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Journal

Archives of Gynecology and Obstetrics, 299(6)

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Publication Date

2019-06-01

DOI

10.1007/s00404-019-05147-3

Peer reviewed

HHS Public Access

Author manuscript

Arch Gynecol Obstet. Author manuscript; available in PMC 2024 May 03.

Published in final edited form as:

Arch Gynecol Obstet. 2019 June ; 299(6): 1683–1690. doi:10.1007/s00404-019-05147-3.

Tumor-targeting *Salmonella typhimurium* A1-R overcomes nab-paclitaxel resistance in a cervical cancer PDOX mouse model

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Abstract

Purpose—Cervical cancer is a recalcitrant disease. To help overcome this problem, we previously established a patient-derived orthotopic xenograft (PDOX) model of cervical cancer. In the previous study, we found the tumor to be resistant to nab-paclitaxal (nab-PTX). We also previously developed the tumor-targeting bacteria *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R). The aim of the present study was to investigate the efficacy of *S. typhimurium* A1-R to overcome nab-PTX resistance in the cervical cancer PDOX model.

Methods—Cervical-cancer tumor fragments were implanted orthotopically into the neck of the uterus of nude mice. The cervical-cancer PDOX models were randomized into the following four groups after the tumor volume reached 60 mm^3: G1: untreated group; G2: nab-PTX (i.v., 10 mg/kg, biweekly, 3 weeks); G3: *Salmonella typhimurium* A1-R (i.v., 5×10^7 CFU/body, weekly, 3 weeks); G4: nab-PTX combined with *Salmonella typhimurium* A1-R (nab-PTX, 10 mg/kg, i.v., biweekly, 3 weeks; *S. typhimurium* A1-R, 5×10^7 CFU/body, i.v., weekly, 3 weeks). Each group

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Author contributions KM: Project development, data collection, data analysis, manuscript writing; TM, TM, MZ, TK, KK, KI, MM, TML, CH, SK, TK: data collection, data analysis; MB, KS, SRS, IE: data analysis; SRS : data analysis, manuscript writing and revision; RMH project development, data collection, data analysis, manuscript writing.

Conflict of interest KM, TK, KK, KI, MM and RMH are or were unsalaried associates of AntiCancer, Inc. MZ is an employee of AntiCancer Inc.. CH, SK and TK are unsalaried associates of AntiCancer Japan. AntiCancer Inc. and AntiCancer Japan use PDOX models for contract reserach. There are no other competing commercial interests.

Ethical approval All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873–1. For patient studies, an informed consent was obtained, and PDOX studies were approved by the Institutional Ethics Committee of Kawasaki Medical School.

comprised eight mice. All mice were sacrificed on day 22. Tumor volume was measured on day 0 and day 22. Body weight was measured twice a week.

Results—Nab-PTX and *Salmonella typhimurium* A1-R did not show significant efficacy as monotherapy compared to the control group ($P = 0.564$ and $P = 0.120$, respectively). In contrast, nab-PTX combined with *Salmonella typhimurium* A1-R significantly suppressed tumor growth compared to the untreated control group and nab-PTX group ($P < 0.001$ and $P = 0.026$, respectively).

Conclusions—*Salmonella typhimurium* A1-R has potential future clinical application to overcome drug resistance in cervical cancer.

Keywords

Cervical cancer; *S. typhimurium* A1-R; Patient-derived orthotopic xenograft; Nude mice; Bacterial therapy

Introduction

Squamous cervical cancer is the second most frequently occurring cancer in women worldwide, with over 200,000 deaths per year [1]. Despite advances in screening and treatment strategies, a significant number of patients die after first-line therapy. First-line chemotherapy for cervical cancer includes nanoparticle albumin-bound (nab)-paclitaxel (nab-PTX) [2]. Bevacizumab in combination with cisplatinum–paclitaxel was approved as first-line therapy for advanced cervical cancer [3]. Minnion et al. [4] have demonstrated that nab-PTX with bevacizumab could be used for recurrent cervical cancer after failing platinum–taxane or topotecan chemotherapy. Cediranib and pazopanib showed promise in advanced cervical cancer [5, 6]. In a Phase II trial, PTX together with nedaplatinum showed some promise for patients with advanced, recurrent, or metastatic cervical cancer [7]. Recently, chemo-radiation followed by PTX and carboplatinum was well tolerated in women with locally advanced cervical cancer [8]. However, there is no standard treatment option available for metastatic cervical cancer [9].

