Am J Cancer Res 2023;13(12):6290-6312
www.ajcr.us /ISSN:2156-6976/ajcr0149916

Original Article

ONC201/TIC10 plus TLY012 anti-cancer effects via apoptosis inhibitor downregulation, stimulation of integrated stress response and death receptor DR5 in gastric adenocarcinoma

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Received March 1, 2023; Accepted November 13, 2023; Epub December 15, 2023; Published December 30, 2023

Abstract: Gastric adenocarcinoma typically presents with advanced stage when inoperable. Chemotherapy options include non-targeted and toxic agents, leading to poor 5-year patient survival outcomes. Small molecule ONC201/TIC10 (TRAIL-Inducing Compound #10) induces cancer cell death via ClpP-dependent activation of the integrated stress response (ISR) and up-regulation of the TRAIL pathway. We previously found in breast cancer, pancreatic cancer and endometrial cancer that ONC201 primes tumor cells for TRAIL-mediated cell death through ISR-dependent upregulation of ATF4, CHOP and TRAIL death receptor DR5. We investigated the ability of ONC201 to induce apoptosis in gastric adenocarcinoma cells in combination with recombinant human TRAIL (rhTRAIL) or PEGylated trimeric TRAIL (TLY012). AGS (caspase 8-, KRAS-, PIK3CA-mutant, HER2-amplified), SNU-1 (KRAS-, MLH1-mutant, microsatellite unstable), SNU-5 (p53-mutant) and SNU-16 (p53-mutant) gastric adenocarcinoma cells were treated with ONC201 and TRAIL both in cell culture and *in vivo*. Gastric cancer cells showed synergy following dual therapy with ONC201 and rhTRAIL/TLY012 (combination indices < 0.6 at doses that were non-toxic towards normal fibroblasts). Synergy was observed with increased cells in the sub-G1 phase of the cell cycle with dual ONC201 plus TRAIL therapy. Increased PARP, caspase 8 and caspase 3 cleavage after ONC201 plus TRAIL further documented apoptosis. Increased cell surface expression of DR5 with ONC201 therapy was observed by flow cytometry, and immunoblotting revealed ONC201 upregulation of the ISR, ATF4, and CHOP. We observed downregulation of anti-apoptotic cIAP-1 and XIAP in all cells except AGS, and cFLIP in all cells except SNU-16. We tested the regimen in an organoid model of human gastric cancer, and in murine sub-cutaneous xenografts using AGS and SNU-1 cells. Our results suggest that ONC201 in combination with TRAIL may be an effective and non-toxic option for the treatment of gastric adenocarcinoma by inducing apoptosis via activation of the ISR, increased cell surface expression of DR5 and down-regulation of inhibitors of apoptosis. Our results demonstrate *in vivo* anti-tumor effects of ONC201 plus TLY012 against gastric cancer that could be further investigated in clinical trials.

Keywords: ONC201, TRAIL, DR5, ISR, ATF4, CHOP, imipridone, integrated stress response, gastric cancer, cIAP1, XIAP

Introduction

Advanced gastric cancer remains a deadly disease with little progress in therapeutic advances [1-5]. There is much morbidity and mortality from the disease that requires research efforts to improve patient outcomes. While some progress has been made with targeted therapy for example against Her-2 [6-8], this only helps a small minority of patients with gastric cancer and is not curative. Immunotherapy has thus far had limited impact on advanced gastric cancer

although clinical development is ongoing [4, 9-12].

The Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) is a part of the innate immune system that suppresses cancer and its metastases [13-15]. The TRAIL pathway has been and remains under investigation as a cancer therapeutic modality due to its specific for cancer and transformed cells but not normal cells [15]. We previously discovered and cloned TRAIL death receptor DR5 as a novel pro-apoptotic gene and mediator of p53-dependent apoptosis through direct transcriptional upregulation by p53 protein [16-18]. Our subsequent studies uncovered that the TRAIL gene is also a direct p53-regulated gene [19]. We conducted chemical library screening that led to the discovery of TRAIL-Inducing Compound #10 (TIC10) through p53-independent mechanisms [20].

TIC10/ONC201 has been tested in clinical trials and has shown responses in patients with H3K27M-mutated diffuse midline gliomas, non-H3K27M-mutated neuroendocrine tumors including paragangliomas and pheochromocytomas, as well as in some patients with prostate or endometrial cancer [21-27]. Previous studies have implicated various molecular mechanisms in the anti-tumor efficacy of ONC201/TIC10 [21]. Consistently observed activities of the drug have included upregulation of the TRAIL pathway through ClpP-dependent engagement of the integrated stress response with upregulation of ATF4, CHOP, and TRAIL death receptor DR5 [21, 28-30]. A variety of tumor cells also downregulate pAkt and pERK that has been correlated with nuclear localization of Foxo3a and transcriptional upregulation of the TRAIL gene [20]. ONC201/TIC10 has demonstrated effects against cancer stem cells and immune stimulatory effects towards natural killer (NK) cells [31-33]. An inflammatory profile in ONC201-treated patients has been observed and associated with a longer progression free survival [27].

Our preclinical studies have pursued strategies to further potentiate the anti-tumor activity of ONC201 [21]. For example, we showed that there is synergy between ONC201 or its analogues and EZH2 or HDAC inhibitors [34, 35], anti-angiogenic agents [36], radiation and temozolomide (https://www.eventscribe.com/2019/posters/SNO/SplitViewer.asp?PID=NjQ-1ODAwMjM4NzY) [37, 38], lurbinectedin [39], everolimus or enzalutamide [40] or glycolysis inhibitors [41]. For several years, we have explored reasons as to why apoptosis may not occur in some ONC201-treated cells [28, 42, 43]. Our previous studies in breast and other cancers revealed that ONC201 upregulates DR5 in treated tumor cells but this alone is not sufficient to induce cell death, apparently due to limited production of TRAIL that was the goal of the original discovery of TIC10 as a TRAIL-inducing compound [43]. As such, we have previously tested the idea that ONC201 treatment of breast or other tumor cells primes the cells for TRAIL-mediated cell death and that such an activity may be exploited in cancer therapy through a combination of ONC201/TIC10 plus TRAIL [43-45].

In the present study, we have explored the ONC201 cancer cell priming towards cell death hypothesis by evaluating the sensitivity of various human gastric cancer cell lines to treatment by ONC201 alone or in combination with recombinant human TRAIL (rhTRAIL), as well as a more recent PEGylated trimeric TRAIL formulation (TLY012). We found that indeed in human gastric cancer cells, DR5 is upregulated after ONC201/TIC10 treatment, with little change in cell surface TRAIL expression. The addition of rhTRAIL or TLY012 activated massive apoptosis in the ONC201-treated 'apoptosis-primed' tumor cells. The apoptotic cell death was documented in various assays and the priming was also shown to involve downregulation of anti-apoptotic proteins in addition to the ISR-mediated DR5 upregulation. The studies were extended to a gastric cancer organoid model and to in vivo studies using two human gastric cancer xenografts. Our results suggest that ONC201 plus TLY012 which may be a general strategy to target difficult to treat cancers may be helpful in gastric adenocarcinomas that carry a very poor prognosis.

Materials and methods

Cell culture and reagents

We purchased human gastric adenocarcinoma cell lines AGS, SNU-1, SNU-5 and SNU-16 from American Type Culture Collection (ATCC). We confirmed cell lines to be mycoplasma free by morphology and PCR. Cell lines were cultured in appropriate media (AGS in F-12K, SNU-1, SNU-5 and SNU-16 in RPMI-1640) supplement-

ed with 10% (v/v) (AGS, SNU-1 and SNU-16) or 20% (v/v) (SNU-5) fetal bovine serum and 1% Penicillin/Streptomycin at 37°C within a 95% humidified 5% CO_2 incubator. Cells were harvested or passaged when they were ~80% confluent.

ONC201 was supplied by Chimerix, Inc., rhTRAIL was generated in-house using a protocol previously developed by our lab and detailed in Kim et al., and TLY012 was provided by D&D Pharmatech. ONC201 was dissolved in DMSO and TLY012 was dissolved in phosphate buffered saline (PBS) for use in in vivo experiments.

Cell viability assays

Tumor cells were seeded in 96-well plates at a concentration of 5×10^5 cells/10 μL media and ONC201 was added 24 hours later. After 72 hours of treatment with ONC201, rhTRAIL was added and cells were allowed to intubate for another six hours. CellTiterGlo bioluminescence agent (Promega Corporation, Madison, WI) was added per the manufacturer's recommendation to determine cell viability, and plates were imaged to assess cell viability. Synergy and combination indices were determined using Compusyn, which uses the ChouTalalay method for determining synergy.

Western blots

Cells were plated with approximately 5×10^5 cells in each cell of a 6-well plates for 24 hours for the AGS cell line to allow for adherence and suspension cells (SNU-1, SNU-5 and SNU-16) were plated immediately before treating with ONC201 for 24-72 hours as indicated in each figure, followed by rhTRAIL for six additional hours prior to processing for analysis. At this time, one mL of media containing floating cells was harvested for each sample. Cells were crushed and collected into the remaining 1 mL of media, and this total solution was pelleted for 5 minutes at 400 Rcf prior to being washed in PBS once. Cells were then lysed using RIPA buffer (Sigma-Aldrich) with 1X protease inhibitor and 1X phosphatase inhibitor (Roche), at which time samples were stored at -20°C. Protein density was quantified using a BCA Assay (Thermo Scientific).

Lysates were then centrifuged at 13,000 RPM at 4°C for approximately 20 minutes, were then boiled for 10 minutes and loaded in pre-cast NuPAGE 4-12% Bis-Tris gels (Thermo Scientific). Polypeptides were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corp.). Membranes were blocked with 10% non-fat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies: PARP (ATCC CS9542S), Caspase 8 (CST 9496S), Ran (BD Biosciences 610341), BID (CST 2002S), ATF4 (CST 11815S), CHOP (CST 2895S), p-FOX03a (CST 9466S), pAKT (CST 4060S), AKT (CST 2964), pERK (CST 9106S), ERK (CST 9102S), CLPX (BD Biosciences 168338), CLPP (ab124822), DR5 (CST 3969S), CIAP1 (CST 70008S), XIAP (CST 2042S), FLIP-L (CST 56343S), FLIP-S (CST 56343S), BAX (CST 2774S), BCL2 (CST 4223S), BCL-XL (CST 2762S), Survivin (CST 2803S).

