first-line, platinum-based chemotherapy, typically combined with etoposide (response rates of up to 80% [1]), responses are short-lived, and the recurrence of disease is virulent and aggressively metastasizes. Further, even despite the initial efficacy of first-line treatments, these agents often lead to cause toxicities in patients, including hair loss, high-grade fatigue, cytopenias, nausea and diarrhea, making treatment even more difficult in those facing bleak prognoses. While the absence of available biomarkers makes finding breakthrough therapies difficult, it is increasingly important to investigate additional first- and second-line treatment options for the patients. The toxicity of the platinum-based chemotherapies, along with SCLC's high likelihood of developing resistance, validates the emphasis in investigating novel therapeutic combinations. This not only improves patients' outcomes but also improves their quality of life. Imipridones ONC201/TIC10 and other small molecule inhibitors show promise in various solid tumors and could be further tested in SCLC where there is an unmet clinical need for better therapeutics.

ONC201/TIC10, originally discovered as a TRAIL pathway inducing compound, is under investigation in multiple solid tumor types in *in vivo* and *in vitro* studies. Further, it is currently involved in FDA approved clinical trials in gliomas, lymphomas, leukemias, colorectal cancer, breast cancer, endometrial cancer, and paragangliomas. ONC201 has effective antitumorigenic properties, and it is well-tolerated by patients. As an oral drug, it offers a less invasive option for patients. ONC201 is administered weekly at a recommended phase 2 dose of 625 mg based on pharmacokinetics and pharmacodynamics in phase 1 testing, and preclinical dose intensification studies [4]. SCLC cells indicate a particular sensitivity to ONC201 due to its ability to induce extrinsic apoptosis via the induction of TRAIL and DR5, as well as activated caspase-8 [5]. It is noteworthy that DR5 is induced by activation of ATF4 and CHOP through the integrated stress response [6]. ONC201 has recently been reported to have clinical activity against neuroendocrine tumors including pheochromocytoma, medullary thyroid cancer and paraganglioma [7].

Lurbinectedin is currently an increasingly popular option for SCLC patients. It is used as second line therapy in recurrent or progressive disease, and patients report less toxicity when receiving treatment. As of June 2020, the FDA granted accelerated approval for the use of lurbinectedin in patients with progressive or recurrent SCLC. This novel therapy is a small molecule inhibitor that works by preventing RNA polymerase II, an enzyme commonly hyperactivated in SCLC, from aiding in increased tumor cell proliferation [8]. Additionally, lurbinectedininduces doubled-stranded DNA breaks in cells that result in apoptosis, by covalently binding a central guanine in trinucleotide triplets in the minor groove of DNA [8]. This acute DNA damage suffered by the tumor cells results in an increase in expression of cleaved PARP, ATF4, phosphorylated Chk1 and γ-H2AX proteins, among others. Lurbinectedin is currently under investigation for its efficacy in solid tumors such as prostate cancer and Ewing Sarcoma. It is also involved in three FDA-approved clinical trials in SCLC and is being investigated for synergies with other agents in additional cancer cell lines. This therapy is becoming key to treating patients who have acquired resistance to traditional chemotherapies. The unique efficacy of lurbinectedin in these patients indicates an efficient and robust mechanism of preventing tumor growth. We explored the novel combination of lurbinectedin plus ONC201/TIC10 in preclinical studies in SCLC and report synergistic effects along with some novel observations regarding biomarker

and mechanism-based alterations in treated cells.

Materials and methods

Cell lines and culture conditions

The H1048 Small Cell Lung Cancer Cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). This cell line was harvested from a 53-year-old female patient's pleural effusion from metastatic SCLC and harbors the R273C mutation in TP53 (c.817C>T), PIK3CA H1047R (c.3140A>G), and the R552Ter in RB (c.1654C>T). This cell line was classified as having microsatellite instability (https://web.expasy.org/cellosaurus/CVCL_1453) [9]. The H1048 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 2.50 mM L-Glutamine and 15 mM HEPES buffer. Added to this medium was B-estradiol, fetal bone serum and penicillin-streptomycin, hydrocortisone, insulin, and sodium selenite. These cells were incubated at 37 degrees Celsius and were monitored and counted via cell-based counting.

The H1882 Small Cell Lung Cancer Cell line is an attached cell line obtained from American Type Culture Collection (ATCC, Manassas, VA). This cell line was harvest from a 59-year-old male with bone marrow metastasis. The H1882 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 2.50 mM L-Glutamine and 15 mM HEPES buffer. Added to this medium was B-estradiol, fetal bone serum and penicillin-streptomycin, hydrocortisone, insulin, and sodium selenite. These cells were incubated at 37 degrees Celsius and were monitored and counted via cell-based counting.

The H1105 Small Cell Lung Cancer Cell line contains SCLC cells suspended in medium in floating clusters. This cell line was harvested from a 73-year-old male from lymph node metastasis. The H1105 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 2.50 mM L-Glutamine and 15 mM HEPES buffer. Added to this medium was B-estradiol, fetal bone serum and penicillin-streptomycin, hydrocortisone, insulin, and sodium selenite. These cells were incubated at 37 degrees Celsius. The H1417 Small Cell Lung Cancer Cell line contains SCLC cells suspended in medium in floating clusters. This cell line was harvested from a 61-year-old female with Stage E small cell lung cancer. The H1105 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 2.50 mM L-Glutamine and 15 mM HEPES buffer. Added to this medium was B-estradiol, fetal bone serum and penicillin-streptomycin, hydrocortisone, insulin, and sodium selenite. These cells were incubated at 37 degrees Celsius.

Healthy lung epithelial cells were also obtained commercially

The therapeutic agents being examined were dissolved, sterilized, and stored at -20 degrees Celsius according to the manufacturer's recommendations.

Cell viability and combination index analysis

Cells were plated on a 96-well plate. Cells initially were analyzed using single treatment regimens and were dosed with either ONC201 or lurbinectedin at decreasing concentrations and re-incubated for 72 hours. After the 72-hour incubation period, the cells were analyzed using CellTiter-Glo (CTG) technology to evaluate cell viability. The relative number of live cells remaining in each well as calculated and analyzed. An IC50 value was found for each of the compounds in the before-mentioned cell line. The IC50 data for ONC201 and lurbinectedin were both well within the range of previously published IC50 values in SCLC cells.

Using this data, a combination index analysis was performed, using a 96-well plate and testing combinations of the drugs, beginning with a control for each combination and increasing in concentrations of each compound. Using the CTG technology, we were able to create a cell viability assay and analyze for synergies, using the combination index software, Combenefit [10].

