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Tumor-targeting *Salmonella typhimurium* A1-R decoys quiescent cancer cells to cycle as visualized by FUCCI imaging and become sensitive to chemotherapy

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Abbreviations: FUCCI, fluorescence ubiquitination-based cell cycle indicator; *S. typhimurium*, *Salmonella typhimurium*.

Quiescent cancer cells are resistant to cytotoxic agents which target only proliferating cancer cells. Time-lapse imaging demonstrated that tumor-targeting *Salmonella typhimurium* A1-R (A1-R) decoyed cancer cells in monolayer culture and in tumor spheres to cycle from G_0/G_1 to $S/G_2/M$, as demonstrated by fluorescence ubiquitination-based cell cycle indicator (FUCCI) imaging. A1-R infection of FUCCI-expressing subcutaneous tumors growing in nude mice also decoyed quiescent cancer cells, which were the majority of the cells in the tumors, to cycle from G_0/G_1 to $S/G_2/M$, thereby making them sensitive to cytotoxic agents. The combination of A1-R and cisplatinum or paclitaxel reduced tumor size compared with A1-R monotherapy or cisplatinum or paclitaxel alone. The results of this study demonstrate that A1-R can decoy quiescent cancer cells to cycle to $S/G_2/M$ and sensitize them to cytotoxic chemotherapy. These results suggest a new paradigm of bacterial-decoy chemotherapy of cancer.

Introduction

The phase of the cell cycle can determine whether a cancer cell can respond to a given drug. Monitoring of real-time cell cycle dynamics of cancer cells throughout a live tumor intravitally using a fluorescence ubiquitination-based cell cycle indicator (FUCCI),[1] we previously demonstrated approximately 90% of cancer cells in the center and 80% of total cells of an established tumor are in G_0/G_1 phase. Longitudinal real-time imaging demonstrated that cytotoxic agents killed only proliferating cancer cells at the surface or near blood vessels and, in contrast, had little effect on quiescent cancer cells.[2]

With FUCCI imaging, we also previously observed that cancer cells in G_0/G_1 phase in Gelfoam histoculture migrated more rapidly and further than cancer cells in $S/G_2/M$ phases. Cancer cells ceased migrating when they entered $S/G_2/M$ phases and restarted migrating after cell division when the cells re-entered G_0/G_1. Migrating cancer cells also were resistant to cytotoxic chemotherapy, since they were preponderantly in G_0/G_1.[3]

The OBP-301 telomerase-dependent adenovirus decoyed quiescent cancer cells to $S/G_2/M$ phases where they became chemosensitive in tumors in vivo and tumor spheres in vivo, visualized with FUCCI imaging.[4]

Records for >200 y have documented cancer patients going into remission after a bacterial infection.[5] In the late 19th century and early 20th century, William B. Coley at New York Cancer Hospital, the precursor of Sloan-Kettering Memorial Cancer Center, treated cancer patients with *Streptococcus pyogenes*.[6]

S. typhimurium, is a facultative anaerobe which confers important advantages, compared to obligate anaerobes, in that a facultative anaerobe can grow in the oxic viable region of tumors as well as necrotic regions.[7] Attenuated auxotrophic mutants of *S. typhimurium* retained their tumor-targeting capabilities.[8]

In a Phase I clinical trial on patients with metastatic melanoma and renal carcinoma, the *S. typhimurium* strain tested (VNP20009), attenuated by msbB, amino-acid, and purI mutations, was safely administered to patients, but did not sufficiently colonize the patients' tumors, perhaps because this strain was overattenuated.[9]

The *S. typhimurium* A1-R strain developed by our laboratory has high tumor colonization efficacy and antitumor efficacy. *S. typhimurium* A1-R is auxotrophic for Leu-Arg, which prevents

