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Antitumorigenic effect of combination treatment with ONC201 and TRAIL in endometrial cancer *in vitro* and *in vivo*

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ABSTRACT

ONC201 demonstrated promising activity in patients with advanced endometrial cancer in a Phase I clinical trial. ONC201 activates the integrated stress response (ISR) and upregulates TRAIL and its receptor DR5. We hypothesized ONC201 upregulation of DR5 could sensitize tumors to TRAIL and combination of ONC201 and TRAIL would lead to enhanced cell death in endometrial cancer models. Five endometrial cancer cell lines AN3CA, HEC1A, Ishikawa, RL952, and KLE as well as a murine xenograft model were treated with ONC201 alone or in combination with TRAIL. ONC201 decreased the cell viability of all five endometrial cancer cell lines at clinically achievable low micro-molar concentrations (2–4 μM). ONC201 activated the ISR and induced protein expression of TRAIL and DR5 at the cell surface. Pretreatment with ONC201 sensitized endometrial cancer cell lines to TRAIL, leading to increased cell death induction compared to either agent alone. Tumor growth was reduced *in vivo* by the ONC201/TRAIL combination treatment in the xenograft model of endometrial cancer (*p* = .014). Mice treated with combination treatment survived significantly longer than mice from the three control groups (*p* = .018). ONC201 decreased cell viability in endometrial cancer cells lines primarily through growth arrest while the combination of ONC201 and TRAIL promoted cell death *in vitro* and *in vivo*. Our results suggest a novel cancer therapeutic strategy that can be further investigated in the clinic.

Introduction

Endometrial cancer is the most common gynecologic malignancy among women in the United States, with the incidence and mortality rate continuing to rise over recent years.[1] Curative treatment is feasible when the disease is caught early; however, 30% of cases will recur, leading to over 11,000 deaths from uterine cancer each year. For women with advanced stage or recurrent endometrial cancer, the prognosis is poor[2,3] as current treatment at this stage has had limited efficacy.

The current treatment approach for women with recurrent, metastatic, or high-risk disease consists mainly of chemotherapy with or without radiation. There are only a handful of potentially effective therapies for these women and these are therapies with high toxicity profiles that provide a median progression free survival of around only 13 months. These results have led the National Comprehensive Cancer Network (NCCN) to strongly encourage participation in clinical trials for these patients.[4] Identification of a new effective therapy in endometrial cancer would therefore be of great impact clinically.

ONC201 is a well-tolerated, orally active small molecule in the imipridone class that has demonstrated anti-cancer activity in various advanced solid tumors in preclinical cancer models without significant toxicity.[5] Its promising activity in patients with advanced endometrial cancer in a Phase I clinical trial has led to it currently being tested as a single agent in multiple Phase II trials.[5] Clinical trials of ONC201 are also being conducted in patients with breast cancer. Our group has recently shown efficacy of ONC201 in breast cancer cell models (17) however not all cell lines were responsive to ONC201 as a single agent. Importantly, in a recent publication we were able to show that breast cell lines resistant to ONC201 alone were sensitive to ONC201 when used in combination with TRAIL (12). Gynecologic cancers often have limited response to single-agent therapies. We are therefore hopeful that this combination would be efficacious in endometrial cancer models as well.

ONC201 has not been well elucidated in endometrial cancer; however, its mechanism of action had been well studied in a variety of other model systems. ONC201 is able to induce

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transcription of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through inhibition of the ERK and AKT pathway in a p53 independent manner.[6,7] TRAIL ligand can then activate cell death by binding to death receptor 4 and 5 (DR4, DR5) which activates the extrinsic pathway leading to apoptosis.[8,9] Importantly, TRAIL has been shown to selectively target transformed cells[10] leading to the appeal of TRAIL activation as an anticancer therapeutic. In addition to increasing TRAIL expression, ONC 201 has the ability to upregulate the TRAIL receptor, DR5, through activation of the integrated stress response (ISR).[11] Both upregulation of TRAIL and its receptor DR5 may prime the cell for entry into the cell death pathway.

Our goal was to explore the efficacy of ONC201 in endometrial cancer in preclinical studies and to elucidate the mechanism of action in endometrial cancer in order to choose combination therapies that may improve clinical outcomes. We hypothesized that ONC201 upregulation of DR5 could sensitize tumor cells to exogenous TRAIL, as recently demonstrated in preclinical breast cancer models,[12] and that combination treatment of ONC201 with recombinant human TRAIL (rhTRAIL) would facilitate enhanced cell death in ONC201-treated endometrial cancer cells.

We demonstrate that ONC201 decreases cell viability in endometrial cancer cells lines primarily through growth arrest while the combination of ONC201 and rhTRAIL promotes cell death in endometrial cancer cells. Our results suggest a novel cancer therapeutic strategy that can be exploited in the clinic.

Results

ONC201 decreases the viability of various endometrial cancer cell lines at µM concentrations

In order to determine the effectiveness of ONC201 against endometrial cancer we first conducted cell viability experiments *in vitro*. Five different endometrial cell lines with variation in disease pathology, AN3CA, HEC1A, KLE, Ishikawa and RL952, were subjected to vehicle or increasing doses of ONC201 at µM concentrations, and cell viability was determined by Cell Titer Glo assay. Dose response curves were then generated to determine the GI_{50}, the concentration of drug that inhibits the growth of cancer cells by 50% (Figure 1a, Table 1). GI_{50} for AN3CA, HEC1A, and KLE was approximately 2.5 µM and GI_{50} for Ishikawa and RL952 was approximately 4.0 µM. Endometrial cancer cell lines are therefore sensitive to ONC201 at micromolar doses previously shown in the first-in-human trial to be clinically achievable in advanced solid tumors.[5] Phase contrast imaging, shown in Figure 1b, demonstrates the effect of ONC201 at its GI_{50} concentration for each cell line. Next the impact of ONC201 at the GI_{50} on clonogenic survival was assessed. This assay is used to determine if a single cell can propagate and form a colony under the given condition and evaluates its long-term survival. Low-density single cells were plated for each cell line and treated with vehicle or with ONC201 at its GI_{50} for 72 hours followed by an additional 10 days in drug-free media (Figure 1c). In all five cell lines treated, long-term colony formation was completely abolished after treatment with ONC201 at low (i.e. $\leq$4 µM) concentrations.

