Am J Cancer Res 2023;13(1):307-325
www.ajcr.us /ISSN:2156-6976/ajcr0147236

Original Article

Synergistic activity of ABT-263 and ONC201/TIC10 against solid tumor cell lines is associated with suppression of anti-apoptotic Mcl-1, BAG3, pAkt, and upregulation of pro-apoptotic Noxa and Bax cleavage during apoptosis

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Received October 25, 2022; Accepted December 24, 2022; Epub January 15, 2023; Published January 30, 2023

Abstract: A major underlying cause of the resistance of solid tumor cells to cancer therapy is the evasion of cell death following anti-cancer drug treatment. We explored the combination of TRAIL-inducing compound ONC201/TIC10 and Bcl-xL/Bcl-2 inhibitor ABT-263 to target the extrinsic and intrinsic apoptotic pathways, respectively, in solid tumor cell lines (N = 13) derived from different tissues (colon, prostate, lung, breast, ovary, bladder). We found an IC50 range of 0.83-20.10 μM for ONC201 and 0.06-14.75 μM for ABT-263 among the 13 cancer cell lines. We show that combination of ONC201 and ABT-263 produces a strong synergistic effect leading to tumor cell death, and that the combination is not toxic to human fibroblast cells. In OVCAR-3 ovarian cancer cells, 2.5 μM ONC201 and 1.25 μM ABT-263 yielded 37% and 27% inhibition of viability, respectively, while the combination of the two agents yielded 92% inhibition of viability, resulting in a high synergy score of 52; conversely, the same combination in the HFF-1 human fibroblast cells yielded 2.45% inhibition of viability and a synergy score of 6.92 (synergy scores were calculated using SynergyFinder; scores greater than 10 are considered synergistic). We also found that the combination of these two agents resulted in synergistic caspase activation and PARP cleavage consistent with induction of apoptosis. Combination therapy-induced cell death correlated with decreased levels of Mcl-1, BAG3, pAkt, and upregulation of Noxa along with Bax cleavage during apoptosis at 48 hours, and ATF4, TRAIL, and DR5 induction at 24 hours. There was some heterogeneity in the cell lines with regard to these responses. Our data provide evidence for synergy from the combination of ONC201 and ABT-263 against human solid tumor cell lines associated with alterations in cell death and pro-survival mediators. The combination of ONC201 and ABT-263 merits further exploration *in vivo* and in clinical trials against a variety of solid malignancies.

Keywords: Apoptosis, targeted therapy, drug synergy, ONC201, ABT-263, Mcl-1, BAG3, Akt, Noxa

Introduction

Despite remarkable progress over the last two decades in the development of targeted cancer therapy, immunotherapy, and precision medicine, according to the ACS there will be over 600,000 deaths from cancer in the US in 2022. While it is a goal of the field of oncology to prevent or diagnose cancer early, the presentation of cancer at a late stage continues to be associated with a poor prognosis. A general feature of advanced cancer that contributes to this poor

prognosis is resistance to therapy that may be intrinsic or acquired. As such, there is an urgent need for the development of novel therapeutic approaches that can address the complex nature of cancer, including tumor heterogeneity, evolution, and resistance to anti-cancer therapy.

A major underlying cause of the resistance of tumors cells to cancer therapy is the evasion of cell death following anti-cancer drug treatment [1]. Cell death resistance is a hallmark of cancer and is characterized by various phenotypes including upregulation of anti-apoptotic proteins, increased activation of pro-survival factors, and downregulation of expression or activity of pro-apoptotic mediators [2]. A great deal of progress has been made in the discovery and early development of cancer therapeutics that target cell death through the intrinsic or extrinsic cell death pathways.

ABT-263 is a small molecule that targets the intrinsic apoptotic pathway. It induces tumor cell death by directly binding to Bcl-xL and Bcl-2 and blocking their anti-apoptotic function. The ABT-263 targeting of Bcl-xL has been found to cause an on-target dose-dependent thrombocytopenia *in vivo* and in the clinic [3-5]. Consequently, ABT-199 was developed to specifically target Bcl-2 [6]. Although ABT-199's selectivity for Bcl-2 results in greater patient tolerance and has led to its approval by the FDA for the treatment of chronic lymphocytic leukemia (CLL) [7] and acute myeloid leukemia (AML) [8], it is ineffective in cell lines that preferentially express Bcl-xL over Bcl-2 [9]. Thus, despite the limitations of ABT-263, tumor heterogeneity keeps it of interest to the field.

ONC201/TIC10 is a first-in-class small molecule that targets the extrinsic apoptotic pathway. It was originally discovered as a TRAIL-inducing compound from a cell-based screen of an NCI library [10, 11]. Subsequent studies demonstrated that ONC201 not only upregulates the cytotoxic ligand TRAIL but also its pro-apoptotic receptor DR5, and showed activity mediated by upregulation of the integrated stress response [12]. ONC201 has been found to target cancer stem cells [13, 14] and to upregulate an immune response involving natural killer cells which act in part through TRAIL production as part of the host innate immune response against cancer [15]. More recently, ONC201 and its analogues ONC206 and ONC212 have been found to bind to mitochondrial caseinolytic protease ClpP to inhibit oxidative phosphorylation as part of the mechanism of its anti-cancer effect [10, 16]. This mitochondria-mediated cell death by ONC201 may involve the suppression of Mcl-1, an anti-apoptotic Bcl-2 family protein [17].

A major mechanism of resistance to ABT-263 involves the upregulation of anti-apoptotic Mcl-1 in ABT-263-treated solid tumors [18]. Moreover, overexpression of Bcl-2 has been found to limit the efficacy of ONC201 [17]. Given that ONC201 has been found to decrease Mcl-1 expression and that Bcl-2 is a target of ABT-263, the combination of ONC201 and ABT-263 should in theory have great synergistic potential. Synergistic combination therapies between ABT-263 and other anti-cancer molecules may allow for the use of lower concentrations of ABT-263 in the clinic, suppressing the dose-dependent thrombocytopenia and allowing for greater clinical success. The combination of ONC201 and ABT-263 has been tested in glioblastoma [19] and the combination of ONC201 and ABT-199 has been tested in hematological malignancies [17]. However, ABT-263 is not able to cross the blood-brain barrier [20], and the combination of ONC201 and ABT-263 has not been tested in other solid tumors. Thus, there remains a gap in the field to test this combination therapy in tissue types to which ONC201 and ABT-263 are bioavailable.

We investigated the activity of ONC201 and ABT-263 against solid tumor cell lines as single agents and in combination. We identified potent synergy between the two cancer therapeutic agents against a broad range of solid tumor cell lines and provided experimental evidence for alterations in cell death mediators as potential explanations for the observed preclinical synergy. Our results support further preclinical and clinical studies of this drug combination in cancer therapy.

Methods

Reagents

ABT-263 was purchased from MedKoo Biosciences (Morrisville, NC, USA) and was solubilized in DMSO at a storage concentration of 20 mM. ONC201 was supplied by Oncoceutics/

Chimerix, Inc (Philadelphia, PA, USA) and was reconstituted in DMSO at a storage concentration of 20 mM. Both reagents were stored at -20°C.

Cell cultures and growth conditions

All cell lines but one were obtained from the American Type Culture Collection (ATCC). The IGROV-1 cell line was obtained from the Fox Chase Cancer Center Cell Culture Facility. All cell lines were confirmed to be mycoplasma-free using PCR testing methods. HT29 and HCT116 cells were cultured in McCoy's 5A modified medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. SW480, MDA-MB-231, MDA-MB-436, H1048, and SKOV-3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. HFF-1 cells were cultured in DMEM supplemented with 15% fetal bovine serum and 1% Penicillin/Streptomycin. PC3, 22RV1, OVCAR-3, and IGROV-1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. UM-UC-3 and J82 cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. All cell cultures were stored in an incubator at 37°C within a 95% humidified atmosphere containing 5% carbon.

Cell viability assays

Cells were plated in opaque-walled 96-well plates at a density of 3000 cells per well and incubated overnight in 100 µL culture medium. After 24 hrs, single-agent ONC201 and ABT-263 or combined ONC201 and ABT-263 were added and treatment was continued for 72 hrs. 20 µL of CellTiterGlo bioluminescence agent (Promega Corporation, Madison, WI) was then added into each well and the contents were mixed for 2 min on a plate shaker to induce cell lysis. Cell viability was determined using the CellTiterGlo assay. Single-drug dose response curves were created using GraphPad Prism. Synergy scores were calculated using SynergyFinder.

Colony formation assays

With variations between cell lines, cells were plated at a density of 200-500 cells per well of a 12-well plate and allowed to grow overnight. After 24 hrs, the cells were treated with 1.25 µM ONC201, 5 µM ABT-263, or the combination of the two reagents. After an additional six days of incubation, wells were washed with phosphate-buffered saline (PBS), fixed by methanol for 20 min, and stained by Coomassie Brilliant Blue for 10 min. Colonies were imaged and counted using ImageJ.