Clinically-relevant mouse models of cancer can be used for tailor-made therapy based on the patient-derived tumor. We established the patient-derived orthotopic xenograft (PDOX) nude mouse model for major tumor types [9–28], which has many advantages over subcutaneous transplant models [29].

Previously, we developed a tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R), which is auxo-trophic for leu-arg [30]. *S. typhimurium* A1-R was effective in the PDOX models of many cancer types [18, 20–23, 26, 28].

We previously also developed a PDOX model of human epidermal growth factor receptor-2 (HER-2)-positive cervical cancer in which the most active regimen was the combination of tumor-targeting A1-R and trastuzumab [31]. In another cervical cancer PDOX model we previously developed, we demonstrated that nab-PTX was ineffective [9].

In the present study, we determined whether *S. typhimurium* A1-R could overcome nab-PTX resistance in a cervical cancer PDOX model.

Methods

Mice

In the present study, athymic nu/nu nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used. Animal housing, their diet, surgical procedures, and imaging were performed as previously described [23–25]. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed daily and humanely sacrificed by CO_2 inhalation if they met the humane endpoint criteria as previously described [23–25]. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873–1.

Establishment of the cervical cancer PDOX model

The 57-year-old patient with primary cervical cancer previously received a radical hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy at Kawasaki Medical School Hospital, Japan [9]. The tumor was diagnosed as squamous cell carcinoma (grade 2). The patient did not receive any neoadjuvant therapy. Informed consent was obtained, and PDOX studies were approved by the Institutional Ethics Committee of Kawasaki Medical School. A fresh resected tumor specimen was initially implanted sub-cutaneously in nude mice. The established tumors were cut into 5 mm^3 fragments for surgical orthotopic implantation (SOI). A 6 mm lower abdominal midline incision was made under anesthesia. The neck of the uterus was exposed, and a single fragment was implanted by SOI using 8–0 nylon sutures (Ethicon, Inc., NJ, USA). The wound was closed with 6–0 PDS II (polydioxanone) sutures (Ethicon, Inc., NJ, USA) [23–25].

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

Salmonella typhimurium A1-R expressing green (GFP) (AntiCancer, Inc., San Diego, CA) was cultured 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 twice by PBS, and then diluted in PBS up to 5×10^8 CFU/ml. *S. typhimurium* A1-R was administered by tail-vein injection. *S. typhimurium* A1-R (5×10^7 CFU/100 μl) was injected in each mouse weekly [30, 31].

Treatment protocol for the cervical cancer PDOX model

The cervical cancer PDOX models were randomized into four groups when the tumor volume reached 60 mm^3: G1: untreated group; G2: nab-PTX (i.v., 10 mg/kg, biweekly, 3 weeks); G3: *S. typhimurium* A1-R (i.v., 5×10^7 CFU/body, weekly, 3 weeks); G4: nab-PTX combined with *S. typhimurium* A1-R (nab-PTX, 10 mg/kg, i.v., biweekly, 3 weeks; *S. typhimurium* A1-R 5×10^7 CFU/body, i.v., weekly, 3 weeks). Each group comprised eight mice. All mice were sacrificed on day 22. Treatment doses, routes, and schedules were based on our previous reports [9]. Tumor volume and body weight were measured twice a

week using the following formula: tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. All mice were sacrificed on day 22.

Bacterial culture from tumor tissue

To demonstrate *S. typhimurium* A1-R tumor targeting, the cervical cancer PDOX tumors treated with *S. typhimu rium* A1-R were resected on day 22 for bacteria culture. The tumor specimens were homogenized and suspended in 1 ml PBS. The suspension was diluted 10 times each up to 1:10,000, then cultured in LB agar medium for 12 h. *S. typhimurium* A1-R colonies expressing GFP were observed with the OV100 Small Animal Imaging System (Olympus, Tokyo, Japan) [30, 31].

Histology

10% formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and rehydrated in an ethanol series. H&E staining was performed using standard protocol. A BHS system microscope (Olympus Corp., Tokyo, Japan) was used for histological examination.