Western blot analysis

A western blot analysis was conducted to examine the effects of the combination of lurbinectedin and ONC201 on the protein expression in H1048, H1105, and H1882 SCLC, cells while being treated with different combinations of the therapeutic agents. H1048, H1105 and H1882 cells were treated in 6-well plates with a combination of lurbinectedin and ONC201 at five concentrations, serially diluted to evaluate

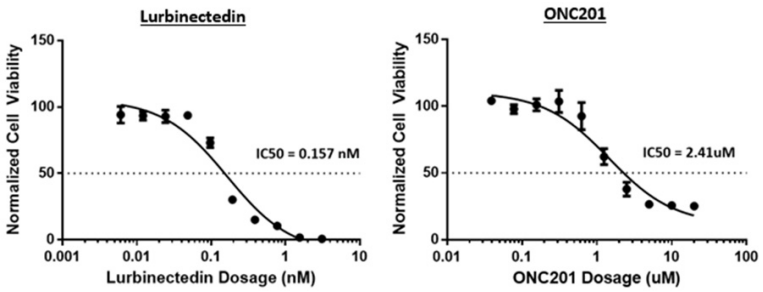

Figure 1. Lurbinectedin & ONC201 dose-response cell viability curves with IC50 values-H1048. Lurbinectedin was administered to the wells containing approximately 5,000 H1048 SCLC cells and the cells were returned to the incubator with the treatment for 72 hours. The highest dose administered was at a concentration of 3.1087 nM. This dosage was serially diluted to a dosage of 0.0061 nM. The curve on the left was used to generate an IC50 value of 0.157 nM, indicating the concentration of lurbinectedin that kills 50% of the H1048 cells. This sub-nanomolar IC50 value indicates an exceptional specificity that this agent has for SCLC cells. The data was obtained using CellTiter-Glo (CTG) imaging technology and the kill curve and IC50 calculation were generated using Prism software by GraphPad. The IC50 value produced in this experience is well within the range of previously reported IC50 values for lurbinectedin in SCLC cells. ONC201 was administered to each well, containing approximately 5,000 H1048 SCLC cells. These cells were exposed to the ONC201 treatment for 72 hours and then cell viability was analyzed via CTG imaging technology. Using the data from the CTG, the on the right kill curve was and an IC50 value of 2.41 μM was calculated. This indicates that the concentration at which 50% of the cells was killed by ONC201. The IC50 value produced in this experience is well within the range of previously reported IC50 values for ONC201 in SCLC cells.

the effects in a range of potencies. Additionally, there was a control well with no treatment added. After adding the various drug doses and a 24-hour period in the 37-degree incubator. Afterwards, the proteins were harvested and quantified before loading into NuPAGE 4 to 12% 1.5 mm Bis-Tris Plus gels and running at 140V for two hours before transferring to the membrane at 0.25 amps for 2 hours. The primary antibodies used in this Western Blot analysis were anti-cleaved PARP, anti-DR5, anti-ATF-4, anti-Chk-1, anti-γ-H2AX and anti-ClpP, all from Cell Signaling Technologies. β-Actin was used as a loading control to ensure precise protein quantification and loading. Anti-rabbit was used as a secondary antibody. All antibodies were diluted in 5% milk.

Results

Cell viability reduction following single agent treatment of H1048 SCLC cells with lurbinectedin

H1048 cells were treated at increasing concentrations of single agent lurbinectedin on one-half of a 96 well plate, for 72-hours, before being analyzed via CTG technology and producing a cell viability assay. The SCLC cells were plated at 5,000 cells per well, and the serially diluted concentrations started at 3.1 nM and decreased to a control concentration with no treatment added. This resulted in an estimated IC50 value of 0.157 nM. This extreme sensitivity of the SCLC cells to lurbinectedin is reflective of the drug's activity in clinical settings as an effective second line agent. **Figure 1** shows a dose-response drug sensitivity analysis, performed using PRISM software, a GraphPad product. This demonstrates lurbinectedin's toxicity the H1048 SCLC cells *in vitro* at various concentrations of lurbinectedin. Additionally, **Figure 2** visually reflects the data from the kill curve using CTG technology, as concentration is increased in each well there are fewer living cells remaining.

Cell viability reduction following single agent treatment of H1048 SCLC Cells with ONC201/TIC10

On a separate half of the 96-well plate, 5,000 H1048 SCLC cells per well were treated with single agent ONC201. Concentrations of ONC201 increased from a control with no treatment added, to a concentration of 20 μM. This resulted in an estimated IC50 value of 2.41 μM for ONC201 in H1048 SCLC cells. This indicates the potential efficacy of ONC201 in treating SCLC tumors at nontoxic dosages. This result is shown in **Figure 1**, a dose-response drug sensitivity curve reflecting ONC201's efficacy at a range of concentrations. **Figure 2** is a visual representation of the cytotoxicity of ONC201 towards H1048 SCLC cells *in vitro* using CTG technology.

Single agent treatment of SCLC cell lines H1048, H1105 and H1882 cell lines show evidence of cell death, upregulation of integrated stress response with ONC201/TIC10 and DNA damage response proteins with lurbinectedin

Western blot imaging revealed the increased expression of DNA damage-related and apopto-