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it from mounting a continuous infection in normal tissues. *S. typhimurium* A1-R has no other apparent attenuating mutations in contrast to VNP20009 and, therefore, has very high tumor-targeting capability. *S. typhimurium* A1-R was able to eradicate primary and metastatic tumors as monotherapy in nude mouse models of prostate,[10,11] breast,[12] lung,[13,14] pancreatic[15,16] and ovarian[17] cancers, as well as sarcoma[18,19] and glioma,[20] all of which are highly aggressive tumor models. *S. typhimurium* A1-R also targeted pancreatic cancer stem-like cells[21] and pancreatic cancer patient-like orthotopic xenograft (PDOX) models.[22]

In the present report, we demonstrate that *S. typhimurium* A1-R can decoy quiescent G_0/G_1 cancer cells to cycle to $S/G_2/M$ and become chemosensitive.

Results and Discussion

S. typhimurium A1-R stimulates cell cycle transit of quiescent cancer cells in monolayer culture

Time-lapse imaging of *S. typhimurium* A1-R interacting with quiescent FUCCI-expressing MKN45 cancer cells in monolayer culture demonstrated that *S. typhimurium* A1-R targets quiescent cancer cells and induces their cell cycle transit from G_0/G_1 to $S/G_2/M$ phase (**Fig. 1**). Before *S. typhimurium* A1-R treatment, approximately 95% of the cancer cells were in G_0/G_1 (**Fig. 1**). After *S. typhimurium* A1-R treatment, the percentage of cancer cells in G_0/G_1 was reduced to less than 40% with approximately 60% in $S/G_2/M$.

S. typhimurium A1-R stimulates cell cycle transit in quiescent tumor spheres

Time-lapse imaging of quiescent FUCCI-expressing MKN45 tumor spheres on agar demonstrated that *S. typhimurium* A1-R targeted quiescent tumor spheres and stimulated cell cycle transit, of the cancer cells within the spheres, from G_0/G_1 to $S/G_2/M$ phases (**Fig. 2**). Before *S. typhimurium* A1-R treatment, approximately 95% of the cancer cells were in G_0/G_1. After *S. typhimurium* A1-R treatment, approximately 30% of the cancer cells were in G_0/G_1 and 70% in $S/G_2/M$ (**Fig. 2**).

S. typhimurium A1-R mobilizes the cell cycle transit of quiescent cancer cells in tumors in vivo

Before *S. typhimurium* A1-R treatment, FUCCI-expressing MKN45 tumors had approximately 95% of the cancer cells in G_0/G_1 after 35 d growth in nude mice. Thirty-five d after treatment with *S. typhimurium* A1-R, approximately 30% of the cancer cells were in G_0/G_1 and 70% in $S/G_2/M$ (**Fig. 3**).

S. typhimurium-decoyed tumors became sensitive to chemotherapy

FUCCI-expressing MKN45 cells were injected subcutaneously into the left flanks of mice. When the subcutaneous tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered *S. typhimurium* A1-R (iv) alone, or in combination with cisplatinum (4 mg/kg) or in combination with paclitaxel (5 mg/kg, ip) for 5 cycles every 3 d *S. typhimurium* A1-R sensitized the tumors to chemotherapy due to cell-cycle decoy of the cancer cells within the tumor (**Fig. 4**). Cisplatinum or paclitaxel alone had only modest growth inhibition on the MKN45 tumor. *S. typhimurium* had a larger growth inhibition effect than the chemotherapy drugs. The greatest effect was the combination of by *S. typhimurium* A1-R with either of the chemotherapy drugs (**Fig. 4**).

FUCCI cell cycle imaging showed that in tumors treated with cisplatinum or paclitaxel, the percentage of cancer cells in G_0/G_1 increased to over 95% from approximately 80% before treatment. In contrast, *S. typhimurium* treatment reduced the percentage of cancer cells in G_0/G_1 to approximately 30%. The combination of *S. typhimurium* A1-R and chemotherapy decreased the percentage of cancer cells in G_0/G_1 to 15% or less. The percentage of $S/G_2/M$ cells in tumors treated in combination with *S. typhimurium* A1-R and either cisplatin or paclitaxel approached 90% (**Fig. 4**).

