

ONC201 induces cell death in pediatric non-Hodgkin's lymphoma cells

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Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; TNF, tumor necrosis family; NHL, non-Hodgkin's lymphoma.

ONC201/TIC10 is a small molecule initially discovered by its ability to coordinately induce and activate the TRAIL pathway selectively in tumor cells and has recently entered clinical trials in adult advanced cancers. The anti-tumor activity of ONC201 has previously been demonstrated in several preclinical models of cancer, including refractory solid tumors and a transgenic lymphoma mouse model. Based on the need for new safe and effective therapies in pediatric non-Hodgkin's lymphoma (NHL) and the non-toxic preclinical profile of ONC201, we investigated the *in vitro* efficacy of ONC201 in non-Hodgkin's lymphoma (NHL) cell lines to evaluate its therapeutic potential for this disease. ONC201 caused a dose-dependent reduction in the cell viability of NHL cell lines that resulted from induction of apoptosis. As expected from prior observations, induction of TRAIL and its receptor DR5 was also observed in these cell lines. Furthermore, dual induction of TRAIL and DR5 appeared to drive the observed apoptosis and TRAIL expression was correlated linearly with sub-G1 DNA content, suggesting its potential role as a biomarker of tumor response to ONC201-treated lymphoma cells. We further investigated combinations of ONC201 with approved chemotherapeutic agents used to treat lymphoma. ONC201 exhibited synergy in combination with the anti-metabolic agent cytarabine *in vitro*, in addition to cooperating with other therapies. Together these findings indicate that ONC201 is an effective TRAIL pathway-inducer as a monoagent that can be combined with chemotherapy to enhance therapeutic responses in pediatric NHL.

Introduction

Lymphoma is the third most common childhood malignancy, with non-Hodgkin's Lymphoma (NHL) accounting for about 7% of new cancer cases in children, most of which are high grade.^{1–3} Current treatment approaches rely heavily on intensive multi-agent chemotherapy regimens such as the cyclophosphamide–doxorubicin–vincristine–prednisone (CHOP) regimen, in addition to the anti-CD20 antibody rituximab. Despite significant progress in 5-year survival rates for pediatric NHL, there is still a need for improved therapies to treat advanced-stage, relapsed, and/or refractory disease.

The TNF-related apoptosis-inducing ligand (TRAIL) is an endogenous protein expressed by several immune cells to selectively induce apoptosis in cancer cells as a critical effector mechanism in the immune surveillance of cancer.^{4,5} The ability of TRAIL to confer apoptosis in various malignant cells while leaving normal cells unharmed has highlighted this ligand and its receptors as exciting potential therapeutic targets.⁶ While early-phase clinical trials have investigated the role of recombinant TRAIL and TRAIL death receptor-agonist antibodies in several

malignancies, pediatric lymphoma remains uninvestigated.^{7–11} Preclinical studies have found that recombinant TRAIL is active in NHL when combined chemotherapy.^{12–14} Interestingly, a prior study in Burkitt's lymphoma found that TRAIL is highly active by a paracrine mechanism in a xenograft model as compared to recombinant protein administration.¹⁵ Thus TRAIL-based therapies have been recognized and remain as a therapeutic intervention for further investigation in pediatric and adult lymphomas.¹⁶

The clinical development of protein-based TRAIL therapeutics has been stymied following clinical efficacy results in lead indications that were less robust than anticipated, despite a benign safety profile. The limited clinical activity of these TRAIL-based agents has been ascribed to intrinsic tumor cell attributes such as O-glycosylation enzymes,^{17,18} the tumor microenvironment,^{19,20} and limitations of the therapeutic agents themselves such as suboptimal death receptor clustering and poor chemical characteristics.²¹ Nonetheless, TRAIL possesses ideal therapeutic properties as an endogenous, natural, anti-cancer protein that was evolved as part of the immune system.⁵

The power of the immune system in general and TRAIL pathway in particular in cancer suppression motivated our effort to

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identify small molecules that harness the therapeutic potential of the endogenous death receptor pathway without the limitations of exogenous protein mimetics.²² Small molecule screening led to the identification of ONC201, a small molecule with a unique pharmacophore that induces TRAIL in a p53-independent manner as well as its proapoptotic receptor DR5, leading to the coordinate activation of apoptosis in tumor cells while sparing normal cells. Mechanistic studies revealed a late-stage inactivation of Akt and ERK that activates Foxo3a, which transcriptionally regulates the TRAIL gene. The involvement of these mechanisms as well as additional early-stage signaling that is currently under investigation supports the strong efficacy and safety characteristics of ONC201 that extends the scope of prior TRAIL-based therapies.