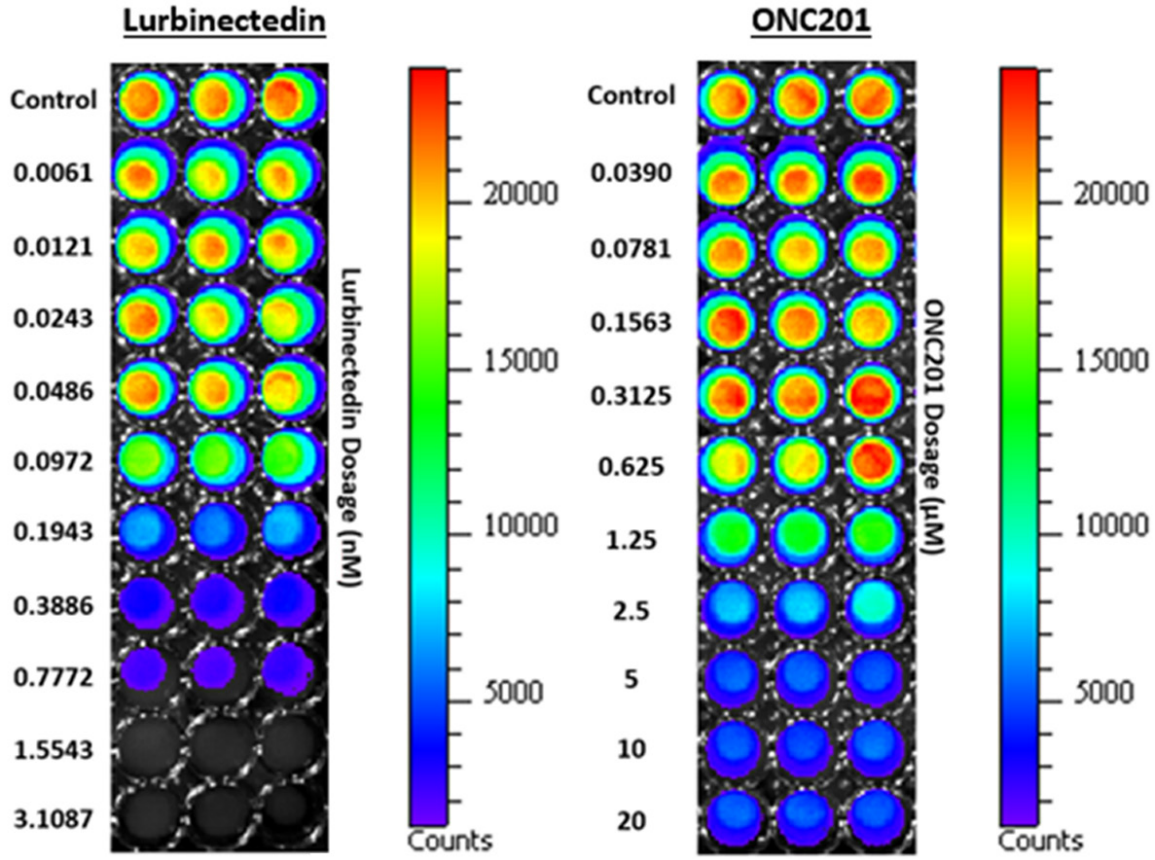

Figure 2. Lurbinectedin & ONC201 CTG imaging-H1048. The visual representation of lurbinectedin's efficacy at killing SCLC cells is displayed on the left. The color panel to the right of each CTG image indicates the relative number of cells in each well, relating to the color shown on the image. It is clear that there are higher relative amounts of live cells at the lower concentrations than at the higher concentrations. These are indicated with red, yellow, and green. The higher concentrations produce wells appearing dark blue and black, indicating more dead or killed cells. ONC201's range of killing cancer cells in the H1048 SCLC cell lines is displayed on the right, indicating the relative amount of live or dead cells in each well. It is clear that there are more live cells at the lower concentrations than at the higher concentrations in the ONC201 single agent treatment. This reduced viability at higher concentrations is the result of a possible increase in killing of cancer cells, reduced cellular proliferation, or both.

sis pathway-related proteins in single agent treatments. While the synergy of the two agents when used in combination results in a more potent antitumor treatment, the expression of DNA damage-related protein phosphorylated Chk-1 and self-induced apoptosis pathway-related protein ATF4 are elevated by single treatments using both ONC201 and lurbinectedin as individual agents (**Figure 3**). The cell lines were treated with individual dosages of each agent. Lurbinectedin was administered at concentrations of 0.05 nM and 1.6 nM. ONC201 was administered at concentrations of 0.16 μM and 2.5 μM.

The H1048 cell line showed increased expression of cPARP, indicating double stranded DNA breakage at the highest concentration of lurbinectedin (**Figure 3**). This cell line also indicated an increased expression of ATF4 at the highest concentration of ONC201 (**Figure 3**).

The H1105 cell line showed a robust expression of phosphorylated Chk-1 at the highest concentration of lurbinectedin (**Figure 3**). Similarly, to H1048 cells, H1105 cells showed a significant increase in the expression of ATF4 at the highest concentration of ONC201 (**Figure 3**), indicating an emphasis on ONC201's effective mechanism of inducing the intrinsic apoptosis pathway to cause tumor cell death.

The H1882 cell line showed an increased expression of both cPARP and Wee1, both indi-

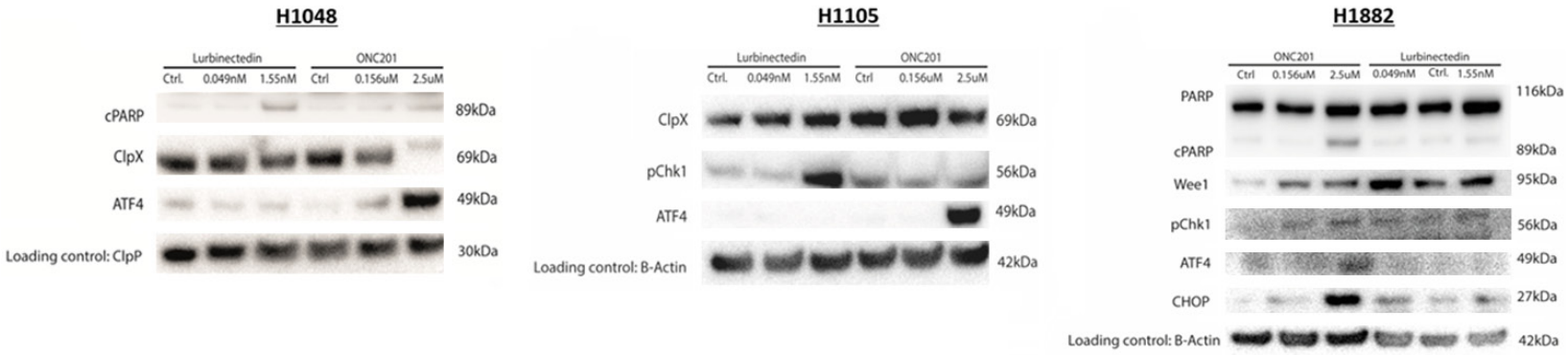

Figure 3. Lurbinectedin and ONC201 single agent treatment-H1048, 1105, and H1882 western blots. Single agent treatment in the H1048 cell line shows an increased expression in cPARP at higher concentrations when treated with lurbinectedin and ONC201, individually. This highlights the DNA damage induced by these treatments in SCLC cells as cPARP is a DNA-damage repair related protein, as more DNA is damaged, this protein is expressed accordingly. Additionally, treatment with ONC201 shows a significant increase in ATF4, underscoring the significance of ONC201's induction of the stress-induced apoptotic pathway in these tumor cells. H1105 cell line shows an increased expression of phosphorylated Chk-1 at the stronger concentration of lurbinectedin in the single agent treatment. This correlates with the double stranded DNA breakages and associated DNA damage induced by lurbinectedin in SCLC cells. In the higher concentration single agent treatment with ONC201, ATF4 is clearly expressed in greater magnitude. This is related to ONC201's unique mechanism of inducing the intrinsic apoptosis pathway in SCLC cells *in vitro*. The H1882 cell line further emphasizes the mechanisms in which both of these agents, individually, are able to effectively kill tumor cells. ONC201 shows effective mechanisms via increased expression of ATF4 and CHOP at higher doses, indicating induction of the intrinsic apoptosis pathway in these cells. Further, there is also increased expression of cPARP at the highest concentration of ONC201, indicating the agent is damaging the DNA in these cells. Similar to its effects in other SCLC cell lines, lurbinectedin shows strong expression of DNA damage-related proteins Wee1 and phosphorylated Chk1, indicating DNA damaged caused by treatment with this single agent.

cating a response to DNA damage induced by the therapeutic agent ONC201 (**Figure 3**). These cells also showed an elevation in expression of ATF4 and CHOP when treated with ONC201 alone, indicating induction of the intrinsic apoptosis pathway.

