Abstract. We have developed the genetically-modified Salmonella typhimurium A1-R strain that selectively targets tumors. S. typhimurium A1-R is auxotrophic for Leu and Arg, which precludes it from growing continuously in normal tissues but allows high tumor virulence. We report here the efficacy and safety of three different routes of S. typhimurium A1-R administration: oral (p.o.), intravenous (i.v.), and intratumoral (i.t.) in nude mice with orthotopic human breast cancer. Nude mice with MDA-MB-435 human breast cancer, expressing red fluorescent protein (RFP), were administered S. typhimurium A1-R by one of the three routes: [p.o.: 2×10^8 colony forming units (CFU)/200 μl; i.v.: 2.5×10^7 CFU/100 μl; i.t.: 2.5×10^7 CFU/50 μl] twice a week. Tumor growth was monitored by fluorescence imaging and caliper measurement in two dimensions. S. typhimurium A1-R targeted tumors at much higher levels than normal organs after all three routes of administration. The fewest bacteria were detected in normal organs after p.o. administration, which suggests that p.o. administration has the highest safety. The i.v. route had the greatest antitumor efficacy. There were no obvious toxic effects on the host with any of the routes of administration. The results of this study suggest that p.o. administration was the most safe to the host and the i.v. route was most effective for tumor targeting with S. typhimurium A1-R.

There have been anecdotal reports since the first part of the 19th century that cancer patients who had bacterial infections sometimes had spontaneous regression of their tumors. At the end of the 19th century, W.B. Coley treated cancer patients with bacteria and later used bacterial extracts to treat cancer patients (Coley’s toxins). After Coley’s death in 1936, bacterial treatment fell out of favor (1).

It has been known since the 1940s that anaerobic bacteria can selectively grow in hypoxic and necrotic areas of tumors in animal models (2-17). Recently, several approaches to developing tumor-therapeutic anaerobic bacteria have been described. Yazawa et al. (15, 16) showed that the anaerobic bacterium Bifidobacterium longum was able to selectively grow in the hypoxic regions of solid tumors. Vogelstein and co-workers (18) created a strain of Clostridium novyi, depleted of its lethal toxin, and showed that i.v.-administered C. novyi spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells. Combined with conventional chemotherapy or radiotherapy, intravenous C. novyi NT spores caused extensive tumor damage within 24 h (18).

The facultative anaerobe Salmonella typhimurium (S. typhimurium) was firstly attenuated by purine and other auxotrophic mutation in order to be used for cancer therapy (13, 19, 20). These bacteria replicated in tumors >1,000-fold more, compared with normal tissues (13). Salmonella lipid A was also genetically modified by disrupting the msbB gene to reduce septic shock (13). The msbB mutant of S. typhimurium, termed VNP20009, which also has additional mutations, has been tested in a Phase 1 clinical trial to determine its safety and efficacy on metastatic melanoma (21).

We have developed a genetically modified strain of S. typhimurium, termed S. typhimurium A1-R, that targets many different cancer types. This bacterial strain targets tumors without mounting continuous infections in normal tissue. A1-R is auxotrophic (leucine-arginine-dependent) but apparently receives sufficient nutritional support from tumor tissue and not normal tissue (1, 22-36).
In the present study, in order to prepare for clinical development, we compared the efficacy and safety of three routes of *S. typhimurium* A1-R administration [oral (p.o.), intravenous (i.v.) and intra-tumoral (i.t.]) in nude mice with orthotopic human breast cancer.

**Materials and Methods**

*Cancer cells.* The MDA-MB-435 human breast cancer cell line was used. There has been controversy whether this cell line is truly breast cancer, but we have definitively shown it is breast cancer (37). For red fluorescent protein (RFP) gene transduction, 10% confluent human MDA-MB-435 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells confluent human MDA-MB-435 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells producing an RFP gene vector with a neomycin (G418) resistant gene, and RPMI-1640 for 72 h. Fresh medium was replenished at this time. MDA-MB-435 cells were harvested by trypsin-EDTA 72 h post transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/ml G418. The level of G418 was increased to 1000 μg/ml stepwise. The brightest MDA-MB-435 cell clones expressing RFP were selected, combined, and then amplified and transferred by conventional culture methods. In order to obtain dual-color cells, the histone H2B-green fluorescent protein (GFP) fusion gene was introduced to the MDA-MB-435 cells using similar methods as noted above (38, 39).