Western blot analysis

Cells were plated in triplicate at a density of 5×10^5-1×10^6 cells per well of a 6-well plate and allowed to grow over night. Cells were treated with single-agent ONC201 and ABT-263 or the combination. After 24 or 48 hrs of treatment, the cells were washed with PBS and lysed using protein lysis buffer. Proteins were quantified using the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA). Cell lysates were electrophoresed through 4-12% SDS-PAGE then transferred to PVDF membranes. The PVDF membrane was blocked using 5% non-fat milk. The membranes were incubated overnight at 4°C with primary antibodies diluted in 5% non-fat milk according to the suggested datasheet dilutions. Invitrogen Goat anti-Rabbit IgG (H+L) Secondary Antibody and HRP #31460 and Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP #31430 were diluted 1:10,000 in 5% non-fat milk. Each membrane was probed with a loading control once and stripped and reprobed for additional proteins no more than one time.

Immunofluorescence analysis

Cells were plated in triplicate at a density of 30 k cells per well of an 8-chamber slide and treated with single-agent ONC201 and ABT-263 or the combination. After 24 or 48 hrs, the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 2% BSA. The cells were incubated with ATF4 primary antibody diluted in 0.1% BSA overnight at 4°C, after which they were washed with PBS and incubated with Alexa Fluor 488 donkey anti-rabbit secondary antibodies for 45 min. The cells were washed with PBS and mounted using a mounting medium containing DAPI stain. The coverslips were sealed and the plates were imaged using a fluorescent microscope.

Statistical analysis

Cell viability assay and colony formation assay data are all represented as the mean ± SD.

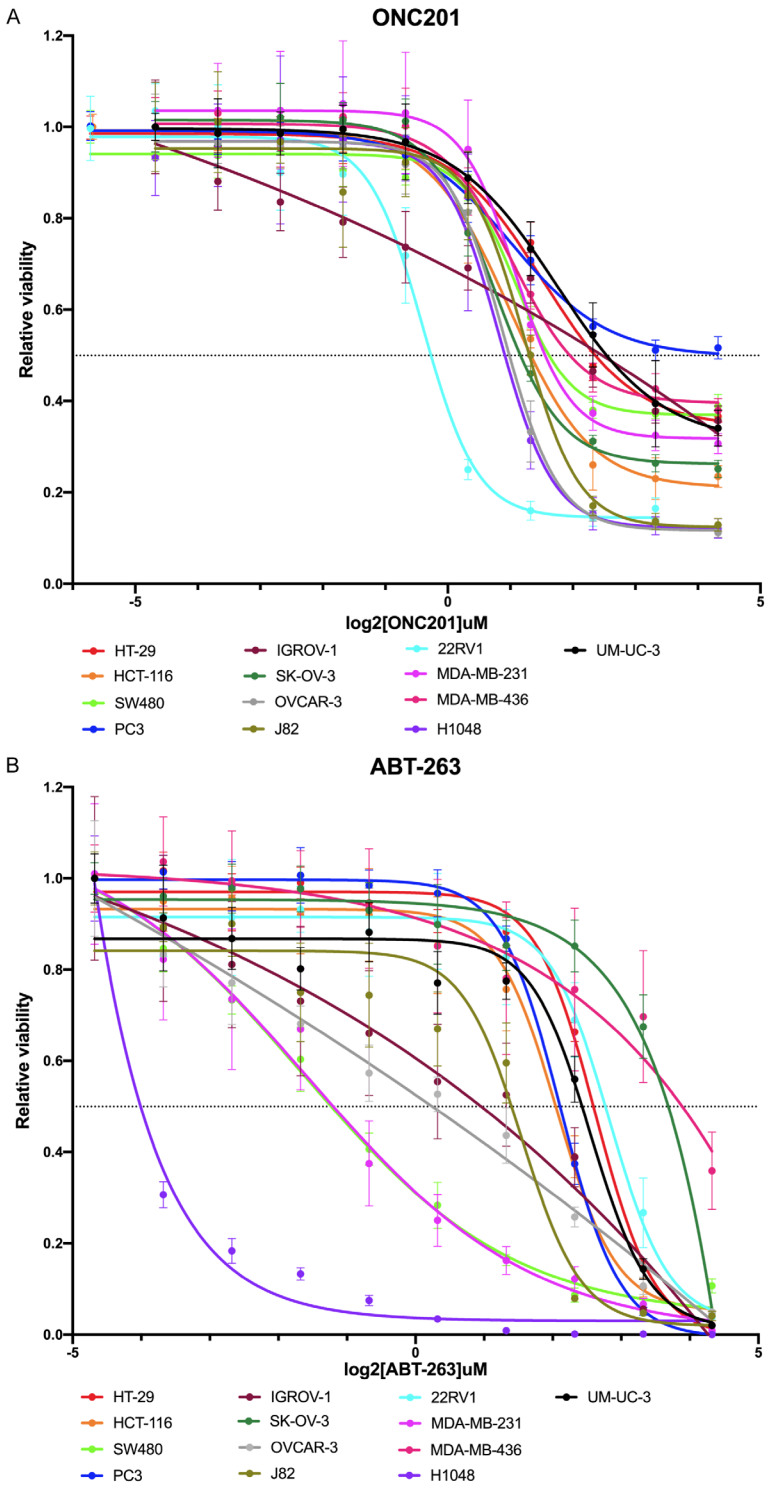


Figure 1. Concentration-dependent effect of ONC201 and ABT-263 on cell viability. 13 solid tumor cell lines were treated with DMSO or varying concentrations of either ONC201 (A) and ABT-263 (B) for 72 hrs. Cell viability was then determined using the CellTiter-Glo Luminescent Cell Viability Assay.

Single-drug dose response curves were generated by nonlinear regression analyses using GraphPad Prism. The treatment groups for the colony formation assay were compared using ordinary one-way ANOVA tests; *P* values < 0.05 were considered to be statistically significant. HSA model synergy scores for the two-drug viability assays were determined using SynergyFinder (Network Pharmacology for Precision Medicine in the Research Program of System Oncology, Faculty of Medicine at University of Helsinki, Helsinki, Finland); synergy scores < -10 indicate antagonism, between -10 and 10 indicate an additive effect, and > 10 indicate synergism. Single-drug viability assays were conducted in replicates of six; two-drug viability assays, colony formation assays, and western blot analyses were conducted in replicates of three.

Results

TRAIL pathway inducer ONC201/TIC10 and Bcl-xL/Bcl-2 inhibitor ABT-263 show cytotoxicity towards solid tumor cell lines (N = 13) derived from different tissues (colon, prostate, lung, breast, ovary, bladder)

To investigate the activity of cell death-targeting therapeutics against solid tumor cell lines, we treated a panel of human cancer cell lines (N = 13) with varying doses of TRAIL pathway inducer ONC201/TIC10 and Bcl-xL/Bcl-2 inhibitor ABT-263 (**Figure 1** and **Table 1**). Dose response relationships are shown for ONC201 (**Figure 1A**) and ABT-263 (**Figure 1B**). Loss of viability was observed at a range of drug concentrations in the panel of cell lines. IC50 values along with tissue of origin and

Table 1. Tissues of origin, mutational profiles, IC50 concentrations of selected solid tumor cell lines

Cell Line	Tissue	Mutational Profile					ABT-263 IC50	ONC201 IC50
		KRAS	BRAF	PIK3CA	PTEN	TP53		
HT29	Colon	wt	V600E	P449T	wt	R273H	6.19 μM	5.07 μM
HCT116	Colon ascendens	G13D	wt	H1047R	wt	wt	4.24 μM	2.54 μM
SW480	Colon	G12V	wt	wt	wt	R273H, P309S	0.43 μM	3.14 μM
PC3	Prostate	wt	wt	wt	Null	Null	4.39 μM	20.10 μM
22RV1	Prostate	wt	L597R	Q546R	wt	Q331R	6.91 μM	0.83 μM
H1048	Lung	wt	wt	H1047R	wt	R273C	0.06 μM	1.89 μM
MDA-MB-231	Breast	G13D	G464V	wt	wt	R280K	0.43 μM	2.96 μM
MDA-MB-436	Breast	wt	wt	wt	wt	E204fs	14.75 μM	3.94 μM
SKOV-3	Ovary	wt	wt	H1047R	wt	Null	13.04 μM	2.28 μM
IGROV-1	Ovary	wt	wt	R38C	Y155C, T319*	Y126C, S90Lfs*59	1.95 μM	5.55 μM
OVCAR-3	Ovary	wt	wt	wt	wt	R248Q	1.20 μM	1.98 μM
UM-UC-3	Urinary tract; bladder	G12C	wt	wt	Null	F113C	5.46 μM	5.86 μM
J82	Urinary tract; bladder	wt	wt	P124L	Null	E271K, K320N	2.64 μM	2.45 μM

(*) denotes a mutation that results in the creation of a translation stop codon.

known genomic driver gene alterations are listed in **Table 1**.

Following treatment with ONC201, the most sensitive cell lines within the 13-cell line panel were 22RV1 prostate cancer cells (ONC201 IC50 = 0.83 μM), H1048 small cell lung cancer (ONC201 IC50 = 1.89 μM), and OVCAR-3 high grade serous ovarian carcinoma cells (ONC201 IC50 = 1.98 μM). The cell lines most sensitive to ABT-263 were H1048 small cell lung cancer (ABT-263 IC50 = 0.06 μM), SW480 colon cancer cells (ABT-263 IC50 = 0.43 μM), and MDA-MB-231 triple negative breast cancer cells (ABT-263 IC50 = 0.43 μM).