Combination treatment using ONC201 and lurbinectedin in SCLC cells leads to synergistic killing of SCLC cells

After confirming that the IC50 for both lurbinectedin and ONC201 was well within range of previous preclinical studies, these two agents were combined in increasing concentrations. The results in **Figure 4** indicate the relative amount of live versus dead cells in each well at increasing concentrations of lurbinectedin and ONC201 in the H1048 cell line. As the concentration of each agent was increased, there were significantly fewer live cells left in each successive well. This finding was replicated in the H1105, H1882 and H1417 cell lines (**Figure 4**), indicating significantly reduced cell viability in these assays, consistent across each SCLC cell line.

A combination index analysis was performed and indicated which combinations of drugs produced the greatest synergistic effects. **Figure 5** displays a table which indicates the relative synergy of each dose in the H1048 cell line. The combinations which showed the highest synergistic effect were ONC201, administered at a concentration of 0.16 μM, and lurbinectedin, administered at a concentration of 0.05 nM. Synergy values for each combination are represented numerically and visually. Darker blue and higher positive values indicate increased levels of synergy and are indicated in **Figure 5**. **Table 1** offers a mathematical representation of the synergistic cytotoxic effects of the two-drug combination toward H1048 SCLC cells. Values lower than 1.0 indicate synergistic efficacy of killing SCLC cells relative to the control sample.

Western blot analysis of double treatment using ONC201 and Lurbinectedin in SCLC cell lines

After 24 hours of treatment and incubation at 37 degrees Celsius, protein was harvested quantified and run using gel electrophoresis for western blotting. After transfer to a polyvinylidene difluoride (PVDF) membrane and incubation in primary then secondary antibodies, imaging was performed to analyze protein expression in each treatment group. A loading control was also imaged, as β-Actin expression confirmed that protein quantification performed correctly. Imaging revealed an increased expression of c-PARP, phosphorylated Chk-1, ATF4, γ-H2AX, ClpP at higher doses. **Figure 6** reveals the Western Blot imaging for each of the mentioned proteins. This increased expression of ATF4 and phosphorylated Chk-1 was replicated in the H1105 and H1882 cell lines (**Figure 7**).

c-PARP, γ-H2AX and phosphorylated Chk-1 indicate increased double-strand DNA breakage within the cells, destabilizing their ability to proliferate. ONC201 and Lurbinectedin offer a uniquely robust combination in the expression of phosphorylated Chk-1. At higher concentrations of the agents in combination, the significantly increased expression of phosphorylated Chk-1 indicates the presence of DNA damage in the H1048, H1105 and H1882 cell lines (**Figures 6** and **7**). The western blot images of the protein expression in the samples treated with the higher concentrations of these agents clearly demonstrate an increase relative to the samples treated with lower concentrations (**Figures 6** and **7**).

ATF4 upregulation indicates an increased in the integrated stress response of the H1048 SCLC cells to higher treatment doses, which, in turn, results in stress-induced apoptosis (**Figure 6**). The samples treated with higher concentrations of the agents display more ATF4 expression, indicating these cells are more likely to induce the ATF4-related apoptotic pathway in tumor cells.

The relative increased expression of ClpP in the H1048 samples receiving stronger treatment (**Figure 6**) highlights ONC201's unique and effective mechanism of action. ClpP is allosterically modified by ONC201 to open substrate channel areas and alter the conformation of its active site causing hyperactivation of ClpP's proteolytic activity [5]. This results in the degradation of cellular subunits in the election transport chain, impairing oxidative phosphorylation, which in turn, increases cellular stress levels, triggering apoptosis. These results are similarly