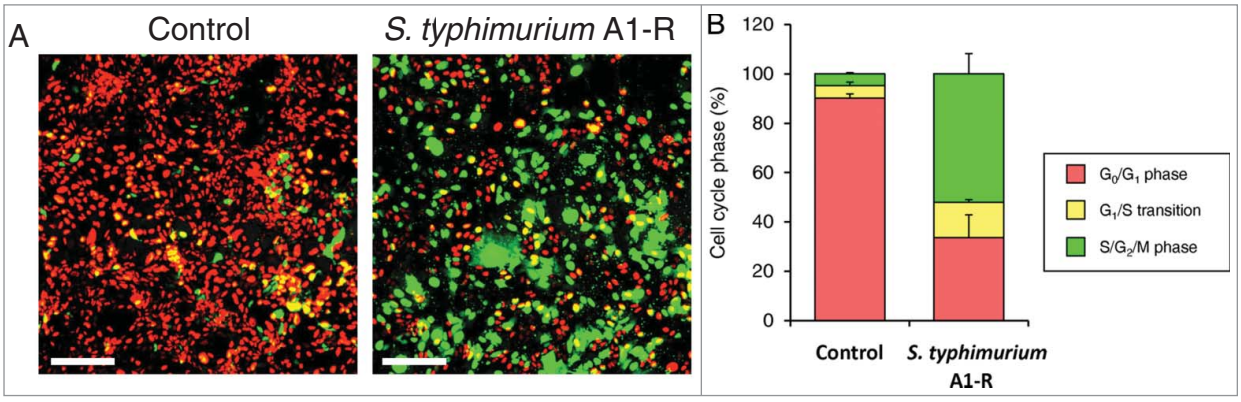

Figure 1. *S. typhimurium* A1-R stimulates cell cycle transit of quiescent cancer cells in monolayer culture. *S. typhimurium* A1-R targeted quiescent cancer cells and stimulates cell cycle transit from G_0/G_1 to $S/G_2/M$ phases. (**A**) Representative images of control cancer cells and cancer cells treated with *S. typhimurium* A1-R. (**B**) Histogram shows cell cycle distribution in control and *S. typhimuriam* A1-R-treated cultures. Scale bar: 500 μm.