In endometrial cancer cell lines, the effect of ONC201 is predominantly antiproliferative, associated with cell cycle arrest and only slightly increased cell death

Decreased cell viability can result from either cell death or growth arrest. Therefore, we next performed cell death and cell cycle assessing assays on our treated cell lines to determine their response to ONC201. Cell death was only slightly increased following treatment with ONC201 for 72 hours, as demonstrated by the modest increase in Sub-G_1 DNA content (increase by 9% with 8 µM ONC201 compared to control in AN3CA) and by the only slight increase in cleaved Parp by western blot (Figure 2, a,b). Cleaved caspase 3 and 8 could not be detected following ONC201 treatment in the cell lines tested. Further assessment of the cell cycle following propidium iodide staining revealed that the cells arrested in the G_0/G_1 phase following ONC201 treatment, with the % of cells in G1 phase increasing from 40 to 55% in the AN3CA cell line and 32 to 48% in the HEC1A cell line when treated with 4 µM ONC201 (Figure 2c). This was associated with an increase in expression of cell cycle inhibitor p27 and a decreased expression of cyclin D1 and pRb, consistent with a G_1 phase arrest (Figure 2d). Taken together these data suggest that ONC201 has a predominantly anti-proliferative effect on endometrial cancer cell lines when given as a single agent. This is in contrast to the strongly apoptotic effect ONC201 has demonstrated in a number of other cancer cell lines including colorectal and uterine serous cancer.[7,11,13]

ONC201 activates the integrated stress response and induces protein expression of TRAIL and DR5 at the cell surface

The apoptotic effect of ONC201 has been shown in prior studies to result from an increased expression of the endogenous tumor suppressor TRAIL as well as an increased expression of its receptor DR5.[7,11] Increased cell surface expression of DR5 is mediated by ONC201 through activation of the integrated stress response involving activation of ATF4 and CHOP. Whereas increased expression of TRAIL is mediated by ONC201 through phosphorylation and subsequent cytoplasmic sequestration of Foxo3a.[7] We set out to determine if these pathways were activated by ONC201 in the endometrial cancer cell lines tested. We show by western blot in Figure 3a that ONC201 increases protein expression of ATF4, CHOP, and DR5 in endometrial cancer cell lines after 24, 48, or 72 hours of ONC201 exposure, signifying activation of the integrated stress response. We also observed an increase in both DR5 and TRAIL cell surface protein expression after ONC201 treatment compared with vehicle alone (Figure 3b, c). Of note, the increase in DR5 expression was less obvious in the AN3CA cell line as compared to in HEC1A or KLE. These results show that ONC201 activates both pathways in endometrial cancer cell lines and is consistent with ONC201 being able to prime the cell for TRAIL-mediated apoptosis.
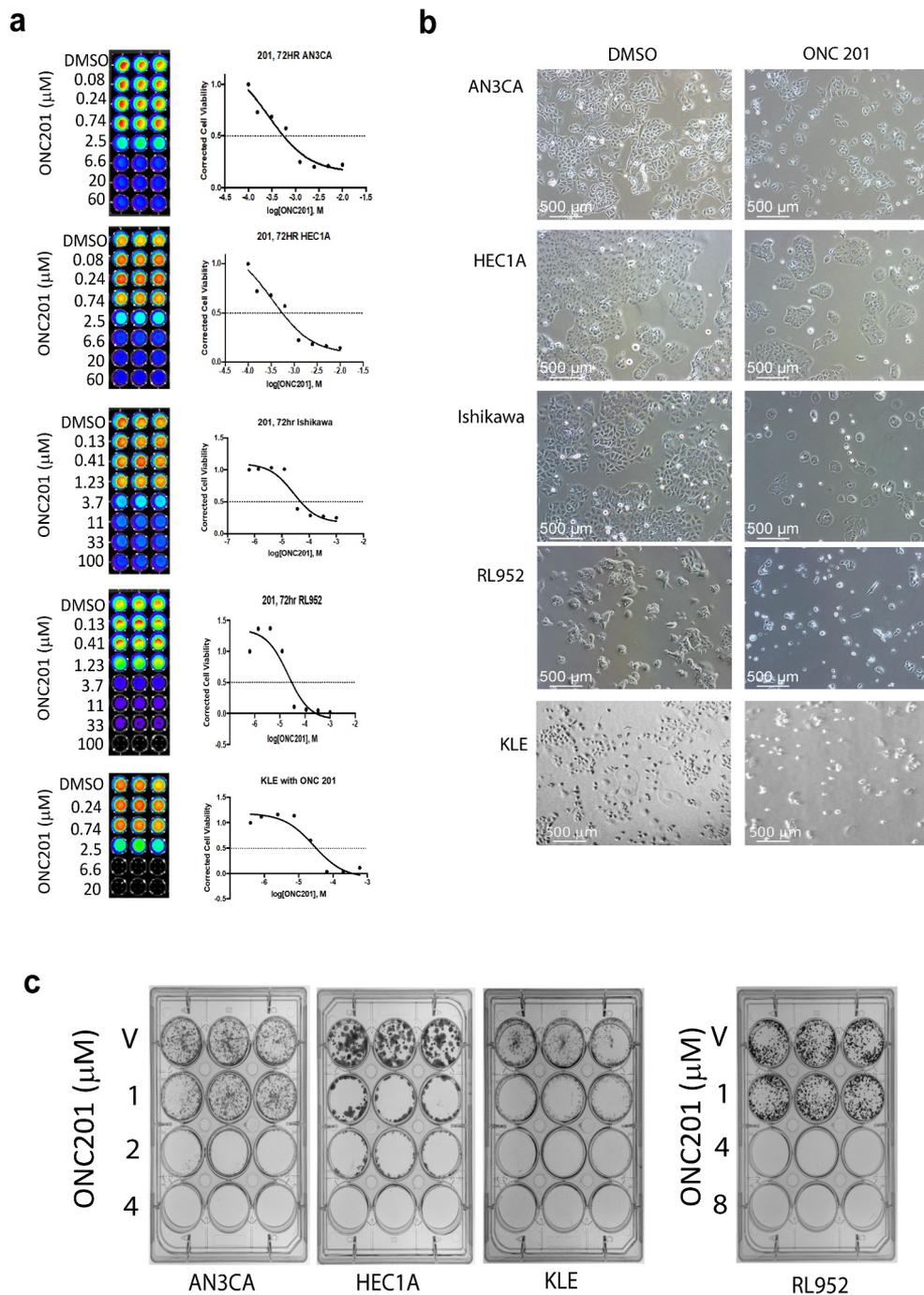
Figure 1. ONC201 decreases the cell viability of endometrial cancer cells at μM concentrations. AN3CA, HEC1A, KLE, Ishikawa, and RL952 endometrial cancer cell lines were treated with varying concentrations of ONC201 for 72 hours. (a) Cell viability was determined using CellTiterGlo reagent and dose-response curves were generated. GI_{50} for AN3CA, HEC1A and KLE was approximately 2.5 μM and GI_{50} for Ishikawa and RL952 was approximately 4.0 μM. (b) Phase contrast imaging taken at 10× after 72 hours of treatment with either vehicle or the GI_{50} concentration of ONC201. AN3CA, HEC1A, and KLE treated with ONC201 2.5 μM and Ishikawa and RL952 were treated with ONC201 4.0 μM. (c) Clonogenic survival was assessed after a 72-hour treatment with ONC201 followed by 10 days in drug-free media. Doses of ONC201 were chosen around the $G1_{50}$ as well as doses more dilute and more concentrated, to evaluate for trends. AN3CA, HEC1A and KLE treated with ONC201 1, 2, and 4 μM and RL952 was treated with ONC201 at 2, 4, and 8 μM.