Combination of ONC201 and ABT-263 produces strong synergy with loss of cell viability in solid tumor cell lines

Having observed single agent cytotoxicity with monotherapy, we explored whether the combination of ONC201 and ABT-263 produced synergistic effects. We performed cell viability assays with the solid tumor cell lines using increasing concentrations of both drugs in combination. The results shown in **Figure 2** and **Table 2** reveal combinatorial interactions of the drugs suggesting strong synergy, with scores greater than 10 found in all cell lines across the 13-cell line panel.

The cell lines with the highest synergy scores were the OVCAR-3 ovarian cancer cells, the SKOV-3 ovarian cancer cells, and the HT29 colon cancer cells. The synergy score for the OVCAR-3 cell line was 52.23 at 2.5 μM ONC-201 and 1.25 μM ABT263, with a two-drug percent inhibition of 91.80% as compared to the single-drug percent inhibition of 36.57% for ONC201 and 26.52% for ABT-263. The synergy score for the SKOV-3 cells was 48.29 at 5 μM ONC201 and 10 μM ABT-263, with a two-drug percent inhibition of 89.43% as compared to 40.83% and 33.72% inhibition with ONC201 and ABT-263, respectively. Finally, the synergy score for the HT29 cells was 35.41 at 5 μM ONC201 and 5 μM ABT-263, with a two-drug percent inhibition of 80.18% as compared to 45.93% and 33.32% inhibition with ONC201 and ABT-263, respectively.

ONC201 and ABT-263 are not toxic to human fibroblast cell lines

Having observed high levels of synergy across the panel of solid tumor cell lines, we sought to determine the sensitivity of normal fibroblast cells to ONC201 and ABT-263 as single agents and in combination. We performed cell viability assays using HFF-1 human foreskin fibroblast cells.

As seen in **Figure 3A** and **3B**, in comparison to the solid tumor cell lines, the HFF-1 cells were relatively insensitive to ONC201 and ABT-263 as single agents: even at the highest concentration tested for each drug (10 μM), IC50 was not reached. As shown in **Figure 3C**, when the two drugs were combined, sensitivity remained

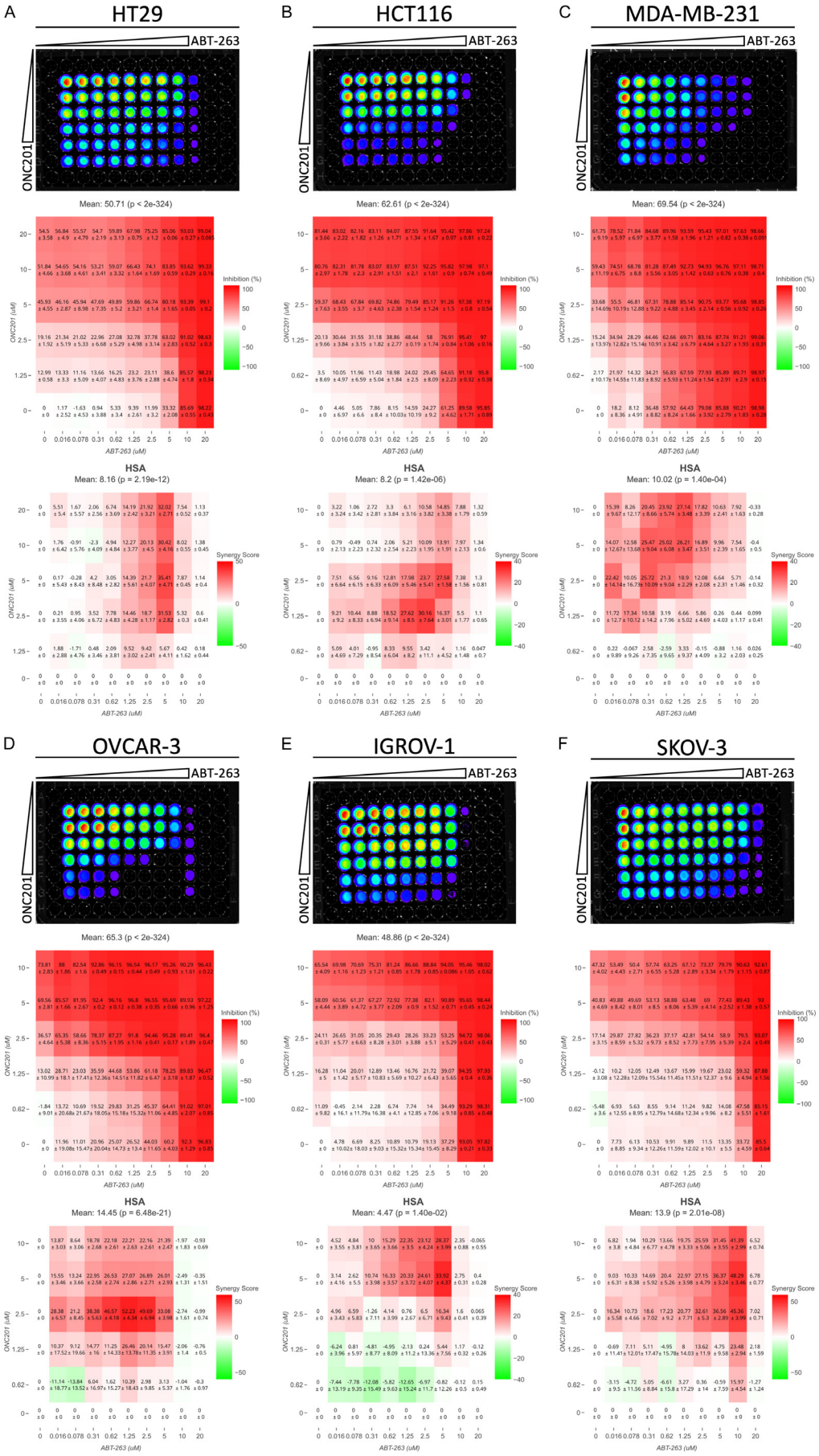

A
HT29
B
HCT116
C
MDA-MB-231
D
OVCAR-3
E
IGROV-1
F
SKOV-3
ABT-263
ONC201
Mean: 50.71 (p < 2e-324)
Mean: 62.61 (p < 2e-324)
Mean: 69.54 (p < 2e-324)
Mean: 65.3 (p < 2e-324)
Mean: 48.86 (p < 2e-324)
HSA
Mean: 8.16 (p = 2.19e-12)
Mean: 8.2 (p = 1.42e-06)
Mean: 10.02 (p = 1.40e-04)
Mean: 14.45 (p = 6.48e-21)
Mean: 4.47 (p = 1.40e-02)
Mean: 13.9 (p = 2.01e-08)
Inhibition (%)
Synergy Score
ONC201 (uM)
ABT-263 (uM)

Figure 2. ONC201 and ABT-263 synergize to reduce cell viability in solid tumor cell lines. HT29 cells (A), HCT116 cells (B), MDA-MB-231 cells (C), OVCAR-3 cells (D), IGROV-1 cells (E), and SKOV-3 cells (F) were treated for 72 hrs. with the indicated concentrations of ONC201 and ABT-263. Viability is visualized (top) on a rainbow scale using the CellTiter-Glo reagent as described in the methods, and inhibition is indicated numerically (middle). HSA synergy scores are shown (bottom) for the combination of ONC201 and ABT-263 at various non-constant concentration ratios. Scores < -10 are considered antagonistic, scores between -10 and 10 are considered additive, and scores > 10 are considered synergistic. Combination of ONC201 and ABT-263 resulted in high synergy scores across all cell lines.

Table 2. Percent inhibition and top synergy scores for cells treated with ONC201 and ABT-263

Cell Line	ONC201		ABT-263		Combination	
	% inhibition	[ ]	% inhibition	[ ]	% inhibition	Score
HT29	45.93±4.55	5 μM	33.32±2.08	5 μM	80.18±1.65	35.41
HCT116	20.13±9.66	1.25 μM	24.27±9.20	2.5 μM	58.00±1.74	30.16
SW480	46.71±3.49	2.5 μM	39.46±3.94	0.31 μM	69.92±1.15	23.15
PC3	47.18±3.38	2.5 μM	13.19±1.6	2.5 μM	71.23±2.06	24.47
22RV1	20.38±8.12	1.25 μM	31.03±4.72	5 μM	50.87±5.19	19.88
H1048	19.09±1.14	1.25 μM	28.90±2.75	0.02 μM	55.05±2.45	25.01
MDA-MB-231	61.65±9.19	10 μM	64.43±1.66	1.25 μM	93.59±1.96	27.14
MDA-MB-436	50.04±4.43	5 μM	29.84±8.44	10 μM	71.26±3.69	25.42
SKOV-3	40.83±4.69	5 μM	33.72±4.59	10 μM	89.43±1.38	48.29
IGROV-1	58.09±4.44	5 μM	37.29±8.29	5 μM	90.89±0.71	33.92
OVCAR-3	36.57±4.64	2.5 μM	26.52±13.4	1.25 μM	91.80±1.16	52.23
UM-UC-3	55.09±13.23	2.5 μM	30.20±23.94	5 μM	70.44±14.77	13.56
J82	59.47±10.19	10 μM	24.99±13.2	10 μM	86.12±5.21	23.87

low, with a maximum inhibition of 67% at the highest concentrations of ONC201 and ABT-263. Although scores indicating synergy were seen at some drug concentration combinations, the great majority of combinations yielded no synergy (scores between -10 and 10) or antagonism (scores less than -10). More specifically, dose combinations of ONC201 and ABT-263 that yielded high synergy scores in solid tumor cell lines yielded no synergy or antagonism in the fibroblast cell line: 2.5 μM ONC201 combined with 2.5 μM ABT-263 synergy scores of 23.7, 49.7, and 2.44 in the HCT116, OVCAR-3, and HFF-1 cells, respectively.

