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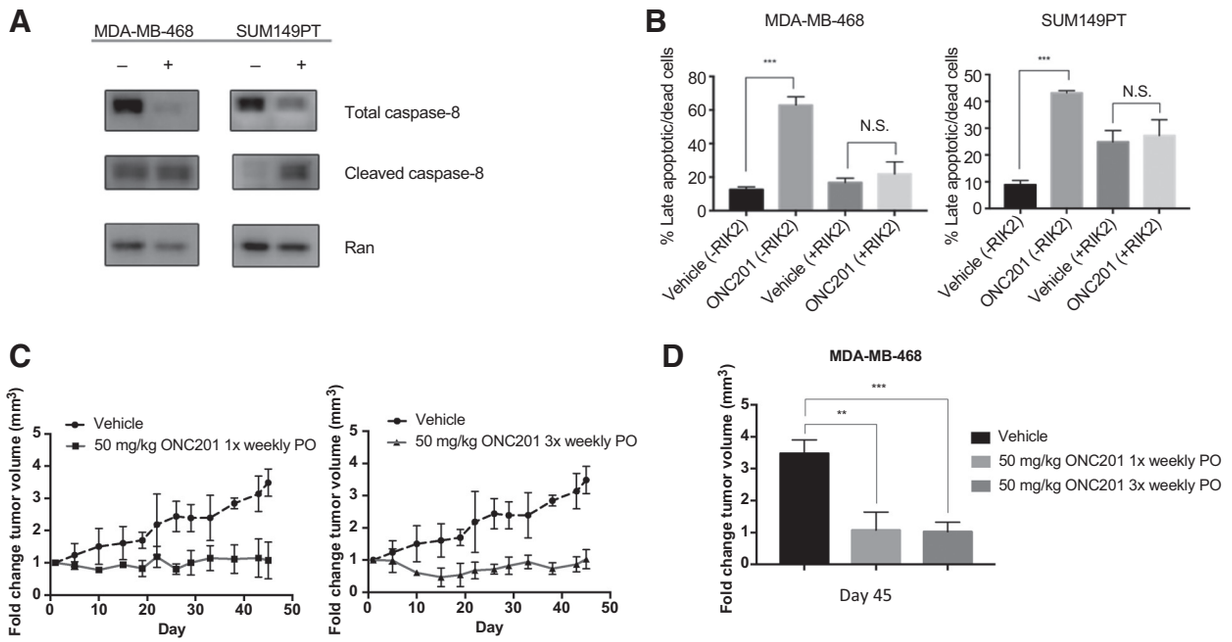

Figure 2.

The proapoptotic effects of ONC201 in some TNBC cells involve the extrinsic pathway, are TRAIL dependent, and translate to efficacy in the MDA-MB-468 breast cancer xenograft model. **A,** Western blot analysis of MDA-MB-468 and SUM149PT TNBC cells treated with a vehicle control or 10 μmol/L ONC201 for 72 hours to show caspase-8 cleavage. **B,** Annexin-V/PI double positive cells were quantified using flow cytometry following a 72-hour treatment with a vehicle control or ONC201 (MDA-MB-468: 5 μmol/L, SUM149PT: 10 μmol/L), with or without 1 μg/mL RIK2 TRAIL blocking antibody ($n = 2$ experiments for each cell line). **C,** Fold change tumor volume measured over time in nude mice bearing MDA-MB-468 xenografted tumors treated orally (PO) with a vehicle control ($n = 3$), 50 mg/kg ONC201 once per week ($n = 3$), or 50 mg/kg ONC201 three times per week ($n = 4$). **D,** Comparison of fold change tumor volume in mice treated with a vehicle control, 50 mg/kg ONC201 once per week, or 50 mg/kg ONC201 three times per week on day 45 of the experiment. ns: $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

coincubation with a TRAIL-neutralizing antibody, RIK2 (Fig. 2B). Importantly, ONC201 showed single-agent efficacy in the MDA-MB-468 xenograft model when given orally at a dose of 50 mg/kg once and three times weekly. Growth of xenograft tumors was prevented by treatment with the compound (Fig. 2C), and the differences in the change in tumor volume at day 45 between mice treated with a vehicle control or ONC201 were significant (Fig. 2D). No difference in efficacy was observed between a once weekly versus three times weekly dosing schedule (Fig. 2D). In addition, ONC201 showed effects that were possibly additive in combination with FDA-approved drugs in MDA-MB-468 TNBC cells (Supplementary Fig. S3A and S3B). These results indicate that the induction of cell death by ONC201 in TNBC cells involves activation of the extrinsic apoptosis pathway, is dependent on TRAIL, and leads to *in vivo* antitumor efficacy of the compound.

