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REPORT

Intra-arterial administration of tumor-targeting *Salmonella typhimurium* A1-R regresses a cisplatin-resistant relapsed osteosarcoma in a patient-derived orthotopic xenograft (PDOX) mouse model

Kentaro Igarashi [a,b,c], Kei Kawaguchi[a,b], Takashi Murakami[a,b], Tasuku Kiyuna[a,b], Kentaro Miyake[a,b], Scott D. Nelson[d], Sarah M. Dry[d], Yunfeng Li[d], Jane Yanagawa[e], Tara A. Russell [e], Arun S. Singh[f], Norio Yamamoto[c], Katsuhiro Hayashi[c], Hiroaki Kimura[c], Shinji Miwa[c], Hiroyuki Tsuchiya[c], Fritz C. Eilber [e], and Robert M. Hoffman[a,b]

[a]AntiCancer, Inc., San Diego, CA, USA; [b]Department of Surgery, University of California, San Diego, CA, USA; [c]Department of Orthopaedic Surgery, Kanazawa University, Kanazawa, Japan; [d]Department of Pathology, University of California Los Angeles, CA, USA; [e]Division of Surgical Oncology, University of California, Los Angeles, CA, USA; [f]Division of Hematology-Oncology, University of California, Los Angeles, CA, USA

ABSTRACT

Previously, a patient-derived orthotopic xenograft (PDOX) model was established with a lung metastasis from an osteosarcoma patient which developed after adjuvant cisplatinum (CDDP) treatment. In this model, we previously demonstrated the efficacy of trabectedin (TRAB) and temozolomide (TEM) compared with CDDP. In the present report, osteosarcoma tissue was implanted orthotopically in the distal femur of mice which were randomized into the following groups when tumor volume reached approximately 100 mm^3; On day 14 after initiation of treatment, all but CDDP significantly inhibited tumor volume growth compared with untreated controls. Control (G1): 793.7 ± 215.0 mm^3; CDDP (G2): 588.1 ± 176.9 mm^3; *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) intravenous (i.v.) (G3): 269.7 ± 72.7 mm^3; *S. typhimurium* A1-R intra-arterial (i.a.) (G4): 70.2 ± 18.9 mm^3 (CDDP: $p = 0.056$; *S. typhimurium* A1-R i.v.: $p = 0.0001$; *S. typhimurium* A1-R i.a.: $p = 0.00003$, all vs. untreated controls). i.a. administration of *S. typhimurium* A1-R was significantly more effective than either CDDP ($p = 0.00007$), or i.v. administration of *S. typhimurium* A1-R ($p = 0.00007$) and significantly regressed the tumor volume compared with day 0 ($p = 0.001$). The new model of i.a. administration of *S. typhimurium* A1-R has great promise for the treatment of recalcitrant osteosarcoma.

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Introduction

Toward the goal of precision oncology, our laboratory pioneered the patient-derived orthotopic xenograft (PDOX) nude mouse model with the technique of surgical orthotopic implantation (SOI), including pancreatic,[1-4] breast,[5] ovarian,[6] lung,[7] cervical,[8] colon,[9-11] and stomach cancer,[12] sarcoma,[13-17] and melanoma.[18-20]

In a previous study, we evaluated the efficacy of trabectedin (TRAB) and temozolomide (TEM) compared with cisplatinum (CDDP) on a lung-metastatic osteosarcoma PDOX mouse model.[21] TEM and TRAB, but not CDDP, significantly inhibited tumor volume compared with the untreated controls. The results of the previous study showed that a PDOX model of an osteosarcoma lung-metastasis that recurred after adjuvant CDDP-treatment, had identified potentially, highly-effective drugs for this recalcitrant disease, while precisely maintaining the CDDP resistance of the tumor in the patient.[21]

However, more effective therapeutics are needed for metastatic osteosarcoma. Toward this goal, our laboratory developed tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R). *S. typhimurium* A1-R was effective against primary and metastatic tumors as monotherapy in nude mouse models of major cancers, including prostate,[22,23] breast,[24-26] lung,[27,28] pancreatic,[1,29-32] ovarian,[33, 34] stomach,[35] and cervical cancer.[36] In addition, *S. typhimurium* A1-R was effective against patient-derived orthotopic models (PDOX) of pancreatic cancer,[1,32] sarcoma,[13,15,37] and melanoma.[18-20]

