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REPORT

Tumor-specific cell-cycle decoy by *Salmonella typhimurium* A1-R combined with tumor-selective cell-cycle trap by methioninase overcome tumor intrinsic chemoresistance as visualized by FUCCI imaging

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ABSTRACT

We previously reported real-time monitoring of cell cycle dynamics of cancer cells throughout a live tumor intravitally using a fluorescence ubiquitination cell cycle indicator (FUCCI). 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 FUCCI imaging demonstrated that cytotoxic agents killed only proliferating cancer cells at the surface and, in contrast, and had little effect on the quiescent cancer cells. Resistant quiescent cancer cells restarted cycling after the cessation of chemotherapy. Thus cytotoxic chemotherapy which targets cells in $S/G_2/M$, is mostly ineffective on solid tumors, but causes toxic side effects on tissues with high fractions of cycling cells, such as hair follicles, bone marrow and the intestinal lining. We have termed this phenomenon tumor intrinsic chemoresistance (TIC). We previously demonstrated that tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) decoyed quiescent cancer cells in tumors to cycle from G_0/G_1 to $S/G_2/M$ demonstrated by FUCCI imaging. We have also previously shown that when cancer cells were treated with recombinant methioninase (rMETase), the cancer cells were selectively trapped in S/G_2, shown by cell sorting as well as by FUCCI. In the present study, we show that sequential treatment of FUCCI-expressing stomach cancer MKN45 in vivo with *S. typhimurium* A1-R to decoy quiescent cancer cells to cycle, with subsequent rMETase to selectively trap the decoyed cancer cells in S/G_2 phase, followed by cisplatinum (CDDP) or paclitaxel (PTX) chemotherapy to kill the decoyed and trapped cancer cells completely prevented or regressed tumor growth. These results demonstrate the effectiveness of the praradigm of "decoy, trap and shoot" chemotherapy.

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cancer; cell-cycle; cisplatinum; decoy; FUCCI; methioninase; nude mice; paclitaxel; *Salmonella typhimurium* A1-R; stomach cancer; trap

Introduction

We previously reported intravitally monitoring of real-time cell cycle dynamics of cancer cells throughout a live tumor using a fluorescence ubiquitination cell cycle indicator (FUCCI) .[1,2] In a mature tumor, 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 FUCCI imaging demonstrated that cytotoxic agents killed only proliferating cancer cells at the surface and, in contrast, had little effect on quiescent cancer cells, the vast majority of an established tumor. Resistant quiescent cancer cells restarted cycling after the cessation of chemotherapy. We have termed this phenomenon tumor intrinsic chemoresistance (TIC).[1]

We previously developed the tumor-targeting bacterial strain *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R).[3] *S. typhimurium* A1-R is auxotrophic for Leu—Arg, which prevents it from mounting a continuous infection in normal tissues. *S. typhimurium* A1-R was able to inhibit primary and metastatic tumor growth as monotherapy in mouse models of major cancers,[4] including prostate,[5,6] breast,[7-9] lung,[10,11] pancreatic,[12-16] ovarian,[17,18] stomach,[19] and cervical cancer,[20] as well as sarcoma cell lines[21-24] and glioma,[25,26] as well as on pancreatic cancer[15] and sarcoma[24] patient-derived orthotopic xenograft (PDOX) models, all of which are highly aggressive tumor models.

Time-lapse FUCCI imaging demonstrated that tumor-targeting *S. typhimurium* A1-R decoyed quiescent cancer cells in tumors growing in nude mice to cycle from G_0/G_1 to $S/G_2/M$, thereby acquiring chemosensitivity.[19]

We previously demonstrated a selective growth arrest of cancer cells by depletion of their source of methionine in vitro. This growth arrest resulted in a reduction in the percentage of mitotic cells. Fluorescence-activated cell sorting demonstrated that the cells were arrested in the S and G_2 phases of the cell cycle.[27] Methionine depletion of co-cultures of cancer and normal cells enabled the selective elimination of the cancer cells by chemotherapy drugs.[28]

Subsequently we induced the tumor-specific cell cycle block in S/G_2 in vivo by depriving Yoshida sarcoma-bearing nude

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mice of dietary methionine. Methionine depletion caused the tumor to eventually regress.[29]

Cancer cells treated with recombinant methioninase (rMETase), were also selectively trapped in S/G_2 as visualized with FUCCI imaging. rMETase-induced S/G_2-phase blockage and sensitized the cancer cells to doxorubicin (DOX), cisplatinum (CDDP), or 5-fluorouracil (5-FU).[30] Cancer cells may be generally methinoine dependent compared to normal cells.[31-33]

In the present study, we show that sequential treatment of FUCCI-expressing MKN45 human stomach cancer in vivo with *S. typhimurium* A1-R to decoy quiescent cancer cells to cycle; rMETase to selectively trap the decoyed cancer cells in S/G_2 phase; and CDDP or paclitaxel (PTX), completely prevented or regressed tumor growth, demonstrating the effectiveness of the paradigm of "decoy, trap and shoot" chemotherapy.