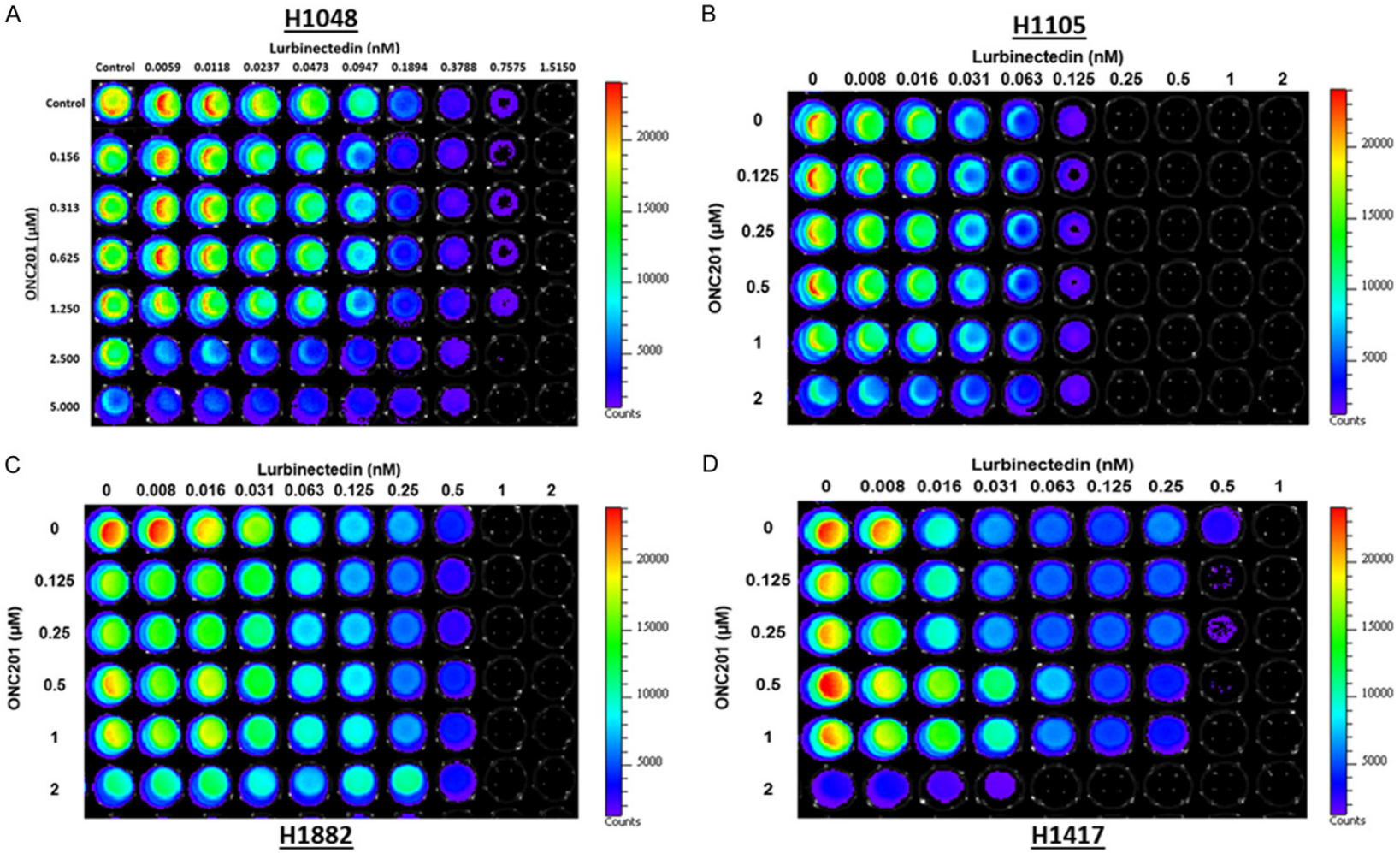

Figure 4. Lurbinectedin and ONC201 combination CTG imaging-H1048, H1105, H1882, and H1417 cell lines. Lurbinectedin and ONC201 administered in combination at increasing concentrations in a 96-well plate to approximately 5,000 cells per well. As concentration increases to the right and down on the plate, it is clear that there are fewer live cells. This combination treatment is the first usage of ONC201 and lurbinectedin and its efficacy and sensitivity for SCLC cells is demonstrated in the image. A. Reduced cellular proliferation, increased cell death or a combination of the two can result in the reduced viability, displayed in the CTG imaging in the H1048 cell line. The highest concentration of lurbinectedin used was 1.5150 nM and was serially diluted to the lowest concentration of 0.0059 nM. ONC201 was serially diluted from a maximum concentration of 5.000uM to the lowest concentration of 0.156 uM. B. Lurbinectedin and ONC201 treatment in combination, suggesting effectiveness in killing tumor cells, reduced cellular proliferation, or both, replicating the trend from the H1048 cells in the H1105 cell line. C. Lurbinectedin and ONC201 treatment in combination in the H1882 cell line. D. Lurbinectedin and ONC201 combination treatment in the H1417 cell line. These results represent the trend of possible increased killing of SCLC cells, reduced cellular proliferation, or both, *in vitro,* at higher concentrations of the double-agent treatment. The top left well in each image serves as a control and did not receive either agent.

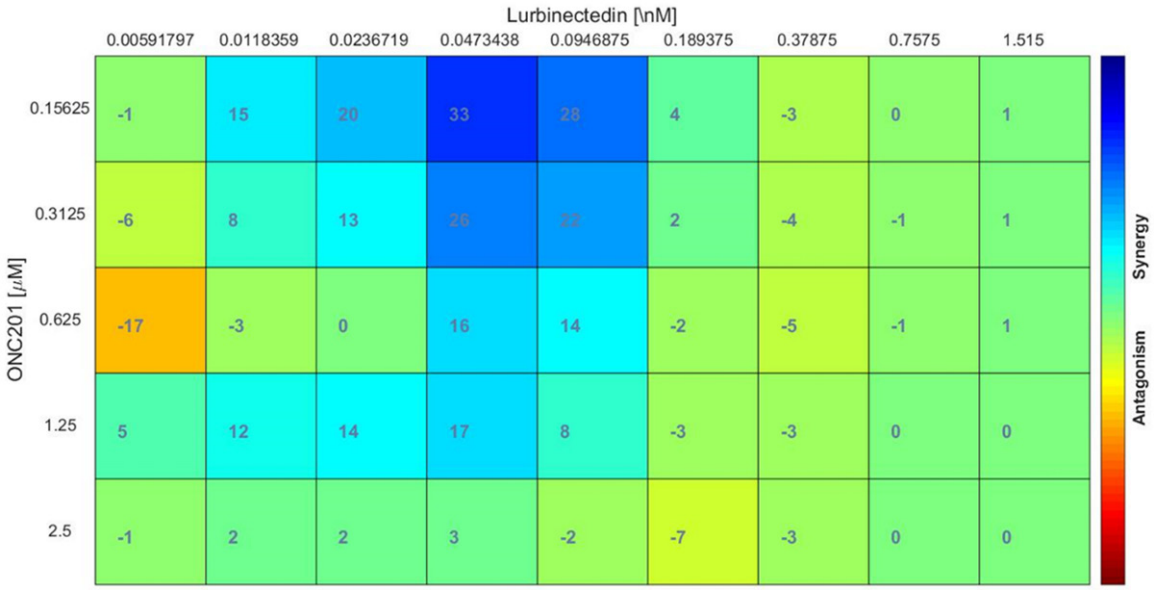

Figure 5. Lurbinectedin and ONC201 combination index. The combination index analysis was performed using the Combenefit [10] software revealed significant synergies at various concentrations. The most synergistic combination occurred at a lurbinectedin concentration of 0.04734 nM and an ONC201 concentration of 0.15625 μM. There were also notably high levels of synergy at the lurbinectedin concentration of 0.09469 nM and ONC201 concentration of 0.15625 μM, as well as a lurbinectedin concentration of 0.04734 nM and an ONC201 concentration of 0.3125 μM. Combenefit uses the *in vitro* data from the experiment and compares it to dose-responses for non-synergistic combinations [10]. The resulting figure is a mathematical representation of which doses of these drugs have the most synergistic effects in killing SCLC cells of the H1048 cell line. It is clear from this image and the data in **Table 1** that there are significant synergies of these two agents, even when combined and administered at low doses of less than 0.1 nM and 0.5 μM, for lurbinectedin and ONC201, respectively.