Membranes were washed three times for five minutes and incubated with species-specific secondary antibodies horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences) for 1 h at room temperature. Blots were washed three times for five minutes, and developed by the Enhanced Chemiluminescence system (Amersham Biosciences).

Sub-G1 analysis

Cells were plated in 6-well plates for 24 hours for the AGS cell line to allow for adherence and suspension cells (SNU-1, SNU-5 and SNU-16) were plated immediately before treating with ONC201 for 72 hours, followed by rhTRAIL for six additional hours prior to processing for analysis. At this time all floating and adherent cells were harvested. They were centrifuged and washed with PBS to remove the phenol red from the cell culture medium. Once thoroughly washed, cells were resuspended and fixed in 70% cold ethanol and stored at 4°C. After at least 24 hours to allow for fixation, cells were stained with propidium-iodide to bind DNA. Samples were then evaluated using an Epics Elite (Beckman Coulter) flow cytometer, then analyzed with FlowJo to determine the percentage of cells in the sub-G1 phase of the cell cycle.

Cell surface analysis

Cells were plated in 6-well plates for 24 hours for the AGS cell line to allow for adherence and suspension cells (SNU-1, SNU-5 and SNU-16)

were plated immediately before treating with ONC201 for 24-72 hours as indicated in each figure, followed by rhTRAIL for six additional hours prior to processing for analysis. At this time adherent cells (AGS) were dislodged from cell plates by using a dissociation buffer (ThermoFischer Scientific 13151014), then all cells were resuspended in growth media, centrifuged, then blocked in PBS and 10% FBS. Cells were then suspended with the appropriate antibody (DR5 BioLegend 307406, TRAIL BioLegend 308206, mouse IgG1 BioLegend 400112) prior to being vortexed then centrifuged in PBS with 1% FBS. Fluorescent conjugated secondary antibodies were then added to allow for detection of cell surface bound primary antibodies. After being vortexed again and another wash, cells were fixed by being resuspended in 4% paraformaldehyde. Samples were evaluated using an Epics Elite (Beckman Coulter) within one week, and results were analyzed with FlowJo to determine amount of cell surface bound antibody.

Organoids

Human-derived gastric adenocarcinoma organoid (L97 837-ZL9-T-W-Organoid) sample was obtained from the National Cancer Institute (NCI). The organoid was grown, passaged and harvested according to the NCI designated organoid protocols as published online (https://pdmr.cancer.gov/sops/ SOP30101, SOP40102, SOP40103, SOP40104).

For experiments they were plated in Matrigel. Cell viability assays followed the protocol as designated above for cell culture. Immunofluorescence experiments were performed with the LIVE/DEAD viability/cytotoxicity kit (Invitrogen L3224) which results in staining live cells green and dead cells red upon fluoroscopic examination.

In vivo studies

All in vivo studies conducted for this manuscript were approved by the Brown University IACUC. For in vivo tumor xenograft studies, we used female, athymic nude mice acquired from Taconic Biosciences (NCR-Nu-F, genotype: sp/sp). Mice were aged 5-8 weeks at the time of tumor inoculation. Cells were mixed in a 50:50 Matrigel (Corning):PBS solution and mixed at various dilutions. Total inoculation volume was 200 µL, irrespective of tumor model or number of cells inoculated. ONC201 was always administered via oral gavage, and doses were titrated such that mice only received 100 µL of solution. The vehicle is a solution of 20% Cremophor EL (SigmaAldrich), 70% PBS, and 10% DMSO. TLY012 was administered via intraperitoneal injections. Tumor volumes were determined using Veniper calipers and calculating volume using the following formula: Volume = (width^2 × length)/2.

SNU-5 growth experiment

Tumors were initiated by injecting 5×10^6 cells into the flanks of the mice. Once tumors reached a volume of 150-250 mm^3, mice were randomized, and treatment regimens initiated. ONC201 100 mg/kg was given weekly for the ONC201, TLY012 2 mg/kg was given twice weekly, and for the dual therapy group, TLY012 treatment was given 3 days after ONC201.

For short-term analysis, mice were euthanized 1 week after treatment initiation, and tumors were harvested. They were then fixed in formalin and used for immunohistochemistry analysis.

For long-term analysis, tumor volume and mouse weight was measured 3 times per week, and mice were euthanized for final tumor analysis 30 days after treatment initiation. Tumor mass was determined upon harvest, at which time tumors and organs (heart, lungs, liver, spleen and kidneys) were harvested and stored in formaldehyde and blood was obtained via cardiac puncture for chemical analysis.

AGS growth experiment

Tumors were initiated by injecting 5×10^6 cells into the flanks of the mice. Once tumors reached a volume of 150-250 mm^3, mice were randomized, and treatment regimens initiated. ONC201 100 mg/kg was given weekly for the ONC201, TLY012 1 mg/kg was given once weekly, and for the dual therapy group, TLY012 treatment was given 3 days after ONC201.

For short-term analysis, mice were euthanized 2 weeks after treatment initiation, and tumors were harvested. They were then fixed in formalin and used for immunohistochemistry analysis.

For long-term analysis, tumor volume and mouse weight was measured 3 times per week, and mice were euthanized for final tumor analy-

sis 30 days after treatment initiation. Tumor mass was determined upon harvest, at which time tumors and organs (heart, lungs, liver, spleen and kidneys) were harvested and stored in formaldehyde and blood was obtained via cardiac puncture for chemical analysis.

Immunohistochemistry staining

Immunohistochemistry analysis was performed via a long-standing lab protocol. Tumors were fixed in formalin immediately after harvesting in cassettes. After fixation, cassettes were paraffin embedded. Slides were cut 5 μm thick. Immunohistochemistry was initiated by deparaffinizing slides using xylene. Slides were dehydrated through sequential dilutions of ethanol. The antigen retrieval step was conducted by heating slides for 10 minutes in pH 6.0 citrate acid buffer. Ki67 (MIB-1) antibody was obtained from Cell Signaling Technologies, used at 1:200 dilution. CC3 Antibodies obtained from BD Biosciences, used at 1:100 dilution. Slides were incubated in primary antibodies overnight; respective secondary antibodies were added the following day. Slides were developed using DAB Staining Kit (Vector Labs) and mounted using a xylene-based mount, Cytoseal XYL.

Statistical methods

All statistical analysis for animal models were performed in GraphpadPrism 7 software to perform a one-way ANOVA analysis to assess for differences in the means between groups. We then applied the Turkey's honestly significant procedure analysis in order to apply pairwise comparisons between individual group. Both allowed for us to establish a *p*-value both amongst all of the groups as a whole and between individual groups. We established significance as a *p*-value of ≤ 0.05.

Results

Cytotoxic effects of ONC201 and rhTRAIL as well as synergy of their combination in human gastric cancer cell lines

We hypothesized that the combination of ONC201 and rhTRAIL may be efficacious against human gastric cancer cells due to the previously described cell death priming effect. To investigate the cytotoxic effects and potential synergy. We used 4 different human cancer cell lines including AGS (caspase 8-, KRAS-, PIK3CA-mutant, HER2-amplified), SNU-1 (KRAS-, MLH1-mutant, microsatellite unstable), SNU-5 (p53-mutant) and SNU-16 (p53-mutant) gastric adenocarcinoma cells in treatment experiments with ONC201 and TRAIL individually or in combination. **Figure 1A-D** demonstrates that both ONC201 and rhTRAIL have cytotoxic effects against human gastric cancer cell lines. SNU-16 (IC50 = 1.82 μM), SNU-5 (IC50 = 2.88 μM) and SNU-1 (IC50 = 1.35 μM) appeared to be generally sensitive to ONC201 while AGS appeared relatively resistant (IC50 > 40 μM). All 4 gastric cancer cell lines were sensitive to rhTRAIL with a variation in their sensitivities. SNU-1 (IC50 = 14.5 ng/mL) and SNU-16 (IC50 = 38.9 ng/mL) were the most sensitive while AGS (IC50 = 144.5 ng/mL) and SNU-5 (IC50 = 457 ng/mL) were less sensitive. Normal human cell lines MRC5 and WI38 were relatively resistant to both ONC201 and rhTRAIL (**Figure 1E**, **1F**).

We investigated and found evidence for synergy between ONC201 and rhTRAIL in all four human gastric adenocarcinoma cell lines that were tested (**Figure 2**). The observed synergies in AGS, SNU-1, and SNU-16 appeared comparable while in SNU-5 greater synergy in cell culture were observed with ONC201 plus rhTRAIL.

PARP-, caspase 8- and Bid cleavage and sub-G1 cell cycle content following ONC201 and rhTRAIL treatment of human gastric cancer cell lines

We suspected that given the activation of TRAIL-pathway mediated apoptosis by either ONC201 or rhTRAIL, that markers of extrinsic pathway signaling would be increased. **Figure 3** shows that PARP, caspase 8 and Bid were cleaved in gastric cancer cells treated with ONC201 plus rhTRAIL over a 72-hour time course, although there was less Bid cleavage in SNU-16. As further evidence of synergistic apoptosis induction we found an increase in sub-G1 cell cycle content of gastric cancer ONC201 plus rhTRAIL treated cells (**Figure 4**).