Pretreatment with ONC201 sensitizes endometrial cancer cell lines to TRAIL, leading to increased cell death induction as compared to either agent alone

Next, we asked whether treating the cells with exogenous TRAIL ligand, after they had been primed with ONC201 to upregulate the DR5 receptor, would push the balance from proliferation to apoptosis. To test this, endometrial cancer cell lines were treated with ONC201 for 72 hours followed by treatment with recombinant human TRAIL (rhTRAIL) for 4 hours. Cells were stained with propidium iodide and assessed for $subG_1$ DNA content. Figure 4a shows that in AN3CA, RL952, Ishikawa and KLE cell lines there was a modest increase in cell death with ONC201 alone, but there was a robust increase in the proportion of cells undergoing cell death in the group that received the combination treatment (12-fold

Table 1. GI$_{50}$ of the endometrial cancer cell lines.

Cell line	GI$_{50}$
AN3CA	2.5
HEC1A	2.5
Ishikawa	4
RL952	4
KLE	2.5

increase in AN3CA and a 16-fold increase in RL952 compared to control). TRAIL alone showed only little to modest effect on the cell lines tested. Both the intrinsic and extrinsic apoptotic cascades were activated following combination treatment with ONC201 and TRAIL as seen by increased cleavage of PARP, caspase-3, caspase-9, and caspase-8 (Figure 4b). Together this data show that pretreatment with ONC201 sensitizes endometrial cancer cell lines to TRAIL, leading to increased cell death induction compared to either agent alone.

Of note, neither ONC201 alone or in combination with TRAIL resulted in activation of the apoptotic caspase cascade and showed only minimal increase in cell death by sub G1 in the HEC1A cell line (Figure 4a,b). Cancer cells have developed

numerous mechanisms to overcome cell death. One mechanism includes dysregulating the equilibrium between anti- and proapoptotic family members. We therefore tested a panel of anti-apoptotic proteins by Western blot analysis in our cell lines following dual treatment with ONC201 and TRAIL. As seen in Figure 4c expression of prosurvival proteins were similarly affected by ONC201 and TRAIL in both AN3CA and HEC1A cell lines. Decreased expression of FLIP was noted following dual treatment of ONC201/TRAIL compared to vehicle alone and there was no change in expression in Mcl-1, Bcl-2, Bcl-XL, CIAP, or survivin following treatment (Figure 4c). The differing response to ONC201/TRAIL treatment seen between HEC1A and AN3CA could not be explained by variances in Bcl-2 family modulators and warrants further investigation in future studies.

Tumor growth is reduced *in vivo* by combination treatment with ONC201 and recombinant TRAIL in a xenograft model of endometrial cancer

Next, the antitumorigenic effects of ONC201 with TRAIL were tested *in vivo* using a xenograft mouse model. Female mice yielding xenograft endometrial tumors were treated
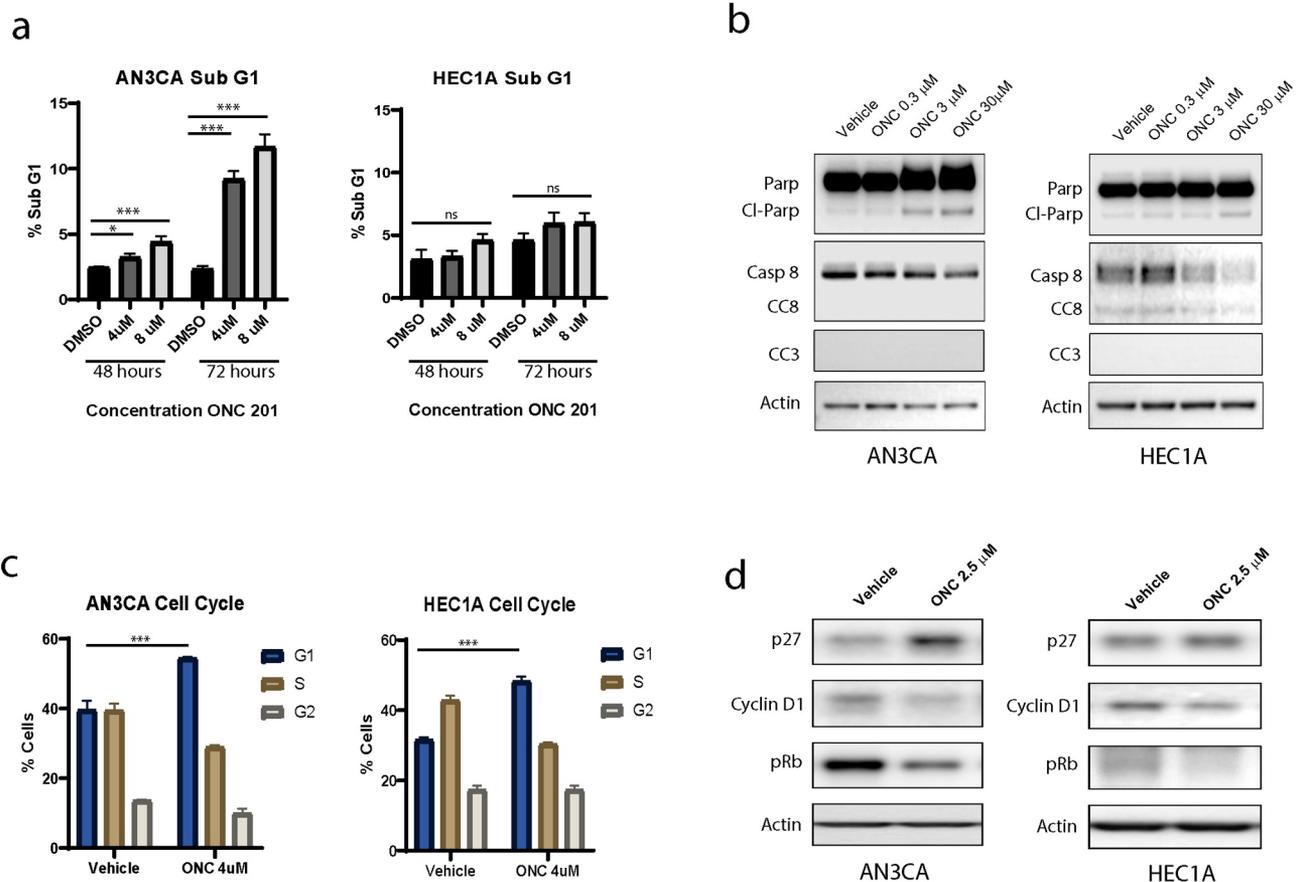
Figure 2. The effect of ONC 201 is predominantly antiproliferative, associated with cell cycle arrest and only slightly increased cell death. AN3CA, and HEC1A endometrial cell lines were treated with a vehicle control or ONC201 for 48 or 72 hours. (a) Cells treated DMSO or with ONC201 at 2, 4, or 8 µM. Cells stained with propidium iodide. Flow cytometric analysis was used to determine the percentage of cells with subG$_1$ DNA content. (b) Western blot analysis shows PARP and caspase 8 cleavage and caspase 3 cleavage, markers of apoptosis. Cell lines were treated with a vehicle control or ONC201 at 0.3 µM (one tenth the GI$_{50}$), 3 µM (around the GI$_{50}$), and 30 µM (10× the GI$_{50}$) for 72 hours to allow for sufficient death to occur. (c) Cells treated DMSO or with ONC201 at 2 or 4 µM (below and above the GI$_{50}$ to evaluate for trends). Cell cycle analysis at 4 µM shown. Cells stained with propidium iodide. Flow cytometric analysis of the cells was used to determine the percentage of cells in each stage of the cell cycle after 48 hours. (d) Cells treated DMSO or with ONC201 at 2.5 µM for 48 hours. Western blot analysis shows p27, cyclin D1 and phosphorylated Rb. Actin is used as a loading control. Samples run in triplicate. Error bars denote SEM. * denotes $p < .05$, ** denotes < 0.002, *** < 0.001, ns denotes nonsignificance.