TNBC cells that do not undergo apoptosis show differential sensitivity to the antiproliferative effects of ONC201

ONC201 treatment decreased the number of viable cells in the TNBC cells that did not undergo apoptosis (Fig. 3A). Two of 6 cell lines tested showed less sensitivity to the compound, characterized by a failure of the cell viability to drop below 50%, even at a dose of 100 μmol/L of ONC201 (Fig. 3A). The observation that ONC201 decreased the number of viable TNBC cells without inducing apoptosis suggested that the compound exerted an antiproliferative effect. Count of viable cells over time showed that cell proliferation was inhibited over time in sensitive MDA-MB-231, MDA-MB-361, and HCC1395 cells (Fig. 3B; Supplementary Fig. S4A) more than in relatively resistant MDA-MB-436 cells (Fig. 3B). Uptake of nucleoside analogue BrdUrd was decreased by greater than 10% at 24, 48, and 72 hours of ONC201 treatment in sensitive MDA-MB-231 cells, but not in relatively resistant MDA-MB-436 cells (Fig. 3C).

In TNBC cells with high sensitivity, the compound activates an ISR and causes cells to accumulate in G_1

Treatment of MDA-MB-231 cells with 5 or 10 μmol/L of ONC201 led to a dose-dependent activation of the ISR characterized by increased phosphorylation of the eukaryotic initiation factor 2α (eIF2α) at serine 51, induction of ATF4, and a decrease in cyclin D1 protein levels as early as 12 hours (Fig. 4A). This was associated with an accumulation of cells in the G_1 phase of the cell cycle, shown by cell-cycle profiling of unsynchronized cells (Fig. 4B). These results show that ONC201 has an antiproliferative effect in cells that do not undergo cell death, and those with high sensitivity activate the ISR and accumulate in G_1.

pRb levels may be important for ONC201 to arrest and maintain cells in the G_1 phase of the cell cycle

Relatively resistant MDA-MB-436 cells did not arrest in G_1 following treatment with ONC201 (Fig. 5A). Expression of pRb was associated with increased sensitivity to the antiproliferative effects of ONC201, and relatively resistant MDA-MB-436 and

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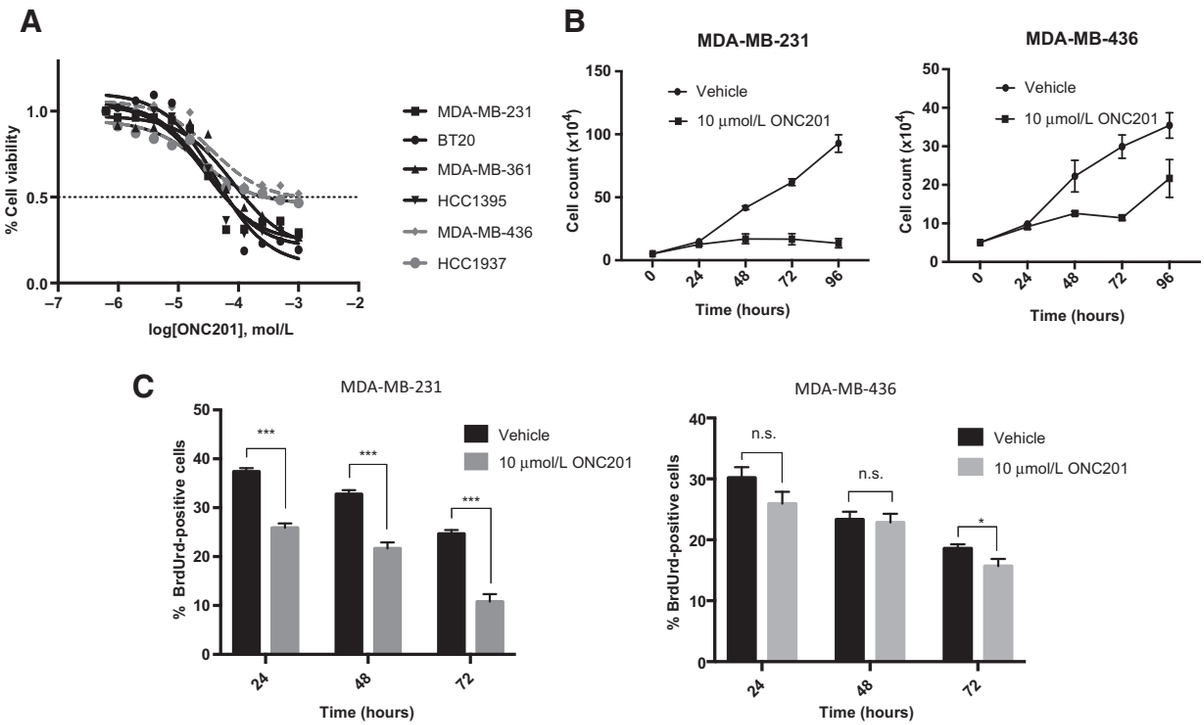