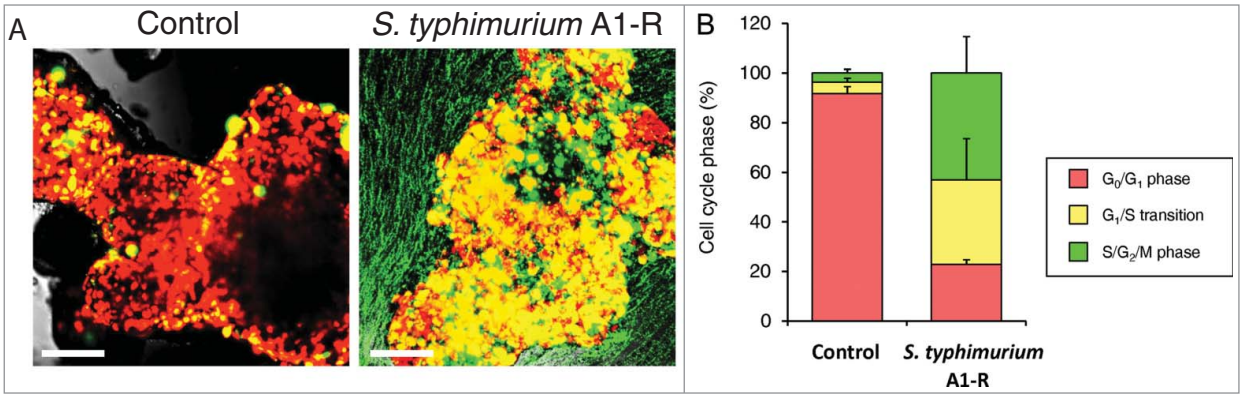

Figure 2. *S. typhimurium* A1-R stimulates cell cycle transit in quiescent tumor spheres in vitro. *S. typhimurium* A1-R stimulated cell cycle transit from G_0/G_1 to $S/G_2/M$ phase. (**A**) Representative images of control tumor spheres and tumor spheres treated with *S. typhimurium* A1-R. (**B**) Histogram shows cell cycle distribution in control and *S. typhimurium* A1-R-treated tumor spheres. Scale bar: 500 μm.

FUCCI imaging demonstrated that the combination of *S. typhimurium* A1-R decoy therapy and chemotherapy can effectively kill quiescent cancer cells that are resistant to conventional chemotherapy. The combination of *S. typhimurium* A1-R and either cisplatinum or paclitaxel decoyed almost all the cancer cells to cycle, greatly enhancing their sensitivity.

We previously compared the cell cycle dynamics of invading and non-invading cancer cells in 3-dimensional Gelfoam histoculture, where cancer cells have in vivo-like behavior. We demonstrated with FUCCI imaging that cancer cells in G_0/G_1 phase can migrate faster and further than cancer cells in $S/G_2/M$ phases. When cancer cells in G_0/G_1 cycled into $S/G_2/M$ phases, they ceased movement and then only restarted migration after re-entry into G_0/G_1 phase after cell division. Chemotherapy had little effect on G_0/G_1 invading cancer cells. Decoy chemotherapy may also be useful to target invasive cancer cells, which may otherwise be highly chemoresistant.[3]

We previously showed with FUCCI imaging that the vast majority of cancer cells in a tumor was in G_0/G_1. We demonstrated that cytotoxic chemotherapy kills only cancer cells in $S/G_2/M$ phases, which are in a minority in an established tumor, and had little effect on cancer cells in G_0/G_1 phase. Moreover, we showed the efficacy of chemotherapy depends not on tumor size, but the cell cycle phase of each cancer cell, which depends on the location in the tumor. We spatially and temporally demonstrated the cell cycle dynamics of individual cancer cells during tumor growth before, as well as during and after treatment with cytotoxic agents, within the same tumors. Our results explained why temporary regression may be often seen in the clinic after chemotherapy, as the drugs are effective only on cells in the outer layer of the tumor or near blood vessels, where cancer cells proliferate. Recurrence takes place when some of the quiescent cells reenter the cell cycle as they replace the cycling cells killed by chemotherapy at the surface or near blood vessels.[2]

We previously demonstrated, using FUCCI imaging, that a genetically-engineered telomerase-specific adenovirus, OBP-301, could decoy the cell cycle of cancer cells in tumor spheres and tumors thereby sensitizing them to chemotherapy.[4]

The present study demonstrated that *S. typhimurium* A1-R can decoy the cell-cycle transit of quiescent cancer cells and sensitize the cancer cells to chemotherapy.

Previously developed concepts and strategies of highly selective tumor-targeting[23-34] can take advantage of spatial–temporal cell cycle imaging of a tumor described in the present report.

Future studies will focus on optimizing decoy chemotherapy with *S. typhimurium* A1-R and to screen for other decoy agents. Decoy chemotherapy is a promising approach to overcome the problem that the majority of cancer cells in most tumors are quiescent and are thereby chemoresistant.