Activation of the integrated stress response (ISR), ATF4, CHOP, DR5 along with suppression of p-Akt, p-ERK, p-Foxo3a and ClpX in ONC201 and rhTRAIL treated gastric cancer cell lines

We further investigated the underlying mechanisms of cell death induction in ONC201

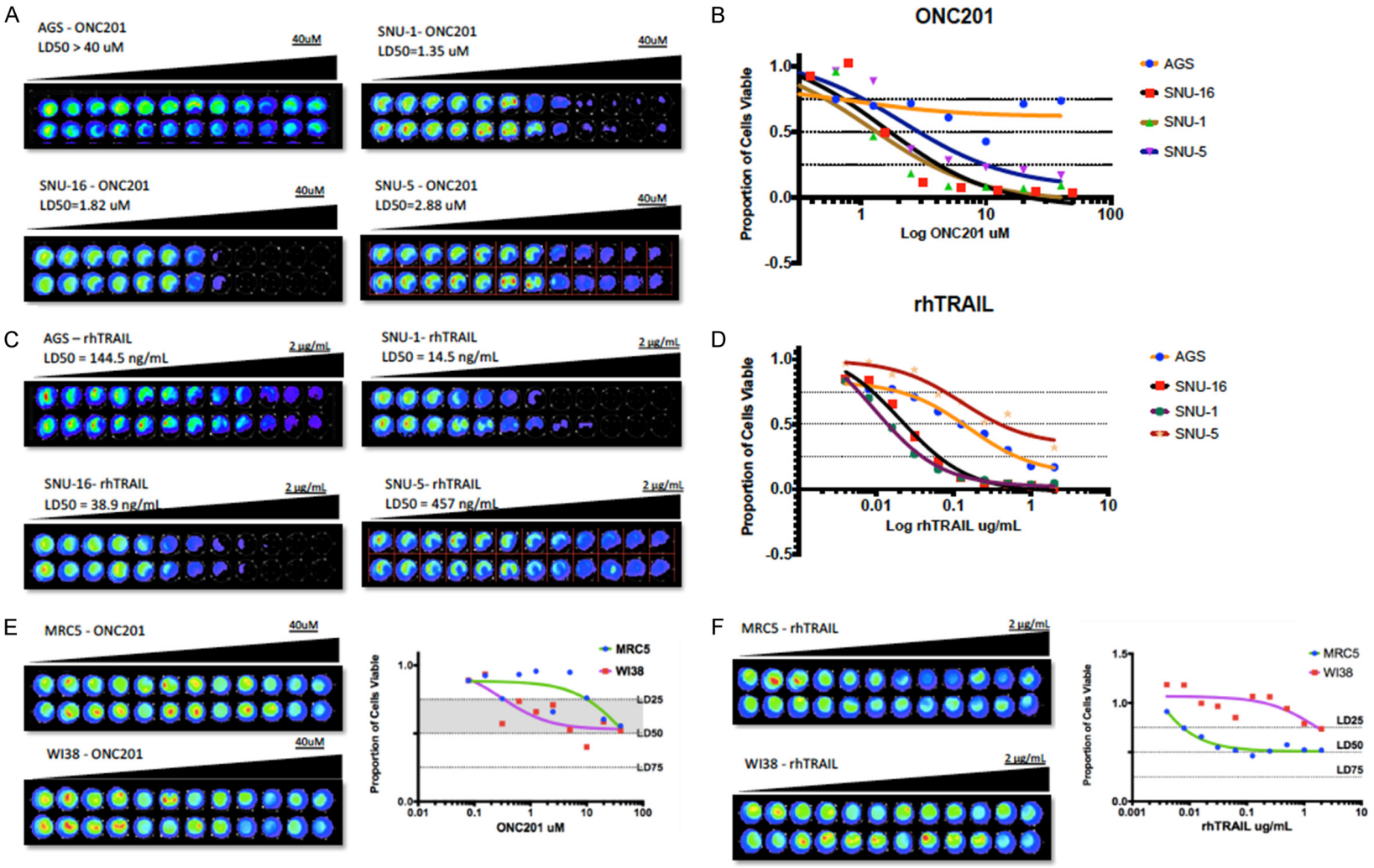
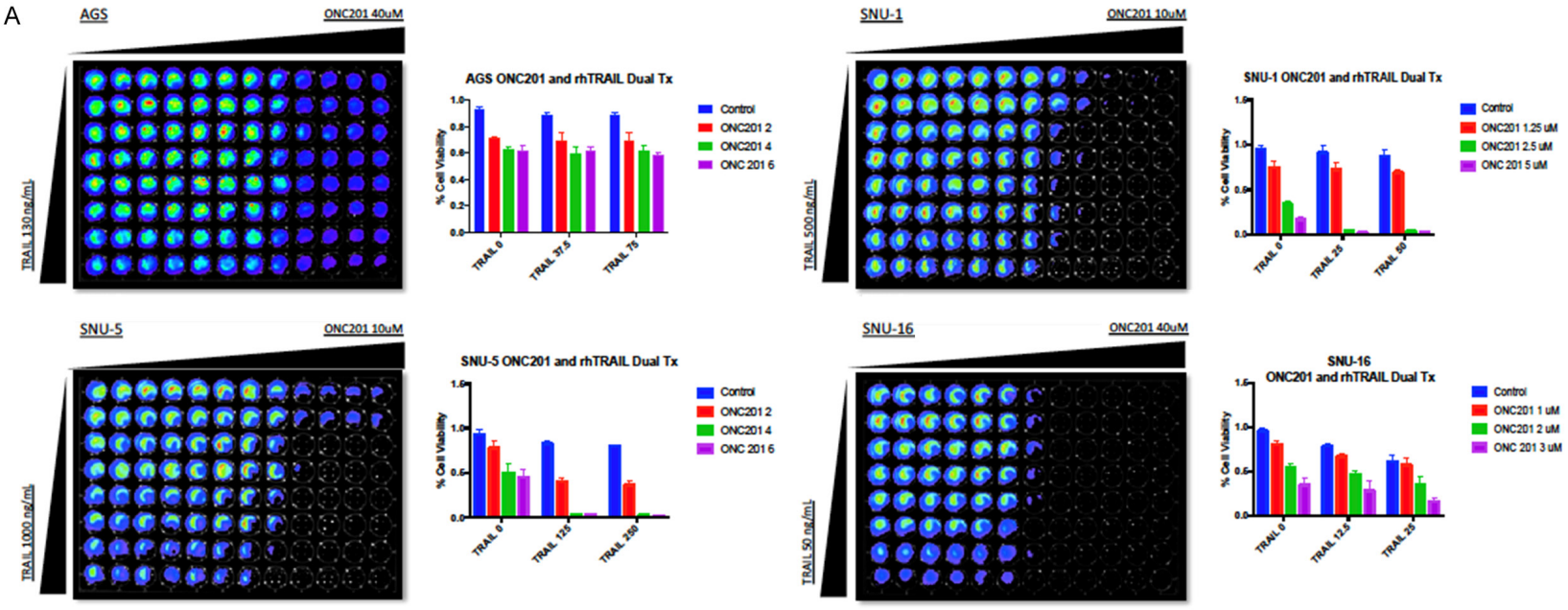


Figure 1. Cytotoxic effects of ONC201 and rhTRAIL in human gastric cancer cell lines. (A, C) Cell Titer Glo (CTG) assay of the viability of AGS, SNU-1, SNU-16 and SNU-5 cells treated with ONC201 (A) or rhTRAIL (C) for 72 hours is shown. Drug doses and cell lines are as indicated. (B, D) Graphical representation of cell viability reduction by ONC201 (B) or rhTRAIL (D) in treated gastric cancer cell lines. (E, F) Lack of significant loss of cell viability in ONC201-treated (E) or rhTRAIL-treated (F) MRC5 or WI38 cultured normal fibroblast cells.

A



B

$$\text{Combination Index} = (D)_1/(D)_2 + (Dx)_1/(Dx)_2$$

Synergy: CI<1

D: dose of drugs 1 and 2 when in combination yields 50% growth inhibition

Dx: GI_{50} of single dose drug

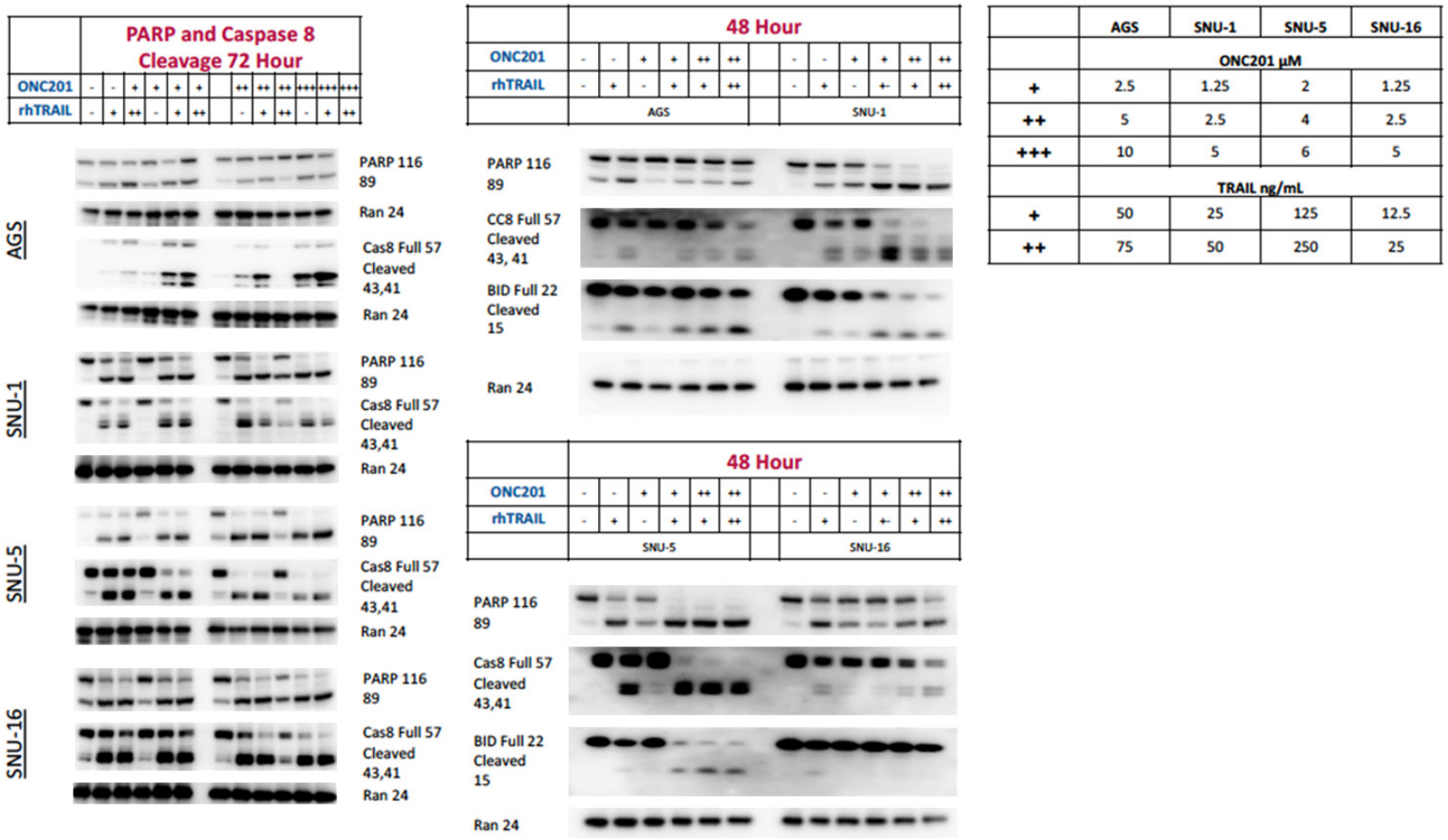
AGS		ONC201 μM		
		2.5	5	10
TRAIL ng/mL	32.5	0.81	0.43	0.53
	65	0.3	0.5	0.46