Figure 3.

Differential sensitives to antiproliferative effects of ONC201 are observed among TNBC cells that do not undergo apoptosis. **A,** Dose–response curves of TNBC cells treated with ONC201 for 72 hours. **B,** Trypan blue exclusion used to determine viable cell count in TNBC cells over time following treatment with a vehicle control or 10 μmol/L ONC201. **C,** Percent BrdUrd-positive cells over time in TNBC cells following treatment with a vehicle control or 10 μmol/L ONC201. ns: $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

HCC1937 did not express detectable levels of the pRb protein (Fig. 5B). ONC201 treatment decreased total pRb protein in MDA-MB-231 cells over time (Fig. 5C). By 72 hours of ONC201 treatment, MDA-MB-231 cells passed through the restriction point from G_1 into S-phase (Fig. 5D), but did not proliferate (Fig. 3B and C). Treatment with 100 mg/kg of ONC201 orally once per week as a single agent did not lead to an antitumor effect in the MDA-MB-231 xenograft model (Supplementary Fig. S4B and S4C). These data show that ONC201 has an antiproliferative effect in TNBC cells that do not undergo apoptosis and that pRb expression may be important for initiation and maintenance of G_1 arrest.

ONC201 synergizes with taxanes in tumor cells with decreased sensitivity to ONC201 as a single agent

We further explored the potential for synergies between ONC201 and FDA-approved anticancer drugs. Combinations with FDA-approved therapies that synergized with ONC201 in TNBC cells with relative resistance to the antiproliferative effects of the compound were identified. Combination indices calculated using the Chou–Talalay method (17) from cell viabilities following drug treatment showed that ONC201 synergized with taxanes docetaxel and paclitaxel in MDA-MB-436 and HCC1937 (BRCA1 deficient) TNBC cell lines (Table 2). These results demonstrate that the combination of ONC201 and taxanes, which are used as first-line treatment of breast cancer in both the adjuvant and metastatic settings, is synergistic.

ONC201 activates caspase-8 cleavage and induces TRAIL-independent cell death in non-TNBC cells

We initially hypothesized that because ONC201 has effects on mechanisms of TRAIL resistance, the compound could show efficacy in non-TNBC cells. Unlike TRAIL, ONC201 induced cell death in non-TNBC cells (Fig. 1A and B). At 72 hours, treatment with 10 μmol/L ONC201 decreased expression of antiapoptotic proteins from the Bcl-2 and IAP families, regardless of whether the cells underwent cell death (Supplementary Fig. S5). Non-TNBC cells SKBR3 and MCF7 showed a decrease in total caspase-8 and an increase in cleaved caspase-8 following treatment with 10 μmol/L ONC201 for 72 hours (Fig. 6A). Although ONC201 induced expression of surface TRAIL and DR5 in control MDA-MB-231 cells, it did not induce these components of the extrinsic apoptosis pathway in SKBR3 and MCF7 cells (Fig. 6B). Unlike the apoptosis induced by ONC201 in TNBC cells, apoptosis in non-TNBC cells was not abrogated by TRAIL-neutralizing antibody RIK2 (Fig. 6C and D). These data indicate that ONC201 may induce cell death in non-TNBC cells through a TRAIL-independent mechanism.