a

b

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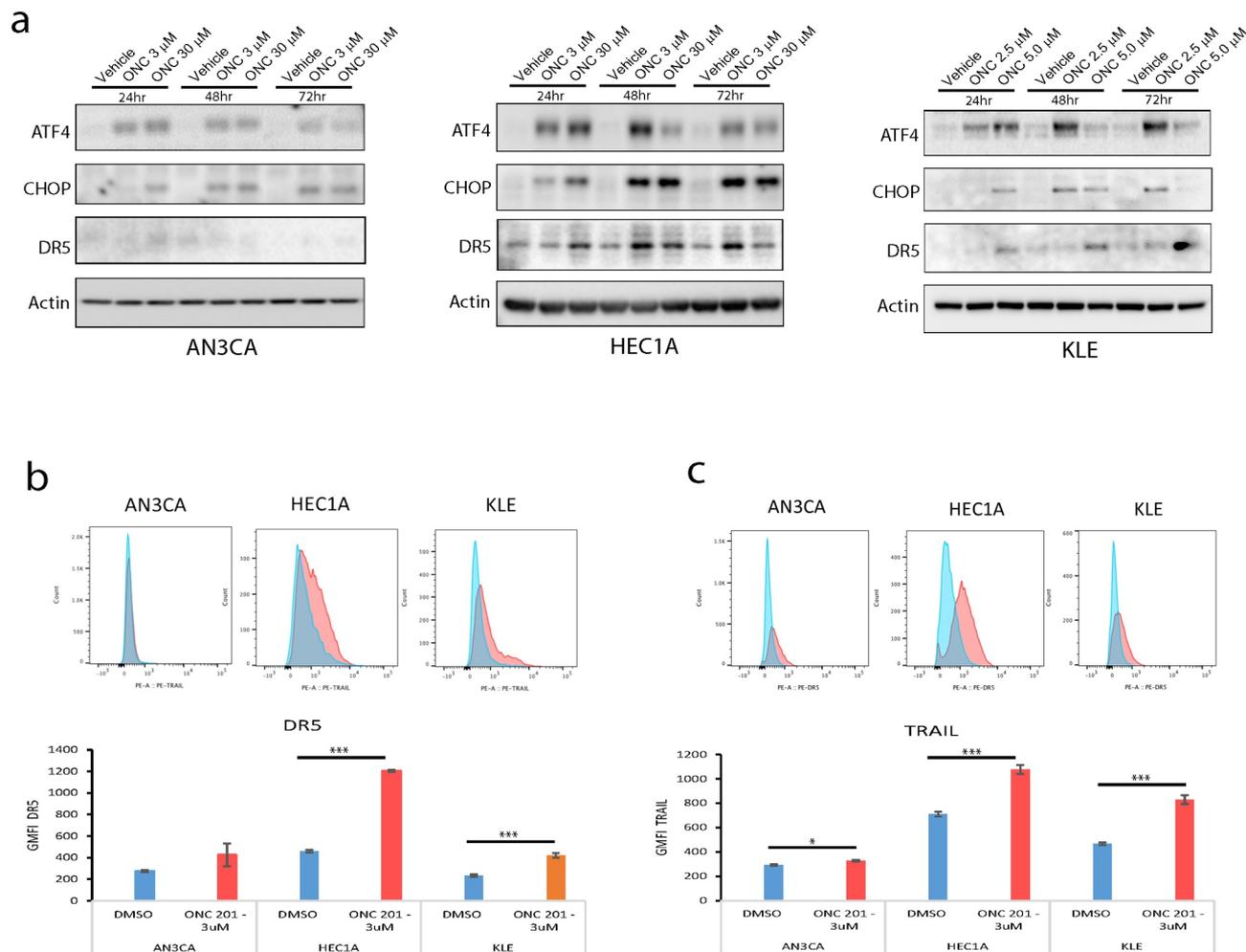
Figure 3. ONC201 activates the integrated stress response and induces protein expression of TRAIL and DR5 at the cell surface. (a) AN3CA, HEC1A, and KLE endometrial cell lines were treated with a vehicle control or ONC201 at 0.3 μM (one tenth the $G1_{50}$), 3 μM (around the $G1_{50}$), and 30 μM (10x the $G1_{50}$) for 24, 48, and 72 hours. Western blot analysis shows ATF4, CHOP, and DR5, mediators of the integrated stress response pathway. Actin is used as a loading control. (b/c) Cells were treated with a vehicle control or approximate GI_{50} doses of ONC201 for 48 hours and then stained with an anti-DR5 (b) or anti-TRAIL (c) antibody. The geometric mean fluorescence intensity was determined using flow cytometric analysis. Representative histograms show the staining. Samples run in triplicate. N = 3. Error bars denote SEM. * denotes $p < .05$, ** denotes < 0.002, *** < 0.001, ns denotes nonsignificance.