Results and discussion

S. typhimurium *A1-R decoys quiescent cancer cells to cycle visualized by FUCCI imaging*

S. typhimurium A1-R treatment significantly decoyed HeLa-FUCCI cells in monolayer culture to cycle from G_0/G_1 to S/G_2 phase (*S. typhimurium* A1-R treatment vs control: 62.3% vs 25.9% in S/G_2, respectively, $p < 0.01$) (Figs. 1A and 1B). In tumor spheres, *S. typhimurium* A1-R treatment significantly decoyed MKN45-FUCCI cells to cycle to S/G_2 phase (*S. typhimurium* A1-R treatment vs control: 62.5 % vs 6.3% in S/G_2, respectively, $p < 0.01$) (Figs. 1C and 1D). *S. typhimurium* A1-R significantly decoyed MKN45-FUCCI cells in tumors in vivo to cycle to late-S/G_2 phase (*S. typhimurium* A1-R treatment vs control; 62.6 % vs 24.6% in S/G_2, respectively, $p < 0.01$) (Figs. 1E and 1F).

Recombinant methionine (rMETase) trap of cancer cells in S/G_2 visualized by FUCCI imaging

Control HeLa cells in vitro continue to divide. In contrast, rMETase trapped HeLa-FUCCI cells in S/G_2 phase before cell division (Fig. 2A). rMETase continued to trap HeLa-FUCCI cells in S/G_2 phase over time without entry into mitosis (Fig. 2B).

Decoy, trap and shoot chemotherapy with CDDP

MKN45 tumor-bearing mice were treated with CDDP; or *S. typhimurium* A1-R; or *S. typhimurium* A1-R and CDDP