Metastatic osteosarcoma is a recalcitrant disease. We previously reported that a patient-derived subcutaneous-transplant nude-mouse model of the ostoeosarcoma lung metastasis that occurred after adjuvant CDDP treatment, was regressed by tumor-targeting *S. typhimurium* A1-R. The osteosarcoma was only partially sensitive to the molecular-targeting drug sorafenib, which did not arrest its growth. *S. typhimurium* A1-R was significantly more effective than sorafenib.[21, 37]

We previously reported the efficacy and safety of intra-portal-vein (iPV) targeting of *S. typhimurium* A1-R on colon cancer liver metastasis in a nude-mouse orthotopic model.[38]

In the present report, we demonstrate the high efficacy of intra-arterial (i.a.) administration of *S. typhimurium* A1-R on the CDDP-resistant, recurrent osteosarcoma in the PDOX model.

CONTACT Fritz C. Eilber fceilber@mednet.ucla.edu Professor of Surgery, Professor of Molecular & Medical Pharmacology UCLA, Division of Surgical Oncology, 10833 LeConte Avenue, Rm. 54–140 CHS, Los Angeles, CA 90095–1782, USA; Robert M. Hoffman all@anticancer.com-or-Fritz AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111, USA.

Results and discussion

Distribution of S. typhimurium *A1-R in the CDDP-resistant relapsed osteosarcoma PDOX mouse model*

Distribution of *S. typhimurium* A1-R was imaged by confocal microscopy with the Olympus FV1000 on day 3 after injection of *S. typhimurium* A1-R (Fig. 1). The mean fluorescence intensity in the PDOX tumor on day 3 after intra-arterial (i.a.) injection of *S. typhimurium* A1-R was 1.76×10^6 compared with 1.49×10^5 after intravenous (i.v.) injection ($p = 0.000013$; Fig. 2A). The fluorescence area in the PDOX tumor on day 3 after i.a. injection of *S. typhimurium* A1-R was $485.4 \pm 103.7\ \mu m^2$ compared with $51.3 \pm 45.7\ \mu m^2$ after i.v. injection ($p < 0.00001$; Fig. 2B). In the tibialis anterior muscle of the tumor-bearing limb, fluorescence intensity after i.a. injection *S. typhimurium* A1-R was 5.18 compared with 2.74 after i.v. injection ($p = 0.50$). The fluorescence area after i.a. injection *S. typhimurium* A1-R was 0.01 μm^2 compared with 0.02 μm^2 after i.v. injection in the tibialis anterior muscle of affected limb ($p = 0.42$).

Efficacy of CDDP, S. typhimurium *A1-R i.v. and* S. typhimurium *A1-R i.a. on the CDDP-resistant osteosarcoma PDOX mouse model*

On day 14 after initiation of treatment, all but CDDP significantly inhibited tumor volume growth compared with untreated control (G1): $793.7 \pm 215.0\ mm^3$; CDDP (G2): $588.1 \pm 176.9\ mm^3$; *S. typhimurium* A1-R i.v. (G3): $269.7 \pm 72.7\ mm^3$; *S. typhimurium* A1-R i.a. (G4): $70.2 \pm 18.9\ mm^3$ (CDDP: $p = 0.056$; *S. typhimurium* A1-R i.v.: $p = 0.0001$; *S. typhimurium* A1-R i.a.: $p = 0.00003$, all vs. untreated controls). i.a. administration of *S. typhimurium* A1-R was significantly more effective than either CDDP ($p = 0.00007$); i.v. administration of *S. typhimurium* A1-R ($p = 0.00007$) and significantly regressed the tumor volume compared with day 0 ($p = 0.001$) (Fig. 3).

There were no animal deaths, limb necrosis or paraplegia in any group. The body weight of treated mice were not significantly different in each group (Fig. 4).