The preclinical efficacy of ONC201 has been reported in a wide range of tumor types as a monoagent and in combination with approved therapies,^{22,23} including extension of survival in a Eu-myc transgenic model of lymphoma. A unique feature of ONC201 is the coupling of its strong efficacy and benign safety features, which is particularly important in pediatric cancer patients where genotoxicity is germane and accompanies many of its standard-of-care therapies.^{24,25} Based on the aforementioned efficacy, mechanistic, and safety characteristics of ONC201 we investigated its activity in pediatric NHL cell lines to determine its therapeutic potential in this indication that is in need of novel effective and safe therapies.

Materials and Methods

Cell lines

Granta, UPN2, NCEB, Karpas299 and Daudi cell lines were a kind gift from Dr. E. Epner at Penn State Hershey Cancer Institute; Ramos and Raji cells were a kind gift from Dr. S. Dovat at Penn State Hershey Medical Center; the BJAB cell line was a kind gift from Dr. D. Tantin at University of Utah. Cells were cultured in ATCC-recommended media and maintained in a humidified incubator at 5% CO₂ and 37°C.

Cell culture

Cell lines (all except Granta) were cultured in RPMI 1640, which was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin (all from Life Technologies, Inc.). Raji cells in addition needed 0.05 mM 2-mercaptoethanol. Granta cells were maintained in DMEM with 10% FBS and 100 units/ml penicillin/streptomycin. All cells were incubated in a fully humidified atmosphere with 5% CO₂ at 37°C. Cell media was renewed every 3–4 d per standard specifications. All experiments were conducted with a cell number of 1×10^6 cells/ml.

Cell viability assays

Cell viability was measured using the CellTiter Glo[®] Luminescent Cell Viability assay kit (Promega). Briefly $1-2 \times 10^4$ cells per well were incubated in fresh media in 96-well black-walled plates (Nunc) at a concentration of 2×10^6 cells per mL and a

volume of 50–100 μ L per well, along with the indicated concentrations of specified drug. Viability assays were performed according to the manufacturer's protocol at 72 hours post-treatment or at otherwise indicated time points. Bioluminescent imaging was recorded on an IVIS imaging system (Xenogen). GI50 values were calculated using non-linear regression curve fitting (GraphPad Prism).

Flow cytometry

For sub-G1 DNA content analyses, cells were harvested at indicated time points and fixed in 80% ethanol at 4°C. Cells were then stained with propidium iodide in the presence of RNase and analyzed on an Epics Elite flow cytometer (Beckman Coulter).

For surface TRAIL expression analyses, cells were harvested and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, incubated with an anti-TRAIL antibody (Abcam, ab2435) at 1:250 overnight, washed and incubated with anti-rabbit Alexa Fluor 488 (Invitrogen) for 30 min, and analyzed. Cells were gated on forward and side scatter to eliminate debris and dead cells from the analysis. Surface TRAIL data are expressed as median fluorescence intensity relative to that of control samples unless indicated otherwise.

For DR5 expression analyses, cells were harvested and fixed in 4% paraformaldehyde to avoid receptor internalization. Cells were then incubated with primary antibodies at 1:250 (DR5, Imgenex Img-120A) for 1 hour, washed in PBS, incubated with Alexafluor 488 as a secondary antibody, and resuspended in PBS for analysis. Surface DR5 data are expressed as median fluorescence intensity relative to that of control samples unless indicated otherwise. RIK-2 (Santa Cruz Biotechnology, sc-56246) was used as a TRAIL sequestering antibody for testing inhibition of ONC201 mediated cytotoxicity per product specifications. Recombinant His-tagged TRAIL was produced as previously described.²⁶

Western blot analysis

Cells were grown in 6-well plates and treated in log-phase as indicated. Cells were harvested then centrifuged and lysed on ice for 2 hours. Samples were electrophoresed under reducing conditions on NuPAGE 4–12% Bis-Tris gels (Invitrogen), transferred to PVDF, and blocked in 10% non-fat milk in TBST for 1 hour. Membranes were then incubated with the primary antibodies overnight: DR5 (Cell Signaling #3696) and Ran (BD Biosciences # 610341 at 1:1000 in TBST overnight at 4°C. Membranes were washed in TBST, incubated with the appropriate secondary antibody for 1 hour, washed in TBST, and visualized using ECL[™] Plus (Amersham) and X-Ray film (Thermo-Scientific) or by Odyssey Li-Cor protocol.