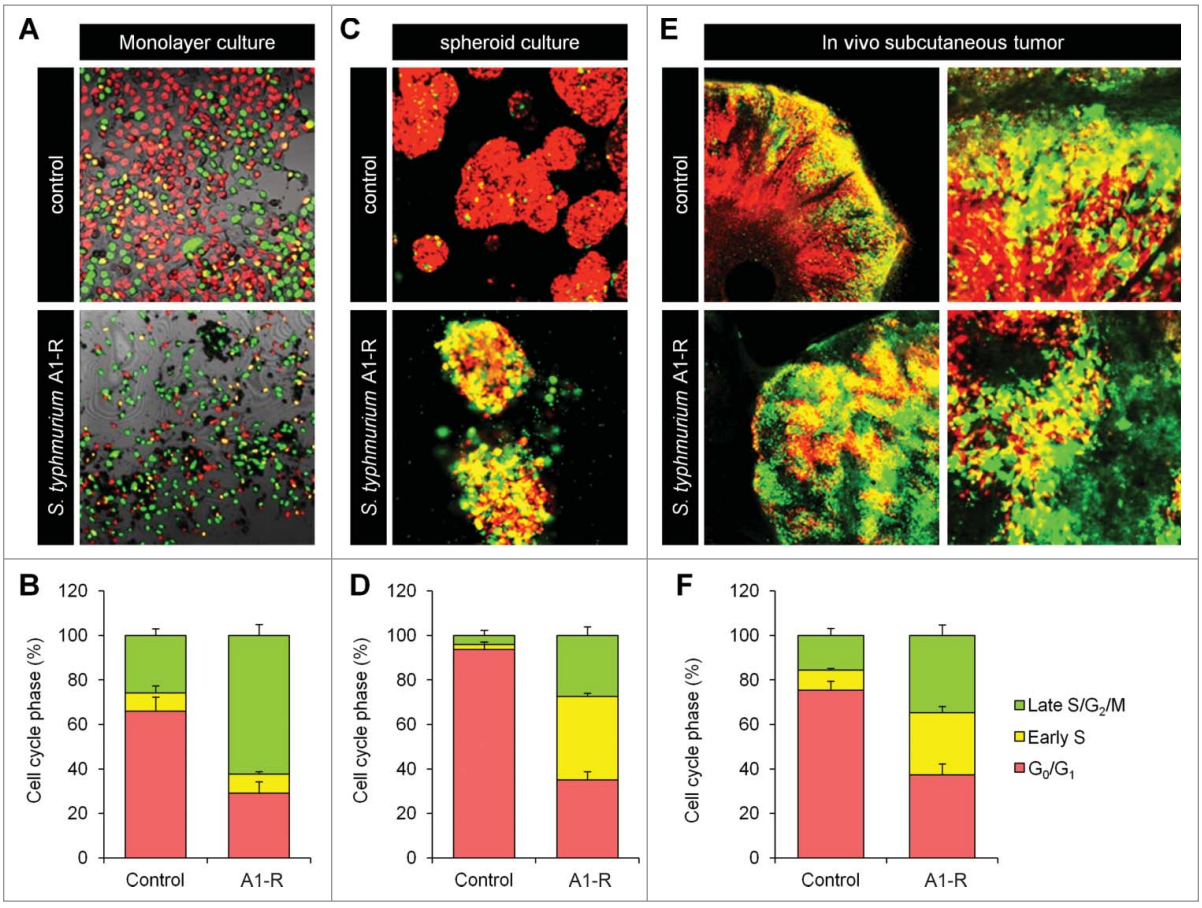

Figure 1. *S. typhimurium* A1-(R) decoyed quiescent cancer cells to cycle. *S. typhimurium* A1-R targeted quiescent cancer cells and decoyed cell cycle transit from G_0/G_1 to S/G_2/M phases. (A) Representative images of control HeLa-FUCCI cancer cells and HeLa-FUCCI cancer cells in monolayer culture treated with *S. typhimurium* A1-R. (B) Histogram shows cell cycle distribution in control and *S. typhimurium* A1-R-treated cultures. Scale bar: 500 mm. (C) *S. typhimurium* A1-R stimulated cell-cycle transit from G_0/G_1 to S/G_2 phase in quiescent tumor spheres formed from MKN45-FUCCI cells in vitro. Representative images of control tumor spheres and and tumor spheres treated with *S. typhimurium* A1-R. (D) Histogram shows cell-cycle distribution in control and *S. typhimurium* A1-R-treated tumor spheres. (E) *S. typhimurium* A1-R decoyed the cell-cycle transit of quiescent cancer cells in MKN45-FUCCI tumors in vivo. Representative images of cross sections of FUCCI-expressing MKN45 tumor xenografts treated with *S. typhimurium* A1-R or untreated control. (F) Histograms show the cell-cycle phase distribution of FUCCI-expressing cells within the tumors treated with *S. typhimurium* A1-R or untreated control. The cells in G_0/G_1, S, or G_2/M phases appear red, yellow, or green, respectively.

