Efficacy of ONC201 in Desmoplastic Small Round Cell Tumor

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Abstract
Desmoplastic Small Round Cell Tumor (DSRCT) is a rare sarcoma tumor of adolescence and young adulthood, which harbors a recurrent chromosomal translocation between the Ewing’s sarcoma gene (EWSR1) and the Wilms’ tumor suppressor gene (WT1). Patients usually develop multiple abdominal tumors with liver and lymph node metastasis developing later. Survival is poor using a multimodal therapy that includes chemotherapy, radiation and surgical resection, new therapies are needed for better management of DSRCT. Triggering cell apoptosis is the scientific rationale of many cancer therapies. Here, we characterized for the first time the expression of pro-apoptotic receptors, tumor necrosis-related apoptosis-inducing ligand receptors (TRAILR1-4) within an established human DSRCT cell line and clinical samples. The molecular induction of TRAIL-mediated apoptosis using agonistic small molecule, ONC201 in vitro cell-based proliferation assay and in vivo novel orthotopic xenograft animal models of DSRCT, was able to inhibit cell proliferation that was associated with caspase activation, and tumor growth, indicating that a cell-based delivery of an apoptosis-inducing factor could be relevant therapeutic agent to control DSRCT.

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Introduction
Desmoplastic Small Round Cell tumor (DSRCT) first described by Gerald and Rosai, is a rare poorly differentiated sarcoma cell of childhood and adolescence that has distinctive histologic, genetic and clinical features [1–3]. DSRCT is characterized as a small round blue cells, with fibroscopic stroma and abundant extracellular material co-expressing epithelial, mesenchymal, myogenic and neural bio-

Abbreviations: DR4/DR5, Death Receptors; DSRCT, Desmoplastic Small Round Cell Tumor; TRAIL, TNF-related apoptosis inducing ligand.

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Materials and Methods

DSRCT patients were initially treated like Ewing sarcoma (ES) with similar multimodality intensive chemotherapy regimens and was considered to correlate with a good prognosis within a short following up [6–8]. However, relapses were acknowledged in the majority of patients after longer following up. The combination treatment strategies including this multimodal intensive chemotherapy, aggressive surgical resection, and radiation therapy that are now considered as the standard care for DSRCT patients with primary disease, can improve a survival benefit, but the overall prognosis remains poor [9–13].

Since this tumor is only recently described, and there are no commercial cell lines, little new information is available. Therefore, further insight into DSRCT tumor inhibition like triggering cell apoptosis may lead to the generation of beneficial specific targeted therapies.

Apoptosis can be induced by TNF-related apoptosis inducing ligand (TRAIL), a pro-apoptotic cytokine from the TNF superfamily. TRAIL can bind to five receptors, two death receptors (DR4 and DR5) triggering TRAIL-induced apoptosis, two decoy receptors that possibly inhibits this cell pathway, and one soluble inhibitor of RANK ligand (osteoprotegrin) at low affinity [12–14].

In the current investigation, we hypothesize that death receptor dependent apoptosis is a mechanism for cell death in DSRCT cells. Apoptosis through the activation of death receptor 4 and 5 has been found to be a promising candidate for the development of new therapeutic approach in the treatment of the most frequent malignant primary bone tumors [15–18], but has not been described for this type of sarcoma.

ONC201 is a novel drug not yet tested in sarcomas. ONC201 was discovered as a p53-independent inducer of TRAIL gene transcription and had an anti-proliferative and anti-apoptotic effects against a broad range of tumor cells [19–22]. The mechanism of action of ONC201 engages PERK-independent activation of the integrated stress pathway, leading to upregulation of DR5 and TRAIL in tumors [23,24]. The mechanisms of the stress response induced differ between hematological malignancies that prompt an atypical integrated stress responses (ISR) associated with p53-independent apoptosis and solid tumors that trigger an ISR dependent on ATF4 activation by specific eIF2α kinases [21,23,25]. A Synergistic anti-tumor effect was observed with the joined inhibition of Bel-2 with ABT-263/ABT-199 and Mcl-1 with ONC201 (through Bag3/UspX inhibition) through the mitochondrial pathway of apoptosis involving caspase activation and PARP-cleavage [23,26].

Here, we demonstrate for the first time the efficacy of ONC201 and its anti-neoplastic mechanism in a new preclinical model of DSRCT, within in vitro cell line culture and in vivo xenograft animal model.

Preparation of Drug Solutions

For in vitro utilization, ONC201 (Oncoceutics, Inc., 3624 Market Street, University City Science Center, Suite 5E, Philadelphia, PA 19104) stock solution (10 mmol/L) was prepared in DMSO and stored at -20°C. For in vivo application, the stock solution of ONC201 (25 mg/ml) was prepared for an oral administration with either low dose (50 mg/kg) or high dose (100 mg/kg) in 1X PBS.

