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REPORT

Salmonella typhimurium A1-R targeting of a chemotherapy-resistant BRAF-V600E melanoma in a patient-derived orthotopic xenograft (PDOX) model is enhanced in combination with either vemurafenib or temozolomide

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ABSTRACT

A metastatic melanoma obtained from the right chest wall of a patient was previously established orthotopically in the right chest wall of nude mice as a patient-derived orthotopic xenograft (PDOX) model. We previously showed that the combination of tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) and chemotherapy was highly effective against the melanoma PDOX. In the present study, we investigated the mechanism of the high efficacy of this combination. Two weeks after implantation, 40 PDOX mouse models were randomized into 4 groups of 10 mice each: untreated control (n = 10); treated with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., once a week for 2 weeks, n = 10); treated with temozolomide (TEM) (25 mg/kg, p.o. for 14 consecutive days) combined with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., once a week for 2 weeks, n = 10); treated with vemurafenib (VEM) (30 mg/kg, p.o., for 14 consecutive days) combined with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., once a week for 2 weeks) (n = 10). On day 14 from initiation, all treatments significantly inhibited tumor growth compared with untreated control (*S. typhimurium* A1-R: $p < 0.01$; TEM combined with *S. typhimurium* A1-R: $p < 0.01$; VEM combined with *S. typhimurium* A1-R: $p < 0.01$). Combination therapy with *S. typhimurium* A1-R was significantly more effective on tumor growth than *S. typhimurium* A1-R alone (with TEM: $p < 0.01$; with VEM: $p < 0.01$). Combination therapy significantly increased *S. typhimurium* A1-R tumor targeting alone (*S. typhimurium* A1-R + TEM: $p < 0.01$, *S. typhimurium* A1-R + VEM: $p < 0.01$), relative to *S. typhimurium* A1-R alone, respectively. In conclusion, chemotherapy drugs promoted targeting of *S. typhimurium* A1-R of melanoma, thereby enhancing efficacy against the melanoma PDOX.

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Introduction

The tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R), developed by our laboratory,[1] is auxotrophic for Leu-Arg, which prevents it from mounting a continuous infection in normal tissues. *S. typhimurium* A1-R was effective against primary and metastatic tumors as monotherapy in nude mouse models of major cancers, including prostate,[2,3] breast,[4-6] lung,[7,8] pancreatic,[9-13] ovarian[14,15] stomach,[16] and cervical cancer[17] In addition, *S. typhimurium* A1-R was effective against patient-derived orthotopic models (PDOX) of pancreatic cancer,[9,13] sarcoma[18-20] and melanoma.[20,21]

S. typhimurium A1-R has been shown to directly kill cancer cells *in vitro*.[2]

We have previously developed a patient-derived orthotopic xenograft (PDOX) nude-mouse model of melanoma with a BRAF-V600E mutation.[22-24] *S. typhimurium* A1-R combined with temozolomide (TEM) was significantly more effective on tumor growth than either *S. typhimurium* A1-R alone or TEM alone. TEM combined with *S. typhimurium* A1-R could regress the melanoma in the PDOX model.[22] We also previously demonstrated that *S. typhimurium* A1-R was significantly more effective than VEM alone, in the melanoma PDOX model.[24]

In the present study, we demonstrate a mechanism of how chemotherapy enhances the anti-melanoma efficacy of *S. typhimurium* A1-R.

Results and discussion

All treatments significantly inhibited tumor growth compared with untreated control (*S. typhimurium* A1-R: $p < 0.01$; TEM combined with *S. typhimurium* A1-R: $p < 0.01$; VEM combined with *S. typhimurium* A1-R: $p < 0.01$; on day 14 after initiation. Combination therapy with *S. typhimurium* A1-R was significantly more effective than *S. typhimurium* A1-R alone with TEM: $p < 0.01$; with VEM: $p < 0.01$ (Figs. 1,2).