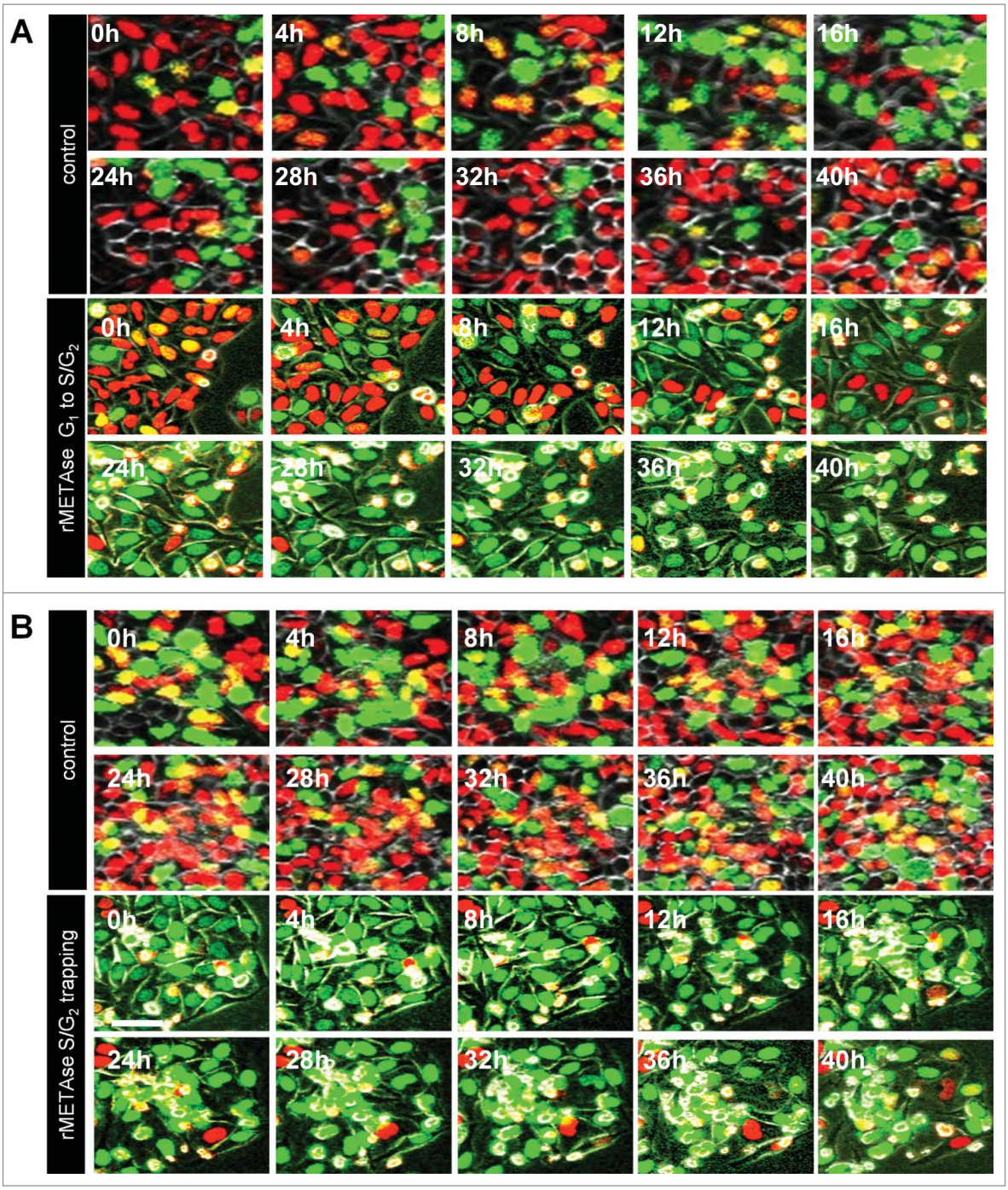

Figure 2. rMETase traps cancer cells in S/G_2 phase. Time-course imaging of HeLa-FUCCI cells treated with rMETase. After seeding on 35 mm glass dishes and culture overnight, HeLa-FUCCI cells were treated with rMETase at a dose of 1.0 unit/ml. (A) Kinetics of rMETase trapping of cells in S/G_2. (B) Maintenance of rMETase trap in S/G_2 over time. All images were acquired with the FV1000 confocal microscope (Olympus, Tokyo, Japan).[81] The cells in G_0/G_1, S, or G_2/M phases appear red, yellow, or green, respectively.

or *S. typhimurium* A1-R, rMETase and CDDP. CDDP inhibited tumor growth ($p < 0.01$). *S. typhimurium* A1-R inhibited tumor growth more than CDDP ($p < 0.01$). *S. typhimurium* A1-R and CDDP combined had a greater inhibition of tumor growth ($p < 0.01$). The sequential combination of *S. typhimurium* A1-R, rMETase and CDDP prevented or regressed tumor growth more than *S. typhimurium* A1-R or CDDP alone or the combination of these two agents ($p < 0.01$) (Fig. 3).

rMETase induces mitotic catastrophe after late-S/G_2 trap visualized by FUCCI imaging

HeLa-FUCCI cells were treated with rMETase for more than 80 hours. HeLa-FUCCI cells trapped in late-S/G_2 phase did not divide and their nuclei turned red, after which they died (Figs. 4A and 4B). These results showed that methionine was indispensable for cell division, and therefore rMETase induced mitotic catastrophe.[34-36]