Statistical Analyses

Pairwise comparisons were evaluated by a Student's 2-tailed *t*-test using Microsoft Excel. The GI50 values and Pearson's correlation were evaluated using GraphPad Prism.

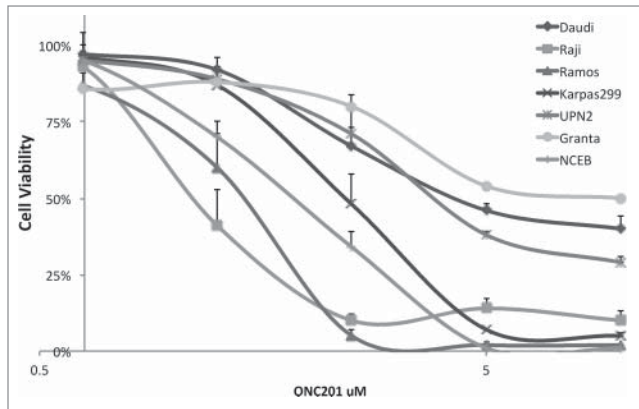


Figure 1. ONC201 induces a dose-dependent reduction in the cell viability of human lymphoma cell lines. Cell viability assays with ONC201 for 72 hours treatment.

Combination indices

Combination indices were computed with CalcuSyn software (Biosoft) using the Chou-Talalay method.

Results

ONC201 induces cytotoxic responses in pediatric NHL cell lines

We selected a panel of human lymphoma cell lines that were primarily derived from pediatric patients, including Burkitt's lymphoma and the Karpas299 T-cell lymphoma cell lines (Table S1) to assess *in vitro* sensitivity to ONC201. In parallel, we examined mantle cell lymphoma as another indication for comparison. Cell viability assays at 72 hours post-treatment with ONC201 demonstrated dose-response curves in this panel that tended to saturate beyond 5 μ M (Fig. 1). The GI50 among this panel ranged from 1.3 to 5.1 μ M, which is comparable to reported activity of ONC201 in other tumor types.²²

To further understand the observed activity of ONC201, we performed sub-G1 analysis by flow cytometry that revealed significant levels of apoptosis at 72 hours post-treatment (Fig. 2A). The Ramos and Karpas299 pediatric cell lines exhibited the strongest levels of apoptosis under the evaluated condi-

tions. The other cell lines that exhibited weaker levels of cell death likely undergo a cytostatic response based on their responsiveness in cell viability assays (e.g. NCEB cells). In general, the induction of cell death was dose-dependent but saturated at 5 μ M for some cell lines such as Ramos and Karpas299. Caspase-mediated apoptosis was confirmed by reduced sub-G1 DNA content that resulted from co-incubation with the pan-caspase inhibitor zVAD-fmk (Fig. 2B).

The TRAIL pathway is induced by ONC201

Due to the prior demonstration of the TRAIL pathway as a critical component of the cytotoxic response to ONC201, we compared the activity of recombinant TRAIL to that of ONC201. Interestingly, BJAB cells were moderately responsive to recombinant TRAIL but were strongly responsive to ONC201 (Fig. 3A). Flow cytometry analysis revealed that pediatric lymphoma cell lines upregulate TRAIL expression on their cell surface in response to ONC201 (Fig. 3B). We noticed that the saturation of TRAIL induction in these experiments occurred