with either a vehicle control or 100 mg/kg ONC201 via oral gavage followed by either a vehicle control or recombinant TRAIL at 5 mg/kg IV 72 hours later. This dosing scheme was repeated weekly. The mice that received the combination therapy grew significantly smaller tumors (Figure 5a,b) compared to those who received vehicle ($p < .001$) or either agent alone ($p = .002$ and $p < .001$) on day 18. Additionally, Kaplan-Meier survival analysis showed a significant survival benefit with the combination treatment ($p = .018$). There was no significant difference found between Vehicle, ONC201 alone or TRAIL alone control groups (Figure 5d). Mouse weights were measured over time as a surrogate measure for drug toxicity. No difference in mouse body weight was seen between treatment groups over time (Figure 5c). In addition, no evidence of liver toxicity was seen by H&E staining following treatment (Figure 5e). This is consistent with a nontoxic profile of either drug alone or combination therapy. Together this data demonstrated that combination treatment with ONC201 with recombinant TRAIL is effective at inhibiting tumor growth and is not toxic *in vivo*.

Discussion

Uterine cancer is the most common gynecologic malignancy among US women and the incidence is on the rise with over 63,000 new cases identified each year. Although prognosis for early stage endometrial cancer is favorable, the prognosis for those with metastatic or recurrent disease remains poor leading to over 11,000 deaths annually. For these patients the optimal adjuvant therapy has yet to be established, and chemotherapy in this group has had only modest response rates. In recent years, there has been a push to identify novel therapeutic approaches for these patients.

One novel drug therapy, ONC201, showed promising activity in patients with advanced endometrial cancer when tested in a variety of solid tumors during a phase I trial.[5] This has steered interest in ONC201 for its use as a therapeutic agent in endometrial cancer.