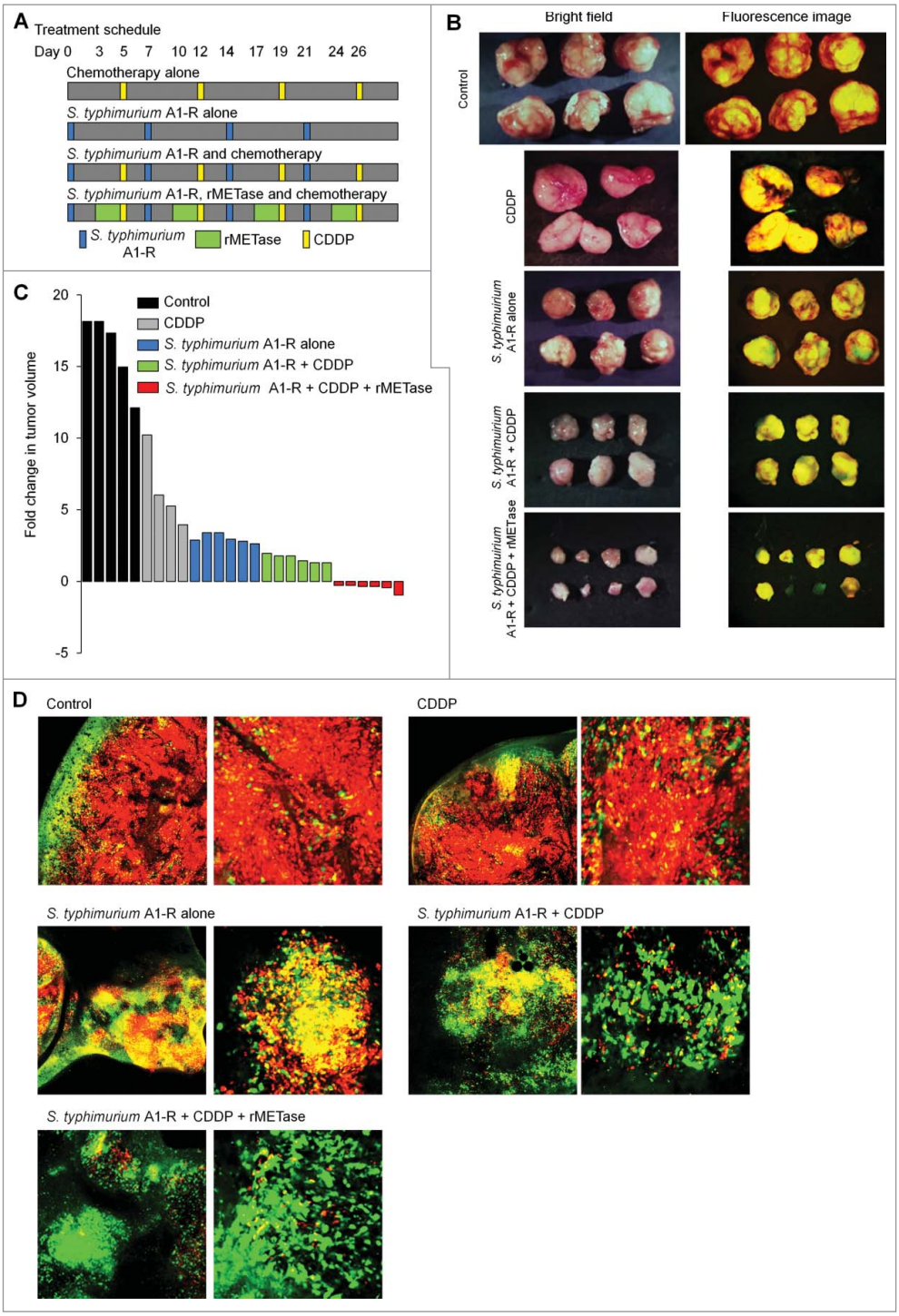

Figure 3. Decoy, trap and shoot chemotherapy with CDDP. (A) Treatment schedule. FUCCI-expressing MKN45 cells (5×10^6 cells/mouse) were injected subcutaneously into the left flank of nude mice. When the tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered iv *S. typhimurium* A1-R alone (5×10^7 CFU/mouse, iv, qW × 4); or cisplatinum (CDDP) alone (5 mg/kg, ip, q3d); or *S. typhimurium* A1-R followed by CDDP; or *S. typhimurium* A1-R, rMETase (200 units/mouse, ip, q d for 3 d × 4) and CDDP in that order. (B) Macroscopic photographs of FUCCI-expressing tumors: untreated control; *S. typhimurium* A1-R-treated; CDDP-treated; *S. typhimurium* A1-R and CDDP-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and CDDP. (C) Waterfall plot indicating fold change in tumor volume: untreated control; CDDP-treated; *S. typhimurium* A1-R-treated; *S. typhimurium* A1-R and CDDP-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and CDDP. (D) Representative images of cross-sections of FUCCI-expressing MKN45 subcutaneous tumors: untreated control; *S. typhimurium* A1-R-treated; CDDP-treated; *S. typhimurium* A1-R and CDDP-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and CDDP.