Combination of ONC201 and ABT-263 synergistically suppresses colony formation of solid tumor cell lines

Having observed synergistic cytotoxicity across the 13-cell line panel in short-term cell viability assays, we investigated whether similar phenotypes of sensitivity and synergy might be observed when using long-term colon formation assays.

Our examples shown in **Figure 4** and **Table 3** suggest that the synergistic effects of ONC201 plus ABT-263 extend to longer-term (7 days) colony formation assays, further supporting the observation that the two drugs have potent anti-cancer effects when combined in the treatment of solid tumor cells. The cell lines that showed the greatest reduction in colony forming ability for combined ONC201 and ABT-263 treatment as compared to single agent treatment were the 22RV1 prostate cancer cells and the HCT116 colon cancer cells, with ordinary one-way ANOVA tests showing P values of < 0.0001 between the ONC201 and ABT-263 combination group and all other treatment conditions (**Figure 4**).

ONC201 and ABT-263 enhance caspase cleavage and apoptosis

Having found high levels of synergy across all cell lines, we sought to explore whether apoptosis was induced following treatment with ONC201 and ABT-263. We performed western blot analyses for several markers of apoptosis, including cleaved PARP, caspase 8, caspase 9,

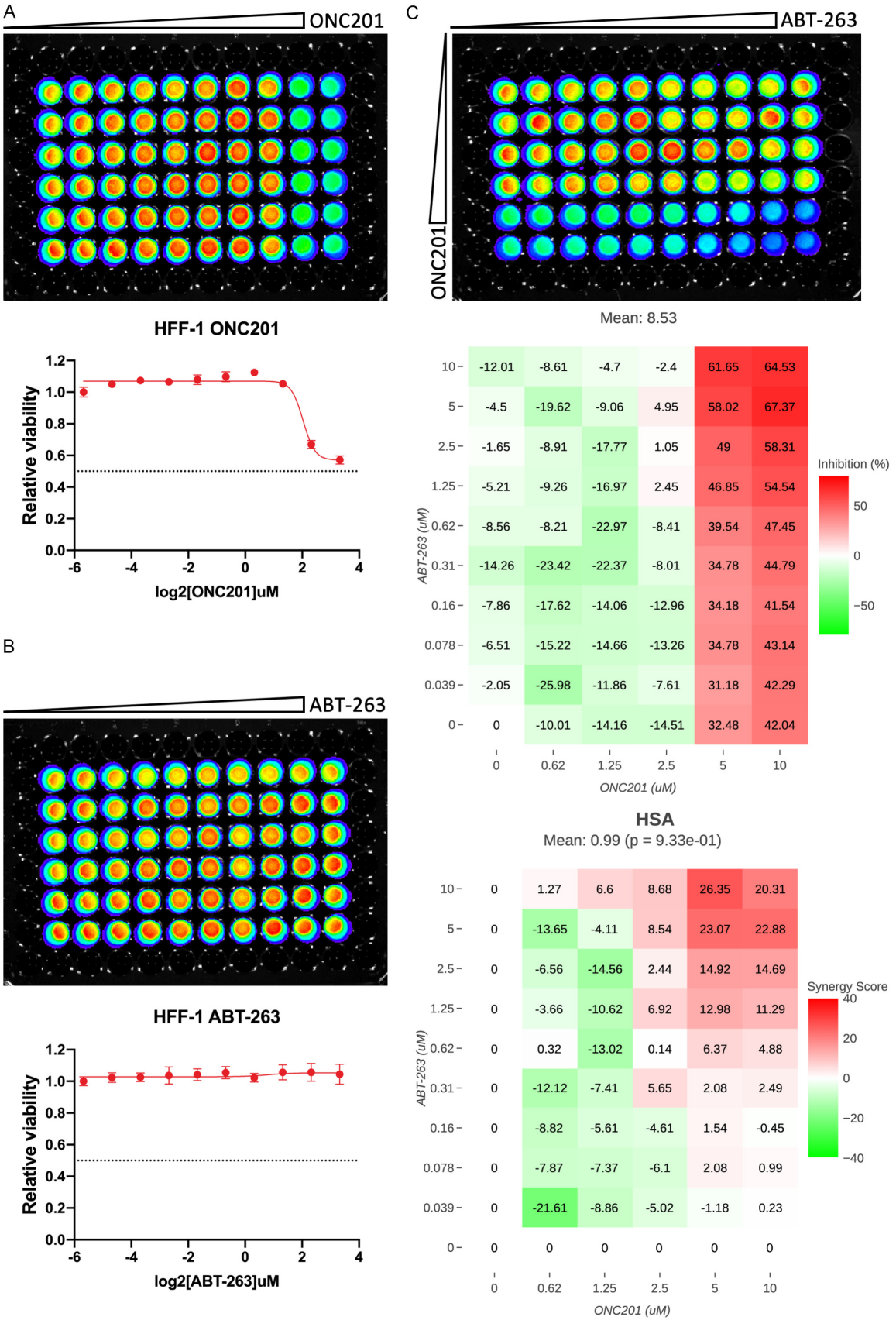

A
ONC201
HFF-1 ONC201
Relative viability
log2[ONC201]uM
B
ABT-263
HFF-1 ABT-263
Relative viability
log2[ABT-263]uM
C
ABT-263
ONC201
Mean: 8.53
ABT-263 (uM)
ONC201 (uM)
Inhibition (%)
HSA
Mean: 0.99 (p = 9.33e-01)
ABT-263 (uM)
ONC201 (uM)
Synergy Score

Figure 3. Sensitivity to ONC201 and ABT-263 as single agents and in combination is low in human fibroblast (normal) cells. HFF-1 cells were treated with varying concentrations of ONC201 as a single agent (A), ABT-263 as a single agent (B), or ONC201 and ABT-263 in combination (C) for 72 hrs. Viability is visualized on a rainbow scale using the CellTiter-Glo reagent as described in the methods. HSA synergy scores are shown (C) for the combination of ONC201 and ABT-263 at various non-constant concentration ratios. Combination of ONC201 and ABT-263 resulted in negative to moderate synergy scores across all cell lines.