Discussion

Here, we demonstrate that ONC201 shows efficacy in a wide range of breast cancer subtypes, independent of their sensitivity to TRAIL. TRAIL has been of interest as a cancer therapeutic due to its ability to induce cell death in cancer cells while sparing normal cells (2), but many breast cancers show resistance (8). ONC201

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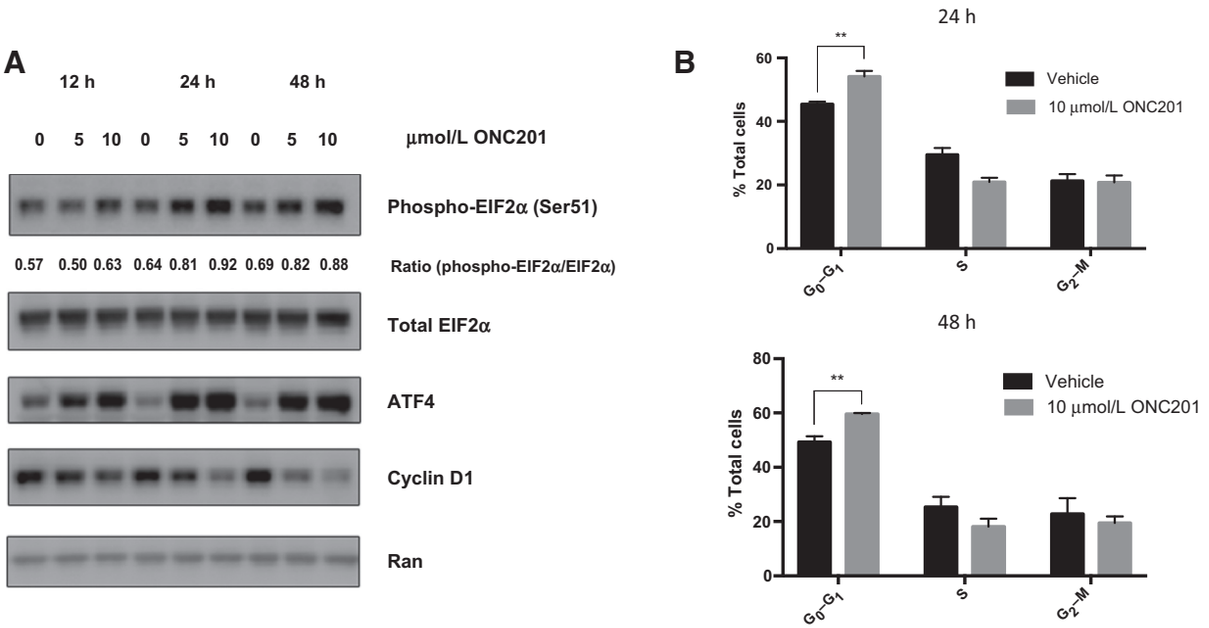

Figure 4.

ONC201 activates an ISR, decreases cyclin D1 expression, and causes an accumulation of cells in G_1 in TNBC cells sensitive to the compounds' antiproliferative effects. **A,** Western blot analysis of MDA-MB-231 cells for EIF2α phosphorylation, ATF4 expression, and cyclin D1 expression and following treatment with a vehicle control, 5 µmol/L, or 10 µmol/L ONC201 for 72 hours. Densitometry was performed using NIH ImageJ software. The ratio represents the intensity of the phospho-EIF2α band divided by the intensity of the total EIF2α band. **B,** Quantification of cells in G_0–G_1, S, and G_2 phases of the cell cycle using flow cytometric analysis of PI-stained MDA-MB-231 cells at 24 and 48 hours following treatment with a vehicle control or 10 µmol/L ONC201. ns: $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

was initially identified as a small molecule inducer of the TRAIL pathway (3) and cell death. In breast cancer cells, we show that ONC201 has antiproliferative and TRAIL-independent effects as well. This is highly relevant to breast cancer, where most subtypes are resistant to TRAIL, and explains the observed broad efficacy profile of ONC201. Most TNBC ($n = 6$) and all non-TNBC ($n = 5$) cells show GI_{50} values in the low micromolar range following treatment with ONC201. This is the first demonstration of ONC201 efficacy in non-TNBC cells and is clearly different than what was observed with TRAIL, where almost all ($n = 10$) breast cancer cells show resistance.

ONC201 was previously shown to induce cell death through activation of the extrinsic apoptosis pathway. We performed Annexin V–PI staining and Western blotting for PARP cleavage to determine whether ONC201 induced cell death in breast cancers. Cell death is induced in a subset of TNBC (2/8) and non-TNBC (4/5) cells. In TNBC cells where ONC201 induces cell death, caspase-8 cleavage indicates that the extrinsic pathway is being activated. Use of a TRAIL-neutralizing antibody abrogates apoptosis and confirms that the cell death is TRAIL dependent. These observations agree with the mechanisms of action of the compound that have been previously established by our laboratory (3, 6). The proapoptotic effects of ONC201 in TNBC translate to an antitumor effect in the *in vivo* MDA-MB-468 xenograft model. In addition, ONC201 has effects that are potentially additive in combination with FDA-approved drugs in MDA-MB-468 cells.