SNU-1		ONC201 μM		
		2	4	6
TRAIL ng/mL	25	0.42	0.44	0.44
	50	0.28	0.34	0.44

SNU-5		ONC201 μM		
		2	4	6
TRAIL ng/mL	125	0.43	0.04	0.03
	250	0.69	0.04	0.03

SNU-16		ONC201 μM		
		1.25	2.5	5
TRAIL ng/mL	6.25	0.48	0.25	0.4
	12.5	0.47	0.28	0.29

Figure 2. Synergistic effects of ONC201 and rhTRAIL in human gastric cancer cell lines. (A) Cell Titer Glo (CTG) assay of the viability of AGS, SNU-1, SNU-16 and SNU-5 cells treated with ONC201 plus rhTRAIL for 72 hours is shown. Drug doses and cell lines are as indicated. (B) Drug synergy analysis. Combination index values are shown for the dose combinations indicated on the right of (B).



	AGS	SNU-1	SNU-5	SNU-16
	ONC201 μM			
+	2.5	1.25	2	1.25
++	5	2.5	4	2.5
+++	10	5	6	5
	TRAIL ng/mL			
+	50	25	125	12.5
++	75	50	250	25

Figure 3. PARP-, caspase 8- and Bid cleavage following ONC201 and rhTRAIL treatment of human gastric cancer cell lines. Western blots are shown using lysates from gastric cancer cell lines at 48 or 72 hours as indicated. Ran is used as a loading control. The protein size is indicated next to its name. Doses of ONC201 or rhTRAIL are as indicated.

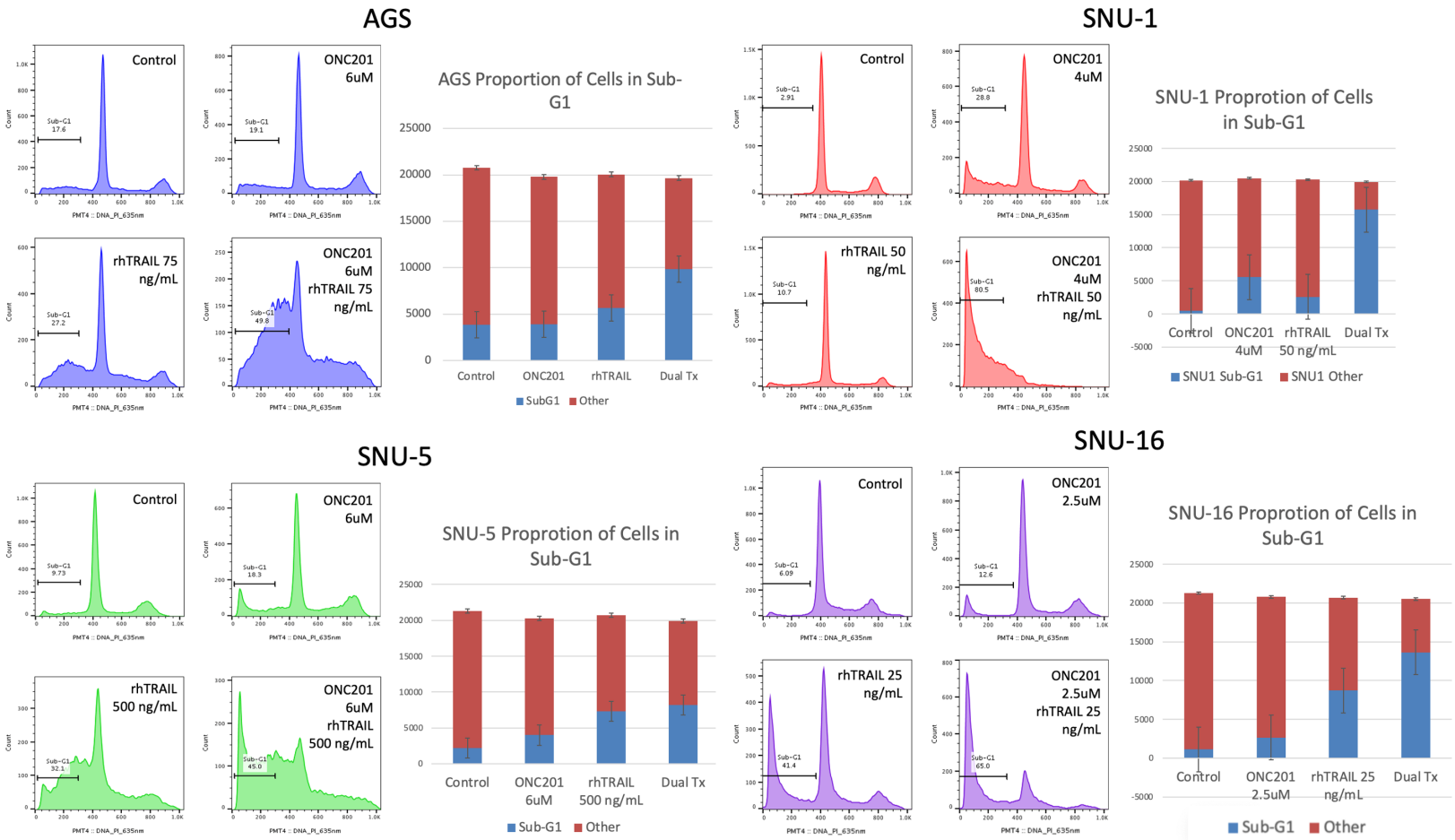


Figure 4. Sub-G1 cell cycle content following ONC201 and rhTRAIL treatment of human gastric cancer cell lines. Flow cytometric analysis for sub-G1 content of gastric cancer cell lines treated with doses of ONC201, rhTRAIL or the combination as indicated. Quantification of sub-G1 content is shown in the graphs.

plus rhTRAIL treated human gastric cancer cells. **Figure 5** shows that the integrated stress response biomarkers ATF4 and CHOP were increased in all four ONC201-treated gastric cancer cell lines. The addition of rhTRAIL appeared to reduce the upregulated ATF4 and CHOP in these experiments, likely as a consequence of cell death. Reductions in Foxo3a, p-Akt, and p-ERK were observed in ONC201 plus rhTRAIL treated gastric cancer cells although there was some variability. For example, p-Foxo3a was reduced in SNU-1 and SNU-5 but not in AGS or SNU-16. Such variations may also correlate with variations in TRAIL induction in treated cells. A reduction in Akt and p-Akt was observed in AGS, SNU-1, SNU-5 but not in SNU-16. Reduction in p-ERK was clearly seen in AGS and SNU-5 but not SNU-1 and while ERK was detectable in SNU-16, p-ERK levels were low in the absence of treatment. ClpX was reduced with ONC201 treatment of all 4 human gastric cancer cell lines.

We observed upregulation of DR5 but not cell surface TRAIL in ONC201-treated human gastric cancer cell lines (**Figure 6**). Cell surface DR5 was reduced in rhTRAIL-treated gastric cancer cell lines, likely due to apoptosis induction (**Figure 6B**). SNU-1 and SNU-5 showed DR5 upregulation by western blotting at the lowest ONC201 doses while AGS showed DR5 induction with higher ONC201 doses and SNU-16 had high levels by western blot regardless of treatment (**Figure 6A**). We also examined cell surface expression of TRAIL under similar treatment conditions, and found it to be unchanged in both AGS and SNU-5 cell lines when treated with ONC201 or rhTRAIL (**Figure 6C**).

Suppression of anti-apoptotic IAP proteins by ONC201 and rhTRAIL in gastric cancer cells

We surveyed gastric cancer cell lines for effects of ONC201 plus rhTRAIL treatment on inhibitor of apoptosis proteins (**Figure 7**). We found that survivin, cIAP-1 and XIAP expression levels were reduced in ONC201 plus rhTRAIL treated cells. Reduction in FLIP levels were observed in SNU-1, SNU-5 and SNU-16. These alterations were associated with apoptosis as shown by PARP cleavage in the treated cells. We noted no changes in Bax, Bcl2 or Bcl-XL in ONC201 plus rhTRAIL treated gastric cancer cells with AGS and SNU-5 showing no detectable Bcl2 expression (**Figure 7**).