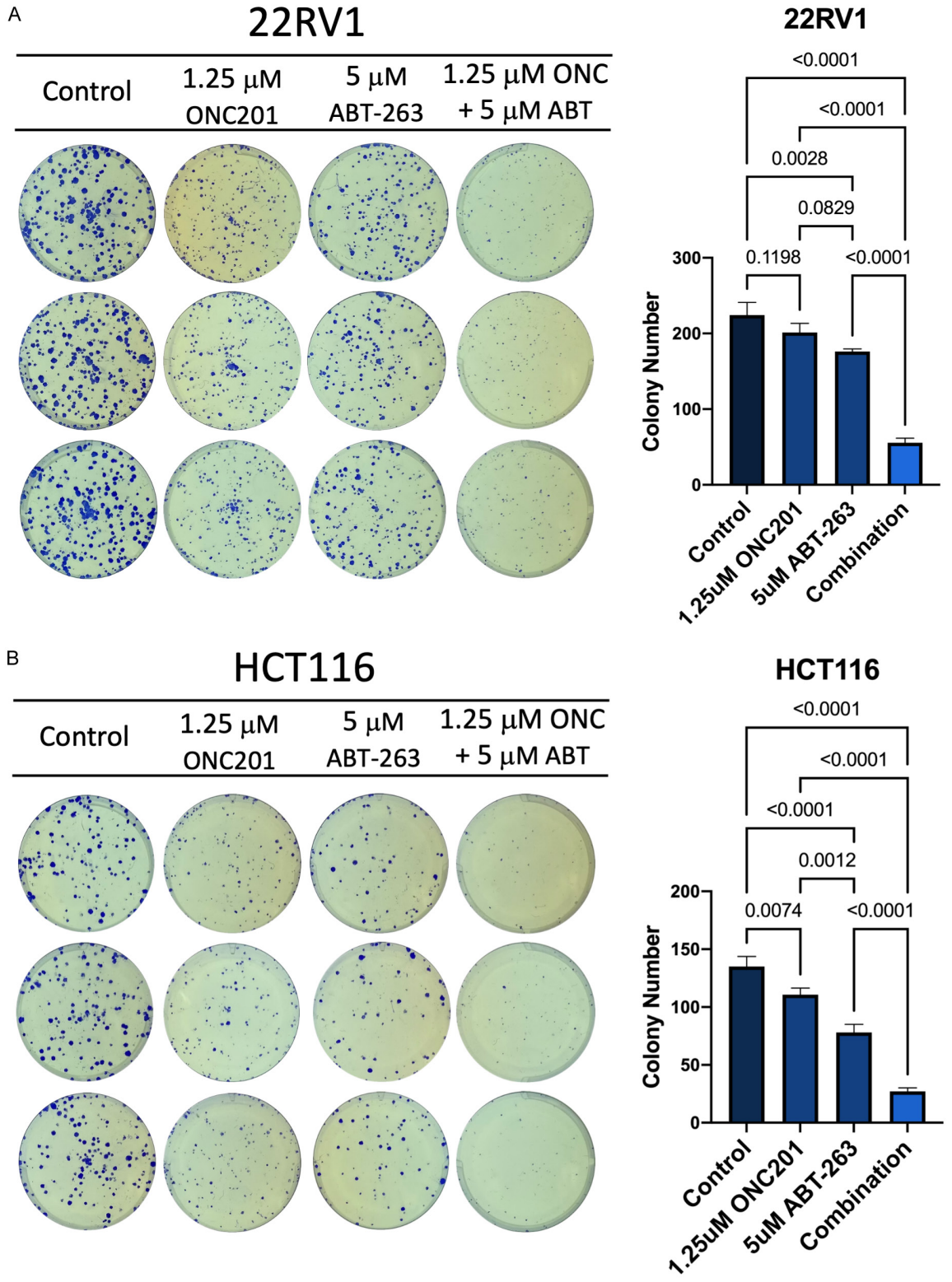


Figure 4. ONC201 and ABT-263 combined reduce colony forming ability of solid tumor cell lines. 22RV1 (A) and HCT116 (B) cells were treated with DMSO, 1.25 μM ONC201, 5 μM ABT-263, or 1.25 μM ONC201 + 5 μM ABT-263 24 hrs. after plating and allowed to grow for six days, after which colonies were stained using Coomassie Brilliant Blue. Images of the colonies are shown (left). Colonies were quantified and expressed as mean ± SD, and ordinary one-way ANOVA tests were used to compare the treatment groups (right). Combination of ONC201 and ABT-263 is shown to significantly reduce the colony forming ability of solid tumor cell lines.

Table 3. Mean colony number ± SD and results of ordinary one-way ANOVA test for cells treated with ONC201 and ABT-263

Cell Line	Control	ONC201	ABT-263	Combo	Combo to Control	Combo to ONC201	Combo to ABT-263
HT29	149±1.528	131±7.550	109±22.81	46±1.528	P < 0.0001	P = 0.0001	P = 0.0009
HCT116	135±8.718	111±5.686	78±7.000	27±3.000	P < 0.0001	P < 0.0001	P < 0.0001
SW480	149±10.44	122±12.17	60±5.859	16±2.082	P < 0.0001	P < 0.0001	P = 0.0012
PC3	139±7.937	116±8.505	97±4.163	53±5.568	P < 0.0001	P < 0.0001	P = 0.0002

and cleaved caspase 3 in three colorectal and two ovarian cancer cell lines. As shown in **Figures 5** and **6**, caspase activation and PARP cleavage consistent with induction of apoptosis was found following treatment of solid tumor cell lines by ONC201 and ABT-263 both as single agents and in combination. In most instances, when comparing the combined agent groups to the single agent and control groups, enhanced PARP, caspase 8, caspase 9, and caspase 3 cleavage were found. These findings are consistent with the high levels of synergism found across the 13-cell line panel.

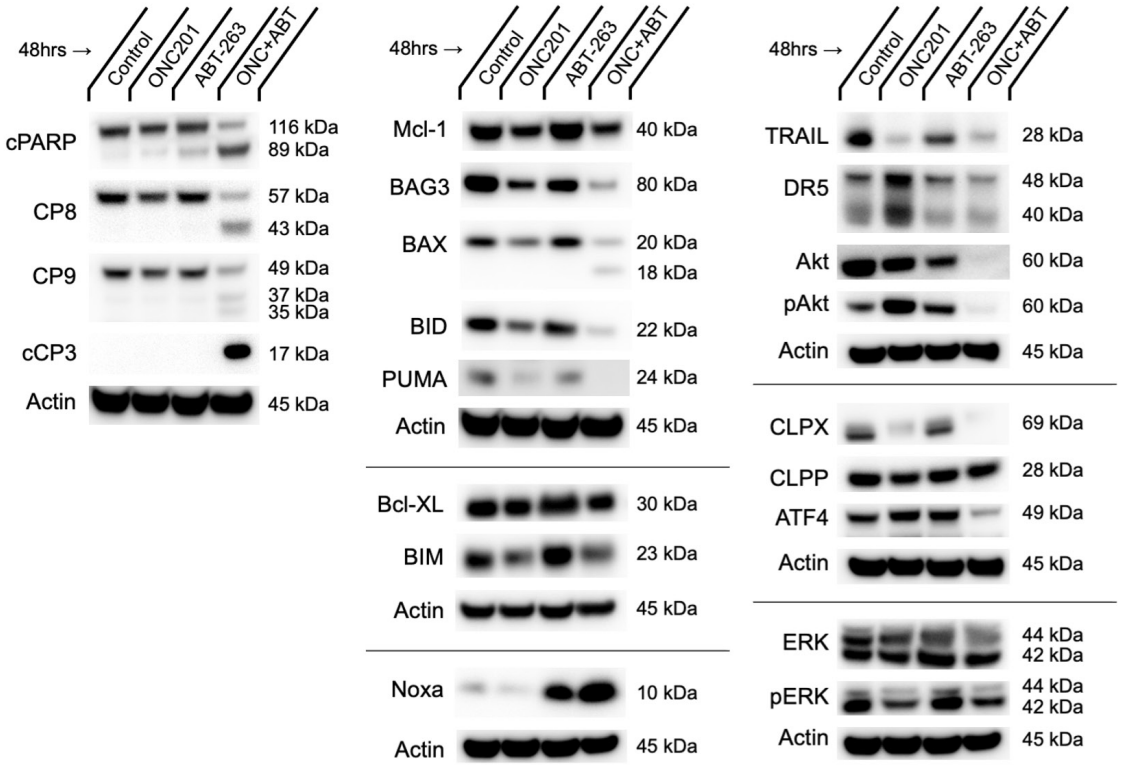
Decreased Mcl-1, BAG3, pAkt, and upregulation of Noxa and Bax cleavage during apoptosis of cancer cell lines following 48-hour treatment with ONC201 and ABT-263

We further investigated the molecular basis for the synergism found between ONC201 and ABT-263. We hypothesized that strong synergy between the two drugs that target the apoptotic pathways might be associated with alterations in relevant anti-apoptotic and pro-apoptotic proteins. To test this hypothesis, we performed western blot analyses across five solid tumor cell lines (**Figures 5** and **6**).

Elevated levels of Mcl-1 are a well-known mechanism of resistance to ABT-263. ONC201 has previously been found to reduce expression of Mcl-1, providing a basis for the combination of the two agents. Indeed, ABT-263 was found to maintain or increase Mcl-1 expression in all tested cell lines, whereas ONC201 was shown to reduce Mcl-1 expression in the HT29, IGROV-1, HCT116, and SKOV-3 cell lines. Combined treatment of ONC201 and ABT-263 at 48 hours was shown in maintain this decrease (HT29, IGROV-1, SKOV-3) or even further reduce (SW480) Mcl-1 expression in comparison to single agent ONC201 treatment. To further explore the mechanism by which Mcl-1 expression is reduced, we decided to look at the expression of BAG3, a protein known to stabilize Mcl-1, following 48-hour treatment. In the HT29, SW480, and HCT116 cell lines, ONC201 and ABT-263 as single agents were found to maintain or decrease BAG3 expression. When ONC201 and ABT-263 were combined in these three cell lines, an enhanced reduction in BAG3 expression was found as compared to the single agent groups.

pAkt is known to inhibit FOXO3a, preventing its translocation to the nucleus and the transcriptional activation of the TRAIL gene. ONC201 is known to cause downstream inhibition of pAkt; as such, we explored the expression of pAkt. In the HT29, IGROV-1, SW480, and SKOV-3 cells, 48-hour combined treatment with ONC201 and ABT-263 was found to greatly reduce pAkt expression as compared to the control and single agent groups.