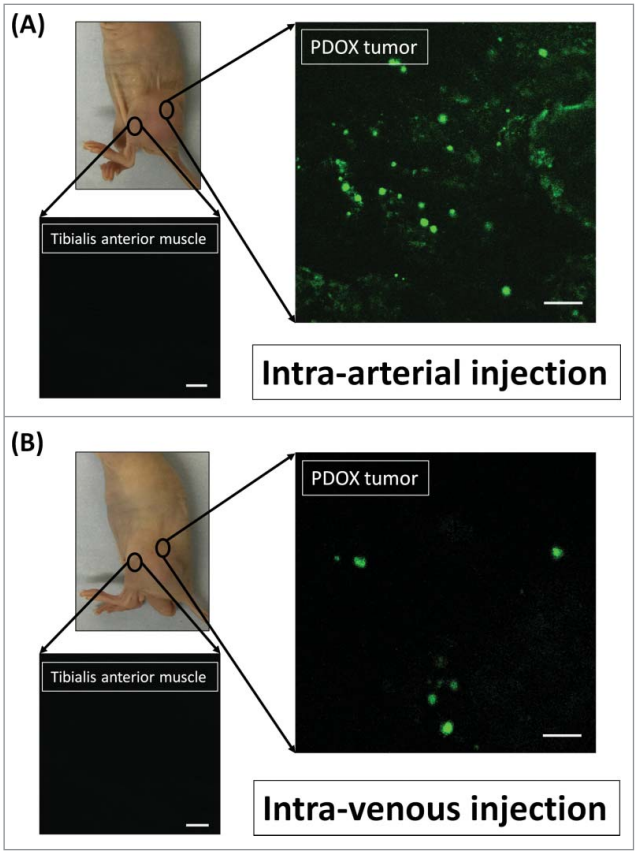

Figure 1. Distribution of fluorescence imaging. (A) *S. typhimurium*-A1-R-GFP targeting the osteosarcoma PDOX after intra-arterial (i.a.) injection. (B) *S. typhimurium* A1-R-GFP targeting the osteosarcoma after intravenous (i.v.) injection. Confocal microscopy imaging with the Olympus FV1000 demonstrated *S. typhimurium* A1-R-GFP targeting the osteosarcoma PDOX. Scale bars: 12.5 μm.

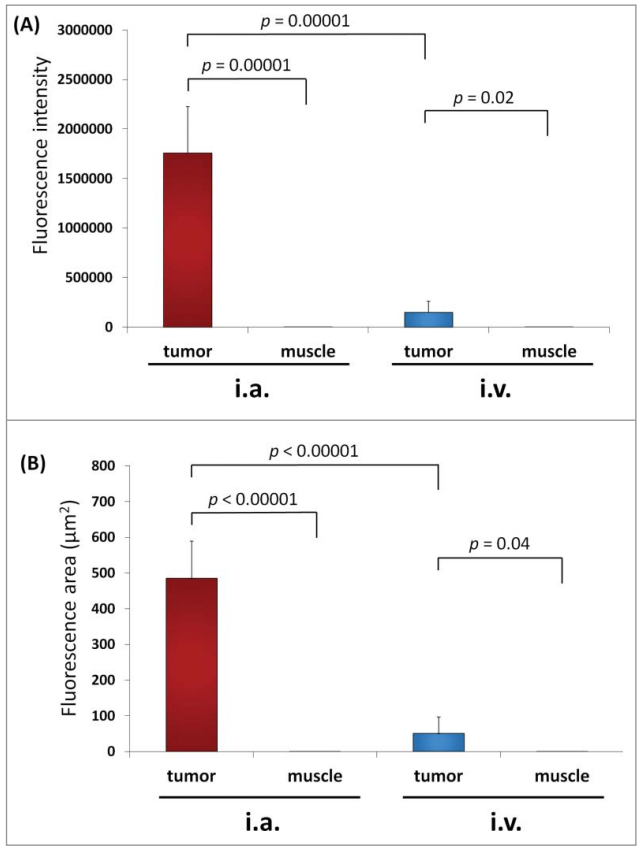

Figure 2. (A) Fluorescence intensity of *S. typhimurium* A1-R-GFP targeting the osteosarcoma PDOX after i.a. or i.v. administration. (B) Fluorescent area of *S. typhimurium* A1-R-GFP targeting the osteosarcoma PDOX after i.a. or i.v. administration.