In vivo synergistic growth suppression, antitumor efficacy and lack of toxicity by combination of ONC201 and TLY012 in SNU-5 human gastric cancer mouse xenograft model

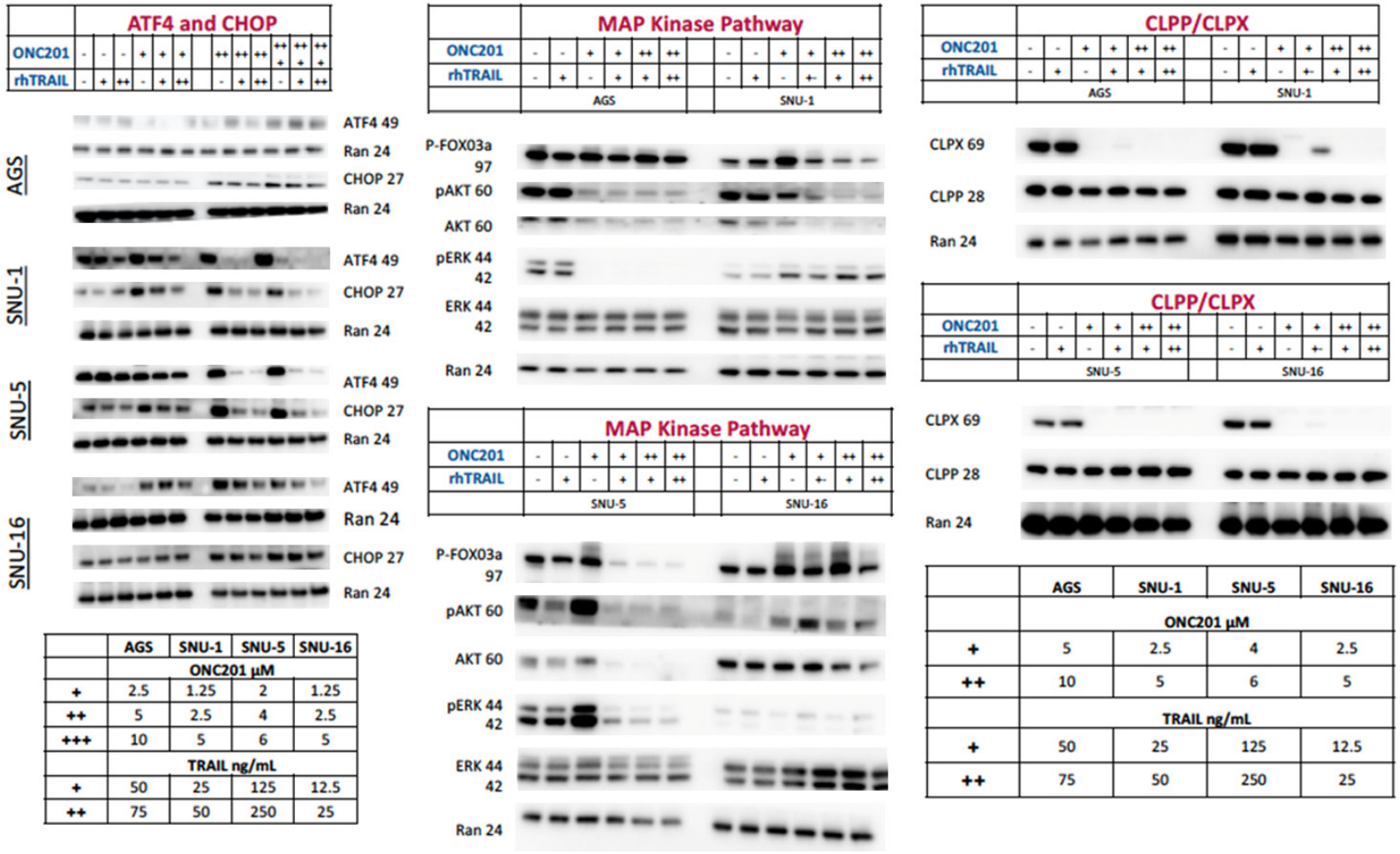
We conducted in vivo studies of the ONC201 plus TLY012 combination in the SNU-5 human gastric cancer mouse xenograft model. There was clear synergy in vivo by the combination of ONC201 plus TLY012 (**Figure 8**). Average tumor volumes are shown over the course of the experiment as well as final tumor volumes and images of the tumors. Individual tumor growth curves are shown in **Figure 8** for all treatment conditions. We found reduction of Ki67 and increase in cleaved caspase 3 in ONC201 plus TLY012 treated SNU-5 xenografts by immunohistochemistry (**Figure 8**). There was no evidence of alteration in mouse weight, creatinine, ALT, or AST liver enzymes or serum albumin in ONC201 plus TLY012 treated SNU-5 tumor-bearing mice (**Figure 9**). No alterations in the cytokines indicated in **Figure 9** were observed but these were at the time of sacrifice of the mice.

Cell death induction by combination of ONC201 and rhTRAIL in a human gastric cancer organoid model

In the course of our experiments we contacted the NCI to access an available gastric cancer organoid that had been established from a patient with gastric cancer. We performed experiments with the gastric cancer organoid that demonstrate synergistic killing by the ONC201 plus TLY012 combination therapy (**Figure 10**).

In vivo synergistic growth suppression, antitumor efficacy and lack of toxicity by combination of ONC201 and TLY012 in AGS as a second human gastric cancer mouse xenograft model

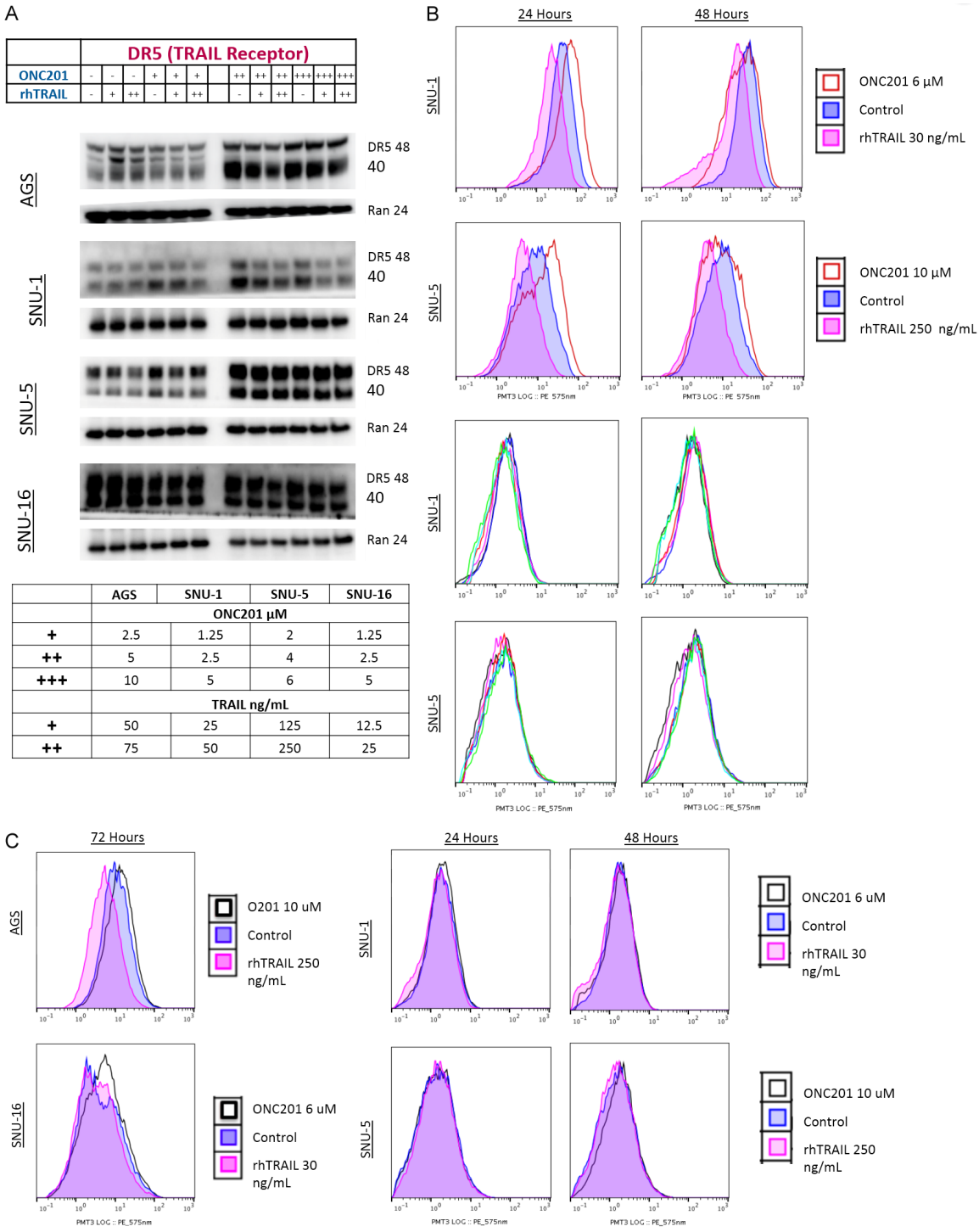
We performed additional *in vivo* experiments of the ONC201 plus TLY012 combination therapy



	AGS	SNU-1	SNU-5	SNU-16
	ONC201 μM			
+	2.5	1.25	2	1.25
++	5	2.5	4	2.5
+++	10	5	6	5
	TRAIL ng/mL			
+	50	25	125	12.5
++	75	50	250	25

	AGS	SNU-1	SNU-5	SNU-16
	ONC201 μM			
+	5	2.5	4	2.5
++	10	5	6	5
	TRAIL ng/mL			
+	50	25	125	12.5
++	75	50	250	25

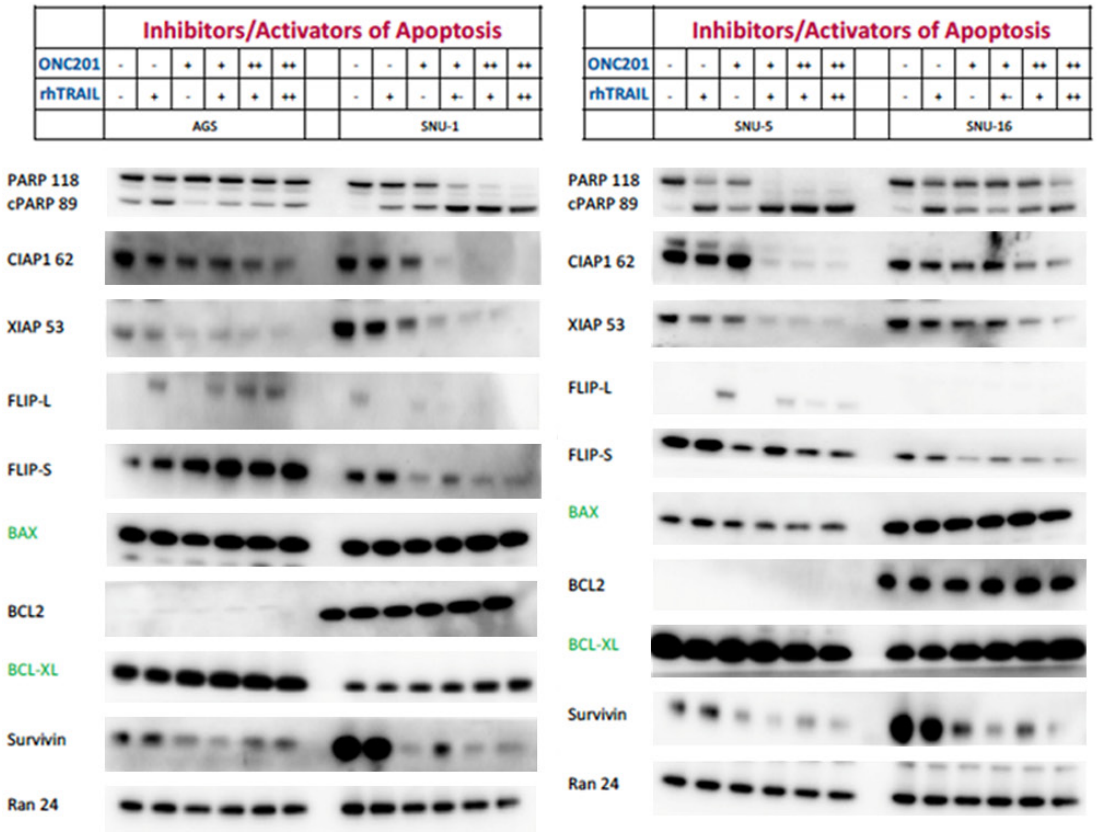
Figure 5. Activation of the integrated stress response (ISR), ATF4, CHOP along with suppression of p-Akt, p-ERK, p-Foxo3a and ClpX in ONC201 and rhTRAIL treated gastric cancer cell lines. Western blots with the indicated antibodies are shown using lysates from gastric cancer cell lines treated as indicated.