ONC201 acts through TRAIL-independent mechanisms in breast cancer as well. In TNBC cells where ONC201 does not induce cell death, the compound has an antiproliferative effect and activates an ISR, decreases expression of cyclin D1, and leads to accumulation of cells in the G_1 phase of the cell cycle. Reduced expression of cyclin D1 may account for the growth arrest of breast tumors cells in G_1 after ONC201 exposure and may be a useful biomarker in posttreatment biopsies in the clinic. The ISR is a cellular response that regulates protein synthesis. Various stresses can trigger phosphorylation of eukaryotic initiation factor 2α (eIF2α), leading to a generalized inhibition of protein translation combined with specific expression of stress-response genes, such as *ATF4* (reviewed by Quirós and colleagues; ref. 18). Activation of the ISR and phosphorylation of eIF2α by kinases PKR and PERK induces proteasome-dependent degradation of cyclin D1 (19). Our laboratory previously showed that the ISR activated by ONC201 involves eIF2α kinases HRI and PKR (6). This suggests that the antiproliferative effects of ONC201, in addition to its proapoptotic effects, may be mediated through the ISR. Future studies will need to elucidate the exact role of the ISR in the antiproliferative effects of ONC201.

Not all TNBC cells are sensitive to the antiproliferative effects of ONC201. In 2 of 6 TNBC cell lines, we observed increased resistance to ONC201, which was associated with decreased expression of pRb at the protein level. pRb is an important tumor suppressor that regulates passage through the G_1–S restriction point of the cell cycle by inhibiting E2F transcription factors (20). Phosphorylation of pRb inactivates the protein and relieves inhibition of the E2F transcription factors, allowing passage through the restriction point and into the S-phase.

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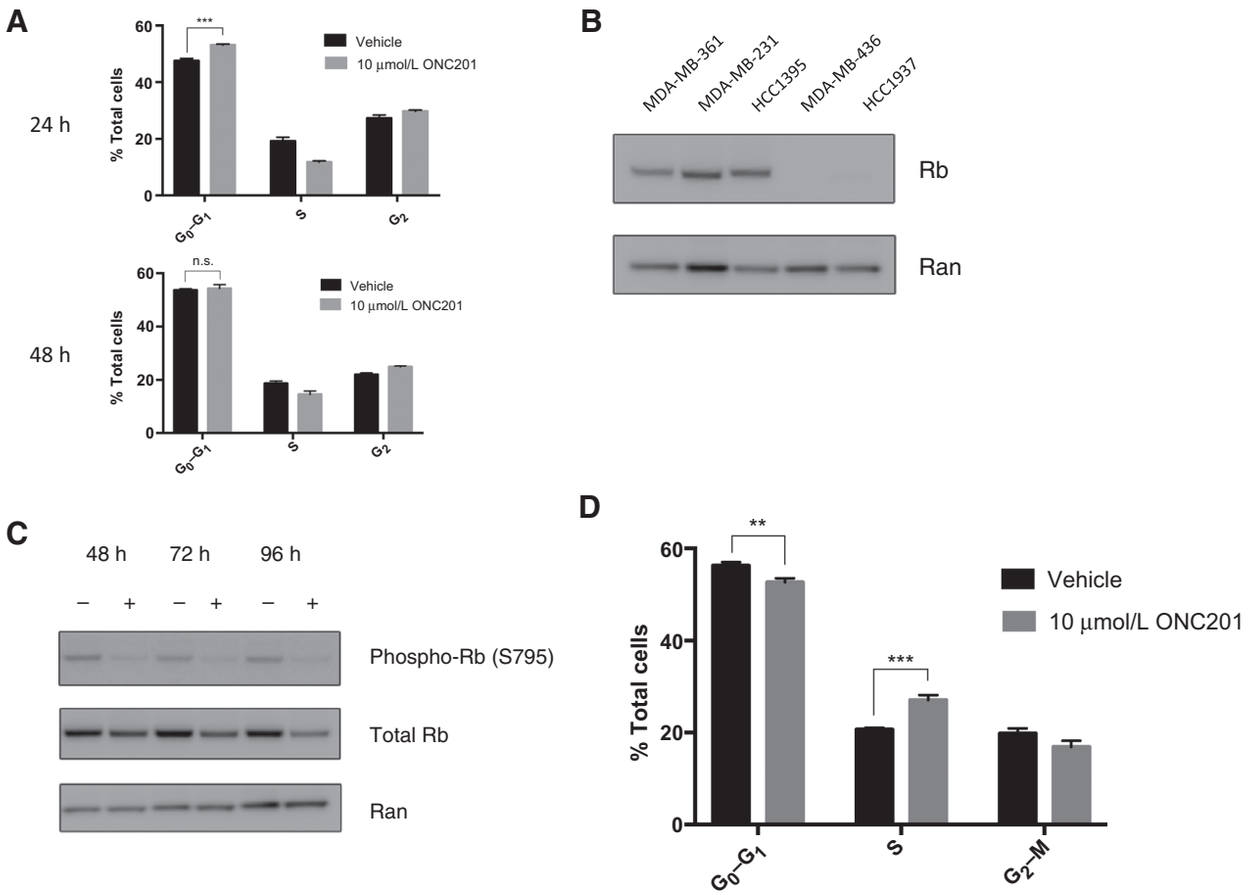