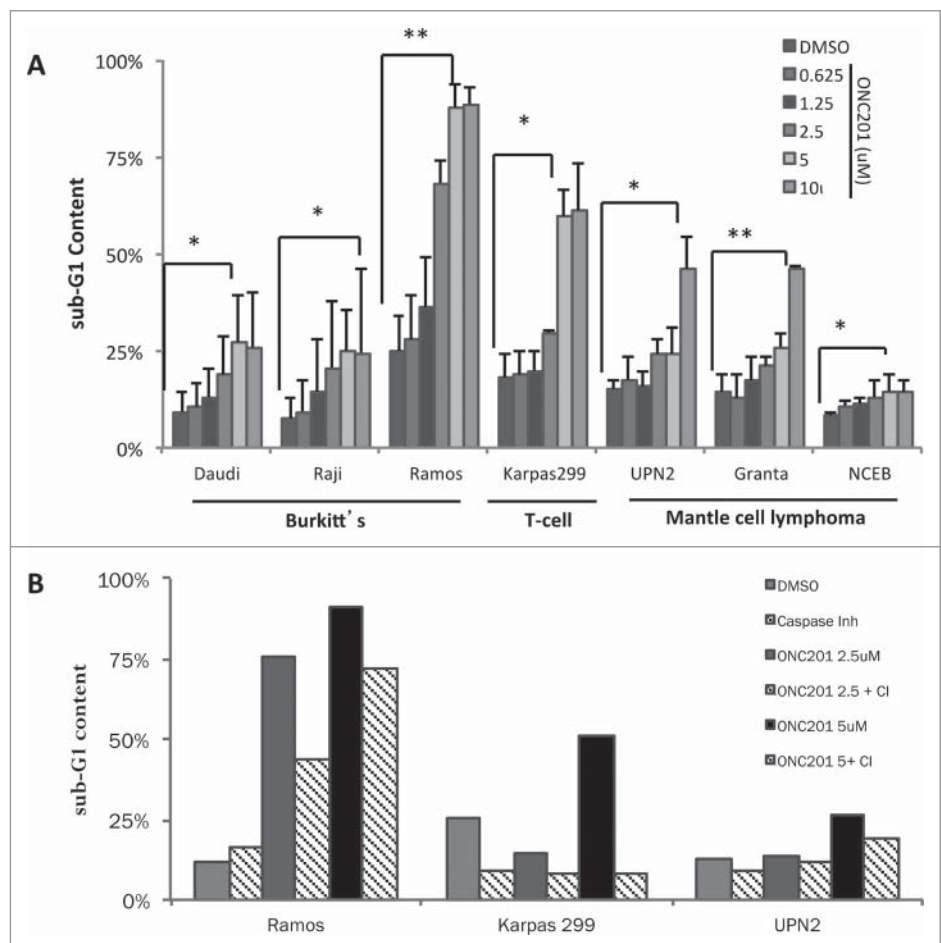


Figure 2. ONC201 induces caspase-dependent apoptosis in human lymphoma cell lines. (A) Sub-G1 DNA content analysis lymphoma cell lines treated with ONC201 at 0.625, 1.25, 2.5, 5 and 10 μ M for 72 hours (n = 3). * P < 0.05; ** P < 0.005. (B) Sub-G1 DNA content analysis of lymphoma cell lines in the presence or absence of the pan-caspase inhibitor zVAD-fmk (CI) and/or ONC201 for 72 hours.

at the same doses where efficacy was saturated in cell viability and cell death assays. Further investigation of this relationship revealed that the induction of TRAIL protein correlates linearly with induction of cell death across the various evaluated lymphoma cell lines and doses (Fig. 3C).

To further investigate activation of the TRAIL pathway by ONC201 and potentially explain its strong cytotoxicity, we examined expression levels of DR5 that is a proapoptotic receptor for TRAIL previously reported to be co-induced by ONC201 along with its ligand.²² In agreement with the prior findings, an increase in surface DR5 expression in response to ONC201 was noted in a dose-dependent manner (Fig. S1A, B). Co-administration of a TRAIL-sequestering antibody reduced induction of cell death, which is indicative of at least a partial role for activation of the TRAIL pathway in ONC201-induced cell death of these pediatric cancer cells (Fig. 3D).

ONC201 cooperates with several pediatric lymphoma chemotherapies

Preclinical studies in adult cancer models support combining TRAIL-based agents with various chemotherapeutic agents. We

recently reported that ONC201 exhibits synergy with several approved targeted agents and chemotherapies that range from multikinase inhibitors, alkylating agents and inhibitors of DNA/nucleoside synthesis.²⁷ We specifically examined the combinatorial efficacy of ONC201 with chemotherapies used to treat pediatric lymphoma (Fig. 4A). While enhanced efficacy was noted with bortezomib, doxorubicin, and bendamustine, ONC201 combined with cytarabine was found to synergistically reduce cell viability as confirmed objectively by combination indices (Table 1).