Table 1. Synergy of the combination treatment with lurbinectedin and ONC201

		Lurbinectedin (nM)									
ONC201 (uM)	H1048	0.0000	0.0059	0.0118	0.0237	0.0473	0.0947	0.1894	0.3788	0.7575	1.5150
	0.000	1.00	1.04	1.06	0.92	0.84	0.58	0.14	0.08	0.03	0.00
	0.156	0.85	0.95	0.80	0.72	0.49	0.23	0.13	0.08	0.03	0.00
	0.313	0.86	0.92	0.78	0.71	0.49	0.24	0.14	0.08	0.03	0.00
	0.625	0.78	0.86	0.71	0.67	0.44	0.23	0.14	0.09	0.02	0.00
	1.250	0.37	0.39	0.32	0.29	0.22	0.16	0.11	0.05	0.01	0.01
	2.500	0.27	0.25	0.22	0.21	0.18	0.15	0.11	0.04	0.00	0.00

The table reflects the concentrations at which synergies between the two agents exists. 1.00 represents all of the cells are alive in the well relative to the control group of no treatment. Any number less than one, indicates increased efficacy in killing H1048 SCLC cells.

displayed and emphasized in the H1105 and H1882 cell lines (**Figure 7**), indicating a clear trend in effectiveness in eliminating tumor cells with this combination therapy's potent mechanisms of action.

Discussion

To our knowledge, this study is the first in combining two promising therapeutic agents to treat SCLC. This combination discovered novel synergy *in vitro* and allowed for the confirmation of the mechanisms in which this combination of therapies kills SCLC tumor cells. This includes verifying increased expression of proteins that indicate double-strand breaks, degradation of electron transport chain components, and increased levels of cellular stress.

Small cell lung cancer landscape

Lung cancer has the highest mortality rate among all diagnoses malignancies, due to its often-late presentation with progressive dis-

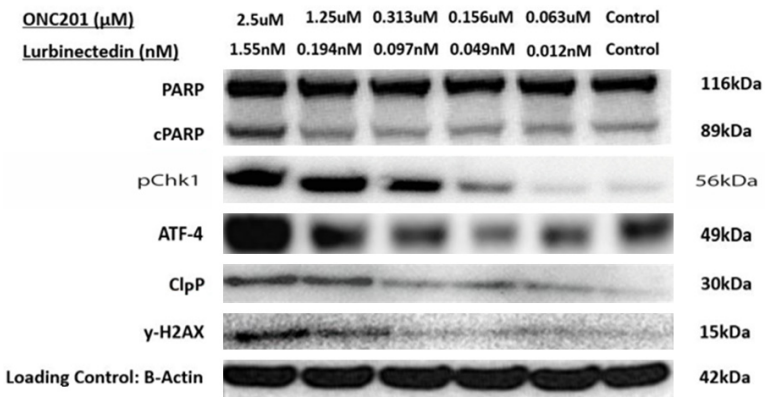

Figure 6. DNA damage, integrated stress response activation and PARP cleavage by combination of Lurbinectedin and ONC201-H1048. The expression of DNA damage and repair-related proteins cPARP, phosphorylated Chk1 and γ-H2AX indicate a greater expression at higher concentrations of the agents. In response to cellular stress, ATF4 expression increases at the higher concentrated treatments. At higher concentrations of ONC201, and agents that induce cellular stress in cancer cells, ATF4 and other stress-induced apoptotic proteins will have increased expression, inducing death of cancer cells in the H1048 cell line. The same trend is valid for the expression of ClpP, at higher concentrations of ONC201. As ONC201 concentration increases the pro-apoptotic TRAIL pathway is induced, and the expression of the mitochondrial protein, ClpP, an electron transport chain subunit degrading-protein, is elevated. The reflection in this figure, demonstrates the mechanism by which this combination kills cancer cells in the H1048 cell line and reflects potential mechanisms for synergy at the biomolecular level.

ease and metastasis. SCLC comprises approximately 15% of all lung cancer diagnoses and is primarily caused by the man-made epidemic resulting from cigarette smoking. This epidemic has led to unnecessary exposure to polycyclic aromatic hydrocarbons, arsenic and other DNA-damaging carcinogens that result in the genetic changes leading to SCLC tumor formation [11].

The virulent nature of SCLC underscores the importance of investigating new therapies for both advanced metastatic and recurrent disease. The lack of long-term effective and tolerable drugs has resulted in innumerable agents being tested, and while several have earned NCCN compendium listing or FDA approval, the lack of available biomarkers has inhibited the ability to refine treatment selection or effectively analyze agents in combination for their synergistic effects.

Efficacy of the novel combination: ONC201 + Lurbinectedin

Both ONC201 and lurbinectedin have been shown to be well-tolerated and less toxic to patients. Lurbinectedin is commonly administered intravenously in clinical settings at a dosage of 3.2 mg/m^2 over 60 minutes every 21 days (Jazz Pharmaceuticals) and has shown fewer toxic effects compared to the platinum series of chemotherapies. ONC201 is administered orally at a dose of 625 mg every three weeks (Oncoceutics/Chimerix). ONC201 is fairly non-toxic to patients and crosses the blood-brain barrier which is advantageous as a potential treatment for SCLC.

Given their safety profiles and promising efficacy in solid tumors, this combination of lurbinectedin and ONC201 was examined in our study for its potential synergistic benefit in killing H1048, H1105 and H1882 SCLC cells *in vitro*. Both agents have shown sensitivity to aggressive solid tumors and their synergy was analyzed to underscore the importance of continuing investigation into this combination as a future treatment option in recurrent or progressive SCLC.

Lurbinectedin showed a uniquely strong sensitivity to the SCLC cells. In the single agent study in the H1048 cell line, a sub-nanomolar IC50 value was obtained (0.16 nM), indicating lurbinectedin's ability to selectively target the cancer cells rather than healthy human cells. Further, ONC201 also displayed selective preference for tumor cells as the single treatment resulted in an IC50 value of 2.41 μM. These low IC50 values indicate treatment options that result in less toxicity for patients and a significant decrease in the adverse effects that traditional therapeutic agents are associated with. This, combined with ONC201's low IC50 value and noninvasive oral administration, further validate this combination's interest as a potential therapy for SCLC patients who are limited in options after first-line platinum-based and traditional chemotherapies do not limit disease progression. This is further displayed when healthy lung epithelial cells were treated with these agents. The CTG results show that there