Decoy, trap and shoot chemotherapy with mitotic inhibitor PTX paclitaxel (PTX)

Based on rMETase prevention of cell division, we tested decoy, trap and shoot chemotherapy on MKN45 tumor bearing mice with paclitaxel (PTX). PTX alone and *S. typhimurium* A1-R alone significantly inhibited tumor growth ($p < 0.05$). *S. typhimurium* A1-R combined with PTX had a similar inhibition of tumor growth compared with PTX alone or *S. typhimurium* A1-R alone. The sequential combination of *S. typhimurium*

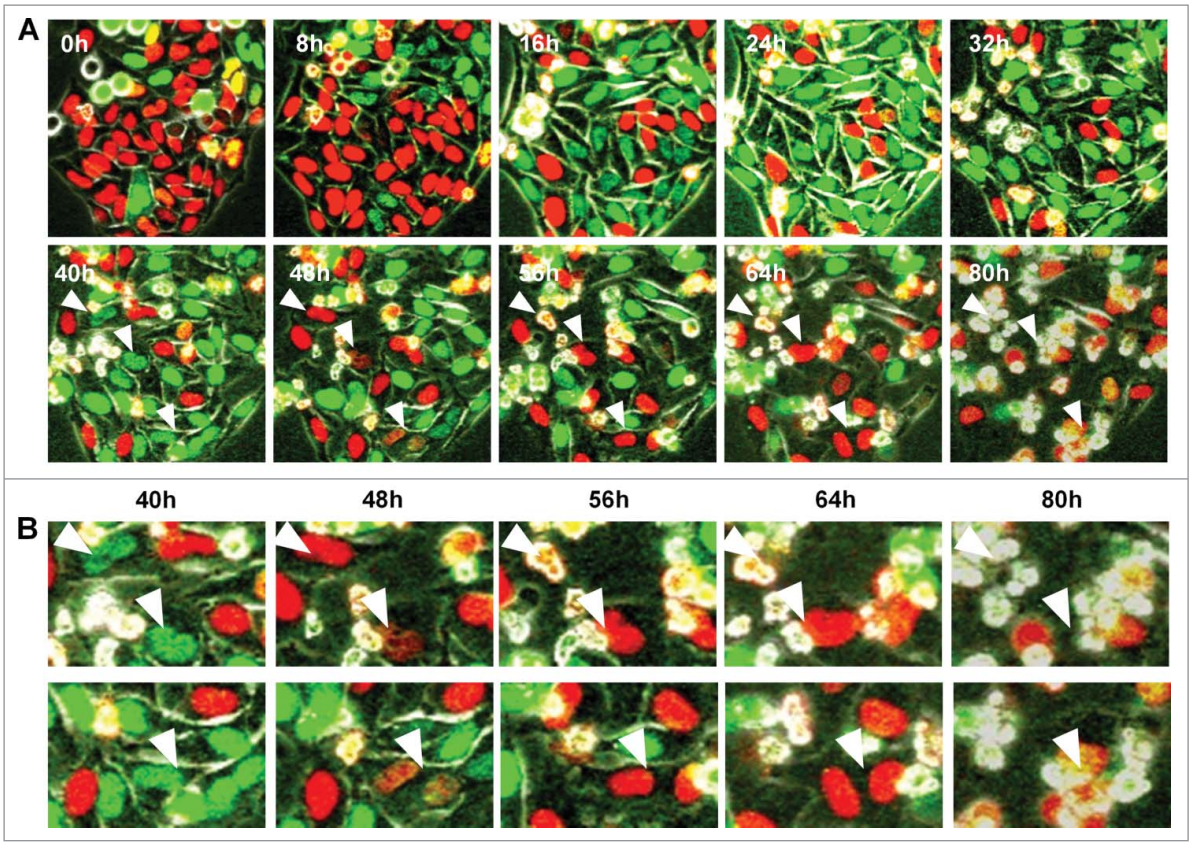

Figure 4. Prolonged administration of rMETase induced mitotic catastrophe after late S/G2 phase blocking. (A) Time-lapse imaging of HeLa-FUCCI cells treated with rMETase. After seeding on 35 mm glass dishes and culture overnight, HeLa-FUCCI cells were treated with rMETase at a dose of 1.0 unit/ml for 80 hours. All images were acquired with the FV1000 confocal microscope (Olympus, Tokyo, Japan). The cells in G_0/G_1, S, or G_2/M phases appear red, yellow, or green, respectively. (B) High magnificent image of A. Arrowheads refer to a cell dying from mitotic catastrophe.