Discussion

In this study, we show that ONC201 is an effective anti-cancer agent against NHL cell lines *in vitro* as a monoagent and in combination with chemotherapies. The significant levels of reduction in cell viability and induction of apoptosis were observed at doses that are achievable in mice²² and in other species in GLP toxicology studies. The fact that ONC201 was effective in both B-cell and T-cell NHL cell lines is in accordance with its broad-spectrum activity that bodes well for its scalability

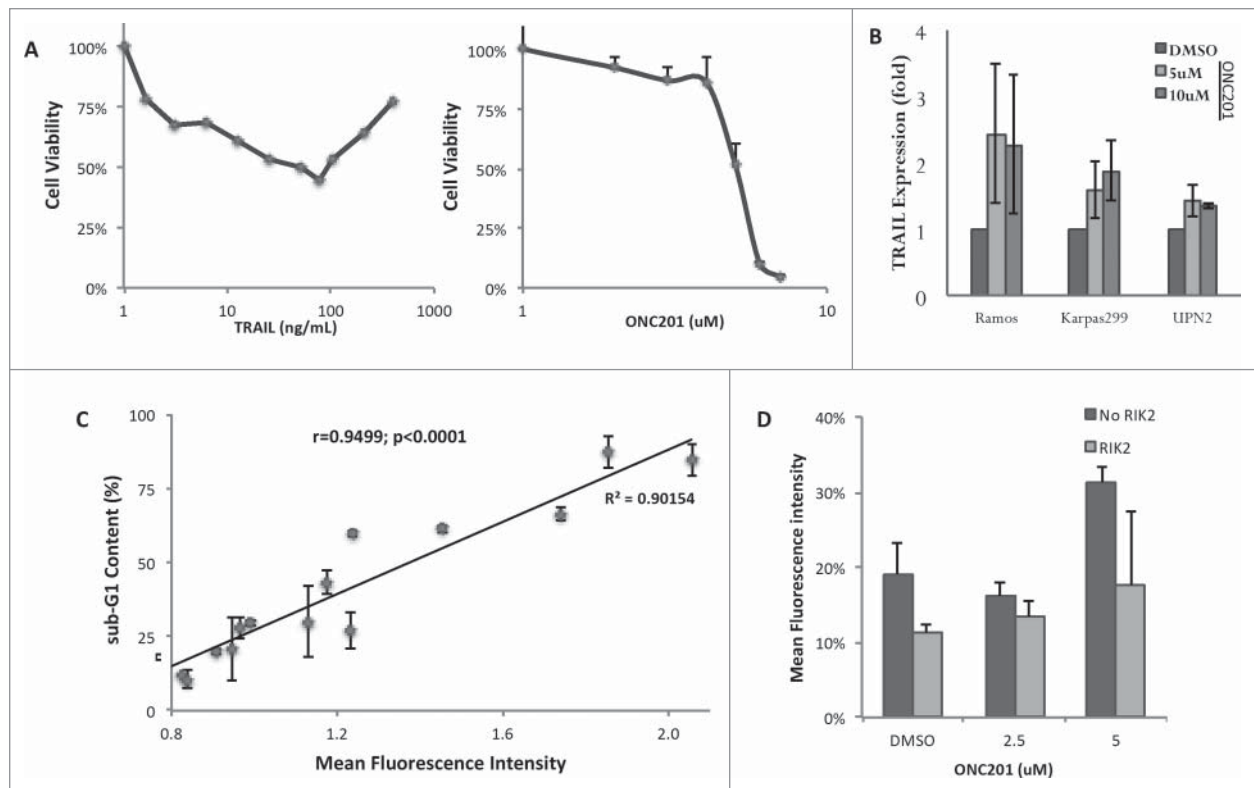


Figure 3. ONC201 induces the TRAIL pathway in human lymphoma cell lines. (A) Dose-response curve of BJAB cells to recombinant TRAIL or ONC201 at 72 hours post-treatment (n = 3). (B) Fold TRAIL expression of lymphoma cell lines at 60 hours post treatment with ONC201 (n = 3). (C) Correlation of surface TRAIL expression by flow cytometry with sub-G1 DNA content of Ramos, Karpas299, and UPN2 lymphoma cells treated with several doses of ONC201 (0.625, 1.25, 2.5, 5, and 10 μ M, n = 3). The Pearson correlation coefficient "r" was 0.9499 with 95% confidence interval of 0.8526 to 0.9836 and a P value of <0.0001. (D) Reduction of cell death by co-incubation with RIK2, a TRAIL-sequestering antibody in Karpas299 cells with 72 hour treatment with ONC201 (n = 2).

