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Tumor-targeting *Salmonella typhimurium* A1-R arrests a doxorubicin-resistant PDGFRA-amplified patient-derived orthotopic xenograft (PDOX) mouse model of pleomorphic liposarcoma

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Abstract

Pleomorphic liposarcoma (PLPS) is a recalcitrant soft-tissue sarcoma (STS) subtype in need of transformative therapy. We have previously established a patient-derived orthotopic xenograft (PDOX) model, of PLPS with PDGFRA amplification, using surgical orthotopic implantation (SOI). In the present study, the PLPS PDOX model was randomized into three groups of seven mice each: untreated control; doxorubicin (DOX)-treated; and treated with *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) expressing green fluorescent protein (GFP). Tumor volume and body weight were monitored during the treatment period. The PLPS PDOX was resistant to DOX. In contrast, the PLPS PDOX was highly sensitive to *S. typhimurium* A1-R. There was no significant body-weight loss among these three groups. Fluorescence imaging demonstrated that *S. typhimurium* A1-R-GFP was very effective to target the PLPS PDOX tumor. The present study demonstrates that a PLPS PDOX, resistant to first-line therapy DOX, was highly sensitive to tumor targeting *S. typhimurium* A1-R.

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Keywords

Pleomorphic liposarcoma; soft tissue sarcoma; patient-derived orthotopic xenograft; PDGFRA amplification; cytogenetics; *S. typhimurium* A1-R; tumor-targeting; GFP

Introduction

Pleomorphic liposarcoma (PLPS) is a heterogenous and recalcitrant disease with a high frequency of local recurrence and distant metastasis.[1] Complete surgical resection is the only known cure for this disease. Both radiation and conventional cytotoxic chemotherapy are largely ineffective for advanced unresectable PLPS.[2] Therefore, precise individualized therapy is necessary to improve outcome of PLPS patients.

Toward this goal, our laboratory pioneered the patient-derived orthotopic xenograft (PDOX) nude mouse model with the technique of surgical orthotopic implantation (SOI), including breast,[3] ovarian,[4] lung,[5] cervical,[6,7] colon,[8–10] stomach,[11] pancreatic,[12–16] melanoma [17–22] and sarcoma.[23–35] The PDOX model, developed by our laboratory over the past 30 years, has many advantages over subcutaneous-transplant models.[36,37]

A PDOX model of a PDGFRA-amplified PLPS was previously established in the biceps femoris of nude mice by surgical orthotopic implantation (SOI) in order to match the patient.[38] The PLPS PDOX was resistant to first-line therapy doxorubicin (DOX). In contrast, trabectedin (TRAB) arrested the PLPS PDOX with no significant body-weight loss. These results suggested that the PLPS PDOX model can precisely identify both effective as well as ineffective drugs for the individual patient. This is of particular importance for a heterogeneous group such as PLPS where standard first-line therapy remains very difficult to determine without individualization.[38]

In a subsequent study, we determined the efficacy of pazopanib (PAZ) which targets the PDGFRA gene amplification[39] of the PLPS compared to temozolomide (TEM) and DOX.[40] The PLPS PDOX was resistant to DOX as previously observed and responded very well to PAZ as well as TEM. The PLPS PDOX model identified an effective targeted drug (PAZ) as well as effective standard therapy (TEM) at the same time, identified an ineffective drug (DOX), even if it is first-line for this disease.[40]

Materials and Methods

Mice

Athymic *nu/nu* male nude mice (AntiCancer, Inc., San Diego, CA), 4–6 weeks old, were used in this study. All mice were kept 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. All animal experiments were performed 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 Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. Anesthesia and analgesics were used for all

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surgical experiments to avoid excessive suffering of the mice. A ketamine mixture (0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate) was used subcutaneously for all mice. The animals were monitored carefully during surgery to keep adequate depth of anesthesia. The animals were observed daily and humanely sacrificed by CO_2 inhalation when they met the following criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop.