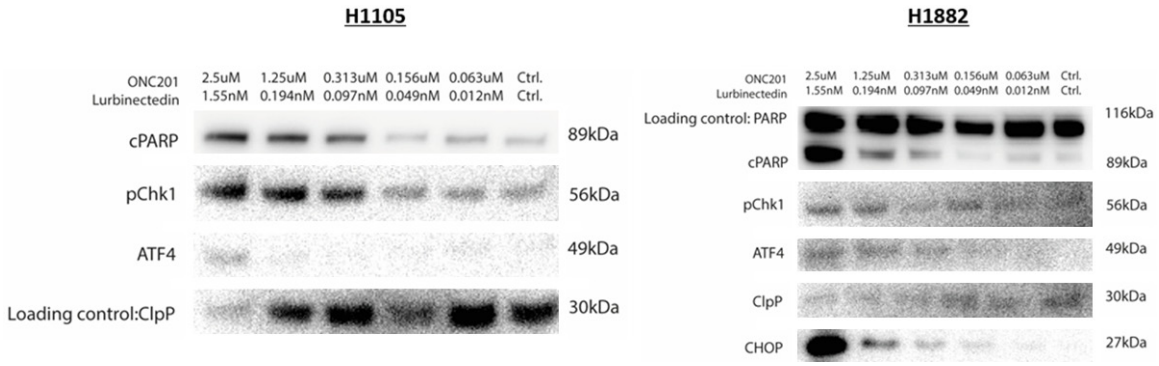

Figure 7. DNA damage, integrated stress response activation and PARP cleavage by combination of Lurbinectedin and ONC201-H1105 and 1882. The left image reflects the western blot results from the combination treatment with lurbinectedin and ONC201 in the H1105 cell line. There is markedly elevated expression of cleaved PARP and phosphorylated Chk-1 at higher doses of the combination therapy. Further, ATF4 is upregulated in this imaging, indicating ONC201 working via the induction of the apoptotic TRAIL pathway. This imaging at 24 hours is early enough in the SCLC cell elimination process to see the expression of these DNA damage-related and apoptosis related proteins increasing in response to exposure to lurbinectedin and ONC201 in the H1105 cell line. The results of the western blot analysis from lurbinectedin and ONC201 combination treatment in the H1882 SCLC cell line, in the right image, reflect the same pattern of pharmacologically induced DNA damage in the SCLC cells as well as increased expression of proteins associated with the pro-apoptotic TRAIL pathway. It is clear that higher dosages of this combination treatment show larger bands, higher levels of expression, of proteins related to these cell-killing mechanisms. This is consistent with the mechanisms in the other cell links which also show an upregulation in TRAIL-related proteins such as ATF4 and CHOP as well as proteins related to the cellular response to DNA damage, such as phosphorylated Chk-1 and cleaved PARP.

are significant levels of cells alive after 24 hours of combination treatment at increasing concentrations (**Figure 8**). The significant number of cells remaining in each well after 24 hours of combination treatment in the HSAEC cell line emphasizes the point that both lurbinectedin and ONC201 show selectivity for cancer cells, sparing healthy epithelial cells from their toxic effects. Compared to when this same combination was used to treat malignant H1048 SCLC cells, there were zero cells remaining in numerous combinations of concentrations using these agents. The quantifiable number of cells remaining, including at the strongest concentrations of ONC201 and lurbinectedin, highlight the selectivity of these agents for malignant cells which cannot evade their cytotoxic effects. Further, this CTG imaging and the quantifiable number of cells alive at higher concentrations of ONC201 and lurbinectedin are consistent with clinical claims from patients who prefer lurbinectedin due to its limited toxicity during treatment when compared to first-line SCLC platinum-based chemotherapeutic agents.

The combination of ONC201 and lurbinectedin showed significant synergies and **Table 1** indicates the synergistic ability of these combined agents to kill the H1048 SCLC cells. As it is shown in **Table 1**, every combination where the concentration of each agent was above zero resulted in increased cell death. These values are shown as less than 1.0 and highlighted in yellow.

The western blot imaging (**Figures 6** and **7**) resulted in clear examples of the mechanisms through which this novel treatment combination may result in synergy when administered together. These synergies resulted in increased expression of DNA damage and repair related proteins, particularly phosphorylated Chk-1, cellular-stress induced apoptosis related proteins, and electron transport chain inhibiting protein.

As lurbinectedin is an inhibitor of RNA polymerase II, triggering its degradation, and an inducer of double-stranded DNA breaks, proteins γ-H2AX, cleaved PARP, and phosphorylated Chk-1 were increasingly expressed. When DNA is damaged and double strands are broken, the histone H2AX is always phosphorylated [12]. This phosphorylated protein would normally recruit repair proteins in health cells. PARP also aids in the recruitment of DNA repair factors and helps to relax the chromatin in the

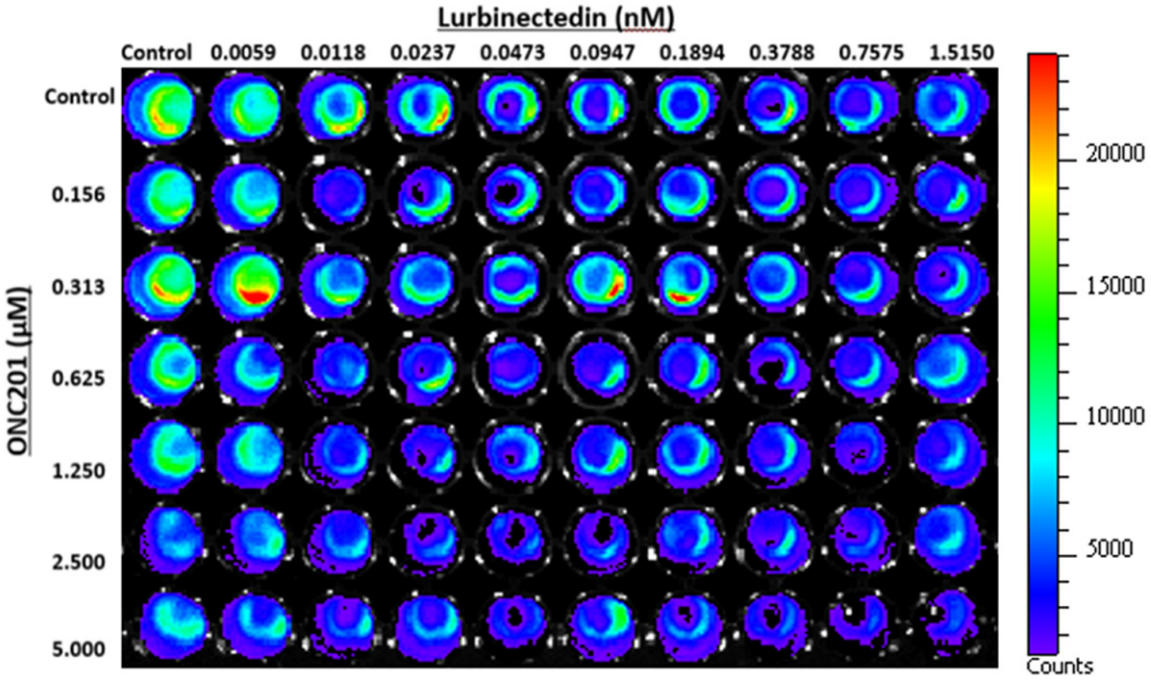

Figure 8. Lurbinectedin and ONC201 combination treatment-CTG imaging-healthy lung epithelial cells. This image reflects the claims that ONC201 and Lurbinectedin selectively target malignant cells in SCLC and spare healthy epithelial cells from their toxic effects. After 24 hours of treatment, CTG analysis reveals that there are quantifiable numbers of live cells in each well of the plate at every concentration, including the highest concentrations of treatment, which left zero cells alive when used in the malignant SCLC cell lines: H1048, H1105 and H1882. This further validates the clinical claims from patients who prefer lurbinectedin due to its limited side effect profile when compared to traditional chemotherapies used in first-line treatment of SCLC.