Confocal microscopy showed that *S. typhimurium* A1-R could directly target the melanoma PDOX. The targeting ability

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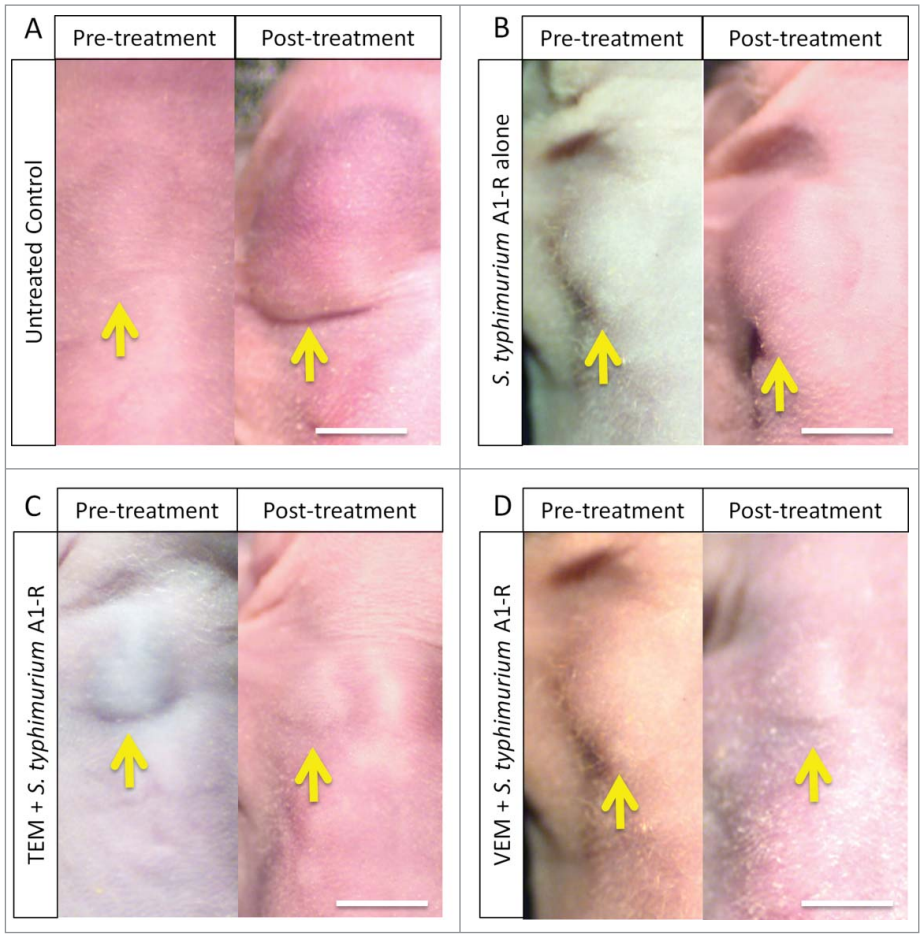

Figure 1. Representative photographs of mice from each treatment group. A. Untreated control. B. Treated with *S. typhimurium* A1-R. C. Treated with TEM and *S. typhimurium* A1-R. D. Treated with VEM and *S. typhimurium* A1-R. Scale bar: 5 mm.

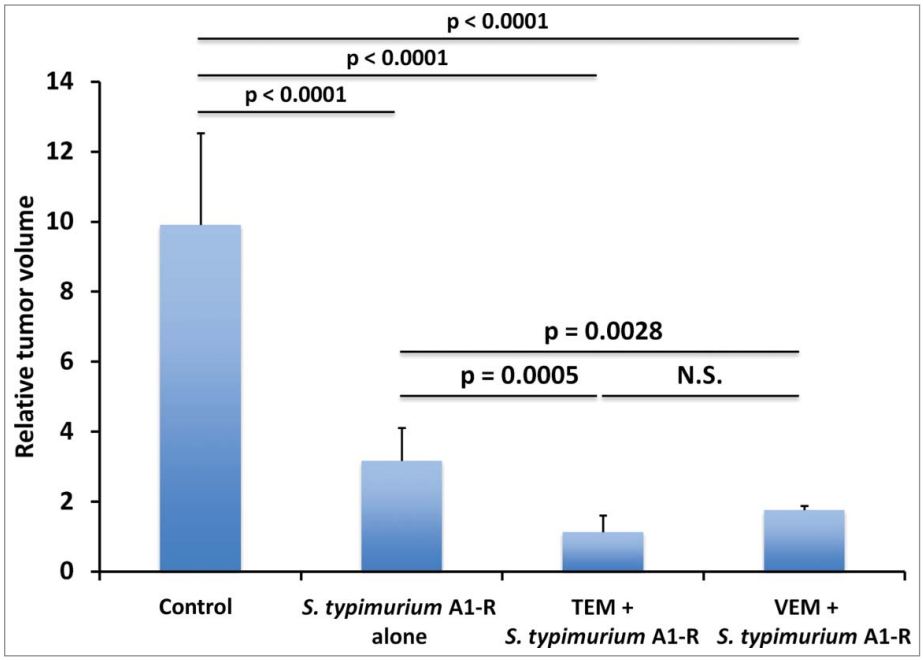

Figure 2. Relative tumor volume in the various treatment groups. Bar graph shows relative tumor volume at post-treatment point relative to the initial pre-treatment tumor volume. Error bars: ± SD.