Statistical analysis

All statistical analyses were performed by statistical software EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 3.4.1) [32]. It is a modified version of R commander (version 2. 4–0) including statistical functions for biostatistics. A normal distribution was evaluated with the Shapiro–Wilk test. A Bartlett's test was used to assess the homogeneity of variances across groups. One-way ANOVA with Tukey HSD for post hoc analysis was used for the parametric test for intergroup comparison. Kruskal–Wallis with Steel–Dwass for post hoc analysis was used for the non-parametric test to compare intergroup. Probability values of $P < 0.05$ were defined as statistical significance.

Results

Effect of treatment on tumor growth

The treatment protocol for the cervical cancer PDOX is shown in Fig. 1. Figure 2 illustrates the estimated tumor volume ratio (post-treatment/pre-treatment). Nab-PTX did not show significant efficacy compared to the control group ($P = 0.564$) as we have previously observed for this PDOX model [9]. There was also no significant difference between the *S. typhimurium* A1-R monotherapy group and the control group ($P = 0.121$). Nab-PTX combined with *S. typhimurium* A1-R was significantly effective compared to the untreated group ($P < 0.001$). Nab-PTX combined with *S. typhimurium* A1-R inhibited the tumor growth more than nab-PTX ($P = 0.026$). The final tumor volume ratios (day 22/day 0) were as follows: untreated control (G1) (5.52 ± 1.59); nab-PTX (G2) (4.63 ± 1.36); *S. typhimurium* A1-R (G3) (3.96 ± 1.03); nab-PTX combined with *S. typhimurium* A1-R (G4) (2.60 ± 0.98).

Effect of treatment on body weight

Body weight was compared in each group. The relative body weight (day 22/day 0) is shown in Fig. 3. No significant difference was observed between the four groups.

Targeting of *S. typhimurium* to the cervical cancer PDOX tumor

Targeting of *S. typhimurium* A1-R-GFP to the cervical cancer PDOX tumor was confirmed by culture in LB agar medium of the homogenized PDOX tumor specimen previously treated with *S. typhimurium* A1-R, and subsequently Fluorescence Imaging (Fig. 4a, b).

Effect of treatment on tumor histology

Figure 5a–d are representative images of hematoxylin and eosin (H&E) staining of tumors from each group resected on day 22. Extensive proliferation of squamous cells was observed in the untreated group (Fig. 4a). The PDOX tumor faithfully replicated the original squamous-cell carcinoma as we reported previously [9]. Nab-PTX did not cause any noticeable histological changes (Fig. 4b). *S. typhimurium* A1-R monotherapy caused slight fibrotic changes (Fig. 4c). Nab-PTX combined with *S. typhimurium* A1-R caused extensive necrosis (Fig. 4d).

Discussion

Cervical cancer is a major problem that requires transformative therapeutic strategies. We have previously established PDOX nude mouse models of this disease and also demonstrated that the highly-touted agent, nab-paclitaxel (NAB-PTX) is ineffective for cervical cancer. Here we demonstrated that *S. typhimurium* A1-R combined with nab-PTX could overcome nab-PTX resistance in cervical cancer.

Various bacterial species such as *Salmonella*, *Listeria*, *Escherichia*, and *Clostridium* have been demonstrated to target and kill solid tumors. Among these bacteria, various strains of *Salmonella* colonize solid tumors and induce anti-tumor immunity [33, 34]. Yoon et al. [35] reported that attenuated *S. typhimurium*-expressing recombinant IFN-γ invaded and directly killed melanoma cells and induced cytotoxicity rather than stable anti-tumor immunity [35].

Salmonella typhimurium A1-R has a number of different effects on tumors. Our previous experiments have shown that *S. typhimurium* A1-R could kill cancer cells directly in vitro [30, 36, 37]. *S. typhimurium* A1-R destroys tumor vessels [38]. *S. typhimurium* A1-R also induces CD8+ T cells into the tumor [39]. *S. typhimurium* A1-R also decoys quiescent cancer cells to cycle from G_0/G_1 to $S/G_2/M$ and makes cancer cells more sensitive to chemotherapy [40, 41]. Colonization of *S. typhimurium* at the tumor site resulted in an elevation of interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) within the tumor mass [42]. Further, a systematic injection of IL2-expressing *S. typhimurium* could reduce angiogenesis and increase necrosis within tumor tissues [43]. Therefore, tumor-targeting bacteria have great potential in treating solid tumors. Recently, we demonstrated that tumor-targeting *S. typhimurium* A1-R together with gemcitabine regresses partially GEM-resistant pancreatic cancer PDOX [44]. *Salmonella* LVR01 in combination with the toll-like receptor agonist, imiquimod, reduced tumor growth and prolonged survival in melanoma-