Materials and Methods

FUCCI (Fluorescence ubiquitination cell cycle indicater)

The FUCCI probe was generated by fusing mKO2 (monomeric kusabira orange2) and mAG (monomeric azami green) to the ubiquitination domains of human Cdt1 and geminin, respectively. These 2 chimeric proteins, mKO2-hCdt1and mAG-hGem, accumulate reciprocally in the nuclei of transfected cells during the cell cycle, labeling the nuclei of G_1 phase cells orange and nuclei of cells in $S/G_2/M$ phase green.[1] Plasmids expressing mKO2-hCdt1 (green fluorescent protein) or mAG-hGem (orange fluorescent protein) were obtained from the Medical and Biological Laboratory. Plasmids expressing mKO2-hCdt1 were transfected into MKN45 cells using Lipofectamine™ LTX (Invitrogen). The cells were incubated for 48 h after transfection and were then trypsinized and seeded in 96-well plates at a density of 10 cells/well. In the first step, cells were sorted into green (S, G_2, and M phase) cells using a FACSAria cell sorter (Becton Dickinson). The first-step-sorted green-fluorescent cells were then re-transfected with mAG-hGem (orange) and then sorted by orange fluorescence.[4]

Cells

MKN45 is a radio-resistant poorly differentiated stomach adenocarcinoma cell line derived from a liver metastasis of a patient.[4]