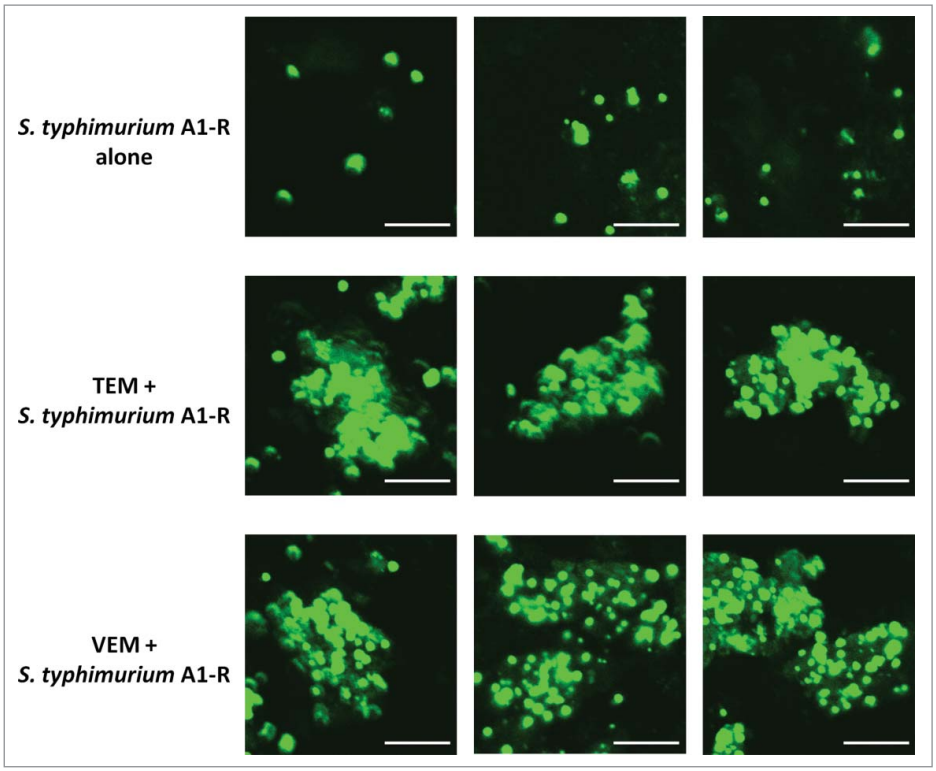

Figure 3. Fluorescence imaging of *S. typhimurium* A1-R-GFP targeting alone and in combination with chemotherapy in the melanoma PDOX. Confocal imaging with the FV1000. Scale bars: 12.5 μm.

for melanoma of *S. typhimurium* A1-R was evaluated by the *S. typhimurium* A1-R-GFP fluorescent area (Fig. 3). Both VEM and TEM significantly increased the *S. typhimurium* A1-R-GFP fluorescent area compared with *S. typhimurium* A1-R alone (TEM: $p < 0.01$; VEM: $p < 0.01$) (Fig. 4).

The histology of the original patient tumor and the untreated PDOX tumor were similar, containing the same types of cells. VEM and TEM caused extensive necrosis in the tumor when each was combined with *S. typhimurium* A1-R and much more extensive than *S. typhimurium* A1-R alone (Fig. 5).[22-24]