bearing mice [45]. Wen et al. [46] demonstrated that TIMP-2 (tissue inhibitor of matrix metalloproteinases 2)-expressing *S. typhimurium* significantly inhibited tumor cell growth and enhanced animal survival, via down-regulating the expression of MMP-2 (matrix metalloproteinase-2).

In the present study, *S. typhimurium* A1-R did not show significant efficacy as a monotherapy. However, *S. typhimurium* A1-R combined with nab-PTX could overcome nab-PTX resistance.

Acknowledgements

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

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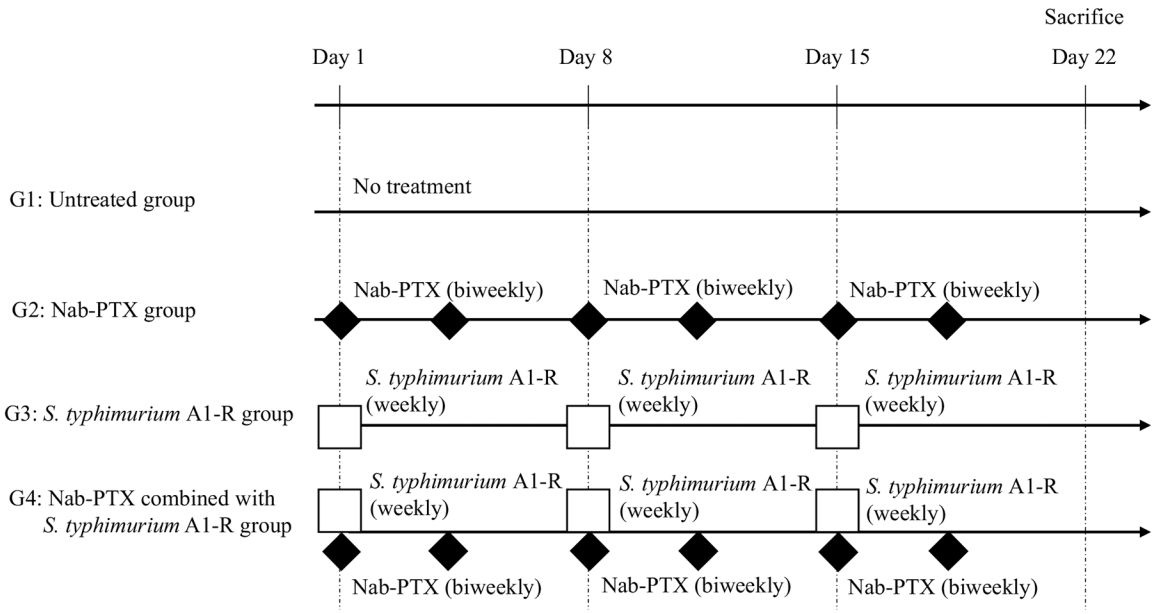

Fig. 1.

Treatment protocol. G1: untreated group; G2: nab-PTX (i.v., 10 mg/kg, biweekly, 3 weeks); G3: *S. typhimurium* A1-R (i.v., 5×10^7 CFU/body, weekly, 3 weeks); G4: nab-PTX combined with *S. typhimurium* A1-R (nab-PTX, 10 mg/kg, i.v., biweekly, 3 weeks; *S. typhimurium* A1-R, 5×10^7 CFU/body, i.v., weekly, 3 weeks). Each group comprised eight mice. All mice were sacrificed on day 22