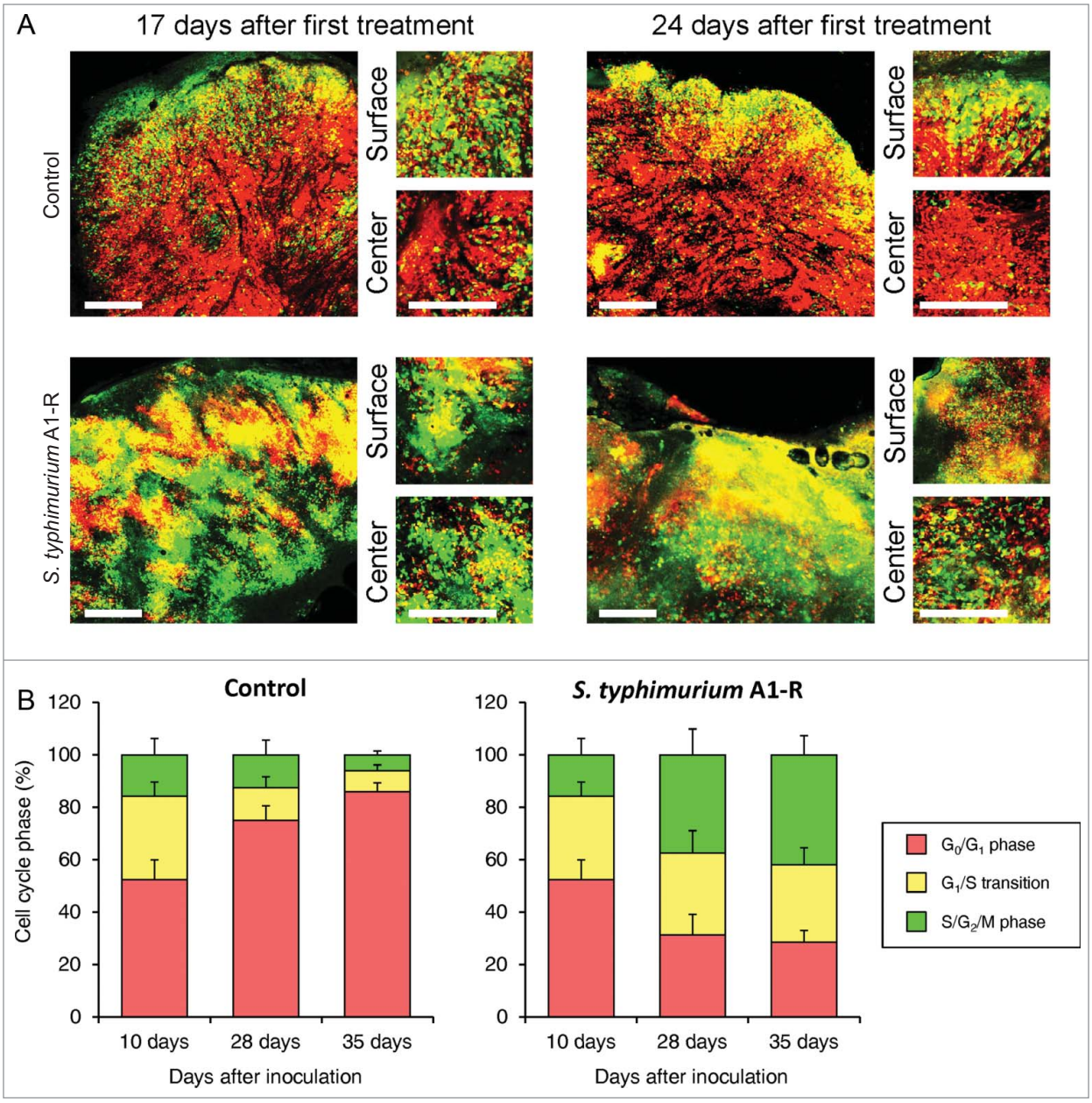

Figure 3. *S. typhimurium* A1-R mobilizes the cell cycle transit of quiescent cancer cells in tumors in vivo. (**A**) Representative images of cross sections of FUCCI-expressing MKN45 tumor xenografts treated with *S. typhimurium* A1-R or untreated control. (**B**) Histograms show the cell cycle phase distribution of FUCCI-expressing cells within the tumor treated with *S. typhimurium* A1-R or untreated control. Scale bars: 500 μm.

Animal experiments

Athymic nu/nu nude mice (AntiCancer, Inc.) were maintained in a barrier facility under HEPA filtration and fed with autoclaved laboratory rodent diet (Teklad LM-485; Harlan). All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873–1.

Tumor model

All animal procedures were performed under anesthesia using s.c. administration of a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl PBS) (Henry-Schein). FUCCI-expressing MKN45 cells were harvested from monolayer culture by brief trypsinization. Single-cell suspensions were prepared at a final concentration of 5×10^6 cells and injected subcutaneously in the left flank of nude mice.

Decoy chemotherapy

When the tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered iv *S. typhimurium* A1-R, alone or in combination with cisplatinum (4 mg/kg ip) or paclitaxel (5 mg/kg ip) for 5 cycles every 3 d.