	AGS	SNU-1	SNU-5	SNU-16
	ONC201 µM			
+	2.5	1.25	2	1.25
++	5	2.5	4	2.5
+++	10	5	6	5
	TRAIL ng/mL			
+	50	25	125	12.5
++	75	50	250	25

Figure 6. Upregulation of cell surface DR5 along in ONC201 treated gastric cancer cell lines. A. Western blots with the indicated antibodies are shown using lysates from gastric cancer cell lines treated as indicated. B. Flow cytometric analysis of DR5 expression in ONC201 or rhTRAIL treated gastric cancer cell lines as indicated (top) and IgG control (bottom). C. Flow cytometric analysis of DR-5 expression in ONC201 or rhTRAIL treated cells in AGS and SNU-16 cell lines at 72 hours (left) and TRAIL binding in SNU-1 and SNU-5 cell lines at 24 and 48 hours (right).

using AGS as a second human gastric cancer mouse xenograft model (**Figures 11-14**). The results reveal similar patterns although less drastic with the ONC201 plus TLY012 combina-



	AGS	SNU-1	SNU-5	SNU-16
	ONC201 uM			
+	5	2.5	4	2.5
++	10	5	6	5
	TRAIL ng/mL			
+	50	25	125	12.5
++	75	50	250	25

Figure 7. Suppression of anti-apoptotic IAP proteins by ONC201 and rhTRAIL in gastric cancer cells. Western blots with the indicated antibodies are shown using lysates from gastric cancer cell lines treated as indicated.

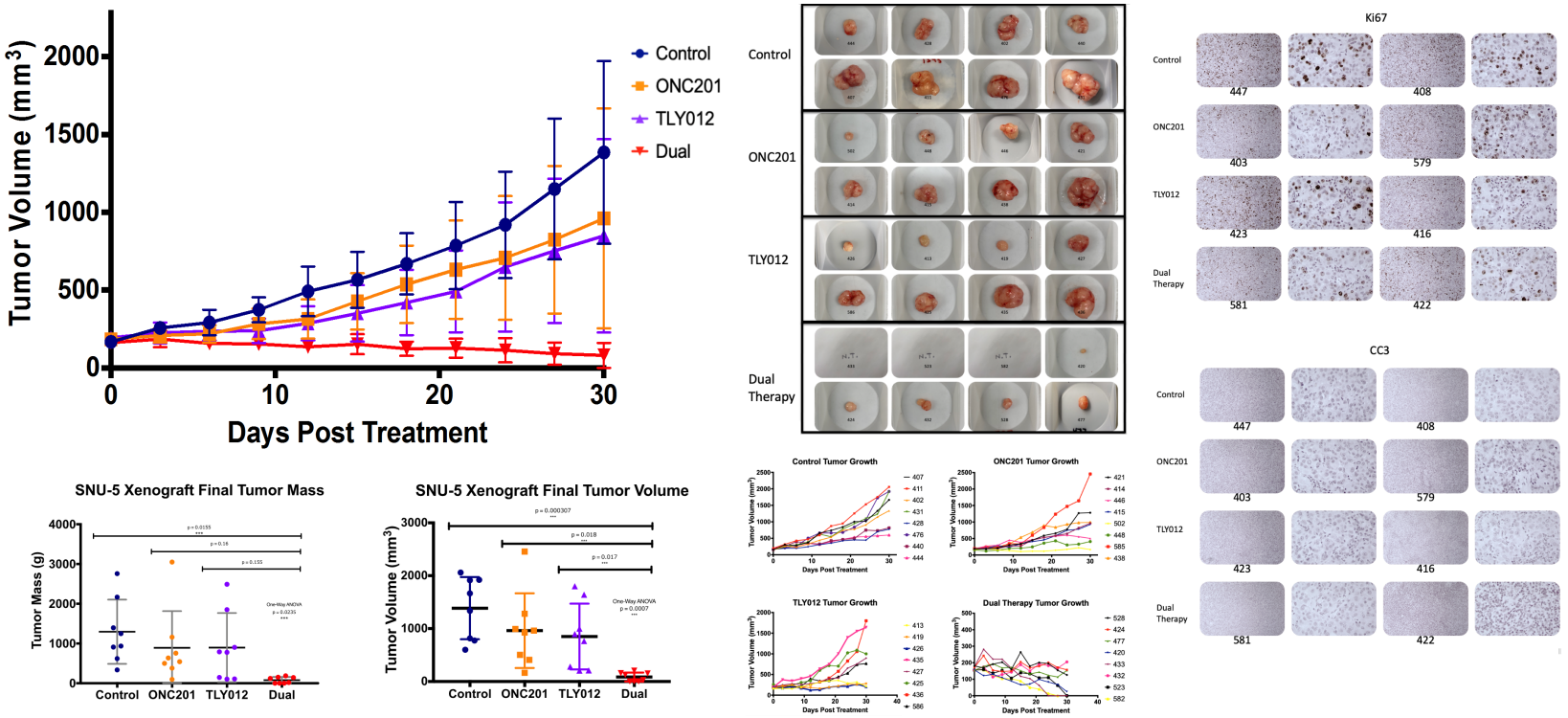


Figure 8. *In vivo* synergistic growth suppression and anti-tumor efficacy by ONC201, TLY012, or combination of ONC201 and TLY012 treatment in SNU-5 human gastric cancer mouse xenograft model. Average tumor volumes are shown over the course of the experiment along with final tumor volumes and images of the tumors. Individual tumor growth curves are shown for all treatment conditions. Ki67 and cleaved caspase 3 in ONC201 plus TLY012 treated SNU-5 xenografts were visualized by immunohistochemistry.

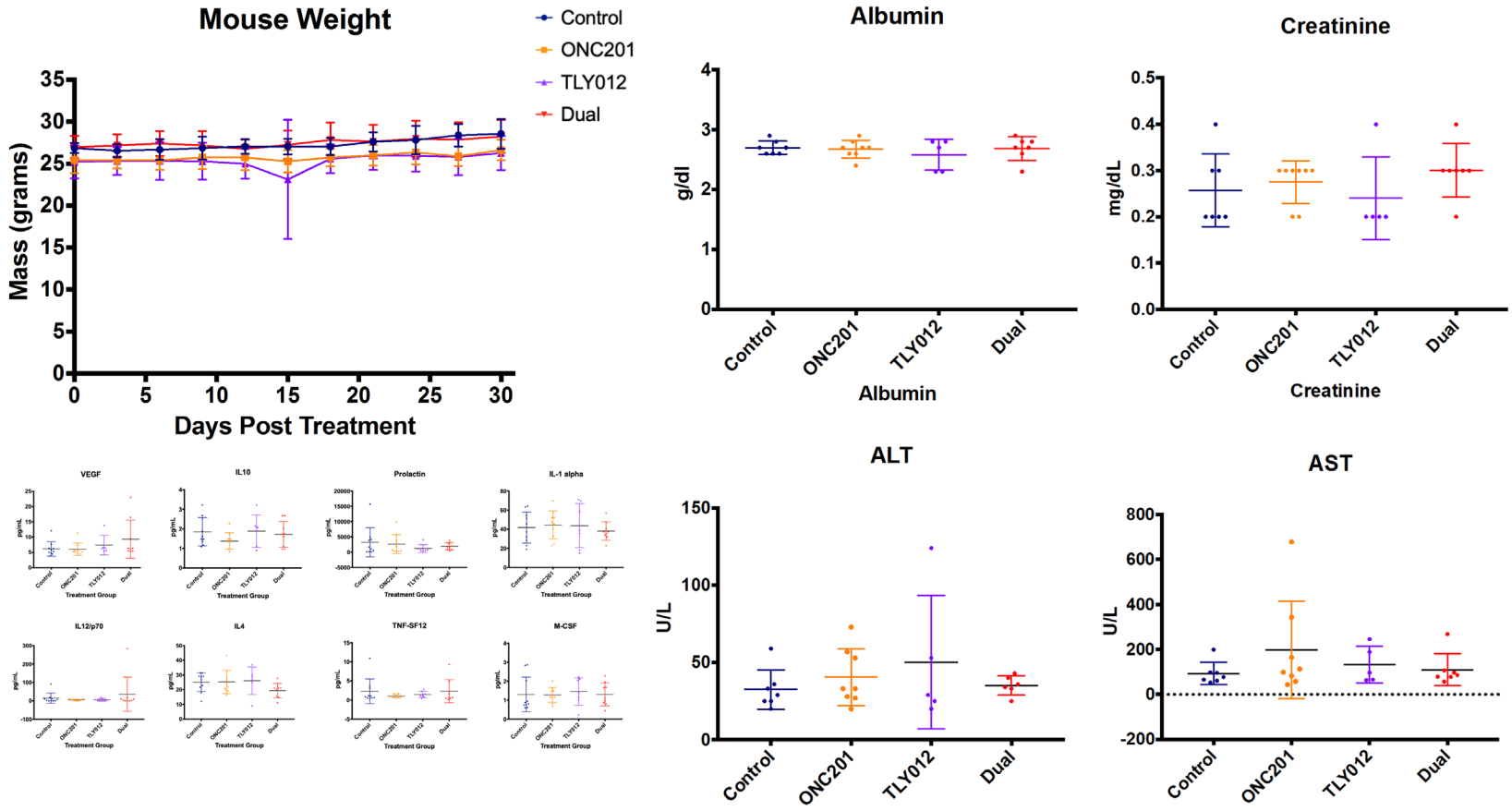


Figure 9. Lack of toxicity by ONC201, TLY012, or combination of ONC201 and TLY012 treatment in SNU-5 gastric cancer xenograft model. Mouse weights, creatinine, ALT, or AST liver enzyme values or serum albumin in control, ONC201, TLY012, or ONC201 plus TLY012 treated SNU-5 tumor-bearing mice are shown. The cytokines indicated were also measured after sacrifice of the mice at the end of the experiment.