Histology of original tumor and PDOX tumors

High power photomicrography of the original patient tumor demonstrated a chondroid matrix occupied by anaplastic cells. The tumor had hypercellular areas populated by anaplastic cells displaying nuclear pleomorphism, coarse and hyperchromatic chromatin and abundant mitotic figures (Fig. 5A). High power photomicrography of the untreated PDOX tumor showed the tumor had a solid and chondroblastic appearance similar to the original patient tumor and had hypercellular areas filled with cancer cells displaying nuclear pleomorphism and mitotic figures (Fig. 5B). The PDOX tumor treated with CDDP comprised viable cells without apparent necrosis or inflammatory changes and had almost the same features as the untreated control (Fig. 5C).

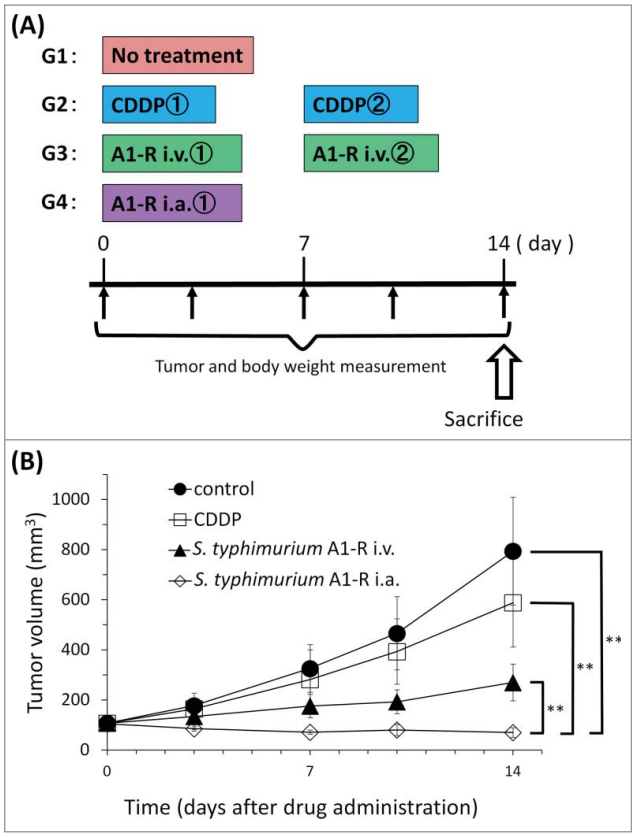

Figure 3. (A) Treatment schema. Mice were treated with CDDP, *S. typhimurium* A1-R i.v. or *S. typhimurium* A1-R i.a. CDDP (6 mg/kg/week i.p. for 2 weeks); *S. typhimurium* A1-R i.v. (5×10^7 CFU/100 μl, i.v., weekly, for 2 weeks); *S. typhimurium* A1-R i.a. (5×10^5 CFU/100 μl, i.a., once). Tumor volume was measured at the indicated time points after the onset of treatment. n = 8mice/group. (B) Treatment efficacy. $^{*}p < 0.05$, $^{**}p < 0.001$.

The PDOX Tumor treated with i.v. injection of *S. typhimurium* A1-R showed changes in cancer-cell shape with a necrotic area (Fig. 5D). The i.a. injection of *S. typhimurium* A1-R-treated tumor showed more extensive tumor necrosis (Fig. 5E).

Previously-developed concepts and strategies of highly-selective tumor targeting can take advantage of molecular targeting of tumors, including tissue-selective therapy which focuses on unique differences between normal and tumor tissues.[39-44]

Materials and methods

Mice

Athymic nu/nu nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. 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. 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. To minimize any suffering of the animals, 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/kg 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 killed by CO_2 inhalation when they met the following humane end point 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

The study was previously reviewed and approved by the UCLA Institutional Review Board (IRB #10–001857).[37] Written informed consent was obtained from the patient as part of the above-mentioned UCLA Institutional Review Board-approved protocol. A 16-year old patient with localized left distal femoral high grade osteosarcoma underwent CDDP-based neo-adjuvant chemotherapy and limb salvage with distal femoral replacement.