Patient-derived tumor

A 68-year-old male patient was previously diagnosed as a recurrent PLPS of the right arm and underwent surgical resection. The patient previously received docetaxel, ifosfamide and radiotherapy to the right arm. Surgical resection for the recurrent PLPS was previously performed by FCE on January 8, 2016. Written informed consent was obtained from the patient as part of a UCLA Institutional Review Board (IRB#10-001857) approved protocol.[38,40]

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

A PDOX model of the PLPS was established subcutaneously in nude mice from the patient's resected PLPS as previously described.[38,40] The subcutaneously-grown tumors were then harvested and cut into 3 mm^3 fragments. After the mice were anesthetized with the ketamine solution described above, a 7 mm skin incision was made on the right upper thigh into the biceps femoris, which was split to make space for the tumor fragment. A single tumor fragment was implanted orthotopically into the space to establish the PDOX model, as previously described.[38,40] The wound was closed with a 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, U.S.A.).

Treatment study design in the PLPS PDOX model

PDOX mouse models were randomized into three groups of seven mice, respectively: Group 1 (G1), untreated control; Group 2 (G2), treated with DOX (3 mg/kg, i.v., weekly, 2 weeks); Group 3 (G3), treated with *S. typhimurium* A1-R (5×10^7 CFU/100 ml, i.v., weekly, 2 weeks) (Fig. 1). Tumor length, width, and mouse body weight were measured twice a week. Tumor volume was calculated with the following formula: Tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. When the tumor volume reached 50 mm^3, treatment was started. The tumor volume ratio is defined as the tumor volume at any given time point relative to the initial tumor volume. Body weight was measured throughout the treatment period. Relative body weight is also defined as the body weight at any given time point relative to the initial tumor weight. All mice included in this study were sacrificed on day 15, and tumors were harvested for further histological evaluation. Data are presented as mean ± SD.

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

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standard protocol. Histological examination was performed with a BHS system microscope. Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada).

Statistical analysis

Statistical analyses were performed with JMP pro version 12 for all statistical analyses. The relative tumor volumes and relative body weights of mouse are expressed as mean ± SD. Significant differences for continuous variables were determined using the Steel-Dwass test for multiple comparison. A probability value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Efficacy of *S. typhimurium* A1-R vs. DOX on the PLPS PDOX

The untreated control group grew 6 times larger than initial tumor volume by day14 (G1: 6.37±2.29). The DOX group did not demonstrate significant tumor growth suppression compared to the control group on day 14 (G2: 4.05±1.21, p= 0.1733). In contrast, tumor-targeting *S. typhimurium* A1-R demonstrated significant tumor growth suppression compared to the control group (G3: 2.03±1.33, p=0.018) and DOX group on day14 (p= 0.047) (Fig. 2). Figure 3 shows representative tumor images at the treatment endpoint for each group.

Tumor targeting in the PLPS PDOX by *S. typhimurium* A1-R

S. typhimurium, expressing green fluorescent protein (GFP), was cultivated in serial dilution from supernatants of tumor homogenates by mincing tumor tissue on agar medium (Fig. 4). Fluorescent bacteria were detected at all dilutions in the tumor obtained from mice treated with by the *S. typhimurium* A1-R. The untreated control group tumors did not contain *S. typhimurium* (Fig. 4).

Effect of treatment on mouse body weight on the PLPS PDOX

Figure 5 shows the relative body weight of all groups. Mice treated with *S. typhimurium* A1-R had slight weight loss at the endpoint. However, there were no significant weight differences among groups, indicating there was not significant adverse effect caused by either agent.

Treatment impact on tumor histology of the PLPS PDOX tumors

Figure 6 represents the tumor histology of all groups and original patient. In both the original patient tumor (Fig. 6A) and control PDOX tumor (Fig. 6B), enlarged and hyperchromatic nuclei with cytoplasm vacuoles were observed. Intact and viable cancer cells were observed in most areas (Fig. 6B). The DOX group tumors did not have significant necrotic areas (Fig. 6C). The *S. typhimurium* A1-R treated tumor had significant necrosis.