Mammary fat pad injection of MDA-MB-435-RFP cells. Twenty 6-week-old female nude mice, bred at AntiCancer Inc. (San Diego, CA, USA), were anesthetized with a 0.03 ml mixture of ketamine, acepromazine and xylazine. MDA-MB-435-RFP cells (5×10⁶/100 μl Matrigel) were slowly injected into the mammary fat pad. The needle holes were pressed in order to prevent any cancer cells overflowing and seeding at the incision site.

*Preparation of S. typhimurium A1-R.* GFP-expressing *Salmonella typhimurium* A1-R bacteria (AntiCancer Inc., San Diego, CA, USA) were grown overnight on Luria broth (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 phosphate buffered saline (PBS) (Omege Sci., San Diego, CA, USA), and then diluted in PBS (22-36).

Targeting breast cancer cells by *S. typhimurium* A1-R in vitro. Dual-color MDA-MB-435 cells, labeled with GFP in the nucleus and RFP in the cytoplasm, were grown on 24-well tissue culture plates to a density of 10⁴ cells per well. *S. typhimurium* A1-R were grown in LB and harvested at late-log phase, diluted in cell culture medium and added to the cancer cells [1×10⁵ CFU per cell]. After 1 h of incubation at 37°C, the cells were rinsed and cultured in medium containing gentamycin sulfate to kill external but not internal bacteria. The interaction between bacteria and cancer cells was observed at different time points by fluorescence microscopy using the Olympus FluoView FV1000 confocal microscope (Olympus Corp., Tokyo, Japan).

Antitumor efficacy of *S. typhimurium* A1-R administered by three different routes. Mice, orthotopically implanted with MDA-MB-435-RFP were randomized into four groups. Group 1: five mice served as untreated controls; group 2: five mice were treated p.o. with 2×10⁸ CFU *S. typhimurium* A1-R/200 μl, twice a week; group 3: five mice were treated i.v. with 2.5×10⁷ CFU *S. typhimurium* A1-R/100 μl, twice a week; group 4: five mice were treated i.t. with 2.5×10⁷ CFU *S. typhimurium* A1-R/50 μl, twice per week. The mice were sacrificed on day 34 after treatment. Tumor, liver and spleen were harvested and homogenized and supernatants were plated on nutrient media. Tissues were also prepared for standard frozen sectioning and H&E staining for histopathological analysis.

Small-animal imaging systems. The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and a DP70 CCD camera (Olympus Corp.), was used for subcellular imaging in live mice (40).

The FluoVivo imaging system (INDEC Biosystems, Santa Clara, CA, USA) was used for whole body and open imaging (41). Whole-body imaging of GFP-expressing tumors was performed once a week after GFP/RFP-visible tumors were established.

The Olympus IV100 microscope is a scanning laser microscope (42, 43) with novel stick objectives (as small as 1.3 mm) for very high resolution images, used for high resolution imaging of excised tumors. A PC running FluoView software (Olympus Corp.) was used to control the microscope. All images were recorded and stored as proprietary multilayer 16-bit tagged image file format files (42).

**Statistical analysis.** The experimental data are expressed as the mean±SD. Statistical analysis of efficacy of *S. typhimurium* A1-R on tumor growth used the two-tailed Student’s t-test, with α equal to 0.05.