Data presented herein show that ONC201, a well-tolerated, orally active small molecule, has antitumorigenic properties in endometrial cancer models. In particular, we show that (1) ONC201 decreases cell viability in endometrial cancer cells
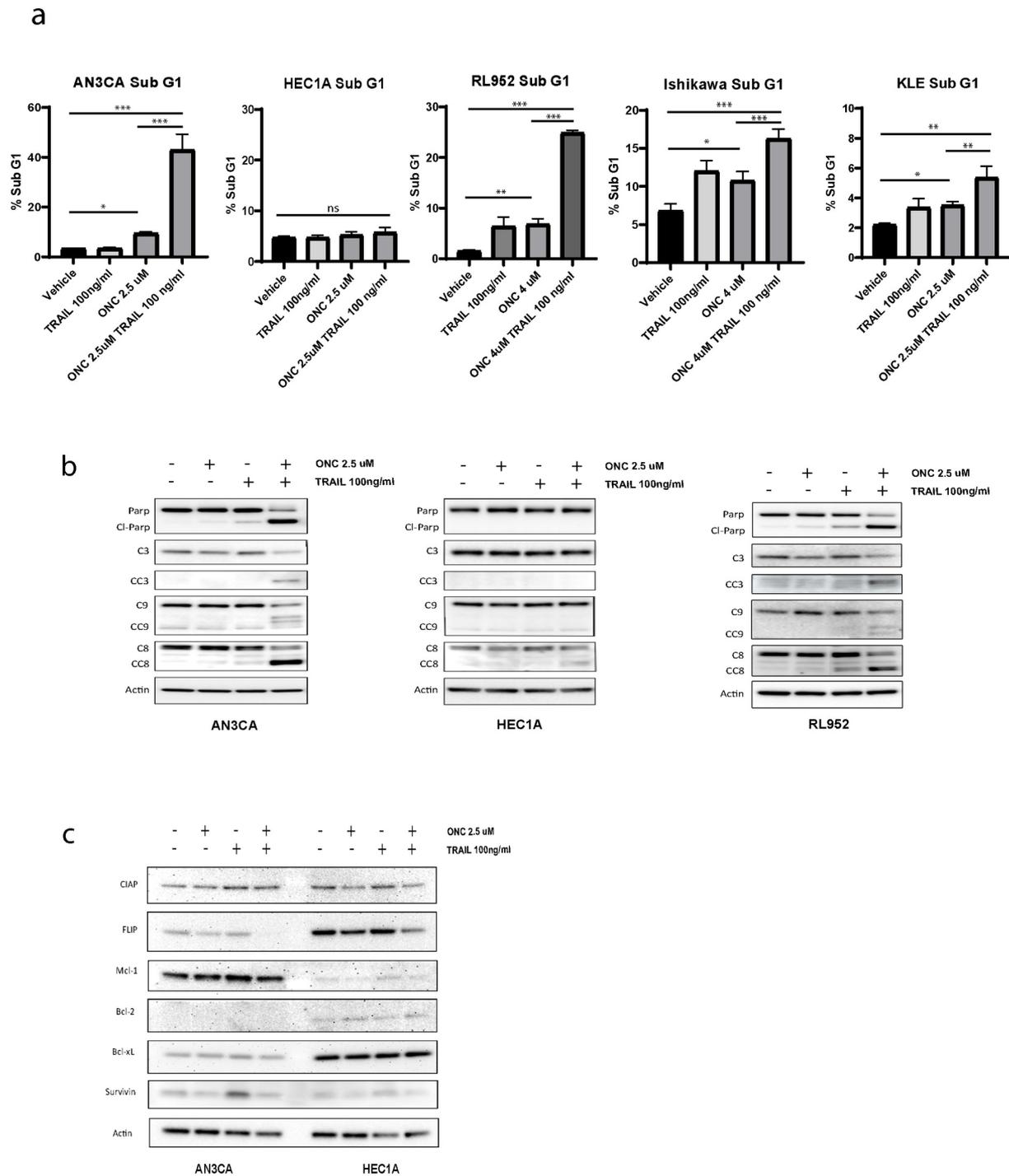
Figure 4. Pre-treatment with ONC201 sensitizes endometrial cancer cell lines to TRAIL, leading to increased cell death induction compared to either agent alone. AN3CA, HEC1A, KLE, RL952 and Ishikawa endometrial cancer cells were treated with either vehicle or approximate GI_{50} concentration of ONC201 for 72 hours followed by vehicle or recombinant human TRAIL (rhTRAIL) for 4 hours. (a) Cells were stained with propidium iodide and Flow cytometric analysis of the cells was used to determine the percentage of cells with $subG_1$ DNA content. Samples run in triplicate. Error bars denote standard deviation. * denotes $p < .05$, ** denotes < 0.002, *** <0.001, ns denotes nonsignificance. Compared to control the combination ONC201+ TRAIL led to an increase of cells in subG1 by 12.6× (AN3CA), 1.1× (HEC1A), 16.6× (RL952), 2.4× (Ishikawa), and 2.4× for KLE (**b**) Western blot analysis shows PARP, caspase-3, caspase-9, and caspase-8 cleavage for the AN3CA, HEC1A, and RL952 cell lines. **c)** Western blot analysis shows protein expression of anti-apoptotic proteins CIAP, FLIP, Mcl-1, Bcl-XL, Bcl-2, and surviving are shown for AN3CA and HEC1A. Actin is used as a loading control.
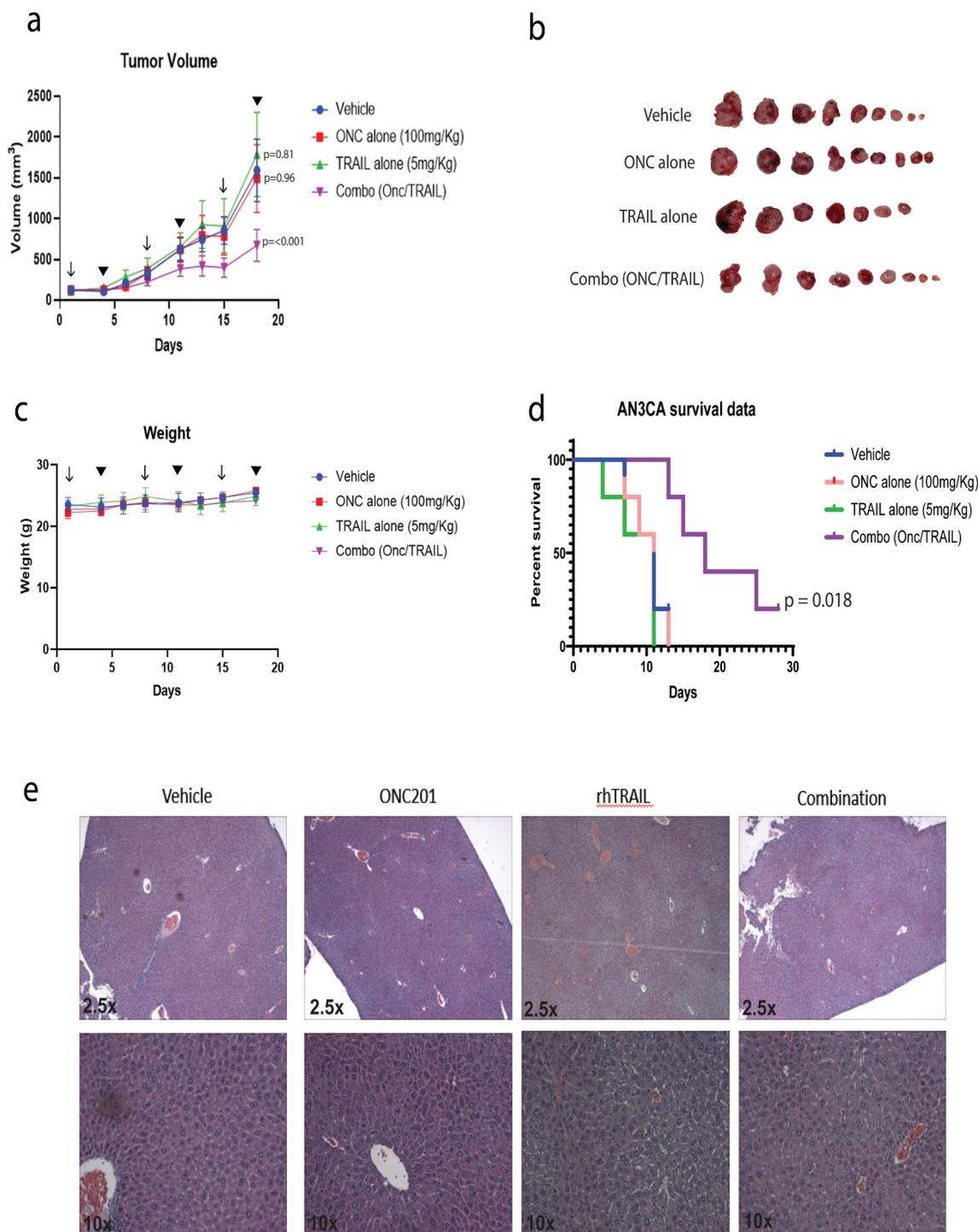
Figure 5. Tumor growth is reduced *in vivo* by combination treatment with ONC201 and recombinant TRAIL in a xenograft model of endometrial cancer. Nude mice bearing AN3CA xenografted tumors. Mice were treated either with a vehicle control (n = 10), 100 mg/kg ONC201 PO once per week (n = 10), recombinant TRAIL 5 mg/kg IV, once per week (n = 10), or a combination of ONC201 PO 100 mg/kg once per week followed by recombinant TRAIL 5 mg/kg IV 72 hours later (n = 10). Tumor volume (a) and mouse weights (c) were measured over time. Tumor size was compared between vehicle control and treatment groups. (a) At the 18 day point the tumors from the combination therapy group were significantly smaller than those in the vehicle control group (p = <0.001) or either agent alone (combo vs TRAIL alone p = .002 and combo vs ONC201 alone p < .001). Mice receiving either agent alone were similar to those from the control group (control vs Trail alone p = .81, control vs ONC201 alone p = .96). (b) At the end of a three-cycle (3 week) treatment course, tumors were excised and are presented to display the size difference between the treatment groups after a three-cycle treatment. One tumor per mouse flank is presented. (c) Mouse weights were measured over the course of the experiments. No significant differences in mouse weights were seen between all four groups at each time point. (d) Mice were treated with either a vehicle control (n = 5), recombinant TRAIL (n = 5), or combination of ONC201/TRAIL (n = 5), and individually euthanized when tumor size reached the size limit specified by the protocol. Kaplan-Meier survival analysis is depicted. Mice treated with combination treatment survived significantly longer than mice from the three control groups (p = .018). No significant difference was found between Vehicle, ONC201 alone or TRAIL alone control groups. (e) H&E stain of mouse liver sections after two cycles of treatment or control. Arrow denotes day of ONC201 treatment, arrowhead denotes day of TRAIL injection. Error bars denote SEM.