We have previously shown that tumor-targeting *S. typhimurium* A1-R has strong efficacy on a PDOX models of Ewing's sarcoma[7] and follicular dendritic cell sarcoma.[32] In a recent study, *S. typhimurium* A1-R was shown to be effective against USTS (2 undifferentiated

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pleomorphic sarcoma [UPS], 1 undifferentiated sarcoma not otherwise specified [NOS] and 1 undifferentiated spindle cell sarcoma [USS]) and was significantly more efficacious than DOX in each case, thereby surpassing first-line therapy.[41]

The scientific premise for the present study is to determine the efficacy of tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) on a patient-derived orthotopic xenograft (PDOX) model of doxorubicin-resistant pleomorphic liposarcoma demonstrating arrest of tumor growth (Figures 2 and 3) and highly-specific tumor targeting of *S. typhimurium* A1-R in the PDOX model (Figure 4). This is the first study that demonstrates efficacy of *S. typhimurium* A1-R on a PDOX model of doxorubicin-resistant pleomorphic liposarcoma and suggests that *S. typhimurium* A1-R can be effective in the clinic in the future in this recalcitrant tumor type.

The present report demonstrates that *S. typhimurium* A1-R is an effective agent for another type of sarcoma, PLPS, and further suggests the generality of *S. typhimurium* A1-R as an effective treatment for sarcoma. Clinical trials of *S. typhimurium* A1-R need to be carried out as soon as possible.

Acknowledgments

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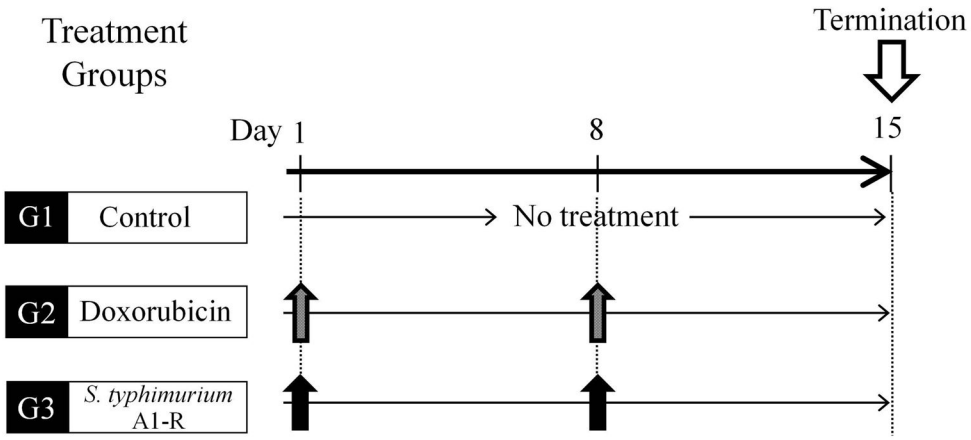

Figure 1. Treatment protocol of pleomorphic liposarcoma (PLPS) PDOX

Treatment protocol. Group 1, untreated control (n =7); Group 2, treated with doxorubicin (DOX) (3 mg/kg, i.v., weekly, 2 weeks, n = 7); Group 3, treated with *S. typhimurium* A1-R (5×10^7CFU/100 ml, i.v., weekly, 2 weeks, n = 7). All mice included in this study were sacrificed on day 15 to harvest tumor for further histological evaluation.

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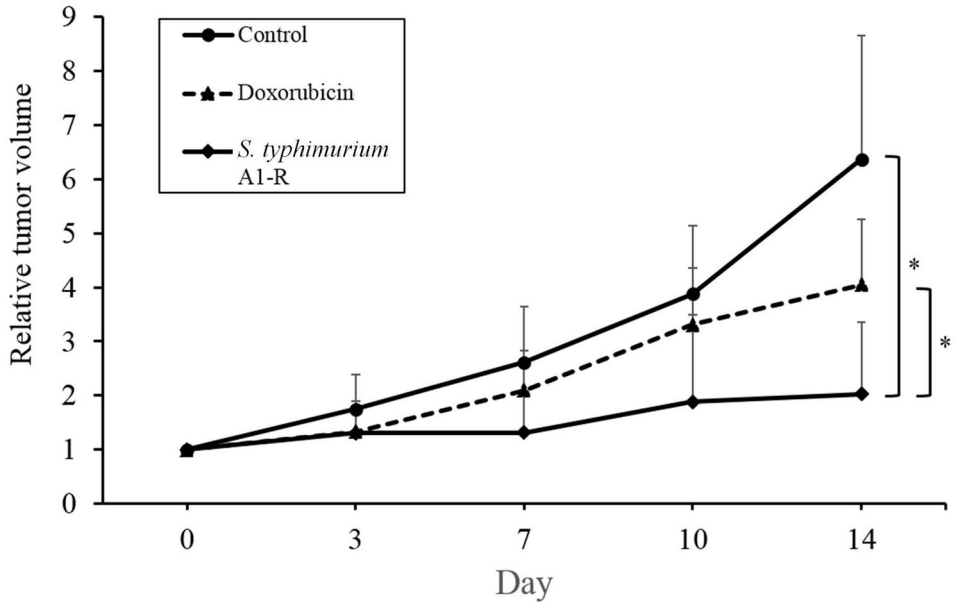