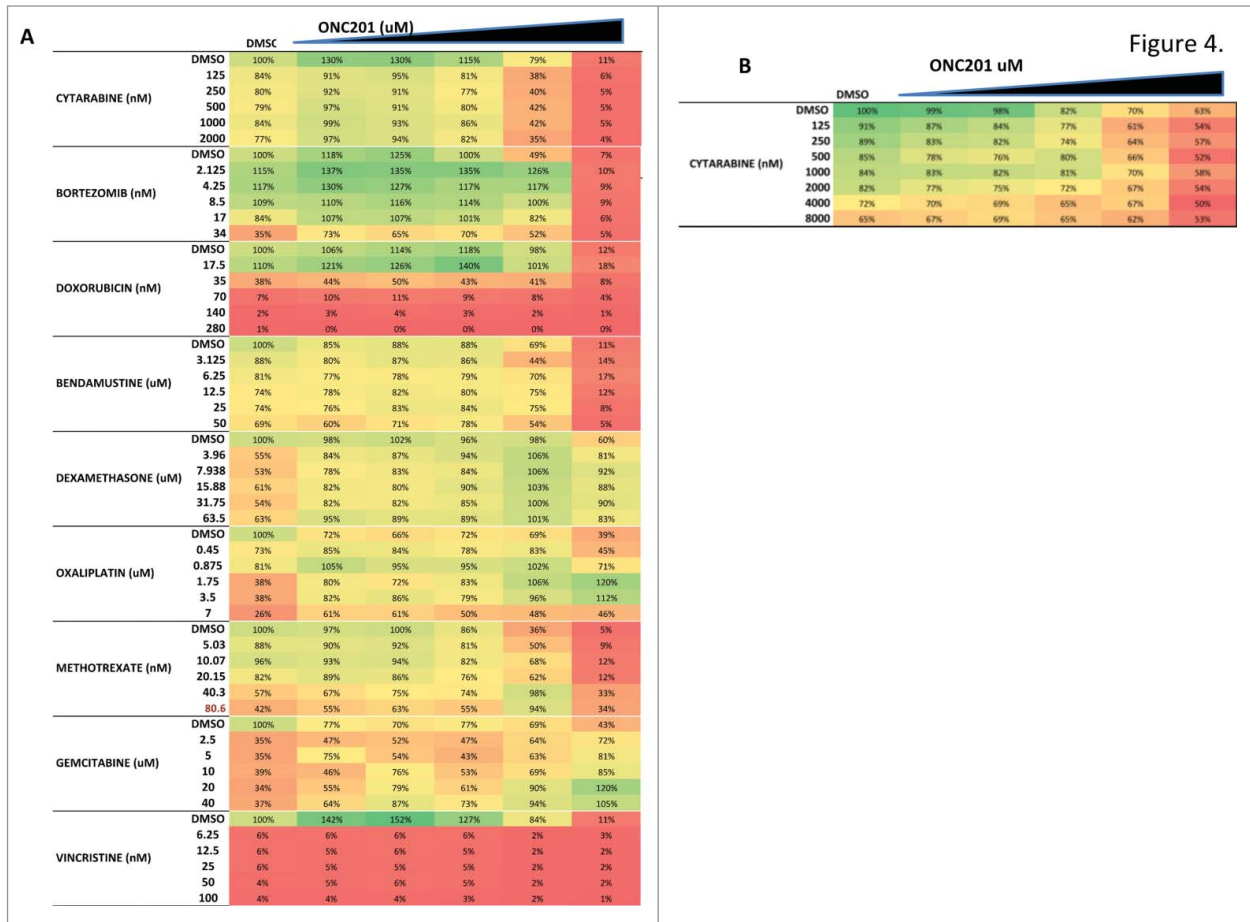


Figure 4. ONC201-induced cytotoxicity is enhanced in combination with pediatric NHL chemotherapy. **(A)** Cell viability assays of Ramos cells following 72 hour treatment with various doses of ONC201 in combination with increasing doses of NHL chemotherapies. 100% cell viability (100%) is represented as darkest green and lowest cell viability (approaching zero) is represented as darkest red. ONC201 doses used were 0.093, 0.1875, 0.375, 0.75, 1.5 μ M and n = 2. **(B)** Cell viability assays of Karpas299 cells following 72 hour treatment with various doses of ONC201 (0.156, 0.3125, 0.625, 1.25, 2.5 μ M) in combination with increasing doses of cytarabine (n = 2).