area of the DNA damage [13]. Phosphorylation of Chk1 leads to the activation of WEE1 and inhibits Cdk1 and results in cell cycle arrest [14]. This halts the cell cycle in the G2 phase as a response to DNA damage [15], such as the damage caused by agents that induce double-stranded breaks. Chk1 also dissociates from chromatin, resulting in Histone H3 lacking acetylation and phosphorylation [16], thus inducing transcriptional repression [16].

ONC201 is particularly effective in selectively killing SCLC cells due to its induction of the TRAIL-mediated apoptotic pathway via its interaction with death receptor DR5 [17]. The TRAIL pathway is inducible in many bodily tissues, including lung tissue. It is also expressed in certain immune cells after cytokine activation [18-20]. It is upregulated due to the inactivation of Akt and Erk. This inactivation results in the nuclear translocation of Foxo3a and transcriptional activation of the TRAIL gene [5]. ONC201 is able to selectively target tumor cells, as Akt and Erk expression is low in healthy cells, as is the integrated stress response [6, 21].

The increased expression of ClpP (**Figure 6**), is a result of ONC201's unique mechanism of action, acting as an agonist of the enzyme. ONC201 allosterically modifies ClpP to change the conformation of its active site and open substrate channels. This modification causes an increase in ClpP's activity, leading to elevated cellular stress, as ClpP degrades critical subunits of the electron transport chain [5]. The impairment of oxidative phosphorylation and subsequent oxidative stress result in the induction of apoptosis. ClpP has also been shown to have activity in the degradation of misfolded proteins and aid in the selective killing of cancer cells, regardless of whether they have a healthy and functioning p53 protein [22]. Mitochondrial ClpP has been shown to activate anti-tumor effects *in vitro* and *in vivo* [22]. The results from the western blot analysis are consistent with these known mechanisms of ClpP. As the concentrations of the agents in the combination treatment were increased, ClpP was more strongly expressed. While the exact mechanisms of the interaction between ClpP and the mitochondrial mechanics remain

to be fully investigated, ClpP inactivation has increased resistance in tumor cells to ONC201 in AML, acute lymphoblastic leukemia (ALL) and breast cancer cells [5].

The western blot analysis (**Figure 6**) also revealed a clear increase in expression of ATF4 in H1048 cells treated with the highest concentration treatment. ATF4 is commonly upregulated in cancer cells and under persistent stress conditions, it induces stress-related apoptosis [23]. The integrated stress response pathway causes upregulation of ATF4 in tumor cells [6], consistent with cellular response to damage induced by therapeutic agents. ATF4 activation also induces DR5 expression via the integrated cellular stress response [6], leading to the signaling through an activated TRAIL pathway. The increased expression of ATF4 in response to cellular stress is magnified in the H1048 cells treated with higher doses of lurbinectedin and ONC201. The cellular stress caused by DNA damage and cell cycle inhibition, coupled with the induction of the death receptor DR5, and the increase in oxidative stress caused by ClpP's degradation of electron transport chain components underscore the synergies this combination offers.

The results from the combination index analysis and western blot analysis are consistent with current knowledge and discoveries involving the single agents lurbinectedin and ONC201. The novelty in this project stems from the analysis of synergies when these two agents are combined. With the combination index mathematically displaying the distinct synergies at various concentration combinations of these agents and the confirmation of the mechanism through which the SCLC cells are being killed via western blot, it is clear that, when administered together, in the H1048, H1105, and H1882 cell lines, *in vitro*, there is observable and clinically relevant synergy that requires further investigation. Both ONC201 and lurbinectedin have efficacy in multiple types of solid tumors. There should be increased emphasis in continuing investigation using these promising agents in additional SCLC cell lines, as well as other solid tumor types.

Limitations and future directions

Limitations of this work include the limited experience with SCLC cell lines, and lack of *in vivo* data at this time. We believe the ONC201 and lurbinectedin combination therapy holds potential and is worth consideration for further study and clinical investigation. Future directions include preclinical studies in additional SCLC and diverse solid tumor cell lines *in vitro*, use of patient- and mouse-derived organoids, and evaluation of this combination and its efficacy and synergy on organoids derived from small cell lung cancer tumors. These can be either grown from patient samples or grown in mouse models. Additionally, there is an opportunity to investigate these agents in combination in various solid tumor cell lines such as prostate cell lines LnCAP and NCI-H660, as well as Ewing's Sarcoma cell lines and ovarian cancer cell lines.

With over 275 clinical trials either active or recruiting that focus on patients with SCLC, there is some optimism surrounding the research into emerging therapeutics. Interestingly, the non-platinum-based therapies, such as the ones outlined in this paper offer effective killing of tumor cells while sparing patients from the toxic effects of the platinum-based chemotherapies which have been the standard of care for the past half-decade. The effects of certain therapies, such as ONC201 and lurbinectedin, are magnified when they are used in combination. These synergies must continue to be monitored going forward and emphasis on testing this regimen in organoids and in mouse models is imperative, with the ultimate goal of reaching human trials.

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Disclosure of conflict of interest

W.S.E-D. is a co-founder of Oncoceutics, Inc., a subsidiary of Chimerix. Dr. El-Deiry has disclosed his relationship with Oncoceutics and potential conflict of interest to his academic institution/employer and is fully compliant with

NIH and institutional policy that is managing this potential conflict of interest.

Address correspondence to: Dr. Wafik S El-Deiry, Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Warren Alpert Medical School, Brown University, 70 Ship Street, Room 537, Providence, Rhode Island 02903, USA. E-mail: wafik@brown.edu; Christopher G Azzoli, Hematology/Oncology Division, Department of Medicine, Lifespan Health System and Brown University, 167 Point Street, Providence, Rhode Island 02903, USA. E-mail: christopher_azzoli@brown.edu

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