Figure 2. Efficacy of *S. typhimurium* A1-R vs. DOX on the PLPS PDOX

The line graphs show relative tumor volume on each time point. Relative tumor is the tumor volume at any given time point at the beginning of the treated period divided by the tumor volume. Error bars: ± SD. * $p < 0.05$.

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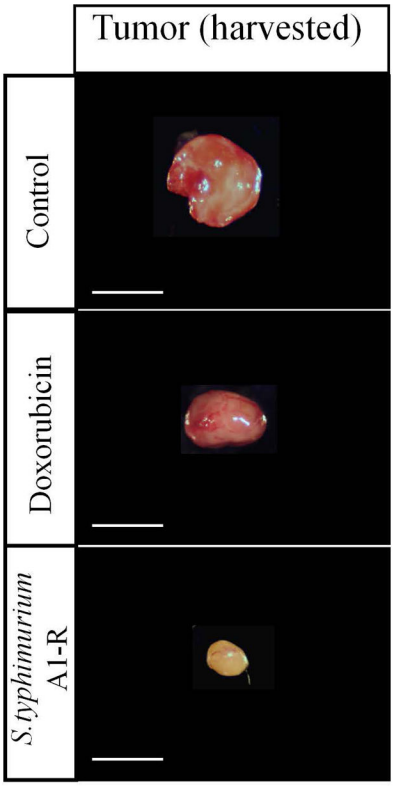

Figure 3. Macroscopic imaging of control and treated tumor

Images were obtained at the end of the treatment period. Scale bar: 10mm.

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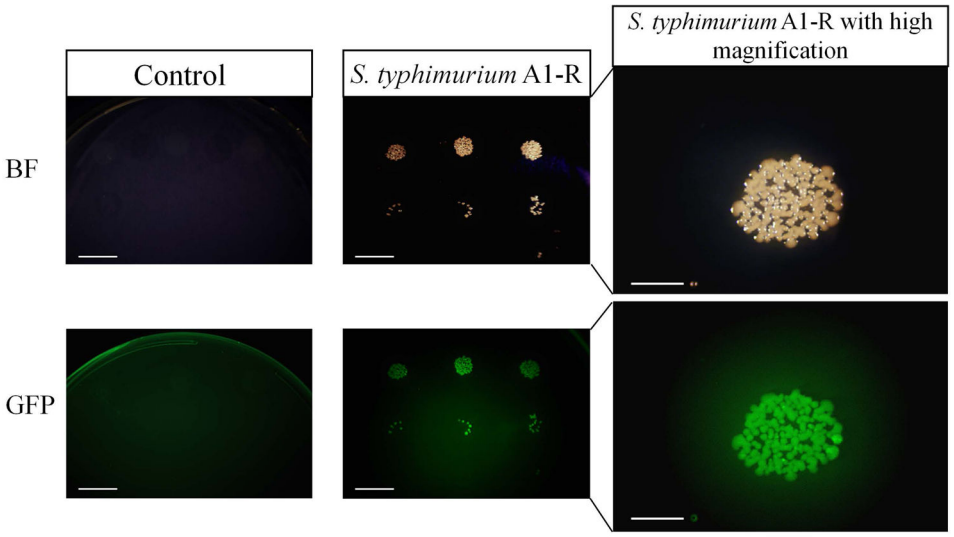

Figure 4. Tumor targeting *S. typhimurium* A1-R-GFP of the PLPS PDOX

S. typhimurium, expressing green fluorescent protein (GFP), was cultivated in serial dilution from supernatants of tumor homogenates by mincing tumor tissue on agar medium. Grown colonies of bacterial were imaged by GFP fluorescence. Fluorescent bacteria were detected at all dilutions in the tumor treated with *S. typhimurium* A1-R. Scale bar: 10 mm, 2.5 mm at high magnification. BF: Bright field, GFP: Green fluorescent protein.