into several indications in the clinic, including pediatric lymphoma. The Ramos pediatric Burkitt's lymphoma cell line exhibited the highest degree of sensitivity to ONC201 in vitro, which is interesting given that Burkitt's lymphoma is myc-driven²⁸ and that c-myc has been linked to multiple aspects of the mechanism of ONC201 such as TRAIL^{29,30} and Ras signaling.³¹

Table 1. Combination indices for ONC201 in combination with cytarabine

	ONC201 (μ M)	Cytarabine(nM)						
		31.25	62.5	125	250	500	1000	2000
Karpas299								
	0.3125	0.55	0.76	0.41	0.38	0.40	1.03	1.01
	0.625	0.54	0.64	0.46	0.50	0.46	1.12	1.01
	1.25	0.58	0.79	0.57	0.57	0.92	1.25	1.03
	2.5	0.70	0.64	0.66	0.72	0.83	1.10	1.13

Combination indices obtained for Karpas299 cells at 72H post combination treatment with increasing doses of ONC201 combined with increasing doses of cytarabine confirm synergistic cytotoxicity.

Pediatric lymphomas remain in need of improved therapies for children and adolescents with advanced-stage disease and for those who relapse after conventional therapy. In pediatric lymphomas, the need for safer therapies is particularly underscored by the incidence of secondary neoplasms caused by the available chemotherapies and radiation that are genotoxic.^{24,25} Thus the lack of toxicity, which includes lack of genotoxicity,³² that accompanies the efficacy of ONC201 is particularly important for pediatric populations that have a longer post-therapy life spans where secondary neoplasms can occur.

The observation that the TRAIL pathway mediates ONC201-induced apoptosis in Karpas 299 cells supports the previously reported contribution of TRAIL to the apoptotic activity of ONC201.^{22,23} Accordingly, surface TRAIL expression was correlated linearly with the apoptotic activity of ONC201. Since surface TRAIL protein is cleaved to form a soluble ligand, assaying serum levels in addition to tumor cell expression of TRAIL is suggested for pharmacodynamic studies in ongoing clinical trials of ONC201.

ONC201 is a first-in-class compound with a unique pharmacophore³³ and associated novel mechanism of action. The mechanism of ONC201 remains an area of active study, particularly the upstream signaling mechanisms that trigger its late anti-cancer effects that appear to involve both cytostatic and cytotoxic phenotypes in a cell-type dependent manner. The apparent involvement of cytostatic mechanisms is evident in cells that respond strongly in cell viability assays to ONC201, but do not undergo apoptosis, such as the NCEB cell line evaluated in this study. Thus, the robust anti-cancer activity of ONC201 appears to be a function of both TRAIL-dependent and independent anti-cancer mechanisms that differentially manifest in a cancer cell type-dependent manner but appear to be well tolerated by normal cells.

A recent preclinical study evaluated a panel of FDA-approved anti-cancer small molecules in solid tumors to reveal that ONC201 exhibits a broad synergistic profile.²⁷ This study investigated a subset of approved small molecules that were curated for NHL relevance. Our limited combinatorial study identified cytarabine as a synergistic combination with ONC201 in pediatric NHL. Synergy between cytarabine and TRAIL, thought to be driven by cytarabine-mediated DR5 induction, has been previously documented in preclinical hematological malignancies.³⁴ While the combination of ONC201 with cytarabine should be further studied mechanistically and *in vivo*, the combination could be translated in the clinic.

In summary, this preclinical study demonstrates that ONC201 induces dose- and caspase-dependent apoptosis and activates the TRAIL pathway at low micromolar concentrations

in pediatric NHL cell lines. While TRAIL-mediated apoptosis appears to play a part in the cytotoxic activity of ONC201, further studies are needed to explain its full spectrum of activity that includes non-apoptotic effects. These preclinical studies bode well for the monoagent activity of ONC201 in clinical trials in lymphoma. The support for TRAIL as a response-dependent biomarker of ONC201 as well as the identification of cytarabine as a synergistic combination provide additional approaches to realize the therapeutic potential of this first-in-class compound. Furthermore, the demonstration of preclinical efficacy with ONC201 in pediatric NHL provides the rationale for clinical investigation of this novel agent in a patient population where its pronounced safety is highly pertinent.

Disclosure of Potential Conflicts of Interest

Joshua Allen is an employee and stockholder of Oncoceutics, Inc. and Wafik El-Deiry is a Founder and stockholder of Oncoceutics, Inc.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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