Noxa is a pro-apoptotic BH3-only protein. In the HT29, IGROV-1, SW480, and HCT116 cell lines, ABT-263 both alone and in combination with ONC201 was found to induce Noxa expression. Furthermore, in the HT29, IGROV-1, and SW-480 cell lines, the combination of ONC201 and ABT-263 was found to induce Noxa expression at higher levels than with single agent ABT-263. Finally, enhanced Bax cleavage was found in


A HT-29
48hrs →
Control
ONC201
ABT-263
ONC+ABT
cPARP
116 kDa
89 kDa
CP8
57 kDa
43 kDa
CP9
49 kDa
37 kDa
35 kDa
cCP3
17 kDa
Actin
45 kDa
Mcl-1
40 kDa
BAG3
80 kDa
BAX
20 kDa
18 kDa
BID
22 kDa
PUMA
24 kDa
Actin
45 kDa
Bcl-XL
30 kDa
BIM
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Actin
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Noxa
10 kDa
Actin
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TRAIL
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DR5
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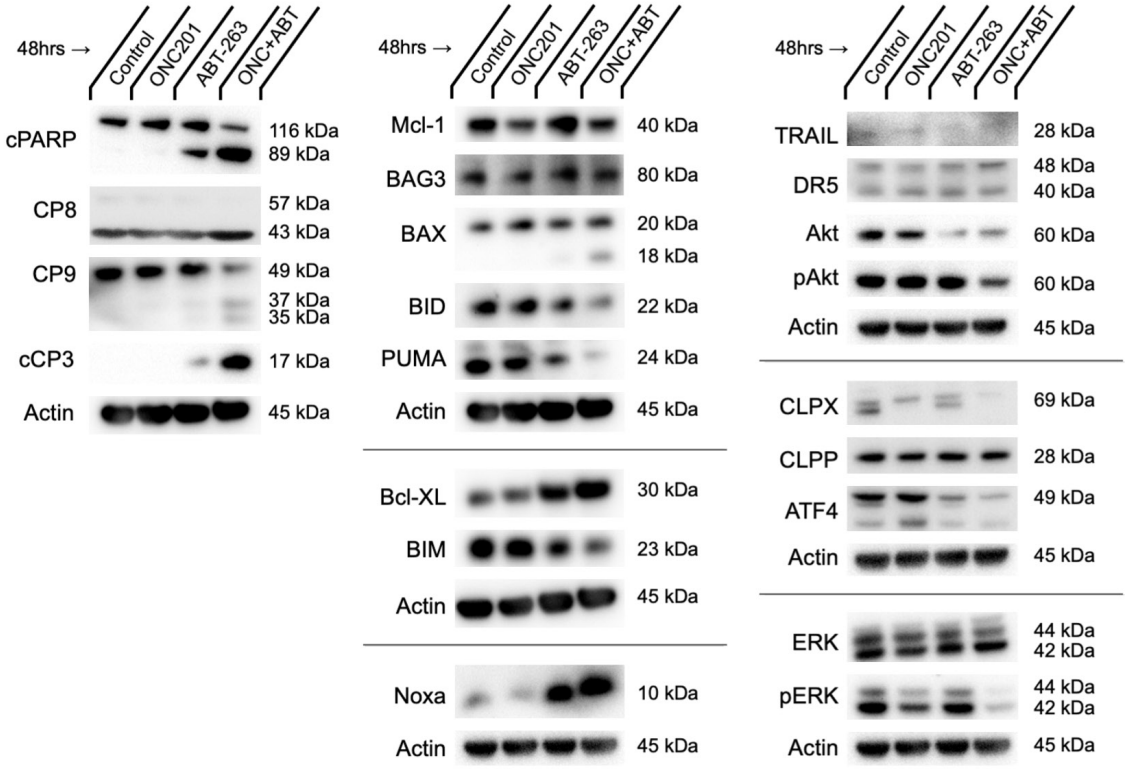
B IGROV-1
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BID
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PUMA
24 kDa
Actin
45 kDa
Bcl-XL
30 kDa
BIM
23 kDa
Actin
45 kDa
Noxa
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TRAIL
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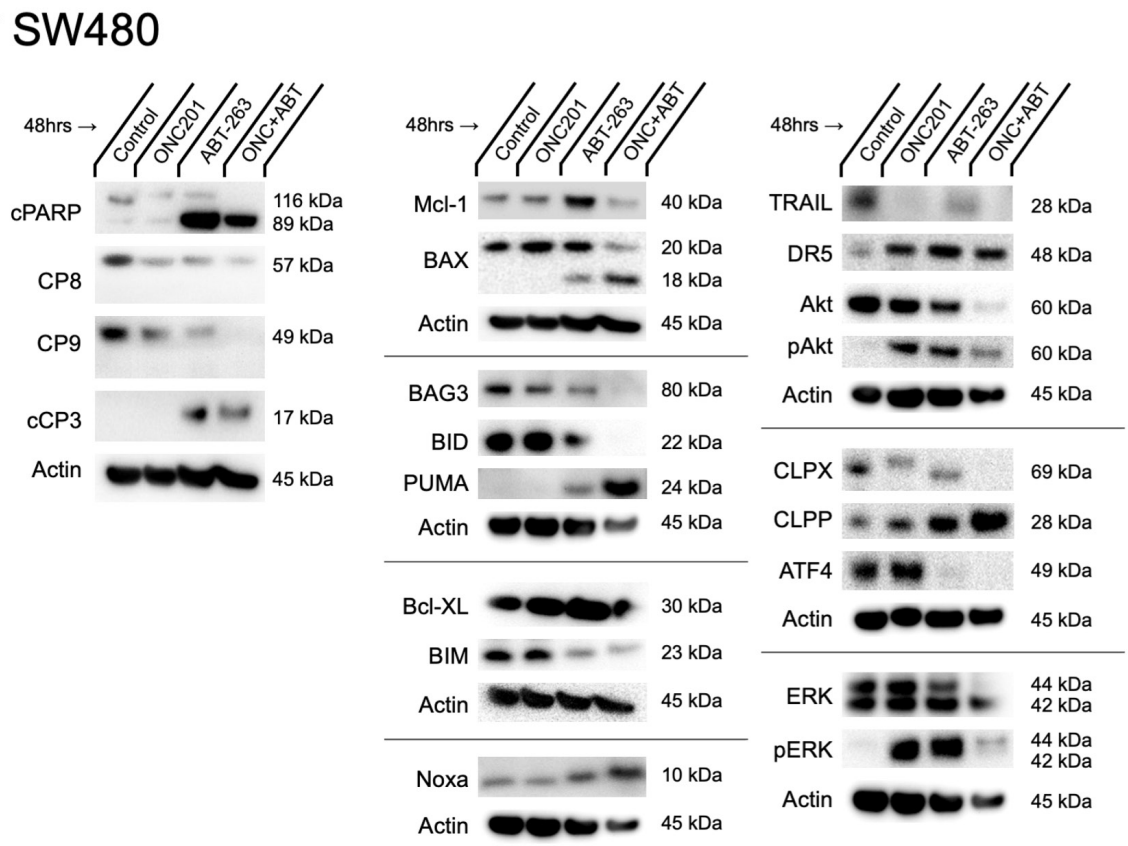

Figure 5. Synergy between ONC201 and ABT-263 is associated with PARP cleavage and caspase activation, decreased BAG3 and Mcl-1, Noxa induction, and BAX cleavage. The expression of various pro- and anti-apoptotic proteins was detected by western blot analysis for the HT29, IGROV-1, and SW480 cell lines. Panels are organized with apoptosis markers in the left column, intrinsic pathway proteins in the middle column, and extrinsic pathway proteins in the right column. A. HT29 cells were treated with DMSO, 5 μM ONC201, 5 μM ABT-263, or the combination for 48 hrs. B. IGROV-1 cells were treated with DMSO, 5 μM ONC201, 1.25 μM ABT-263, or the combination for 48 hrs. C. SW480 cells were treated with DMSO, 5 μM ONC201, 0.625 μM ABT-263, or the combination for 48 hrs. Elevated levels of PARP cleavage, caspase 3/8/9 activation, NOXA expression, and BAX cleavage, as well as reduced levels of Mcl-1, BAG3, and pAkt were observed in comparison to the control and single drug groups across the three solid tumor cell lines. For each cell line, all membranes were produced from a single set of lysates. Each membrane was probed with a loading control once and stripped and reprobed for additional proteins no more than one time. Some loading controls appear twice as they were used for different protein categories. For example, for the HT29 cells, one membrane was first probed for Noxa and Actin and then stripped and reprobed for ERK and pERK, and thus the loading control image is repeated in the middle and right columns. Similarly: for the HT29 cells, one actin was used as a loading control for cPARP, CP8, CP9, cCP3, Mcl-1, BAG3, BAX, BID, PUMA; for the IGROV-1 cells, one actin was used as a loading control for cPARP, CP8, CP9, cCP3, Mcl-1, BAG3, BAX, BID, PUMA and another was used for Noxa, ERK, pERK; for the SW480 cells, one actin was used as a loading control for Noxa, ERK, pERK.

HT29, IGROV-1, SW480, HCT116, and SKOV-3 cells treated with ONC201 in combination with ABT-263 as compared to the single agent groups.