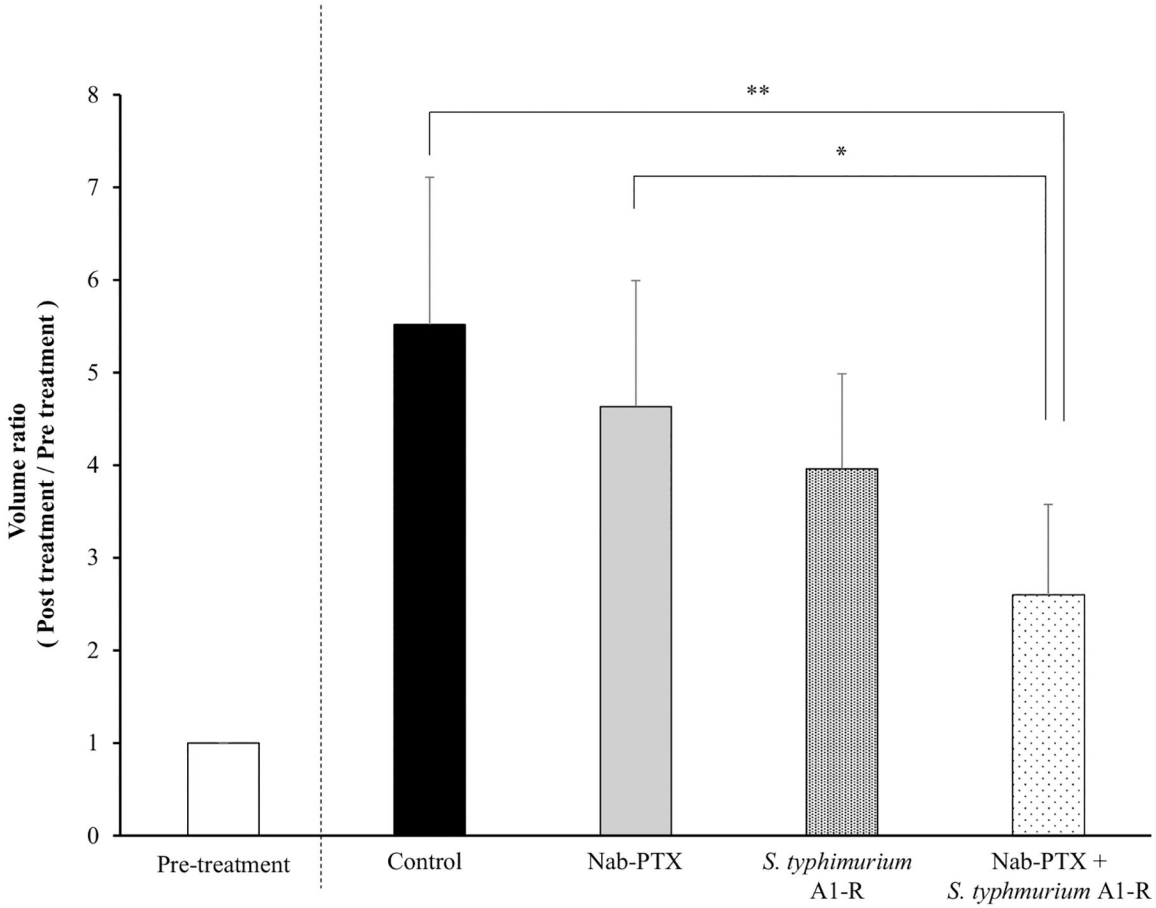

Fig. 2.

Efficacy of treatment on the tumor volume ratio. Bar graphs show tumor volume ratios (post-treatment/pre-treatment). Error bars: ± SD. *$P < 0.05$, **$P < 0.001$

