High efficacy of tumor-targeting Salmonella typhimurium A1-R on a doxorubicin- and dactolisib-resistant follicular dendritic-cell sarcoma in a patient-derived orthotopic xenograft PDX nude mouse model

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ABSTRACT

Follicular dendritic-cell sarcoma (FDCS) is a rare and recalcitrant disease. In the present study, a patient-derived orthotopic xenograft (PDX) mouse model of FDCS was established in the biceps muscle of nude mice. The FDCS PDX was resistant to both doxorubicin (DOX) and NVP-BEZ235, dactolisib (BEZ) an experimental agent which is a dual pan-phosphoinositide 3-kinase-mammalian target of rapamycin inhibitor. However, in contrast to DOX and BEZ, the FDCS PDX was sensitive to the tumor-targeting bacterial strain, Salmonella typhimurium A1-R (S. typhimurium A1-R). The combination of S. typhimurium A1-R and either DOX or BEZ did not increase the antitumor efficacy of S. typhimurium A1-R, indicating that DOX and BEZ were not active in this PDX model. The efficacy of S. typhimurium A1-R in this recalcitrant FDCS gives strong impetus to move bacterial therapy to clinical trials for this disease. The findings of the present study are of particular importance since it demonstrates that S. typhimurium A1-R is effective in a PDX model of FDCS established from a patient who failed DOX therapy.

INTRODUCTION

Follicular dendritic-cell sarcoma (FDCS) is a highly recalcitrant disease. FDCS is rare and arises from follicular dendritic cells [1]. CHOP chemotherapy, which contains cyclophosphamide (CTX), doxorubicin (DOX), vincristine and prednisone, is most frequently used with FDCS with transient, partial responses observed in some patients [2]. Complete responses to CHOP are infrequent [1]. The 5-year overall survival (OS) for localized FDCS is 55% and for metastatic disease is 38% [3]. Therefore, novel approaches to FDCS are needed [1, 2].

The tumor-targeting Salmonella typhimurium A1-R (S. typhimurium A1-R) strain was developed by our laboratory [4]. S. typhimurium A1-R is auxotrophic for Leu–Arg, which prevents it from mounting a continuous infection in normal tissues. S. typhimurium A1-R was able to inhibit or eradicate primary and metastatic tumors as monotherapy in nude mouse models of major cancers [5], including prostate [6, 7], breast [8–10], lung [11, 12],
pancreatic [13–17], ovarian [18, 19] stomach [20], and cervical cancer [21], as well as sarcoma cell lines [22–25] and glioma [26, 27], all of which are highly aggressive tumor models.

Previously, we developed a patient-derived nude-mouse model of soft tissue sarcoma resistant to gemcitabine. However, *S. typhimurium* A1-R significantly inhibited tumor growth compared to the untreated mice. These results suggest tumor-targeting *S. typhimurium* A1-R is a promising treatment for chemo-resistant soft tissue sarcoma [28].

Recently, a patient with high-grade undifferentiated pleomorphic soft tissue sarcoma from a striated muscle was grown in the right biceps femoris muscle of mice to establish a patient-derived orthotopic xenograft (PDOX) model. This sarcoma PDOX was sensitive to DOX and *S. typhimurium* A1-R followed by DOX, could eradicate this tumor [25].

The present study evaluates *S. typhimurium* A1-R efficacy on a DOX-resistant FDCS PDOX model established from a patient who failed DOX therapy.

**RESULTS AND DISCUSSION**

The treatment schedule for the FDCS PDOX is shown in Figure 1. Three weeks after orthotopic implantation, tumors reached 5 mm in diameter and continued to grow rapidly (Figure 1A).

After intraperitoneal (i.p.) administration of *S. typhimurium* A1-R for four weeks, and two subsequent weeks without treatment, the green fluorescent protein (GFP)-expressing bacteria could be visualized by fluorescence imaging in the resected tumor. *S. typhimurium* A1-R was imaged directly as well as by mincing of the tumor and subsequent colony outgrowth from the minced tissue on agar medium (Figure 2).