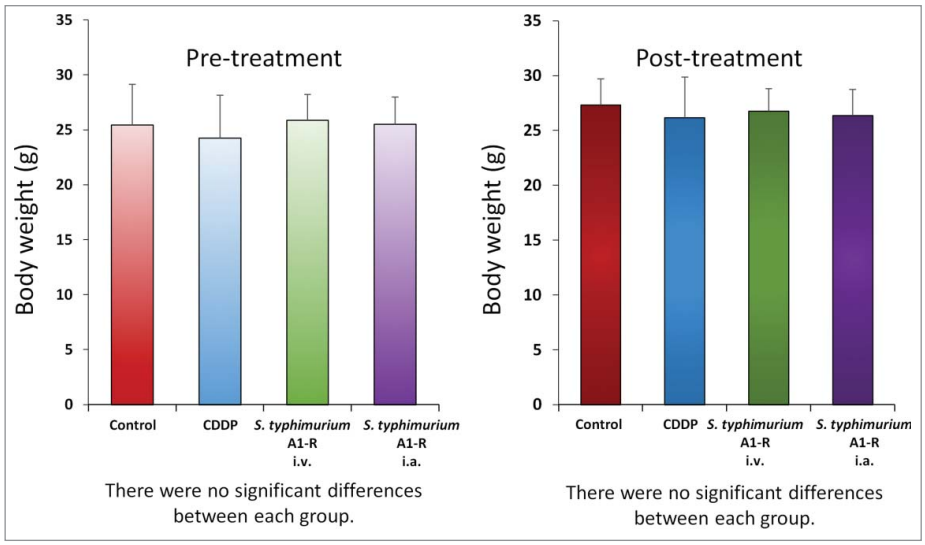

Figure 4. Body weight after various treatments. Bar graphs show body weight in each group at pre-treatment and 2 weeks after drug administration.

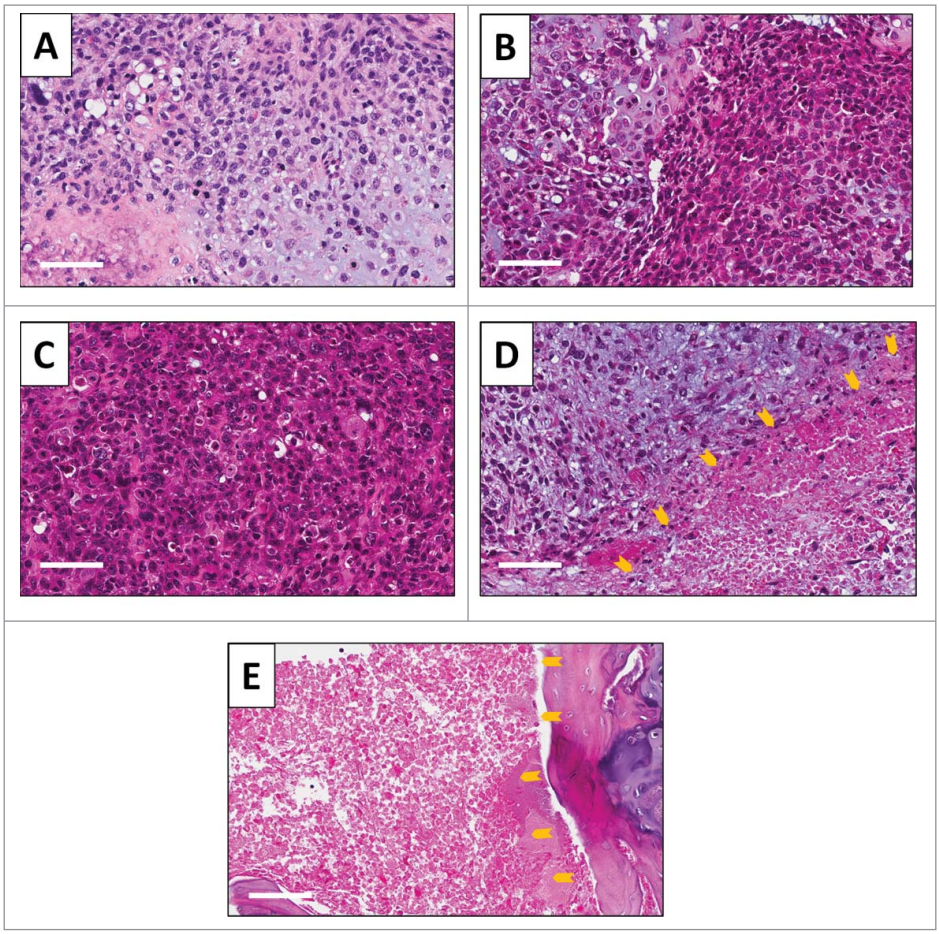

Figure 5. Tumor histology. Hematoxylin and eosin (H&E)-stained section of the (A) original patient tumor; (B) untreated PDOX tumor; (C) PDOX tumor treated with CDDP; (D) PDOX tumor treated with *S. typhimurium* A1-R i.v.; and (E) PDOX tumor treated with *S. typhimurium* A1-R i.a.. Necrotic areas are indicated by yellow arrows. White scale bars: 80 μm.