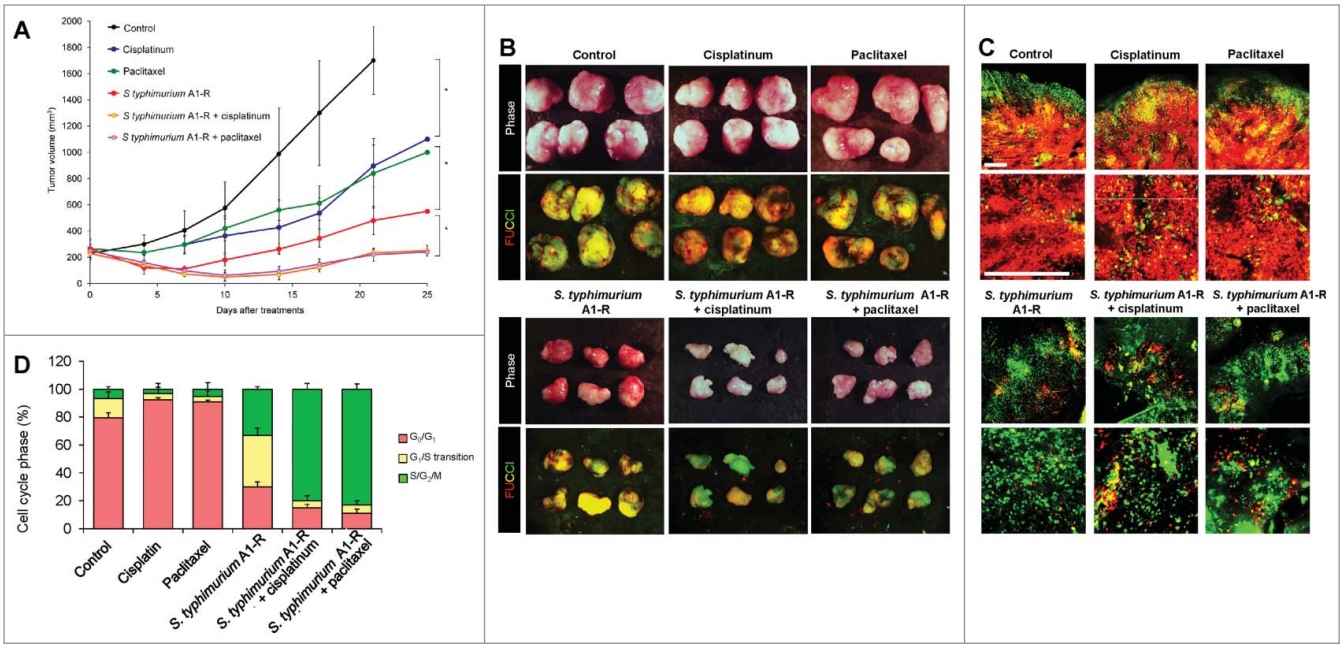

Figure 4. *S. typhimurium* A1-R-decoyed tumors became sensitive to chemotherapy. FUCCI-expressing MKN45 cells (5×10^6 cells/mouse) were injected subcutaneously into the left flank of nude mouse. When the tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered iv *S. typhimurium* A1-R alone, or with cisplatinum (4 mg/kg ip) or paclitaxel (5 mg/kg ip) for 5 cycles every 3 d. (**A**) Growth curves of tumors derived from FUCCI-expressing MKN45 cells after treatment with chemotherapy, *S. typhimurium* A1-R or in combination with *S. typhimurium* A1-R and chemotherapy. The difference between control and cisplatinum-treated: $P < 0.01$; the difference between control and paclitaxel-treated: $P < 0.05$; the difference between control and *S. typhimurium* A1-R: $P < 0.05$; the difference between control and the combination of *S. typhimurium* A1-R and cisplatinum: $P < 0.01$; the difference between control and the combination of *S. typhimurium* A1-R and paclitaxel: $P < 0.01$. (**B**) Macroscopic photographs of FUCCI-expressing tumors, untreated control, *S. typhimurium* A1-R-treated, cisplatinum-treated, paclitaxel-treated, or treated with the combination of *S. typhimurium* A1-R and either cisplatinum or paclitaxel (right). Scale bars, 10 mm. (**C**) Representative images of cross-sections of FUCCI-expressing MKN45 subcutaneous tumors, untreated control: *S. typhimurium* A1-R-treated, cisplatinum-treated, paclitaxel-treated, or treated with the combination of *S. typhimurium* A1-R and either cisplainum or paclitaxel. (**D**) Histogram shows cell cycle phase of FUCCI-expressing MKN45 subcutaneous tumors, including untreated control, *S. typhimurium* A1-R-treated, cisplatinum-treated, paclitaxel-treated, or treated with the combination of *S. typhimurium* A1-R and either cisplatinum or paclitaxel. Scale bars: 500 μm.

Statistical analysis

Data are shown as means ± SD. For comparison between 2 groups, significant differences were determined using the Student t test. For comparison of more than 2 groups, statistical significance was determined with a one-way ANOVA followed by a Bonferroni multiple-group comparison test. $P < 0.05$ was considered significant.

Competing Financial Interests

Y.Z. and M.Z. are employees of AntiCancer Inc. SY, YH, SM, FU, HK, and RMH are or were unsalaried associates of AntiCancer Inc. There are no other competing financial interests.

Authors Contributions

SY and RMH conceived the idea for this project. SY and RMH designed all experiments and wrote the manuscript. SY, YZ, YH, SM, and FU performed all experiments. HK, HT, MZ, MB, and TF provided crucial ideas and helped with data interpretation. YZ and HT provided special technical assistance.

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Dedication

This paper is dedicated to the memory of AR Moossa, MD.

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