The FDCS PDOX was resistant to doxorubicin (DOX) (*p* = 0.11 at day-22 of treatment, Group 3) (Figure 3). The FDCS PDOX was also resistant to NVP-BEZ235 (dactolisib) (BEZ), which is a dual pan-phosphoinositide 3-kinase-mammalian target of rapamycin mTOR inhibitor [29] (*p* = 0.48 at day-18 of treatment, Group 2). In a Phase II trial, investigators reported a durable partial response in a patient with metastatic FDCS treated with ridaforolimus, an mTOR inhibitor [30]. The FDCS PDOX was also resistant to the combination of DOX and BEZ (*p* = 0.14 at day-22, Group 4).

However, in contrast to DOX and BEZ, the FDCS PDOX was sensitive to the tumor-targeting bacterial strain, *S. typhimurium* A1-R (*p* < 0.05 at day-22, Group 5) (Figure 3A, 3B). The combination of *S. typhimurium* A1-R with DOX and BEZ was also effective (Figure 3C).

**Figure 1**: PDOX model of follicular dendritic-cell sarcoma (FDCS) and treatment protocol. A. During the sarcoma transplant procedure in the muscle. B. Three weeks after implantation. C. Treatment protocol.
and either DOX (Group 6) or BEZ (Group 7) did not increase the antitumor efficacy of S. typhimurium A1-R (Figure 3), indicating that DOX and BEZ were not active against this tumor. The tumor-volume ratio in Group 5, S. typhimurium A1-R (3.11 ± 2.05, p < 0.01); Group 6, S. typhimurium A1-R and DOX (2.80 ± 1.72, p < 0.01); and Group 7, S. typhimurium A1-R and BEZ (3.28 ± 4.62, p < 0.05) were significantly lower than in Group 1, untreated control (19.44 ± 6.70) (Figure 3B). There were not significant differences between any other groups. Since BEZ alone was inactive, it is not surprising it also had no effect in combination with DOX. Sequential treatment was given with S. typhimurium A1-R followed by either DOX or BEZ. The goal of this experiment was to determine if S. typhimurium A1-R could sensitize the tumor by decoying the quiescent cells in the tumor to begin to cycle and therefore become more responsive to the chemotherapy [20]. However, the tumor was sufficiently sensitive to S. typhimurium A1-R that no further tumor inhibition could be observed in the combination with either an inactive or slightly active drug compared to S. typhimurium A1-R alone.

There were no significant body-weight differences between the groups (Figure 3C). Actual mouse weights at day-22 for all groups, except Group 2 which was determined day-18, were Group 1 (untreated control): 22.24 ± 1.73; Group 2 (BEZ): 26.13 ± 1.54; Group 3 (DOX): 22.94 ± 2.01; Group 4 (BEZ + DOX): 22.88 ± 1.20; Group 5 (S. typhimurium A1-R): 25.32 ± 2.40; Group 6 (S. typhimurium A1-R + DOX): 25.34 ± 1.52; and Group 7 (S. typhimurium A1-R + BEZ): 23.2 ± 2.21.

**Histology**

The patient’s original tumor (Figure 4A) and untreated PDOX (Figure 4B) showed identical histologic features which are characteristic for FDCS, including relatively uniform ovoid-to-spindle-shaped cells present in short fascicles and storiform patterns with associated lymphocytes sprinkled throughout the tumor. Similar to the original tumor, the PDOX showed a high rate of mitoses (>20/10 High-power fields [HPFs]) (Figure 4B). The patient’s original tumor was positive for CD35 and fascin by immunohistochemistry (data not shown), supporting the diagnosis of FDCS.