lines at clinically achievable low micromolar concentrations, primarily through growth arrest when used as a single agent and that (2) combining ONC201 with TRAIL has the effect of pushing cells toward cell death. Moreover, (3) the effect of combining ONC201 with TRAIL is retained *in vivo* without demonstrated toxicity.

We established that cell proliferation was arrested in all five endometrial cell lines after treatment with ONC201, suggesting that ONC201 is effective universally in endometrial cancer. Moreover, the effective doses of ONC201 were in the micromolar range which have previously been shown to be clinically achievable.[5] This is consistent with the findings in type II serous endometrial cancer cells lines.[13] The effect on proliferation in our cell lines was associated with an arrest in the G1 phase of the cell cycle with an increase in G1 phase inhibitor p27 and decreased cyclin D1 and pRb.

Cyclin D1 plays an important role in activating cyclin-dependent kinases 4/6 to initiate the G1-> S phase transition required for cell proliferation. Aberration in CDK4 expression is seen in 35%–77% of endometrioid endometrial cancers[14] and cyclin D1 over expression has been shown to be associated with poorer prognosis in endometrial cancer.[15] This has led to the development of CDK inhibitors as targeted therapy in endometrial cancer, with clinical trials underway. We demonstrated that ONC201 decreased the expression of cyclin D1 and pRb, similar to the mechanism shown with ONC201 in colorectal cancer (Kline *et al.*, 2016) and in endometrial cancer by CDK4 inhibitors,[16] further supporting a role for ONC201 in endometrial cancer.

Although the cell cycle was arrested after ONC201 treatment, this failed to translate into a significant increase in cell death, an outcome which was similarly found in a subset of breast cancer cell lines.[17] However, Ralff *et al.* demonstrated that in nontriple negative breast cancer cells, the antiproliferative phenotype could be pushed toward apoptosis with the combination treatment of ONC201 and TRAIL.[12]

ONC201's antitumor effect has been shown in nonendometrial cell lines to be p53-independent and mediated through inhibition of ERK and AKT leading to upregulation of TRAIL[7] as well as by activation of the integrated stress response (ISR) leading to DR5 activation.[11] In endometrial tissue, studies have shown that malignant transformation is associated with reduced expression of death receptors DR4/DR5[18] and TRAIL has been shown to have a proapoptotic effect on endometrial cancer cell lines *in vitro*.[19] We demonstrate that in endometrial cancer cell lines ONC201 activates the integrated stress response and induces protein expression of DR5 and TRAIL at the cell surface consistent with other model systems.[7,11] This suggests that ONC201 is able to prime the cell for TRAIL-mediated apoptosis in endometrial cancer. Similar to the breast cancer model[12] when endometrial cancer cells were treated with TRAIL after being primed with ONC201, we were able to push the cells into apoptosis. In a pilot study of AN3CA cell line, pretreatment with ONC201 resulted in decreased cell viability following treatment with a DR5 agonist (data not shown). Our data supports the hypothesis that ONC201 upregulation of DR5 sensitizes tumor cells to TRAIL, leading to conversion from growth arrest to cell death

in ONC201/TRAIL-treated cells. Further exploration of this combination therapy would need to be verified in a clinical trial.

ONC201 advanced from preclinical to clinical trials in 2014[5] and is now the focus of a number of clinical trials in a variety of solid tumors including three phase II clinical trials in advanced endometrial cancer (NCT03099499, NCT03485729, NCT03394027), which have been accruing. Although ONC201 had promising findings as a single agent,[5] it has been accepted in the field of GYN oncology that the future of adjuvant therapy for GYN cancers lies in combination therapy. We are now seeing clinical trials for ONC201 being tested in combination including an upcoming trial of ONC201 with paclitaxel in platinum-resistant refractory or recurrent ovarian cancer (NCT04055649). The data presented here provide preclinical support and rationale for an ONC201/TRAIL combination therapy trial for endometrial cancer.

Current investigative trials for endometrial cancer therapies have been limited by their modest efficacy or toxicity profile.[20] For example, current trials with combined MEK and AKT inhibitors (Trametinib with Uprosertib) or combined mTOR and antiangiogenic agents (temsirolimus with bevacizumab) were discontinued due to toxicity.[21,22] We demonstrate in this paper that both ONC201 alone or in combination with recombinant TRAIL had little toxicity *in vivo*. This is consistent with our preclinical data that established ONC201 does not affect non-neoplastic cells[7] and with data from the first in human phase I trial that showed ONC201 to be to be well tolerated with no drug related > grade 1 adverse events when given as a single agent.[5] Similarly, a recent study by Ralff *et al.* demonstrated little toxicity of the combination treatment of ONC201 with recombinant TRAIL in a mouse model of breast cancer.[12] TRAIL-based therapies have historically been associated with liver toxicity.[23] Given the low levels of recombinant TRAIL required for efficacy when given in combination, there was little toxicity seen in our *in vivo* studies. Furthermore, less toxic forms of recombinant trail and trail receptor antagonists are in development, which may further expand their use clinically.

Although we were able to achieve efficacy with the combination treatment of ONC201 and TRAIL in four of the five endometrial cell lines tested, the HEC1A endometrial cells were only minimally impacted. This suggests that an underlying resistance mechanism exists in a subset of endometrial cancer cells. Cancer cells have developed numerous mechanisms to overcome cell death. One mechanism includes dysregulating the equilibrium between anti- and proapoptotic family members. However, our testing of a number of anti-apoptotic family members showed no differences in expression following drug delivery between the AN3CA and HEC1A cell line, suggesting this was not the resistance mechanism displayed by HEC1A. Additionally, no differences were found in their ability to activate the integrated stress response or upregulate TRAIL expression. Resistance mechanisms toward ONC201 would be clinically relevant and warrant further investigation.