Early increase in ATF4, TRAIL and DR5 expression following 24-hour treatment with ONC201 and ABT-263

The expression patterns for the panel of extrinsic pathway proteins at the 48-hour timepoint were rather unexpected, with paradoxical TRAIL suppression and inconsistent DR5 and ATF4 expression (**Figures 5** and **6**). However, it is known that when it comes to complex pathways that involve many molecular interactions, transcriptional level expression changes, and several cellular compartments, protein expression is timepoint sensitive. Moreover, at later time points, cells that experience early upregulation of pro-apoptotic proteins experience cell death and are consequently eliminated. As the extrin-

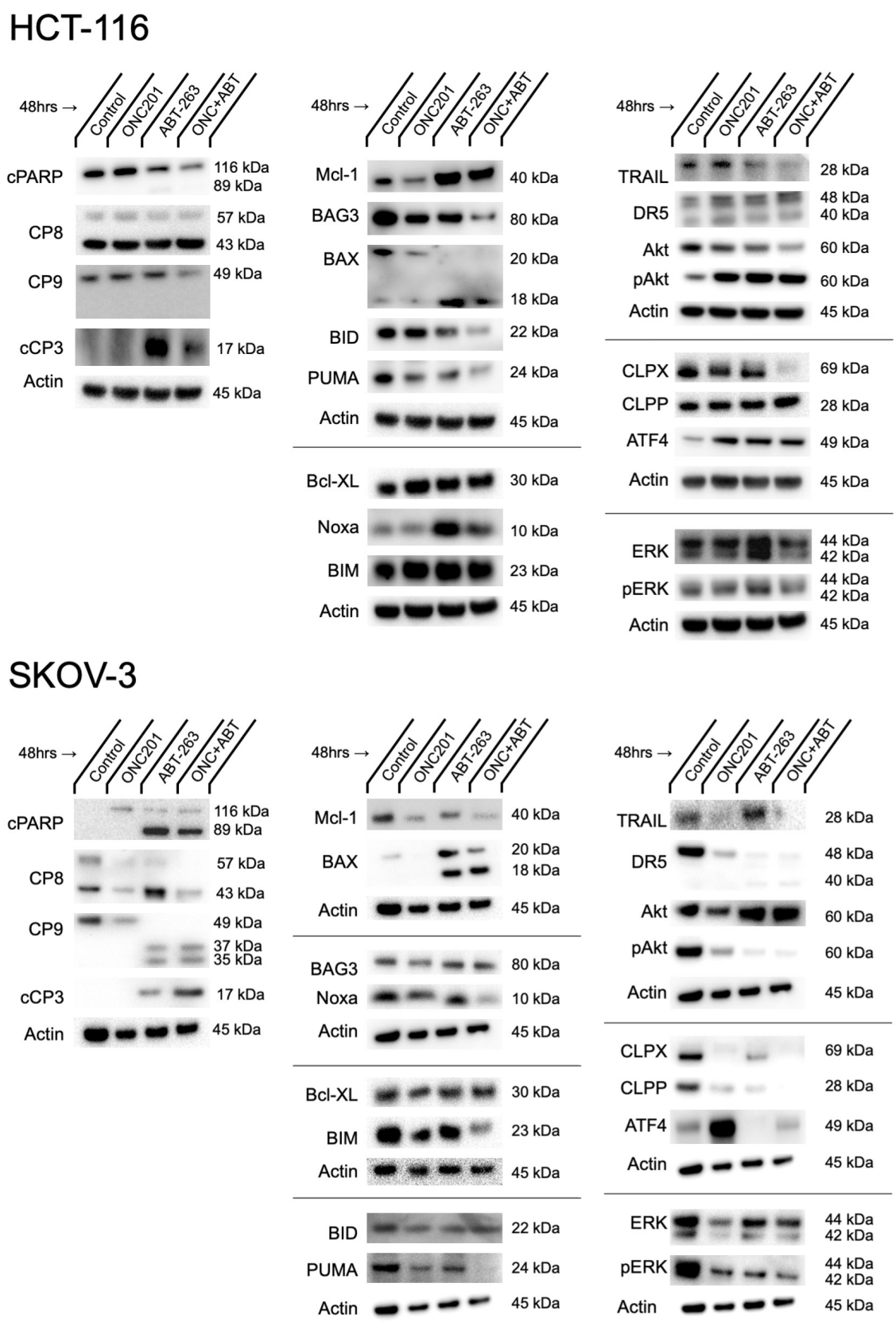

Figure 6. Combined treatment with ONC201 and ABT-263 is associated with changes in anti-apoptotic and pro-apoptotic protein expression at 48 hrs. The expression of various pro- and anti-apoptotic proteins was detected by western blot analysis for the HCT116 and SKOV-3 cell lines. Panels are organized with apoptosis markers in the

left column, intrinsic pathway proteins in the middle column, and extrinsic pathway proteins in the right column. (A) HCT116 cells were treated with DMSO, 2.5 μM ONC201, 2.5 μM ABT-263, or the combination for 48 hrs. (B) SKOV-3 cells were treated with DMSO, 2.5 μM ONC201, 2.5 μM ABT-263, or the combination for 48 hrs. Elevated levels of PARP cleavage, caspase activation, and BAX cleavage were observed in both cell lines. BAG3 reduction and NOXA induction were observed in the HCT116 cells (A). Mcl-1 and pAkt reduction was observed in SKOV-3 cells (B). For each cell line, all membranes were produced from a single set of lysates. Each membrane was probed with a loading control once and stripped and reprobed for additional proteins no more than one time. Some loading controls appear twice as they were used for different protein categories. For example, for the HCT-116 cells, one membrane was first probed for Bcl-XL, Noxa, BIM, and Actin and then stripped and reprobed for ERK and pERK, and thus the loading control image is repeated in the middle and right columns. Similarly: for the HCT-116 cells, one actin was used as a loading control for cPARP, CP8, CP9, cCP3, Mcl-1, BAG3, BAX, BID, PUMA; for the SKOV-3 cells, one actin was used as a loading control for cPARP, CP8, CP9, cCP3, Mcl-1, BAX while another was used for BAG3, Noxa, TRAIL, DR5, Akt, pAkt.

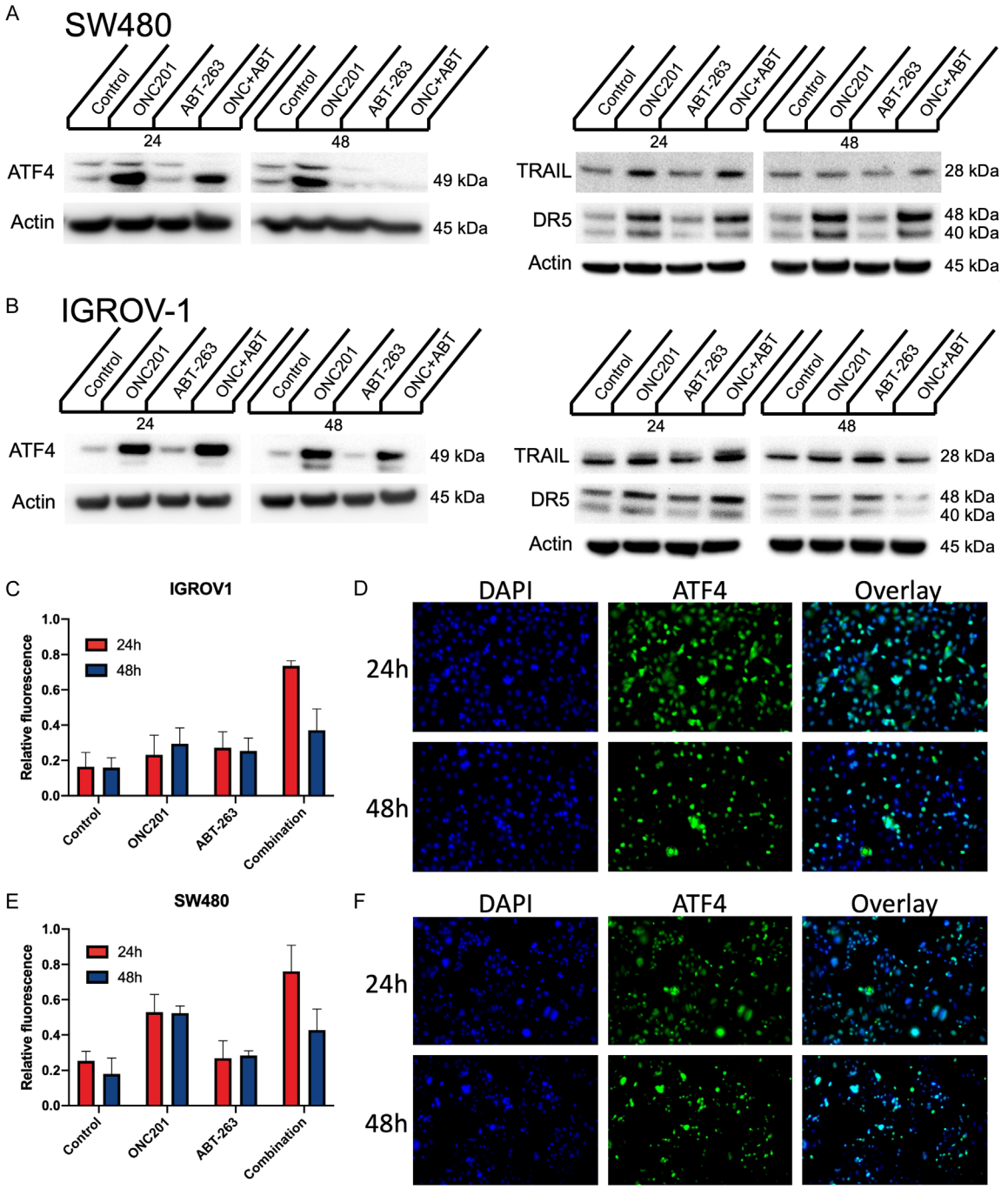

Figure 7. Combined treatment with ONC201 with ABT-263 is associated with induction of key extrinsic pathway proteins at 24 hrs, followed by suppression at 48 hrs. The expression of various extrinsic pathway proteins was detected by western blot and immunofluorescence analysis for the SW480 and IGROV-1 cell lines. A, C, D. IGROV-1 cells were treated with DMSO, 5 μM ONC201, 1.25 μM ABT-263, or the combination for 24 or 48 hrs. B, E, F. SW480 cells were treated with DMSO, 5 μM ONC201, 0.625 μM ABT-263, or the combination for 24 or 48 hrs. An induction of these proteins at 24 hrs was followed by their downregulation at 48 hrs. C, E. Quantification of mean anti-ATF4 fluorescence intensity relative to whole cell staining with DAPI at 24 and 48 hrs. D, F. Images showing ATF4 expression for cells treated with ONC201 and ABT-263 in combination for 24 or 48 hrs.

sic pathway is essential to ONC201 function, we decided to investigate the expression of these key proteins at various timepoints via western blot and immunostaining analysis. We selected two cell lines from the previous five-cell line panel: IGROV-1 and SW480. Results are shown in **Figure 7**.