Review of xenograft sections showed the following approximate amount of necrosis: Group 1: control with PBS (0%, Figure 4B), Group 2: BEZ-235 (5%, Figure 4C), Group 3: DOX (20%, Figure 4D); Group 5: S. typhimurium A1-R (50%, Figure 4E); Group 6: S. typhimurium A1-R and DOX (20%, Figure 4F); Group 7: S. typhimurium A1-R+BEZ (60%, Figure 4G). Group 7 was greatly inhibited by S. typhimurium A1-R. Future studies will examine the extent of necrosis in S. typhimurium A1-R-treated tumors. The BEZ-treated tumors grew extensively and may have outgrown their blood supply and thus became slightly necrotic.

In a previous study, a human patient with advanced leiomyosarcoma was treated with an intra-tumoral injection of Clostridium novyi (C. novyi-NT) spores which reduced the tumor within and surrounding the bone [31], indicating the clinical potential of bacterial therapy of sarcoma. Since S. typhimurium A1-R is a facultative anaerobe, unlike C. novyi-NT which is an obligate anaerobe, it may have more broad application for cancer therapy. S. typhimurium A1-R can greatly potentiate cytotoxic chemotherapy [20]. S. typhimurium A1-R was recently shown to potentiate DOX in a PDOX model of high-grade undifferentiated soft-tissue sarcoma from a striated muscle [25].

**Figure 2:** Imaging tumor-targeting Salmonella typhimurium A1-R in the FDCS PDOX. A. FDCS was resected from a PDOX model after 4 weeks treatment of S. typhimurium A1-R and a subsequent two weeks without treatment. FV1000 confocal microscopy. Scale bar = 100 μm. B. Colonies of S. typhimurium A1-R isolated from the tumor of the bacterially-treated FDCS PDOX after 4 weeks treatment and a subsequent 2 weeks without treatment.
In a Phase I clinical trial of patients with metastatic melanoma and renal carcinoma, the *S. typhimurium* strain tested (VNP20009), attenuated by msbB and purI mutations, was safely administered to patients [32]. The results of the present study suggest *S. typhimurium* A1-R is a candidate for clinical trial to treat DOX-resistant FDCS.

The findings of the present study are of particular importance since it demonstrates that *S. typhimurium* A1-R is effective in the PDOX model of FDCS established from a patient who failed DOX therapy and whose PDOX is DOX-resistant.

Previously developed concepts and strategies of highly selective tumor targeting [33–38] can take advantage of bacterial targeting of tumors, including tissue-selective therapy which focuses on unique properties of normal and tumor tissues [33, 38]. *S. typhimurium* A1-R can possibly overcome de-differentiation of a tumor leading to resistance to targeted chemotherapy, where the targeted protein or pathway may no longer be expressed [38], since *S. typhimurium* A1-R does not depend on such targets [33, 35]. *S. typhimurium* A1-R may also be effectively combined with teratogens which could selectively affect cancer cells that are dedifferentiated.
Figure 4: Photomicrographs of H&E-stained slides from the original patient tumor and the PDOX-grown tumor. 

Since *S. typhimurium* A1-R can decoy quiescent cancer cells to begin to cycle, *S. typhimurium* A1-R could be effectively combined with agents that selectively target proliferating cancer cells [36], where normal cells are protected by agents which induce wild type p53 [37].

**MATERIALS AND METHODS**

**Mice**

Athymic *nu/nu* nude mice (AntiCancer Inc., San Diego, CA), 4-6 weeks old, were used in this study. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals, the use of anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine, 15.2 mg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO₂ inhalation when they met the following humane endpoint criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop. Animals were housed in a barrier facility on a high efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet [25].

**Patient-derived tumor**

A female patient diagnosed with a recurrent extranodal FDACS of the left lower extremity underwent surgical resection. She previously received adjuvant radiotherapy to the left lower extremity following resection of the primary tumor in 2014 and four cycles of chemotherapy with DOX and CTX for her recurrent disease. Chemotherapy was discontinued in April of 2015 due to medical co-morbidities and her inability to tolerate therapy. Surgical resection of the recurrent extra-nodal left lower extremity FDACS was performed by FCE on July 15, 2015. Written informed consent was obtained from the patient as part of a UCLA Institutional Review Board (IRB #10-001857)-approved protocol [25].