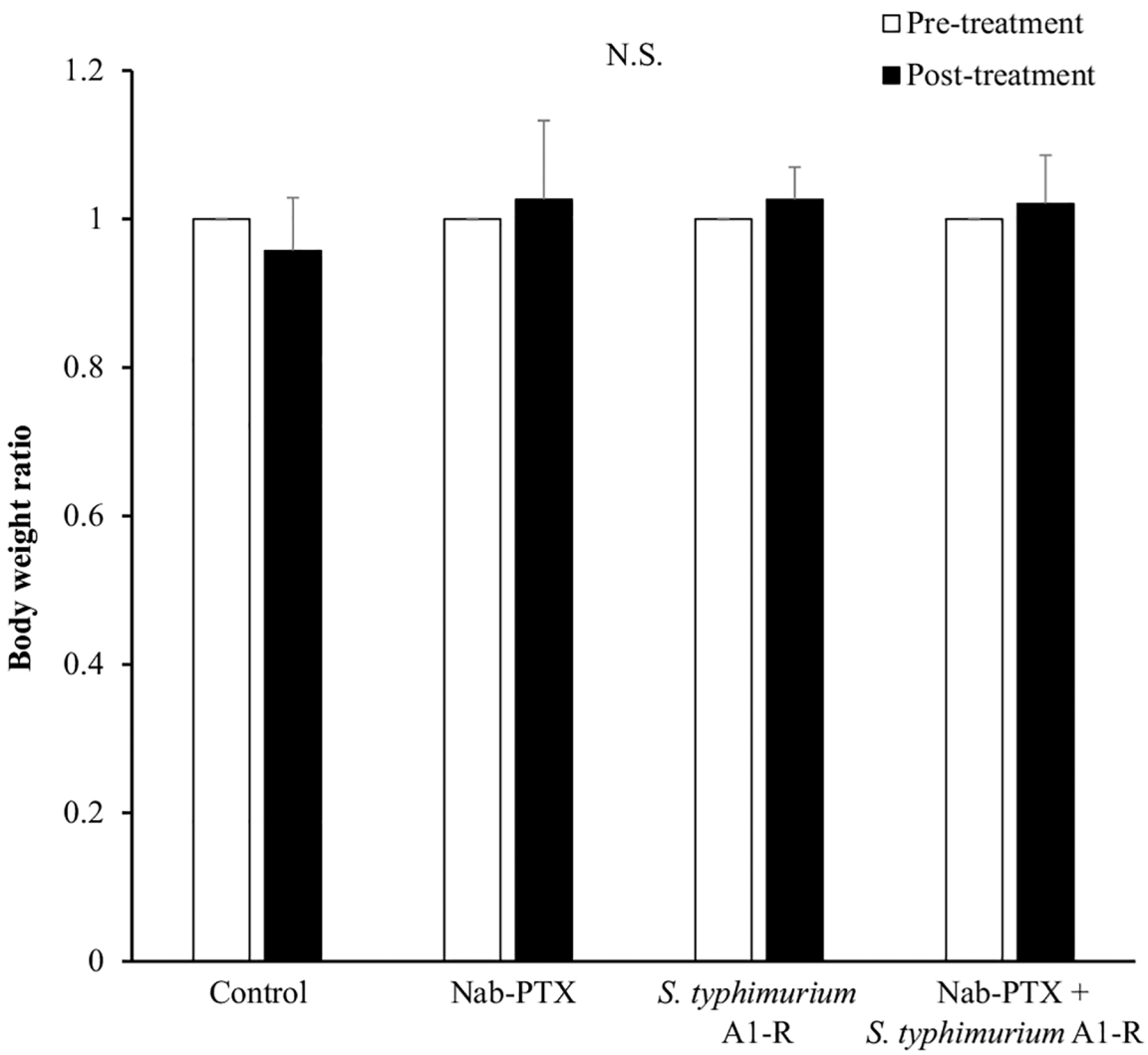

Fig. 3.

Effect of treatment on mouse body weight. Bar graphs show the body weight ratio of each group (post-treatment/pre-treatment). Error bars ± SD