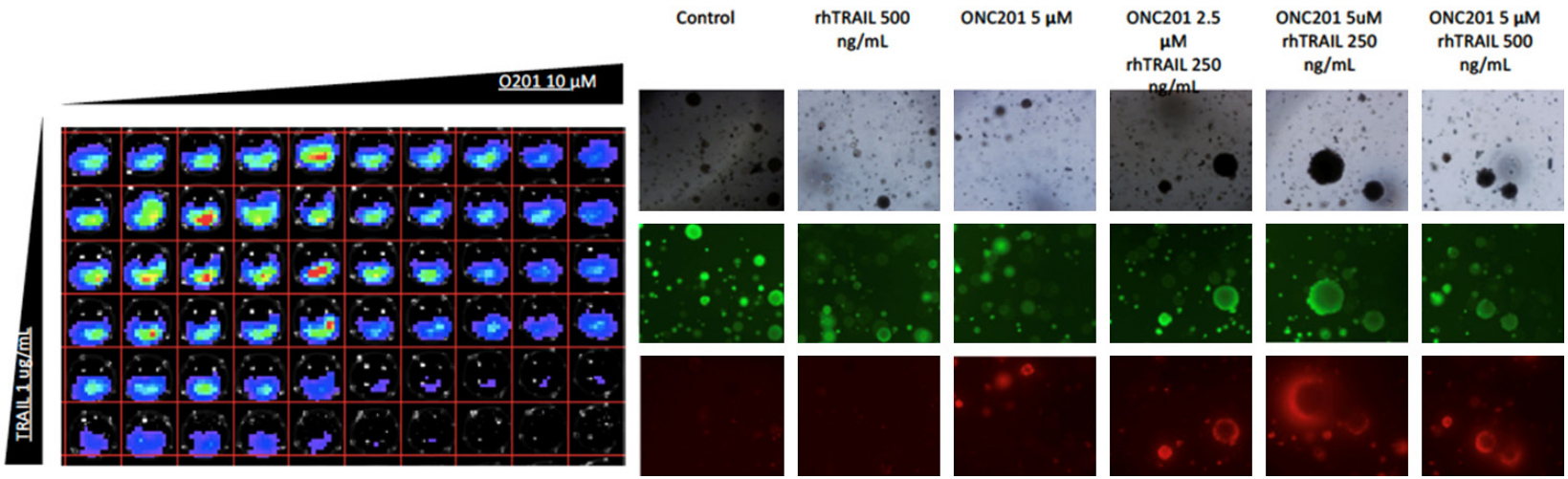


Figure 10. Cell death induction by control, ONC201, TLY012, or combination of ONC201 and rhTRAIL in a human gastric cancer organoid model. CellTiterGlo viability assessment as well as microscopic, immunofluorescence detection of cell death in human gastric cancer organoid treated by ONC201, TLY012, or combination of ONC201 plus TLY012 therapy. Cell death is shown in red in the lower panels to the right.

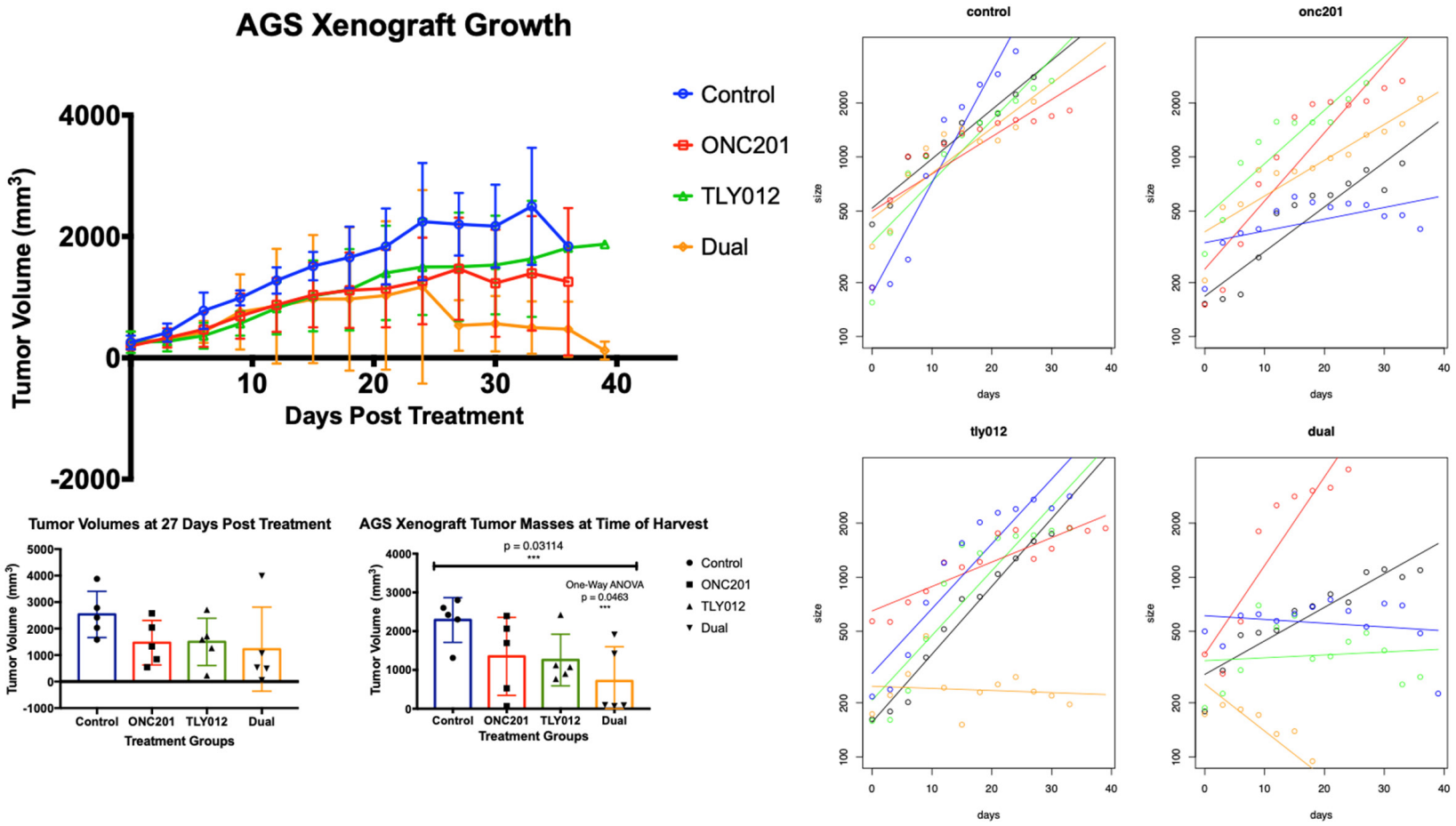


Figure 11. *In vivo* synergistic growth suppression and anti-tumor efficacy by ONC201, TLY012, or combination of ONC201 and TLY012 in AGS as a second human gastric cancer mouse xenograft model. Average tumor volumes are shown over the course of the experiment along with final tumor volumes of the tumors.

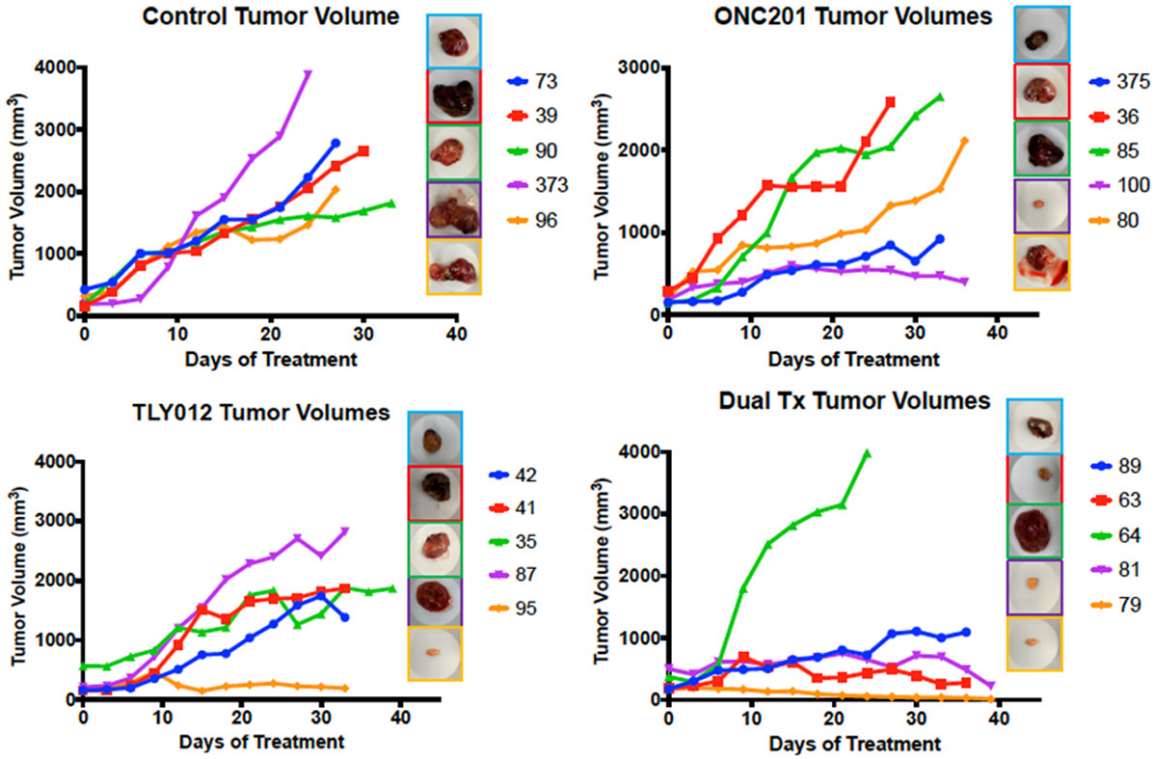


Figure 12. Individual tumor growth by control, ONC201, TLY012, or combination of ONC201 and TLY012 treated AGS gastric cancer xenograft model. Individual tumor growth curves are shown for all treatment conditions.

tion. Nonetheless, the difference between control and ONC201 plus TLY012 combination groups was statistically significant (**Figure 11**). Growth curves for individual AGS tumors growing in individual mice from the treatment cohorts are shown in **Figure 12**. Reduction of Ki67 and increase in cleaved caspase 3 were noted in **Figure 13**, while no reduction in mouse weights or organ histology was observed in the treatment groups (**Figure 14**).