Figure 5.

Rb levels during ONC201-induced growth arrest in the G_1 phase of the cell cycle. **A,** Quantification of cells in G_0–G_1, S, and G_2 phases of the cell cycle using flow cytometric analysis of PI-stained MDA-MB-436 cells at 24 and 48 hours following treatment with a vehicle control or 10 μmol/L ONC201. **B,** Western blot analysis of baseline pRb expression in TNBC cells. **C,** Western blot analysis of MDA-MB-231 cells treated with a vehicle control or 10 μmol/L ONC201 over time probed to examine changes in levels of phosphorylated and total Rb. **D,** Quantification of cells in G_0–G_1, S, and G_2 phases of the cell cycle using flow cytometric analysis of PI-stained MDA-MB-436 cells at 72 hours following treatment with a vehicle control or 10 μmol/L ONC201. ns: $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

In the presence of growth-promoting signals, cells lacking pRb will pass through the G_1–S restriction point in an unrestricted manner, regardless of expression of upstream cell-cycle regulators, such as cyclin D1. Further evidence that pRb may be important for ONC201-mediated G_1 arrest is provided by the observation that ONC201 decreases expression of total pRb in MDA-MB-231 cells, and by 72 hours, the cells that previously had accumulated in G_1 move into the S-phase. Although the cells have entered the S-phase, both cell counting and BrdUrd uptake assays show that they are not actively proliferating. The antiproliferative effect of ONC201 is not sufficient to translate to an antitumor effect *in vivo*, and MDA-MB-231 xenograft tumors do not respond to treatment with 100 mg/kg ONC201 orally once per week. These results prompted a search for a combination with an approved drug in TNBC to potentiate the antitumor effect of ONC201. In TNBC cells with decreased sensitivity to ONC201 as a single agent, the compound synergizes with taxanes paclitaxel and docetaxel. Taxanes are used as first-line chemotherapy agents to treat TNBC in the adjuvant as well as the metastatic disease setting. High levels of antiapoptotic proteins are associated with resistance to chemotherapeutic drugs (reviewed by Pommier and colleagues; ref. 21). The ability of ONC201 to decrease the expression of antiapoptotic proteins (6, 9) may contribute to the observed synergy between the compound and taxane chemotherapy. Although the combination of ONC201 and taxanes in breast cancer needs to be tested *in vivo* and further understood mechanistically, there is potential for translation to the clinic.

SKBR3 and MCF7 non-TNBC cells show induction of caspase-8 and PARP cleavage following treatment with ONC201. This observation is significant given the high levels of TRAIL resistance in these cells. We initially hypothesized that ONC201 could be sensitizing cells to the apoptotic effects of TRAIL through previously characterized effects on mechanisms of TRAIL resistance. ONC201 treatment decreases levels of IAP and Bcl-2 family proteins in non-TNBC cells, high expression of which has been shown to be a regulator of sensitivity to TRAIL-induced apoptosis (16). ONC201 treatment has also been shown to upregulate cell

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Table 2. ONC201 synergizes with docetaxel and paclitaxel in TNBC cells with decreased sensitivity to ONC201