A1-R, rMETase and PTX prevented or regressed tumor growth more than *S. typhimurium* A1-R or PTX alone or the combination of these two agents ($p < 0.05$).

Previously-developed concepts and strategies of highly selective tumor-targeting[37-48] can take advantage of bacterial cell-cycle decoy and rMETase cell-cycle trap described in the present and previous reports.[1,49,50]

Excess thymidine or its analogs have also been used to arrest cancer cells in S-phase, where they are sensitized to S-phase drugs, and after the release of the block, the cancer cells are sensitive to M-Phase drugs.[51-53]

Cytosine arabinoside, methotrexate and hydroxyorea have been used to block cancer cells in S-phase which can sensitize them to an M-phase drug administered after the S-phase block is lifted.[54-58]

Mibefradil, a calcium channel blocker, has been used to synchronize glioblastoma cells at the G_1/S checkpoint sensitizing them to temozolomide.[59] Lovastatin can be used to synchronize cancer cells in G_1.[60,61] The cancer cells can be effectively treated with an S-phase drug after the block is lifted.

PDO332991, a pyridopyrimidine, inhibits cyclin-dependent kinases 4 and 6 and induced early-G_1 arrest in myeloma cells in vitro and in vivo where they become sensitive to cytotoxic drugs.[62] RO-3306, another cyclin-kinase inhibitor, arrests cancer cells in G_2 phase which become sensitive to M-phase drugs after the block is lifted.[63] EGF, G-CSF, and IL-6 can stimulate cancer cell out of G_0 and can sensitize them to cytotoxic chemotherapy.[64-66] Reviews on cell synchronization are available.[67-70]

The critical advantage of *S. typhimurium* A1-decoy and rMETase trapping is that both are tumor specific, unlike the methods listed above, and can overcome tumor intrinsic chemoresistance (TIC).[27,28,32,71-79]

Materials and methods

FUCCI (Fluorescence ubiquitination cell cycle indicator)

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 (orange fluorescent protein) or mAG-hGem (green fluorescent protein) were obtained from the Medical and Biological Laboratory. Plasmids expressing mAG-hGem 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 cell sorter. The first-step-sorted green-fluorescent cells were then re-transfected with mKO_2-hCdt1 (orange) and then sorted by orange fluorescence.[1,2]