Discussion

The innate immune system has yet to be harnessed to the benefit of patients with cancer. As patients with advanced gastric cancer carry a very poor prognosis, we explored the potential use of novel cancer therapeutic ONC201/TIC10 alone or in combination with recombinant human TRAIL formulations. Our results demonstrate TRAIL death receptor DR5 upregulation in human gastric cancer cells following ONC201/TIC10 treatment and that rhTRAIL or TLY012 activated massive apoptosis in the ONC201-treated 'apoptosis-primed' tumor cells. The apoptosis-priming was shown to involve downregulation of anti-apoptotic proteins in addition to the ISR-mediated DR5 upregulation. Apoptotic effects were observed in a human gastric cancer organoid model and in two *in vivo* human gastric cancer xenograft models. The results suggest that ONC201 plus TLY012 which may not only be a general strategy to target difficult to treat cancers but it may also be helpful in gastric adenocarcinomas that carry a very poor prognosis.

Limitations of the study include a limited number of human gastric cancer cell lines and organoids as well as the use of immune-deficient mouse models and subcutaneous implantation of tumor cells. These limitations could be further addressed in future studies. Nonetheless, we observed anti-tumor *in vivo* efficacy of ONC201 plus TLY012 in two xenograft models of human gastric cancer.

While there was observed heterogeneity in the results among the 4 different human gastric cancer cell lines, there were consistent changes demonstrating the importance of the ISR, extrinsic apoptosis and suppression of anti-

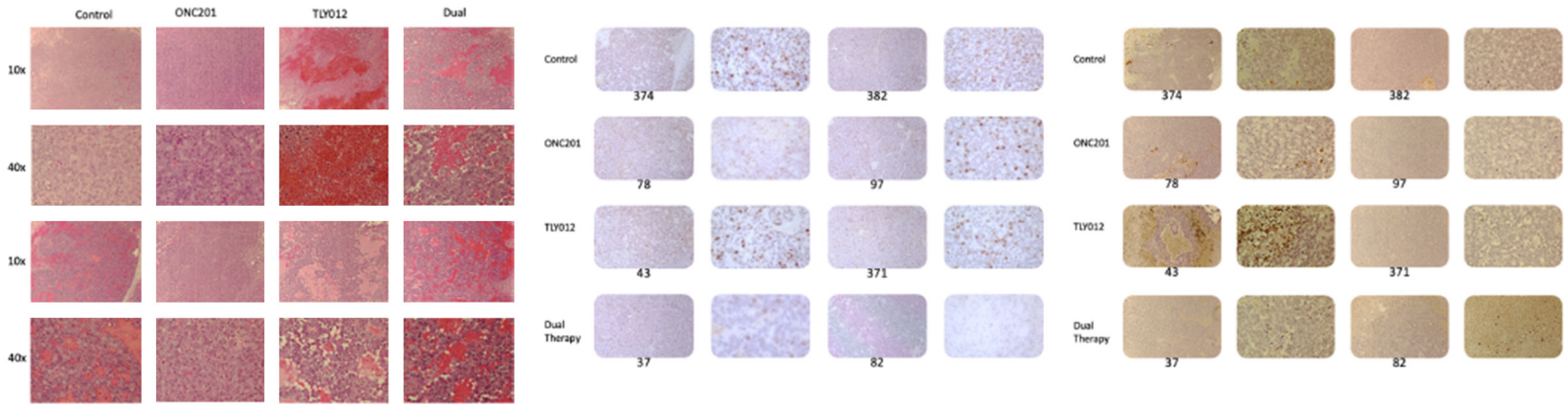


Figure 13. *In vivo* necrosis, proliferation or apoptosis in control, ONC201, TLY012, or combination of ONC201 and TLY012 treated AGS gastric cancer xenograft model. H&E stains as well as Ki67 and cleaved caspase 3 staining by immunohistochemistry are shown for the different treatment groups as indicated.

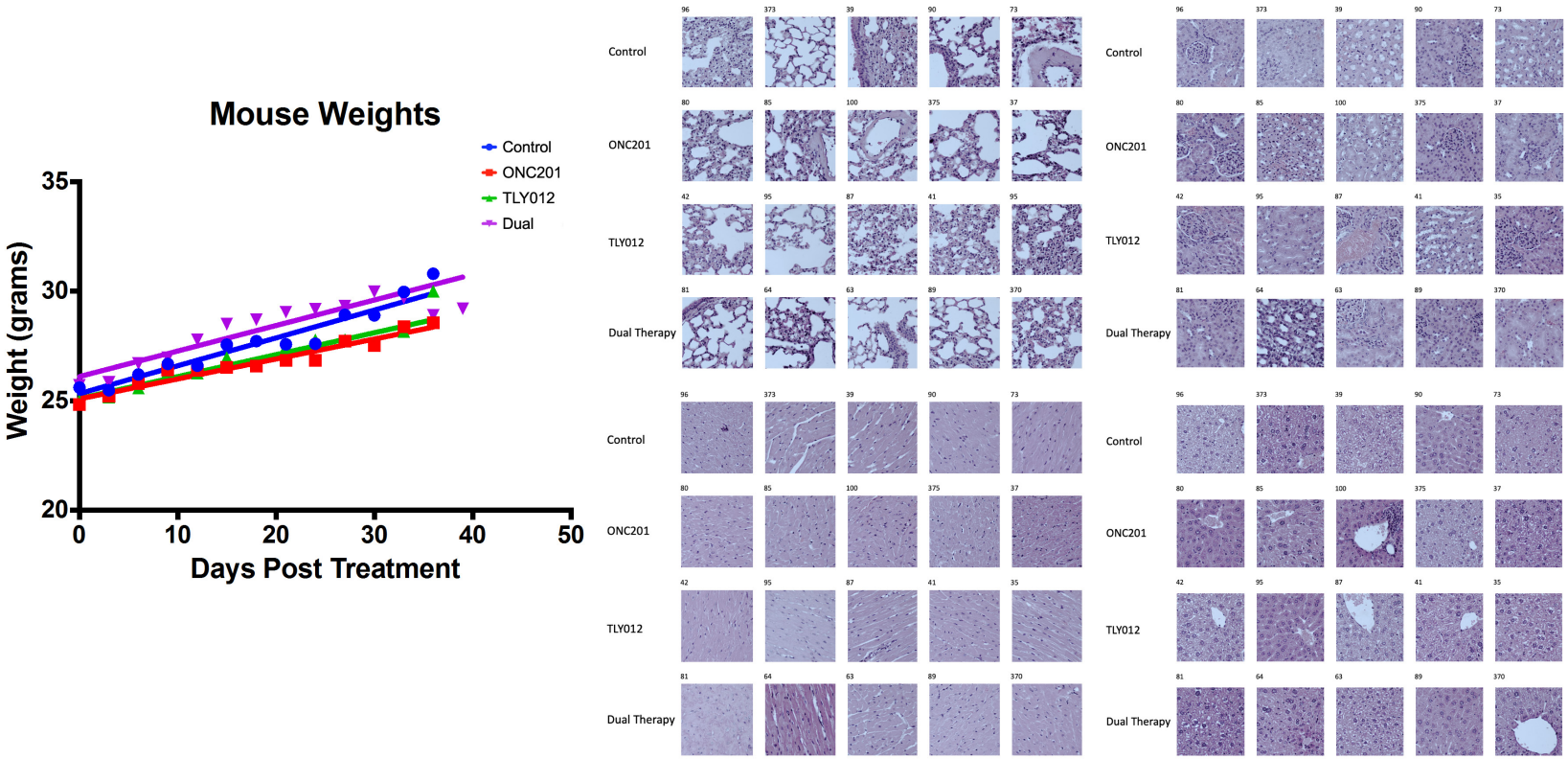


Figure 14. Lack of toxicity by ONC201, TLY012, or combination of ONC201 and TLY012 treatment in AGS gastric cancer xenograft model. Mouse weights in control, ONC201, TLY012, or ONC201 plus TLY012 treated AGS tumor-bearing mice are shown. To the right are shown H&E stains of lungs, kidneys, hearts and livers of mice at time of harvest at 40× magnification.

apoptotic proteins in ONC201 plus rhTRAIL/TLY012 treated cells. The lack of observed toxicity along with anti-tumor efficacy of the ONC201 plus TLY012 combination *in vivo* against gastric cancer suggests that this therapy may be a candidate for further investigation in future human clinical trials.

Acknowledgements

W.S.E-D. is an American Cancer Society Research Professor and is supported by the Mencoff Family University Professorship at Brown University. This work was supported by an NIH grant (CA173453) and by a grant from D&D Pharmatech to W.S.E-D. This work was presented in part at the 2021 meeting of the American Association for Cancer Research.

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/Chimerix 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. V.V.P. is an employee and shareholder at Chimerix.

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