ONC201 induction of ATF4 is thought to be upstream of an increase in DR5, while its effects on Foxo3a increase TRAIL expression. Indeed, following 24-hour treatment with ONC201, an increase in ATF4, TRAIL, and DR5 expression was seen (**Figure 7A** and **7B**). In the IGROV1 cells, combined treatment with ONC201 and ABT-263 appeared to enhance this induction, providing further evidence of synergy. Interestingly, at 48 hours, the expression of these three proteins appeared to drop in the ONC201 and ONC201+ABT-263-treated cells. This resulted in an expression pattern consistent with that seen in **Figures 5** and **6**.

We further examined ATF4 expression via immunostaining at 24 and 48 hours in the IGROV-1 and SW480 cells. As shown in **Figure 7C** and **7E**, when looking at the ratio between ATF4 fluorescence and total cell fluorescence, there was a drop at 48 hours that was specific to the combined agent group, consistent with the above findings. These results are supported visually in **Figure 7D** and **7F**.

Discussion

The treatment of solid tumors remains a great challenge in the field of cancer therapeutics. While surgery is a first-line treatment for most solid tumors, late-stage diagnosis often eliminates it as a viable option. On the other hand, later-stage treatments such as chemotherapeutics or targeted therapies are often limited by tumor cell resistance. One mechanism behind this resistance is tumor cell evasion of programmed cell death through the upregulation of anti-apoptotic molecules and the downregulation of pro-apoptotic molecules. With this, targeting apoptotic pathways has become a focus of the field.

The dual induction of the extrinsic and intrinsic apoptotic pathways has long been recognized as a potentially powerful therapeutic approach. The discovery of ONC201/TIC10 [21] has facilitated the development of such a therapy. Unlike previous inducers of the extrinsic apoptotic pathway, which have been associated with unimpressive patient outcomes [22, 23], issues related to pharmacokinetics [24], and inherent or acquired resistance [25], ONC201 induces TRAIL at the transcriptional level and has shown enhanced tumor control and greater promise in the clinic [10, 11]. Consequently, ONC201 has been combined with BH3-mimetics in glioblastoma [19] and leukemia [17]. However, BH3-mimetics do not cross the blood brain barrier, and solid tumors are more genetically complex than hematological malignancies.

In this study, we tested the combination of ONC201 and ABT-263 against a panel of 13 solid tumor cell lines derived from different tissues, including colon, prostate, lung, breast, ovary, and bladder. This presents the first time ONC201 and ABT-263 have been tested in solid tissues to which they are both bioavailable. We showed that this dual targeting of the extrinsic and intrinsic apoptotic pathways produced a potent anti-tumor effect and high levels of synergy across all tested cell lines. We demonstrated preclinical pro-apoptotic anticancer phenotypes by short term cell viability assays, long-term colony assays, and western blot analysis of apoptotic cell markers such as PARP cleavage and caspase 3/8/9 activation.

Our investigation of the underlying mechanisms of synergy revealed drug effects on relevant pro- and anti-apoptotic mediators as well as cell survival signaling. We showed novel ABT-263-mediated NOXA induction and Bax cleav-

age, both of which were enhanced by the addition of ONC201. Combination therapy-induced cell death was additionally associated with decreased levels of anti-apoptotic Mcl-1, BAG3, and pAkt at 48 hours and earlier induction of pro-apoptotic ATF4, TRAIL, and DR5 at 24 hours. These effects were over and above individual drug effects that may be important for each of the drugs when used alone or in other therapeutic combinations.

ABT-263 selectively inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xL, leaving Mcl-1 free to block pro-apoptotic proteins. It is well-known that innate resistance to treatment with ABT-263 is often caused by the intrinsically enhanced expression of Mcl-1, and that ABT-263 itself paradoxically induces the expression of Mcl-1, leading to acquired resistance [26, 27]. Indeed, combination therapies between ABT-263 and Mcl-1 suppressors are being explored to circumvent this resistance mechanism [28-31]. Our results show that the IGROV-1 and SW480 cell lines express elevated levels of Mcl-1 in the ABT-263-treated cells as compared to the control group, suggesting that even within 72 hours of treatment, mechanisms of acquired resistance are induced. In accordance with previous findings, we show that ONC-201 leads to decreased expression of Mcl-1. Moreover, we show for the first time that Noxa was induced by ABT-263 both as a single agent and in combination with ONC201. Noxa is a pro-apoptotic protein that directly inhibits Mcl-1 [32, 33], and it has been shown that base Noxa expression is a critical determinant of sensitivity to ABT-263 and its analogs [34, 35]. Taken together, these data suggest that ONC201 suppression of Mcl-1 along with ABT-263-mediated inhibition of Mcl-1 via Noxa induction are key components of the mechanism of synergy of ONC201 and ABT-263 combination. Furthermore, we found that BAG3, a chaperone protein known to stabilize Mcl-1 [36-38], was downregulated in an ONC201-dependent manner. BAG3 has previously been suggested as a target for anti-cancer therapy [39], and initial work has been done to develop an anti-BAG3 antibody for the treatment of pancreatic cancer [40]. Our findings support the growing relevance of BAG3 as a significant player in cancer progression.

Interestingly, Bax cleavage was among the most consistent findings in the western blot analysis, with all five tested cell lines showing a lower molecular weight band corresponding to cleaved Bax for the combination treatment groups. Bax is a critical component of the intrinsic mitochondrial apoptotic pathway, as its activation initiates mitochondrial outer membrane permeabilization (MOMP) and the release of key intermembrane space proteins that lead to caspase activation and cell death. A previous study [41] has shown that during stress-induced apoptosis, Bax is cleaved to p18 Bax. p18 Bax is more potent than full-length Bax in its ability to disrupt mitochondrial integrity, and thus its formation leads to accelerated and enhanced apoptosis.

It must be acknowledged that the expression level of some apoptotic proteins was heterogeneous. Further exploration revealed that the variability of extrinsic pathway proteins was likely due to timing-while at 24 hours ONC201 both alone and in combination led to a potent induction of ATF4, TRAIL, and DR5 expression, there was a dramatic decrease in the levels of these proteins at 48 hours. Nevertheless, the question of whether heterogeneity between patients could hinder the success of the ONC201 and ABT-263 combination remains important. Thus, the biological relevance of these findings remains unclear and should be subject to further exploration.

A limitation of ABT-263 therapies is that the drug has been found to induce thrombocytopenia in clinical trials. It is believed that the targeting of Bcl-xL on circulating platelet cells is the cause of this toxicity [42]. Consequently, ABT-199 was developed as a specific inhibitor of Bcl-2. Although ABT-199 is effective in Bcl-2-addicted cells, many cells either do not express Bcl-2 or are addicted to other pro-apoptotic proteins, such as Bcl-xL [9, 43]. Thus, despite its limitations in the clinic, ABT-263 continues to be of interest in pre-clinical studies. Because toxicity to ABT-263 is dose-dependent and caused by effects on non-cancerous cells, the future success of ABT-263 in the clinic is dependent on two factors: first, synergistic combinations between ABT-263 and other compounds that allow for the use of lower concentrations of ABT-263; second, specificity of this synergism to cancerous cells. Here we show that the combination of ONC201 and ABT-263 induces high levels of synergy and cell death at relatively low concentrations of each drug in all tested solid tumor cell lines. Moreover, we show that

synergy of this significance is not reproduced in HFF-1 fibroblast cell line, with the majority of ONC201 and ABT-263 concentration combinations yielding no synergy or antagonistic effects. Although this points to specificity and the potential evasion of toxicity in the clinic, further exploration of this drug combination *in vivo* is needed.

Overall, our data provide evidence for synergy from the combination of ONC201 and ABT-263 against human solid tumor cell lines associated with alterations in cell death and pro-survival mediators. The combination of ONC201 and ABT-263 merits further exploration *in vivo* and in clinical trials against a variety of solid malignancies.

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) to W.S.E-D. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute, the National Institutes of Health, or the American Cancer Society.

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.

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