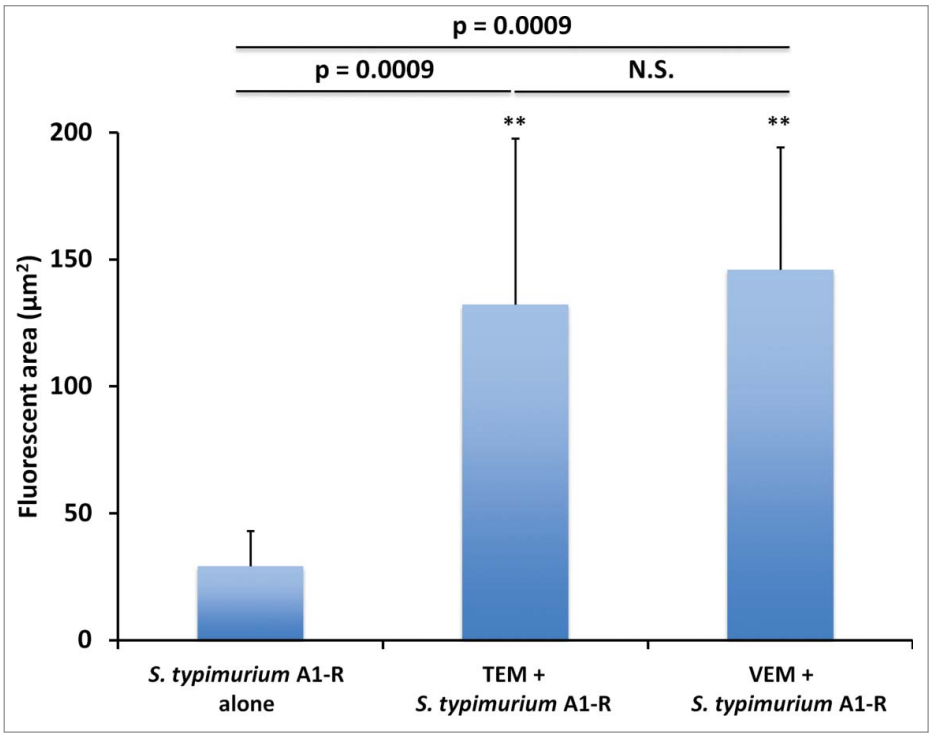

Figure 4. Quantitative tumor targeting by *S. typhimurium* A1-R-GFP alone and in combination with chemotherapy on the melanoma PDOX model. Bar graphs show *S. typhimurium* A1-R-GFP fluorescent area (μm^2) for each treatment group. Error bars: $\pm$ SD.

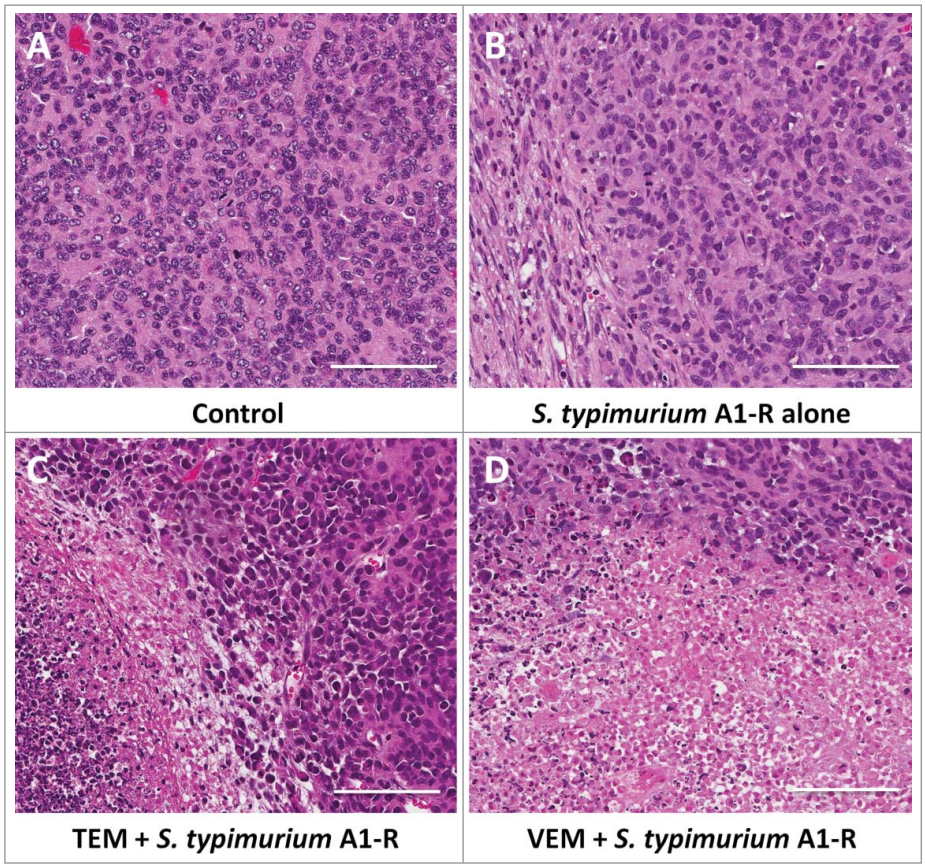

Figure 5. Histological analysis. Hematoxylin and eosin (H&E) stained slides from tumor tissue of each treatment group. A. Untreated control. B. *S. typhimurium* A1-R alone. C. *S. typhimurium* A1-R and TEM. D. *S. typhimurium* A1-R and VEM. Scale bars: 100 μm.