In Vitro Cell Viability Assay

Cell viability was measured using an MTT assay. JN-DSRCT-1 cells were cultured until the log-phase and were subsequently seeded into a 96-well plate at a density of 10^4 cells/well overnight to treatment with different concentrations of ONC201 (0.625–20 μM) or DMSO. Following an incubation of 72 h, the cells were then incubated with medium containing MTT for 4 h and the formazan crystals were dissolved with 150 μl DMSO. The plates were incubated on a shaker for 15 minutes at room temperature and the absorbance was measured at 490nm using a microplate reader (DTX880; Beckman Coulter). The cytotoxicity of the ONC201 was expressed either as percentage cell viability or as ratio of treated/DMSO. IC_{50} values were calculated by sigmoidal dose-response curve fit using Prism GraphPad 6.0.

Immunofluorescent Microscopy

JN-DSRCT-1 cells were cultured on glass coverslips for overnight, and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After washing with PBS, the cells were incubated overnight at 4°C with antibodies to DR5. After washing, the cells were with the secondary antibody for 2 hours at room in dark and humidified chamber. The immuno-stained cells were mounted in mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) for 5 minutes, and washed with PBS. The cells were then visualized under a fluorescence microscope equipped with camera.

Protein Isolation and Western Blot Analysis

The preparation of extract protein from cells for western blotting were prepared by using lysis buffer containing freshly added protease and phosphatase inhibitors via cold incubation. The total lysed proteins were collected after centrifugation, quantified using BCA protein assay kit (Thermo Fisher Scientific), and stored at -80°C until

Materials and Methods

Cell lines and Antibodies

JN-DSRCT-1 and luciferase-transfected human JN-DSRCT-1 cell lines exhibiting a pathognomonic t(11;22)(p13;q12) translocation was generously provided from Dr. M Kikuchi’s laboratory (Fukuoka University, Fukuoka, Japan) [27]. Cell line was banked in multiple aliquots on receipt to reduce risk of phenotypic drift and maintained
Western blot analysis. In which, the proteins were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked using blocking buffer and hybridized with different primary antibodies: PARP, caspase 3, and β-actin. Signals were captured using horseradish peroxidase conjugated secondary anti-rabbit IgG and anti-mouse IgG antibodies (Cell signaling Technology) and visualized using SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific). The level of immunoreactive protein was measured using chemiluminescent Hyperfilm ECL (GE Healthcare) using an automatic Film Processor (AGMEDX-Ray), and quantified for its densitometry using an ImageJ Gel Analysis tool (NIH).

**Human Clinical Samples and Immunohistochemical Staining**

All patients data presented in this work were collected under MDACC institutional review board (IRB)-approved lab protocolLab06-0526. All the patients provided written informed consent prior to surgical resection. Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded (FFPE) tumor patient sections after deparaffinization, antigen retrieval and blockade of endogenous peroxidase activity and total proteins. The primary antibodies diluted in the blocking buffers were added overnight at 4°C for DR4 (BD-Biosciences) and DR5 (BD-Biosciences). Sequentially, the slides were washed and incubated with the secondary antibody. Slides were then developed with 3,3'-diaminobenzidine tetrahydrochloride substrate that includes horseradish peroxidase enzyme and hematoxylin was used for counter staining. Staining was evaluated and scored by HMA. Photomicrographs were captured using a Nikon Microphoto FXA microscope (Nikon Instruments), an Olympus DP70 camera (Olympus America; Jupiter, FL), and the QCapture Suite Plus software (QiImaging; Surrey, British Columbia, CA).