**Results**

Attachment and replication of *S. typhimurium* A1-R in GFP–RFP labeled MDA-MB-435 cells in vitro. Dual-color MDA-MB-435 breast cancer cells were grown in 24-well tissue culture plates with *S. typhimurium* A1-R and then cultured in medium containing gentamycin sulfate to kill external but not internal bacteria. Fluorescence microscopy showed that *S. typhimurium* A1-R attached and invaded MDA-MB-435 cells in vitro within one hour after application (Figure 1B). A1-R replication inside the MDA-MB-435 cells was visualized by 12 h after infection (Figure 1C).

Antitumor efficacy of *S. typhimurium* A1-R delivered via three different routes on MDA-MB-435-RFP human breast cancer growing orthotopically in nude mice. The earliest efficacy was observed in the i.v.-treated group with significant reduction of tumor being found on day 6 after A1-R infection (*p=0.021*). In contrast, significant reduction of tumor in the i.t.-treated mice was found on day 13 after A1-R infection (*p=0.048*), and in the p.o.-treated mice, no significant efficacy was observed on day 16 after A1-R infection (*p=0.127*) (Figure 2).

Distribution of *S. typhimurium* A1-R in different organs after three different routes of administration. *S. typhimurium* A1-R was detected in tumor tissue after administration via all three routes (Figure 3). At 24 h after infection with A1-R, bacterial overflowing and seeding at the incision site.
distribution in tumors was much higher than in spleen and liver in all three routes of infection (Figure 3). Higher levels of A1-R in the tumor in the \textit{i.v.}-treated and \textit{i.t.}-treated groups than in the \textit{p.o.}-treated group were observed. The \textit{p.o.}-treated mice had the lowest bacterial distribution in the liver and spleen. A1-R replicated and survived longer in the tumor than in normal organs after all three of routes of administration (Figures 4, 5). A1-R distribution in normal organs decreased and had almost disappeared by two weeks (Figure 4). By day 34, only a few bacteria were observed in normal tissue after \textit{i.v.} and \textit{i.t.} treatment and no bacteria were found in normal organs after oral treatment in mice (Figure 5).
Discussion

The three routes of delivery of A1-R all showed high tumor-targeting potential. The tumors in the i.v.-treated and i.t.-treated group were earlier responders to A1-R than the p.o.-treated group. Salmonella administered p.o. needs to overcome a series of barriers, such as killing by stomach acid and bactericidal conditions in the small intestine, as well as translocation through the intestine into the general circulation.

All three routes of administration of A1-R delayed tumor growth. Administration of A1-R by i.v. was the most effective route for antitumor efficacy. Administration by i.t. was almost as effective as i.v. Administration by p.o. had the most delayed efficacy but is the safest in terms of the lowest growth in normal organs.

Jia et al. (44) showed significant anticancer efficacy of p.o.-administered VNP20009 to immunocompetent mice with subcutaneous B16F10 melanoma and Lewis lung carcinoma. The results of Jia et al. (44) and the present report suggest that p.o. administration of S. typhimurium A1-R should be further investigated. The prospect of a bacterial tablet for cancer therapy is very intriguing.

Figure 3. Distribution of GFP-labeled A1-R bacteria in tumor and organs at day 1 after three different routes of bacterial administration: (p.o.) (A-C); (i.v.) (D-F); and (i.t.) (G-I). Tissues were removed 24 h after A1-R administration from nude mice with MDA-MB-435 tumors. Bacteria were isolated from the tumor and organs and cultured in LB agar. These results show that A1-R was able to selectively target the MDA-MB-435-RFP tumor in vivo. The number of bacteria in the tumor is much higher than that in the normal organs.
Figure 4. Quantification of S. typhimurium A1-R in tumors and organs over time after three different routes of administration: (p.o.) (A-C); (i.v.) (D-F); and (i.t.) (G-I). The tumors and organs were removed from the mice at various time points after A1-R bacterial administration by three different routes. Bacteria were isolated from the organs and cultured for colony forming units (CFU) quantification. The number of bacteria decreased in all normal organs over time. In contrast, the bacteria grew continuously in the MDA-MB-435 tumors.
**Conflict of Interest**

None of the Authors have a conflict of interest in regard to this study.

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