In conclusion we were able to demonstrate that ONC201 decreases the cell viability of endometrial cancer cells at clinically achievable μM concentrations and that when given in

combination with recombinant TRAIL it promotes cell death *in vitro* and *in vivo*. These preclinical data provide rationale for future human clinical trials.

Materials and Methods

Cell culture and reagents

The cell lines used to generate these data were obtained from the Fox Chase Cancer Center (FCCC) cell culture facility or directly from the American Type Culture Collection (ATCC). The Ishikawa cell line specifically was obtained from Sigma-Aldrich. All cell lines were authenticated and confirmed to be mycoplasma free. The cell lines were cultured in the media specified by ATCC or Sigma-Aldrich guidelines, supplemented with FBS (5 or 10% per guidelines) and 1% penicillin-streptomycin antibiotic. ONC201 was supplied by Oncoceutics, Inc. and was dissolved in DMSO. Recombinant human TRAIL (rhTRAIL) was generated as previously described.[12]

Cell viability assays

Cell viability assays were performed using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions and captured using the *in vivo* imaging system (IVIS). Cells were plated in 96-well black bottom plates (5×10^3 cells/well) and allowed to adhere overnight. Wells were then treated in triplicate with a vehicle control or increasing concentrations of ONC201 for 72 hours. For the combination experiments, the media was removed after 72 hours and replaced with fresh media containing a vehicle control or rhTRAIL at varying doses. Cellular viability was quantitated after 4 hours of rhTRAIL treatment. GraphPad Prism software was used to generate dose-response curves, corrected to the control (DMSO alone).

Clonogenic colony formation assay

Cell were plated in 12-well plates (250–2000 cells/well depending on cell line) and allowed to adhere overnight. Wells were then treated in triplicate with a vehicle control or increasing concentrations of ONC201 for 72 hours. Doses around the GI_{50} were selected as well as $2\times$ and $4\times$ the GI_{50} were used to evaluate for trends. After 72 hours the media was exchanged for drug free media and changed every 3rd day with drug free media day for an additional 10 days of culture. The plates were then stained with 0.25% crystal violet solution and imaged using the IVIS system.

Flow cytometric PI staining

To assess the effects of ONC201 on the cell cycle and on the population of cell in the subG1, pre-treated cells were fixed in 70% ethanol and stained with propidium iodide in the presence of ribonuclease A (RNase A). The cells were then analyzed using an Elite Epics flow cytometer (Beckman Coulter). Cell cycle analysis was performed using FlowJo software. Samples were run in triplicate. For the subG1 and cell cycle experiments

with ONC201 alone various doses of ONC201 were used to evaluate for trends. Doses around the GI_{50} were selected as well as $2\times$ and $4\times$ the GI_{50} were used. For the combination drug experiments, cells were treated with ONC201 at the GI_{50}.

Immunoblotting

Cells were washed with PBS, and lysed with RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was then quantified using the Pierce BCA protein assay kit (Life Technologies) and equal amounts of protein were run on precast polyacrylamide gels (NuPAGE 4–12% Bis-Tris protein gels, Thermo Fischer Scientific) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 hour at room temperature with a 5% nonfat milk powder in TBST and then incubated overnight at 4°C with primary antibodies diluted in TBST with 5% bovine serum albumin (BSA). Horseradish peroxidase (HRP) conjugated secondary antibodies (Thermo Fischer Scientific) diluted in TBST with 5% BSA were then applied and incubated for 1.5 hours. HRP activity was detected with ECL Western blotting substrate (Thermo Fischer Scientific) and chemiluminescent signal was measured using a gel documentation system (SYNGENE). Primary antibodies were purchased from Cell Signaling Technology (s.c) and include: Parp (c.s. 9542), total caspase 8 (c.s. 9746), total caspase 3 (c.s. 9662), cleaved caspase 3 (c.s. 9661), p27 (c.s. 3688), cyclin D1 (c.s. 2978), phosphor-Rb (c.s. 8516), DR5 (c.s. 3696), CIAP (c.s. 4952), FLIP (c.s. 56343), Mcl-1 (c.s. 4572), XIAP (c.s. 2042), Survivin (c.s. 2808), Bcl-XL (c.s. 2872). Actin was purchased from Sigma. Secondary antibodies were horseradish peroxidase conjugated and purchased from The Jackson Laboratory.

Flow cytometric analysis of cell surface TRAIL and DR5 protein levels

AN3CA, HEC1A, and KLE cells were treated with a vehicle control or approximate GI_{50} doses of ONC201 for 48 hours and then stained with an anti-TRAIL or anti-DR5 antibody to assess cellular surface expression of TRAIL and DR5. Cells were harvested with enzyme-free cell dissociation buffer (Life Technologies), washed with PBS and stained with fluorophore conjugated primary or isotype control antibodies. The primary antibodies were obtained from BioLegend (DR5: cat # 307406, TRAIL: cat # 308206, mouse IgG1, k isotype control: cat # 400112) and were diluted in FACS buffer (PBS, 1% FBS, 0.1% sodium azide). Cells were analyzed using an LSR II flow cytometer (BD Biosciences). The geometric mean fluorescence intensity was determined using flow cytometric analysis.

In Vivo experiments

For subcutaneous xenografts, 9-week-old female athymic *nu/nu* mice (Taconic Biosciences) were inoculated subcutaneously with $0.75/2 \times 10^6$ AN3CA cells in each rear flank. Once tumors reached a volume of 150–250 mm^3, treatment was initiated. Mice were treated with either a vehicle control (n = 10),

100 mg/kg ONC201 via oral gavage once per week (n = 10), recombinant TRAIL 5 mg/kg IV tail vein injection, once per week (n = 10), or a combination of ONC201 PO 100 mg/kg once per week followed by recombinant TRAIL 5 mg/kg IV 72 hours later (n = 10). This dosing scheme was repeated weekly, with caliper measurements of tumor volume taken every 2nd or 3rd day for the duration of the experiment. Mice were sacrificed when tumors reached the size limit specified by the protocol or after three cycles depending on experiment. n = the number of independent biological replicates.

Statistical analysis

All statistical analysis was performed using Prism 7 software (GraphPad). The data are presented as the mean -/+ SEM from three or more replicates. Two-way ANOVA was used to compare more than one group. Figures were annotated to indicate significance or lack thereof (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, ****$p \le 0.0001$, ns = nonsignificance).

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