**Evaluation of ONC201 Against JN-DSRCT-1 Tumor Xenografts**

All experiments were conducted in accordance with protocols and conditions approved by the University of Texas MD Anderson Cancer Center (MDACC, Houston, TX) Institutional Animal Care and Use Committee (IACUC Protocol #00000712). Male NOD.Cg-PkdccsidIl2rg<sup>tm1Wjl</sup>/SzJ mice (The Jackson laboratory) were used to generate intraperitoneal (2 × 10<sup>6</sup> cells injected per animal) xenografts with luciferase-JN-DSRCT cells. Cells were injected in the peritesticular region in the lower abdomen of the mice. Mice bearing intra-peritoneal tumors were randomized into treatment and control groups, and received a weekly single p.o. of ONC201 (50 or 100 mg/kg).
or vehicle control on day 7, 14, 21, and 28 of tumor cells injections. The mice were imaged through an in vivo bioluminescence imaging using the Xenogen IVIS-50 imaging system (Perkin Elmer Corporation, Hopkinton, MA, USA) on Day 7, 14, 21, 28, 35, 49, 63 and 70. During bioluminescent imaging, an intraperitoneal injection of 150mg/kg of D-Luciferin was administered to each mouse ten minutes prior to imaging. After the administration of Luciferin, the mice were anesthetized with 2% isoflurane inhalation anesthetic, and placed inside of a Perkin Elmer IVIS Spectrum optical imaging system. Luminescent images were captured on the auto exposure setting to ensure that no pixels within the images were oversaturated. After acquiring all of the images, ROI analysis was performed using the onboard Living Image software platform. The total flux, or photon/sec, values were calculated to determine longitudinal disease progression. The mice were imaged, euthanized and necropsied on day 35.

Results

JN-DSRCT Cells Sensitive to TRAIL Express Death Receptors DR4 and DR5

The expression of TRAIL death receptors was determined in the only available DSRCT cell line at the protein level, respectively, by flow cytometry and immunohistochemistry analyses. Figure 1A shows an example of the results obtained with DSRCT cells at the protein level, showing the expression of TRAIL death and decoy receptors. Flow cytometry reveals a higher expression of DR5 at the cell surface of DSRCT cell than DR4 or Decoy receptor, DCR1-2 (Figure 1A). Through a validation by an immunofluorescence detection of DR5, this receptor expression is also more abundant at the cell surface (Figure 1B, Right). In the present study, in addition to evaluating expression of TRAIL receptors in DSRCT cell line, we evaluated DR4 and DR5 protein expression in tissue sections from a panel of 9 DSRCT human tumors, using an IHC analyses that allowed the use of archival material and morphological correlations. The cellular pattern of IHC staining was cytoplasmic and membranous, whereas the architectural pattern was diffuse for both DR4 (Supplementary Figure 1A) and DR5 receptors (Supplementary Figure 1B). Two representative sections of DR4/DR5 expression within DSRCT tumor sections are shown in Figure 2. Six of nine patient primary tumors showed significant staining of DR-5 and five of 8 showed expression of DR-4. In general, expression of DR-5 on patient tissue was more prominent. DR-4 expression was very weak in all patients (Supplementary Figure 1).

ONC201 Induces Inhibition of In Vitro DSRCT Cell Proliferation and Growth
The effect of ONC201 was investigated on the proliferation and survival of JN-DSRCT cell line using a MTT assay. Treatment with increasing concentrations (0-20 μM) of ONC201 for 72 h decreases cell proliferation in NJ-DSRCT cells with maximum inhibition achieved at 10 μM (IC50=1.66 μM) (Figure 2, A and B). Treatment of cells with 0.5-1 mM ONC201 for 2 weeks induced a dose-dependent decrease in the number of DSRCT clones compared with untreated cells (Figure 2, C and D).

**Caspase Activation in ONC201-Induced Apoptosis**

To determine whether the decrease in the number of JN-DSRCT cellular clones and proliferation was due to induction of cell death, expression levels of cleaved caspase 3 and PARP of the intrinsic pathway of apoptosis was measured in JN-DSRCT cells at increasing doses of ONC201. Expression of cleaved caspase 3 and PARP were found only at the higher doses of treatment with ONC201 (Figure 3). This indicates that ONC201 treatment of DSRCT cells induces cytotoxicity via the intrinsic pathway of apoptosis.

**In Vivo Effect of ONC201 on JN-DSRCT Xenograft Growth**

An animal model accurately representing the cellular and molecular changes associated with the initiation and progression of human DSRCT should have a significant potential to facilitate the development of better method for the treatment of this type of sarcoma. Toward this goal, we developed a novel orthotopic xenograft model of DSRCT using luciferase-transfected JN-DSRCT. Since the organ of origin of DSRCT is unknown, the correct orthotopic position was unclear. Injecting the JN-DSRCT cells in the upper abdomen, or peri-renal area, under the renal capsule or subcutaneous, all failed to result in peritoneal sarcomatosis. Peritoneal sarcomatosis is the pattern of growth and spread in patients with DSRCT. However, the injection of 2 × 10⁶ JN-DSRCT cells in the lower abdominal-peritesticular region of NSG mice resulted in peritoneal sarcomatosis development and metastasis to the liver, pancreas and the intraperitoneal cavity within one month post-injection (Figure 4, C-F). The metastasis into the pancreas and the intraperitoneal cavity was detected by an IHC staining, respectively shown in Figure 4, G and H.