**Establishment of a PDOX model of FDACS by surgical orthotopic implantation (SOI)**

A fresh sample of the FDACS of the patient was obtained and transported immediately to the laboratory at AntiCancer, Inc., on wet ice. The sample was cut into 5-mm fragments and implanted subcutaneously in nude mice. After three weeks, the subcutaneously-implanted tumors grew to more than 10 mm in diameter. The subcutaneously-grown tumors were then harvested and cut into small fragments (3 mm³). After nude mice were anesthetized with the ketamine solution described above, a 5-mm skin incision was made on the right high thigh into the biceps femoris, which was split to make space for the sarcoma tissue fragment. A single tumor fragment was implanted orthotopically into the space to establish the PDOX model. The wound was closed with a 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) [25].

**Preparation and administration of *S. typhimurium* A1-R**

*S. typhimurium* A1-R expressing GFP (AntiCancer, Inc., San Diego, CA, USA) was grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. *S. typhimurium A1-R* was injected intraperitoneally. A total of 2 × 10⁹ CFU *S. typhimurium* A1-R in 50 μl PBS was administered intraperitoneally (i.p.) in the follicular dendritic cell sarcoma-bearing mice [25].

**Treatment study design in the PDOX model of soft tissue sarcoma**

PDOX mouse models were randomized into seven groups of five mice each (Figure 1): Group 1, control with PBS, i.p.; Group 2, treated with BEZ, 50 mg/kg, oral gavage, q5/W for 4 weeks; Group 3, treated with DOX, 2.4 mg/kg, i.p., qW for 4 weeks; Group 4, treated with DOX, 2.4 mg/kg, i.p., qW for 2 weeks; and BEZ, 50 mg/kg, oral gavage, q5/W for 2 weeks; Group 5, treated with *S. typhimurium A1-R*, 2.5 × 10⁷ CFU, i.p., qW for 4 weeks; Group 6, treated with *S. typhimurium A1-R*, 2.5 × 10⁷ CFU, i.p., qW for 2 weeks followed by DOX, 2.4 mg/kg, i.p., qW for 2 weeks; and Group 7, treated with *S. typhimurium A1-R*, 2.5 × 10⁷ CFU, i.p., qW for 2 weeks followed by BEZ, 50 mg/kg, oral gavage, q5/W for 2 weeks. Tumor length, width and mouse body weight were measured twice a week. Tumor volume was calculated with the following formula: Tumor volume (mm³) = length (mm) × width (mm) × width (mm) × 1/2. Data are presented as mean ± SD. The tumor volume ratio is defined at the tumor volume at any given time point relative to the initial tumor volume. All treated mice except Group 2 were sacrificed on day-29, and tumors were resected for further histological evaluation. Mice treated with BEZ were sacrificed on day-18 due to outgrowth of the tumors.

**Histological examination**

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue
sections (5 μm) were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E) staining was performed according to standard protocol. Histological examination was performed with a BHS system microscope. Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada). Grade IV: no viable tumor is detectible [25].

**Imaging of tumor-targeted bacteria**

The FV1000 confocal microscope (Olympus) [39] was used to image resected tumors for the presence of *Salmonella typhimurium* A1-R-GFP. The OV100 (Olympus) variable-magnification fluorescence imager [40] was used to image colonies of *Salmonella typhimurium* A1-R from resected tumors.

**Statistical analysis**

SPSS statistics version 21.0 was used for all statistical analyses (IBM, New York City, NY, USA). Significant differences for continuous variables were determined using the Mann-Whitney U test. A probability value of $P < 0.05$ was considered statistically significant [25].

**DEDICATION**

This paper is dedicated to the memory of A.R. Moossa, M.D. and Sun Lee, M.D.

**CONFLICTS OF INTEREST**

Y.Z. and M.Z. are employees of AntiCancer Inc. T.K., T.M., K.K., K.I. and R.M.H. are unsalaried associates of AntiCancer Inc. R.M.H. is an unsalaried associate of PDOX LLC. There are no other competing financial interests.

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