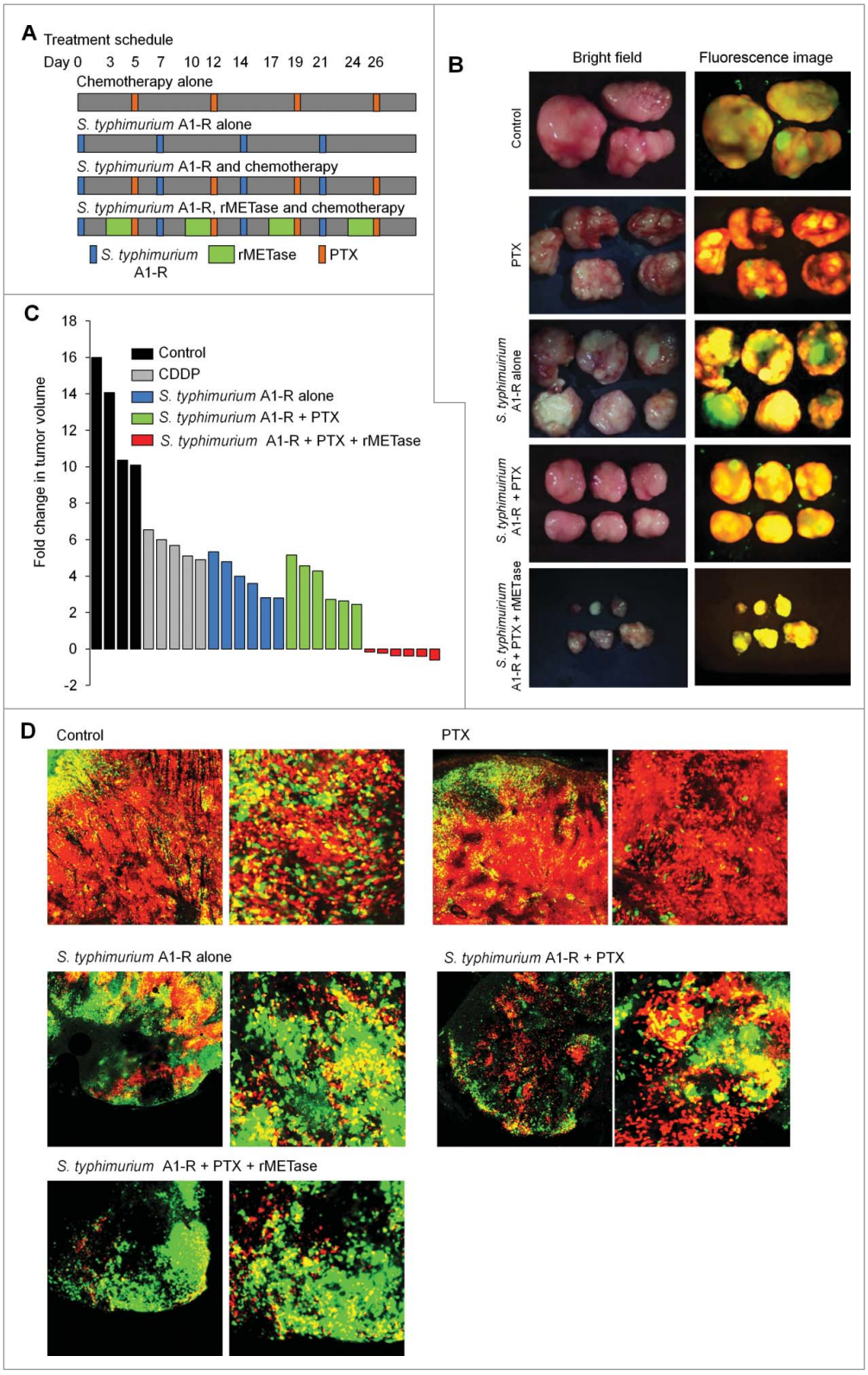

Figure 5. Decoy, trap and shoot chemotherapy with PTX. A). Treatment schedule. FUCCI-expressing MKN45 cells (5×10^6 cells/mouse) were injected subcutaneously into the left flank of nude mice. When the tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered *S. typhimurium* A1-R alone (5×10^7 CFU/mouse, iv, qW × 4), or PTX alone (6 mg/kg, ip, q3d × 4); or *S. typhimurium* A1-R followed by PTX, or *S. typhimurium* A1-R, rMETase (200 units/mouse, ip, q d for 3 d × 4) and PTX sequentially. (B) Macroscopic photographs of FUCCI-expressing tumors: untreated control; *S. typhimurium* A1-R-treated; PTX-treated; *S. typhimurium* A1-R in combination with PTX-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and PTX. Scale bars, 10 mm. (C) Waterfall plot indicating fold change in tumor volume: untreated control; PTX-treated; *S. typhimurium* A1-R-treated; *S. typhimurium* A1-R in combination with PTX-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and PTX. (D) Representative images of cross-sections of FUCCI-expressing MKN45 subcutaneous tumors: untreated control; *S. typhimurium* A1-R-treated; PTX-treated; *S. typhimurium* A1-R in combination with PTX-treated; or treated with the sequential combination of *S. typhimurium* A1-R, rMETase and PTX.

Cells

MKN45 human stomach cancer cells were grown in RPMI 1640 medium with 10% fetal bovine serum and penicillin/streptomycin.[1] HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin.[32]

Mice

Athymic *nu/nu* nude mice (AntiCancer, Inc., San Diego, CA) were maintained in a barrier facility under HEPA filtration and fed with autoclaved laboratory rodent diet (Teklad LM-485; Harlan). 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. 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.[19]

Recombinant methioninase (rMETase)

Recombinant L-methionine α-deamino-γ- mercaptomethane lyase (methioninase, METase) [$_{EC}$ 4.4.1.11] from *Pseudomonas putida* has been previously cloned and was produced in *Escherichia coli* (AntiCancer, Inc.,). rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass.[30,80]

Decoy, trap and shoot chemotherapy

When the tumors reached approximately 8 mm in diameter (tumor volume, 300 mm^3), mice were administered iv *S. typhimurium* A1-R (5×10^7 CFU/mouse, iv, qW $\times$ 4) alone or in combination with cisplatinum (CDDP) (5 mg/kg ip) or paclitaxel (PTX) (6 mg/kg ip) q 3 d $\times$ 5 or the combination of *S. typhimurium* A1-R and either CDDP or PTX, or these combinations with rMETase (200 units/mouse).[19] Please see text and figure legends for dosing schedules.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Dedication

This paper is dedicated to the memory of A.R. Moossa, MD, and Sun Lee, MD.

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