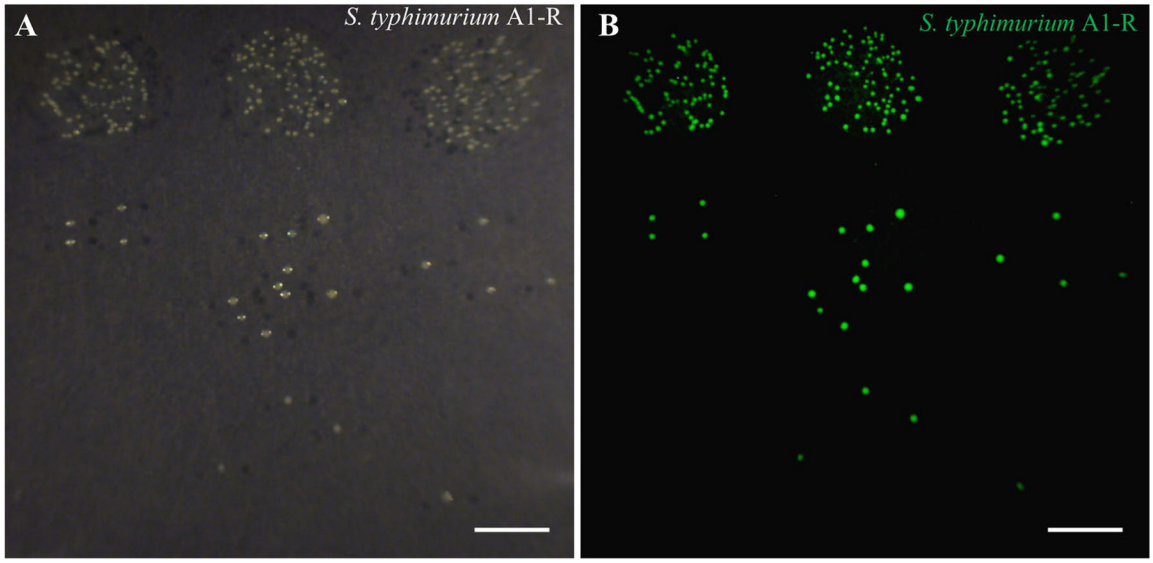

Fig. 4.

Tumor targeting of *S. typhimurium* A1-R to the cervical-cancer PDOX tumor. Bright-field image (**a**) and fluorescence image (**b**) of GFP-expressing *S. typhimurium* A1-R colonies cultured in LB-agar medium from PDOX tumors 24 h after the third treatment with *S. typhimurium* A1-R (day 23). Scale bars: 5 mm

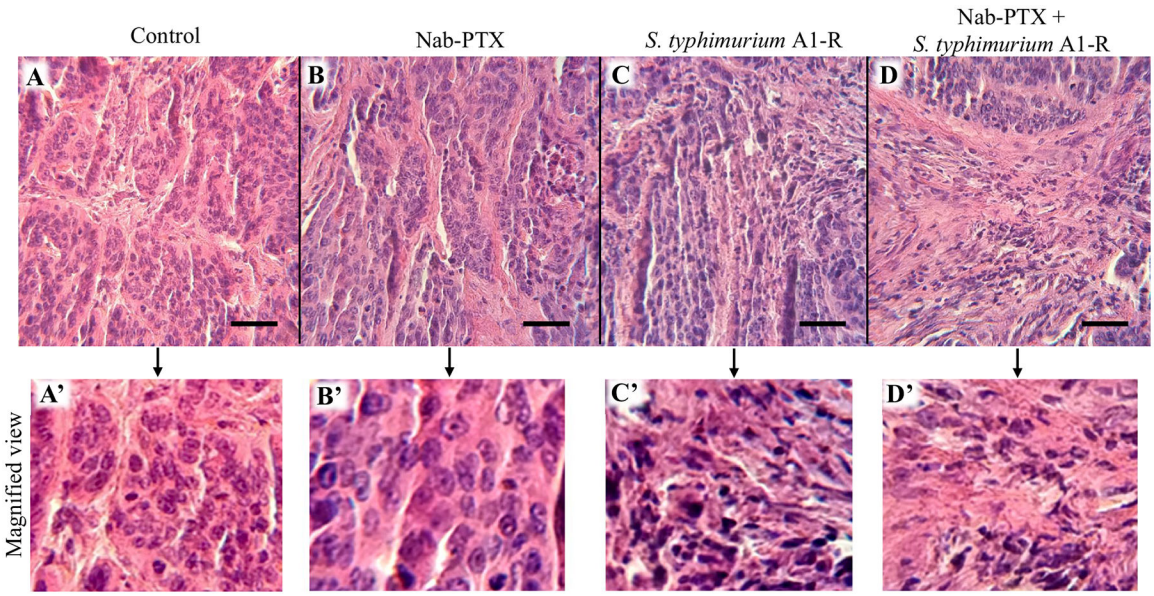

Fig. 5.

Effect of treatment on tumor histology. **a** Hematoxylin and eosin (H&E) staining of the untreated cervical-cancer PDOX tumor. **b** H&E staining of the cervical-cancer PDOX tumor treated with nab-PTX. **c** H&E staining of the *S. typhimurium* A1-R-treated cervical-cancer PDOX tumor. **d** H&E staining of the cervical-cancer PDOX tumor treated with nab-PTX combined with *S. typhimurium* A1-R. **A′–D′** Show magnified images. Scale bar: 50 μm