MDA-MB-436					HCC1937				
CI	μmol/L ONC201				CI	μmol/L ONC201			
nmol/L paclitaxel	2.5	5	10	20	nmol/L paclitaxel	2.5	5	10	20
6.25	0.38	0.56	0.54	0.86	**6.25**	0.55	0.44	0.67	1.18
12.5	0.71	0.52	0.43	0.68	**12.5**	0.53	0.51	0.7	1.16
25	0.59	0.42	0.38	0.41	**25**	0.67	0.68	0.9	1.32
50	0.98	0.66	0.65	0.68	**50**	1.17	1.05	1.24	1.69
CI	**μmol/L ONC201**				**CI**	**μmol/L ONC201**			
nmol/L docetaxel	**2.5**	**5**	**10**	**20**	**nmol/L docetaxel**	**2.5**	**5**	**10**	**20**
6.25	0.73	0.39	0.41	0.63	**6.25**	0.45	0.34	0.44	0.75
12.5	0.58	0.44	0.38	0.55	**12.5**	0.67	0.45	0.55	0.78
25	0.46	0.45	0.46	0.54	**25**	0.84	0.68	0.78	1
50	0.39	0.79	0.78	0.83	**50**	1.62	1.25	1.33	1.49

NOTE: Combination indices (CI) for MDA-MB-436 and HCC1937 cells treated with different concentrations of ONC201 and paclitaxel for 72 hours. Combination indices calculated using the Chou–Talalay method. CI > 1, antagonism; CI = 1, additive effect; CI < 1, synergy.

surface DR5 (3), low levels of which are associated with resistance to TRAIL (15). Surprisingly, treatment with ONC201 did not upregulate surface TRAIL and DR5 in SKBR3 and MCF7 cells as it did in control MDA-MB-231 cells, and TRAIL-neutralizing antibody RIK2 failed to abrogate cell death induced by the compound in the cells. These observations suggest that ONC201 is acting through a TRAIL-independent mechanism of action. Fas ligand (FasL) and TNFα can activate death receptors and thus apoptosis through the extrinsic pathway (reviewed by Tait and colleagues; ref. 22). It is possible that these alternative death receptor ligands are responsible for the effects of ONC201 in these cells or that feedback mechanisms