Salmonella typhimurium (VNP20009) has been previously used for effective therapy of a melanoma.[25-31] VNP20009 was attenuated by a lipid A-mutation (msbB), purine auxotrophy (purI) and amino acid auxotrophy. The tumor-targeting *S. typhimurium* A1-R, developed by our laboratory,[32] is auxotrophic only for Leu-Arg and is less attenuated.

We previously reported that chemotherapy combined with *S. typhimurium* A1-R was effective for the BRAF-V600E mutant melanoma PDOX.[22,24] In the present study, we showed that both VEM and TEM promoted *S. typhimurium* A1-R tumor targeting with elevated accumulation of *S. typhimurium* A1-R that led to extensive tumor necrosis. This is the first report to elucidate the mechanism by which the combination of chemotherapy with *S. typhimurium* A1-R is extremely effective.

Despite progress in melanoma therapy, there is still no cure for stage III and IV disease due to drug resistance, tumor heterogeneity and an immunosuppressive tumor environment.[33-37] In addition, the presence of melanin appears to interfere with chemotherapy and radiotherapy of this recalcitrant disease.[36] The present results suggests that *S. typhimurium* A1-R could potentiate chemotherapy for melanoma. Clinical trials are warranted for this strategy.

The present report demonstrates the enhancement of tumor-targeting of *S. typhimurium* A1-R by chemotherapy drugs. We have previously demonstrated that *S. typhimurium* A1-R can decoy quiescent cancer cells in tumors to cycle and become more sensitive to chemotherapy. Therefore, chemotherapy enhances bacterial targeting and bacterial targeting enhances chemotherapy, a very powerful mutual effect for cancer therapy.

Previously-developed concepts and strategies of highly-selective tumor targeting can take advantage of molecular targeting tumors, including tissue-selective therapy with focuses on unique differences between normal and tumor tissues.[38-43]

Conclusions

The combination of chemotherapy with *S. typhimurium* A1-R was highly effective on a chemotherapy-resistant melanoma in a PDOX mouse model. In future experiments, the powerful combination of chemotherapy and *S. typhimurium* A1-R, described in the present report, will be used to determine increased survival of melanoma PDOX models. This treatment strategy has important future clinical potential, which possibly can be realized in the near future.

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 efficacy particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All mouse surgical procedures and imaging

were performed with the animals anesthetized by subcutaneous injection of a ketamine mixture (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 if 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. 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.

Patient-derived tumor

A 75-year-old female patient was previously diagnosed with a melanoma of the right chest wall. The tumor was resected in the Department of Surgery, University of California, Los Angeles (UCLA). Written informed consent was provided by the patient, and the Institutional Review Board (IRB) of UCLA approved this experiment.[22-24]

Establishment of PDOX models of melanoma by surgical orthotopic implantation (SOI)

After nude mice were anesthetized with the ketamine solution described above, a 5-mm skin incision was made on the right chest into the chest wall, which was split to make space for the melanoma 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).[22-24]

Preparation and administration of S. typhimurium A1-R

GFP-expressing *S. typhimurium* A1-R bacteria (AntiCancer Inc.) were grown overnight on 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. *S. typhimurium* A1-R was injected intravenously. A total of 5×10^7 CFU *S. typhimurium* A1-R in 100 μl PBS was administered to each mouse.[2-4]

Treatment study design in the PDOX model of melanoma

PDOX mouse models were randomized into 4 groups of 10 mice each: untreated control (n = 10); treated with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., qw × 2, n = 10); treated with TEM (25 mg/kg, p.o., qd × 14) combined with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., qw × 2, n = 10); treated with VEM (30 mg/kg, p.o., qd × 14) combined with *S. typhimurium* A1-R (5×10^7 CFU/100 μl, i.v., qw × 2, n = 10). Tumor length and width 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. 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.

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.[44] The tumor fluorescent area was analyzed with UVP software (UVP, Upland, CA).[24,45]

Histological analysis

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 protocols. Histological examination was performed with a BHS System Microscope (Olympus Corporation, Tokyo, Japan). Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada).[22-24]

Statistical analysis

JMP version 11.0 was used for all statistical analyses. Significant differences for continuous variables were determined using the Mann-Whitney *U* test. Line graphs expressed average values and error bar showed SD. A probability value of $P \leq 0.05$ was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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