Therapeutic effect of ONC201 was assessed in immunocompromised NSG mice (>5 mice/group) bearing intra-peritoneal JN-DSRCT cells using two different doses (50 and 100 mg/kg). To visualize DSRCT tumor implantation and monitor the therapeutic responses of ONC201 in these mice by bioluminescence imaging, it was necessary to express a luciferase gene on them.
Subsequently, treatment with low or higher doses of ONC201 resulted in reduced tumor growth (Figure 5).

When treated with ONC201, there were 7 mice in each group: low dose, high dose and no treatment. Two of 7 mice in the high dose group were not evaluable and one in the low dose group was not evaluable. In the low dose group, there were 17% (1/6) PR and 17% (1/6) CR, both who had rebound growth after withdrawal of treatment, and 50% (3/6) without response. In the high dose group,
there were 40% (2/5) CRs without rebound growth, 1/5 20% PR, and 40% (2/5) without response to treatment. Therefore, the response rate in the low dose group is 35% compared to 60% in the high dose group. Also, the mice in the high dose group demonstrated a more durable response after withdrawal of treatment (Figure 6, A-H).

**Discussion**

DSRCT is a rare sarcoma tumor with little progress in therapeutic strategy the last 3 decades, particularly in the case of metastatic disease, which requires novel biotherapeutic strategies. There is an urgent need to develop new biotherapeutic approach to improve DSRCT patient survival. The proapoptotic TRAIL, a member of the TNF superfamily, represents a promising potential therapeutic option by its selectivity to kill tumor cells with minimal toxicity to most normal cells and tissues, as it was demonstrated in several types of cancer, including Ewing sarcoma and osteosarcoma [15–17,28,29].

In this study, by complementary approaches both in vitro and in preclinical model, we investigated the sensitivity of the unique available JN-DSRCT cell line to TRAIL targeted therapy. First, we determined TRAIL receptor expression together with TRAIL agonistic drug ONC201 effect on cell proliferation, cell survival and cell colony formation. DSRCT cell line and tumor tissues expressed high level of DR4 and DR5 (Figures 1 and 2). ONC201 is a new anticancer drug that acts in part through TRAIL pathway induction [26]. To further understand the efficacy and mechanism of action of ONC201, JN-DSRCT cell line was sensitive with an EC$_{50}$ in the 1.66μM range and a maximal inhibition index of proliferation and colony formation in the range of 0.2 (Figure 2). ONC201 was initially reported to transcriptionally induce the expression of TRAIL and its receptor DR5 in a FOXO3a-dependent manner in solid tumors [30]. In DSRCT we found that ONC201 did induce TRAIL and DR5 protein expression, and cell death through the extrinsic pathway of apoptosis (Figure 3). Recent studies have demonstrated that engineered TRAIL-expressing MSC can induce apoptosis in

![Figure 6](image-url)
variety of sarcomas like osteosarcoma, rhabdomyosarcoma and ES by activation of caspase-8, paving the way for TRAIL-based targeted therapy in these aggressive forms of cancer. In addition, another TRAIL signaling pathway has been recently described, involving NFkB, MAPK, PI3K/Akt activation via binding to the same receptors, but leading to increased tumor cell proliferation, survival, migration and invasion [31,32].

As DSRCT is a rare tumor, only first described in 1989, there are no commercial cell lines, and only one available cell line, JN-DSRCT, used in this study [1]. The reasons cell lines are so difficult to generate lies in the histology. As demonstrated in Figure 4H, (pink is desmoplastic component) DSRCT is composed of sheets of desmoplastic stroma interdigitating with small round blue cells. The "small round blue cell" component, is what makes up the majority of the cell line. Lack of cell line availability and the histology of DSRCT is what has limited the development of animal models.

To test the effectiveness of TRAIL therapy in vivo, the first preclinical xenograft animal model of DSRCT, induced by injection of human JN-DSRCT cells was used (Figure 4). This model closely reproduces the clinical development of DSRCT, as the tumor normally develops exclusively in intra-abdominal location and metastases to the liver and lymph nodes (Figure 4, A-F). The sarcomatosis seen in the mice also mimics humans in that the tumors are adherent to the peritoneal surfaces, including the liver. Since testicles are intra-abdominal organs tumor implants were also found on the testicles. Most DSRCT patients have ommental disease. Mice do not have omentum. Therefore, the local and distant metastases in this murine model mimics DSRCT in humans. This model now allows us to more accurately study DSRCT and evaluate novel therapeutics.

Conclusion
In this preclinical investigation, ONC201 has shown single drug efficacy in a novel orthotopic xenograft model of DSRCT. Further clinical trials are planned.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.02.006.

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