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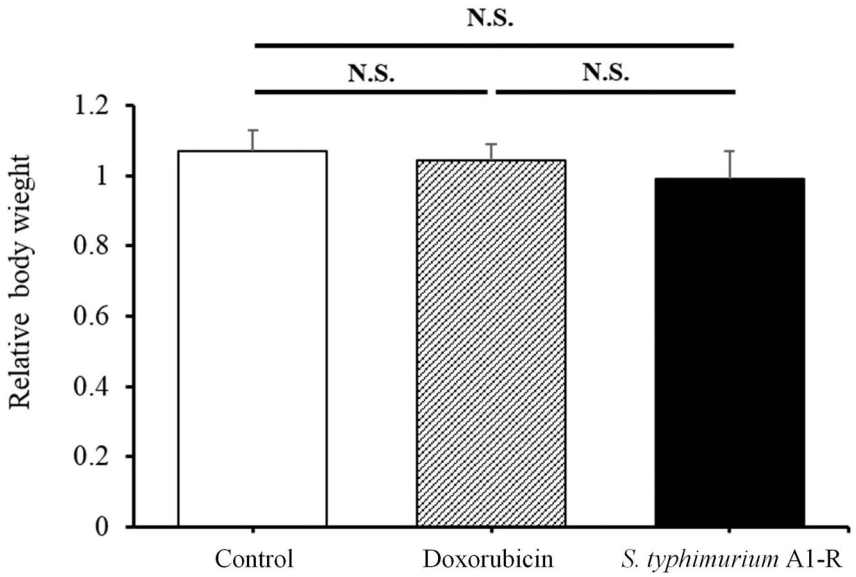

Figure 5. Body weight of treated PLPS PDOX mouse models

Bar graphs show the relative body weight of mice treated with each agent. Relative body weight as the body weight at any given time divided by the inhibited body weight. Error bars: ± SD. N.S.: not significant.

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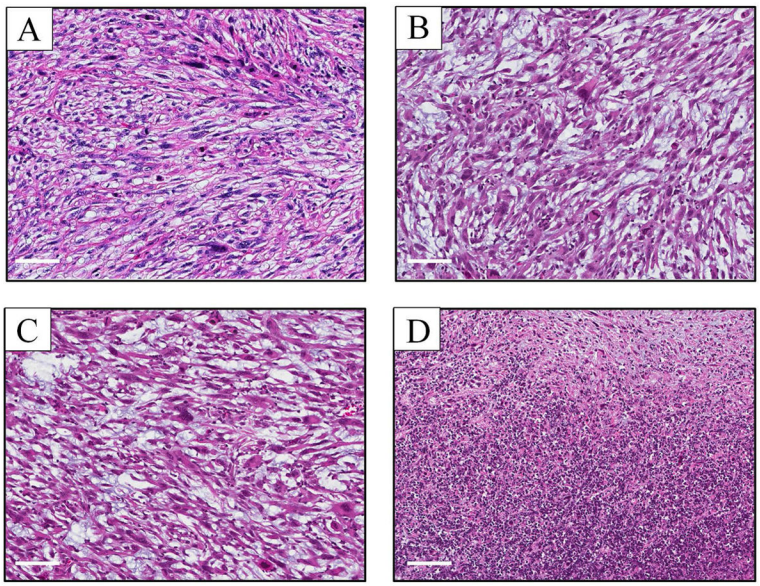

Figure 6. Tumor histology

(A) Control tumor (B) Untreated PLPS PDOX. (C) DOX group. (D) *S. typhimurium* A1-R group. Scale bars: 100μm.