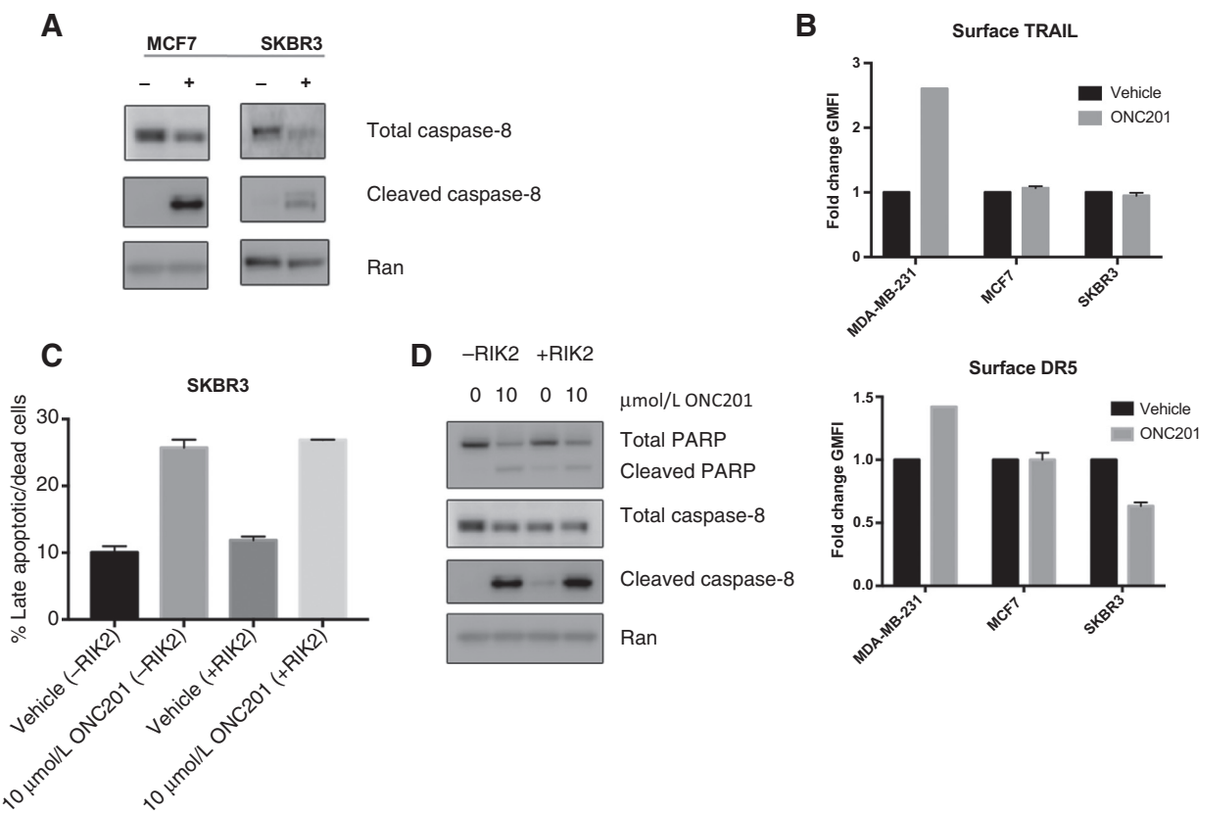

Figure 6.

The proapoptotic effects of ONC201 in non-TNBC cells involve caspase 8 cleavage, but are TRAIL independent. **A,** Immunoblot analysis of SKBR3 and MCF7 cells treated with a vehicle control or 10 μmol/L ONC201 for 72 hours. **B,** Geometric mean fluorescence intensity of TRAIL and DR5 surface staining was determined in MDA-MB-231 and MCF7 cells treated with a vehicle control or 5 or 10 μmol/L ONC201 for 72 hours. **C,** Annexin-V/PI double positive cells were quantified in SKBR3 using flow cytometry following a 72-hour treatment with a vehicle control or 10 μmol/L ONC201 in the presence or absence of 1 μg/mL RIK2 TRAIL-blocking antibody. **D,** Immunoblot of MCF7 cells treated with a vehicle control or 10 μmol/L ONC201 in the presence or absence of 1 μg/mL RIK2 TRAIL-blocking antibody.

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from downstream caspase activation result in caspase-8 cleavage and amplification of a cell death signal independent of involvement of the death receptors. Further experiments are needed to explore this.

In summary, this preclinical study demonstrates that ONC201 shows efficacy in TNBC and non-TNBC cells, including BRCA1-deficient cells, regardless of their sensitivity to TRAIL. Some TNBC cells undergo TRAIL-dependent cell death that translates to an antitumor effect *in vivo*, but the effects of ONC201 in most TNBC cells are antiproliferative rather than proapoptotic (2/8). Expression of pRb may be important for sensitivity to the antiproliferative effects of the compound. This is the first demonstration of the efficacy of ONC201 across a spectrum of breast cancer cell lines, including non-TNBC cells that are TRAIL resistant. ONC201 induces cell death that appears to be TRAIL independent in non-TNBC cells. The heterogeneity of breast cancers observed at the genomic and epigenomic level may explain why ONC201 exerts such a wide range of effects among cells of this tumor type. Further studies are needed to elucidate the exact mechanisms responsible for the TRAIL-independent effects of ONC201. ONC201 has completed its first-in-human trial in advanced solid tumors, where it was shown to be safe and to have preliminary efficacy (11). It is currently being tested in multiple phase II trials of patients with solid tumors and hematologic malignancies. Overall, our findings present a preclinical rationale for clinical testing of ONC201 as a single agent and in combination with approved therapies, including taxanes, in breast cancers.

Disclosure of Potential Conflicts of Interest

V.V. Prabhu has ownership interest (including patents) in Oncoceutics. W. Oster has ownership interest (including patents) in Oncoceutics. W.S. El-Deiry has ownership interest (including patents) in Oncoceutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M.D. Ralff, C.L.B. Kline, W. Oster, W.S. El-Deiry
Development of methodology: M.D. Ralff, C.L.B. Kline, W.S. El-Deiry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.D. Ralff, O.C. Küçükkase, D.T. Dicker, W.S. El-Deiry
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.D. Ralff, D.T. Dicker, W. Oster, W.S. El-Deiry
Writing, review, and/or revision of the manuscript: M.D. Ralff, C.L.B. Kline, B. Lim, V.V. Prabhu, W. Oster, W.S. El-Deiry
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.T. Dicker, W. Oster, W.S. El-Deiry
Study supervision: W.S. El-Deiry
Other (assistance): J. Wagner

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Molecular Cancer Therapeutics

ONC201 Demonstrates Antitumor Effects in Both Triple-Negative and Non–Triple-Negative Breast Cancers through TRAIL-Dependent and TRAIL-Independent Mechanisms

Marie D. Ralff, Christina L.B. Kline, Ozan C. Küçükkase, et al.

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