One year later, the osteosarcoma recurred with 3 bilateral metachronous pulmonary metastases. The patient was treated with curative surgery at the Division of Surgical Oncology, University of California, Los Angeles (UCLA). The patient did not receive chemotherapy or radiotherapy before lung surgery.[37]

SOI for establishment of the PDOX osteosarcoma model

The osteosarcoma from the patient was previously established subcutaneously in mice.[37] Subcutaneously grown tumors were harvested and cut into small fragments (3–4 mm). After nude mice were anesthetized, a 10 mm skin incision was made on the right thigh, the vastus lateralis muscle was opened and the biceps femoris muscle was split to reach the distal femur. An incision was made in the lateral patello-femoral ligament, sparing the knee joint and then the lateral condyle of the femur was resected. A single 3 to 4 mm tumor fragment was implanted orthotopically into this space to establish a PDOX model. The muscle and wound was closed with 6–0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) (Fig. 1).

Treatment design

Primary osteosarcomas are fed by an artery. Therefore, in the present study, we compared i.a. administration of *S. typhimurium* A1-R with i.v. administration in the osteosarcoma PDOX.

The PDOX models were randomized into the following groups when tumor volume reached 100 mm^3: G1, control without treatment, n = 8; G2, CDDP (6 mg/kg, intraperitoneal (i.p.) injection, weekly, for 2 weeks, n = 8); G3, i.v. injection of *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., weekly, for 2 weeks, n = 8); G4, i.a. injection of *S. typhimurium* A1-R (5×10^5 CFU/100 μl, i.a., once, n = 8). Tumor length, width and mouse body weight were measured twice in a week. Tumor volume was calculated by following formula: Tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. Data are presented as mean ± SD.

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

GFP-expressing *S. typhimurium* A1-R bacteria (AntiCancer Inc.,) were grown overnight in LB medium (Fisher Sci., Hanover Park, IL, USA) and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS.[22-24]

i.a. injection of S. typhimurium *A1-R*

Nude mice were anesthetized with the ketamine mixture as described above and placed in a right lateral decubitus position. A 20 mm skin incision was made on the left lateral abdomen followed by exposure of the abdominal aorta. *S. typhimurium*

A1-R (5×10^5 CFU in 100 μl PBS) was injected in the abdominal aorta using a 31G needle. After removal of the needle, bleeding was stopped by gently pressing the puncture site with a cotton swab. After injection, the abdomen was closed with non-absorbable sutures.

Confocal microscopy

The FV1000 confocal microscope (Olympus, Tokyo, Japan) was used for high-resolution imaging. Fluorescence images were obtained using the 20 × /0.50 UPlan FLN and 40 × /1.3 oil Olympus UPLAN FLN objectives.[45]

Distribution of S. typhimurium *A1-R*

Twelve PDOX mouse models were treated with i.v. administration of *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., once) or i.a. administration of *S. typhimurium* A1-R (5×10^5 CFU/100 μl, i.a., once) when the tumor volume reached 500 mm^3, (n = 6 mice each). PDOX tumors or the tibialis anterior muscle of the affected limb were resected on day 3. The distribution of *S. typhimurium* A1-R was determined by confocal imaging with the FV1000.[46] Three random fields were accessed in each specimen.

Histological analysis

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections (3 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).

Conclusions

The new model of i.a. administration of *S. typhimurium* A1-R has great promise for the treatment of recalcitrant osteosarcoma.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

ORCID

Kentaro Igarashi http://orcid.org/0000-0002-4680-4317
Tara A. Russell http://orcid.org/0000-0002-3912-3601
Fritz C. Eilber http://orcid.org/0000-0003-3336-9333

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