IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Medical Sciences

2018; 15(6): 574-579. doi: 10.7150/ijms.23285

Research Paper

Salmonella Overcomes Drug Resistance in Tumor through P-glycoprotein Downregulation

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Received: 2017.10.11; Accepted: 2018.02.14; Published: 2018.03.09

Abstract

Chemotherapy is one of effective methods for the treatment of tumor. Patients often develop drug resistance after chemotherapic cycles. *Salmonella* has potential as antitumor agent. *Salmonella* used in tandem with chemotherapy had additive effects, providing a rationale for using tumor-targeting *Salmonella* in combination with conventional chemotherapy. To improve the efficacy and safety of *Salmonella*, a further understanding of *Salmonella* interactions with the tumor microenvironment is required. The presence of plasma membrane multidrug resistance protein P-glycoprotein (P-gp) is highly relevant for the success of chemotherapy. Following *Salmonella* infection, dose-dependent downregulation of P-gp expressions were examined. *Salmonella* significantly decreased the efflux capabilities of P-gp, as based on the influx of Rhodamine 123 assay. In addition, *Salmonella* significant reduced the protein express the expression levels of phosph-protein kinase B (P-AKT), phosph-mammalian targets of rapamycin (P-mTOR), and phosph-p70 ribosomal s6 kinase (P-p70s6K) in tumor cells. The *Salmonella*-induced downregulation of P-gp was rescued by transfection of cells with active P-AKT. Our results demonstrate that *Salmonella* in tumor sites leads to decrease the expression of P-gp and enhances the combination of *Salmonell*a and 5-Fluorouracil therapeutic effects.

Key words: *Salmonella*, P-glycoprotein, 5-Fluorouracil, tumor, combination therapy

Introduction

The proliferation of drug resistant tumor cells is believed to relapse in most patients after several cycles of chemotherapy. The growing evidences point out that the tumor microenvironment plays an important role in drug resistance [1]. P-glycoprotein 1 (P-gp) is the multidrug resistance protein 1 (MDR1) or ATP-binding cassette, sub-family B member 1 (ABCB1). The P-gp is an important protein of the cell membrane that pumps chemotherapeutic drugs out of cells [2]. Many tumors have higher levels of P-gp that correlates with drug resistance [3].

Salmonella targets to tumor sites and arrests tumor growth [4, 5]. The combination of *Salmonella* and chemotherapy acted additively to delay tumor growth and prolong the survival time of tumor-bearing mice [5- 7]. Previously, we have shown that *Salmonella* increases the efficiency of cisplatin via enhancement of gap junction [8]. Meanwhile, *Salmonella* can target to tumors and modulate numerous signaling pathways of tumor cells [9]. We hypothesize that *Salmonella* not only enhances drug diffusion via upregulation gap junction, but also decreases drug efflux through downregulation P-gp. This study provides evidence for the mechanism of the regulation of P-gp by *Salmonella* and validates the incorporation of *Salmonella* as a novel therapeutic

strategy against tumor.

Material and Methods

Bacteria, cell lines, plasmid, reagents and animals

Salmonella enterica serovar choleraesuis (*S.* Choleraesuis; S.C.) (ATCC 15480) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan) [10]. B16F10 (mouse melanoma) [11] and 4T1 (mouse breast cancer) [12] cells were maintained in culture dish with Dulbecco's Modified Eagle Medium containing 1% antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), 2mM l-glutamine and 10% fetal bovine serum. Rhodamine 123 (Rho-123) and 5-Fluorouracil (5-FU) were purchased from Sigma Aldrich. Constitutively active AKT plasmid was kindly provided by Dr. Chiau-Yuang Tsai (Department of molecular immunology, Osaka University) [13]. Cells were treated with plasmids for 16 h in serum free medium prior to adding *Salmonella* (multiplicity of infection (MOI) = 10) into cells for 90 min. C57BL/6 (B16F10 mouse tumor model) and BALB/c (4T1 mouse tumor model) female mice were purchased from the National Laboratory Animal Center of Taiwan. The animals were maintained in a pathogen-free animal care facility in isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the National Sun Yat-sen University.

Western Blotting Analysis

Bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein content in each sample. Quantified each sample concentration to 60-80 μg and proteins were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK) and detected with antibodies against P-gp (GeneTex, Inc. Irvine, CA, USA), the mammalian target of rapamycin (mTOR) (Cell Signaling, Danvers, MA, USA), phosph-mTOR (Cell Signaling), protein kinase B (AKT) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), phosph-AKT (Santa Cruz Biotechnology, Inc.), p70s6K (Cell Signaling), phosph-p70s6K (Cell Signaling), caspase 3 (Cell Signaling), β-actin (Sigma Aldrich). Rabbit anti-mouse IgG-peroxidase antibody (Sigma Aldrich) and goat anti-rabbit IgG-peroxidase antibody (Sigma Aldrich) were used as the secondary antibody and protein-antibody complexes were visualized by enhanced chemiluminescence system. The Western blotting signals were quantified with ImageJ software (rsbweb.nih.gov/ij/) [14, 15].

P-gp functional assay

B16F10 or 4T1 cells were plated in 12 well culture plates for 24 h, and treated with various MOI of *Salmonella* for 4 h. The cells were washed with PBS and added the antibiotic medium. Retention of Rho-123 was measured to evaluate P-gp functional activity in B16F10 and 4T1 cells. Intracellular Rho-123 was determined from the fluorescence (Ex. 530nm Em. 590 nm) by spectrometer (BMG Labtech, Headquarters Germany). The accumulation of Rho-123 in cells was also analyzed using a fluorescence microscope. Cell proliferation was assessed by the colorimetric WST-8 assay (Dojindo Labs, Tokyo, Japan) according to the manufacturer's instructions [16].

Animal study

Mice were inoculated subcutaneously (s.c.) with 10^6 B16F10 or 4T1 cells at day 0, and at day 7 nodules developed at all injection sites with approximately tumor volume of 80 mm^3. Groups of tumor-bearing mice were injected intraperitoneally (i.p.) with *Salmonella* 2 $\times 10^6$ colony-forming units (cfu) at day 7 followed by 5-FU (40 mg/kg) treatment on days 9, 11, and 13, or with either treatment alone. All of the mice were monitored for tumor growth as previously described [8].

Statistical analysis

The ANOVA was used to determine differences between groups. Any P value less than 0.05 is regarded statistically significant.

Results

Intracellular accumulation of Rho-123 after *Salmonella* infection

To analysis whether *Salmonella* could regulate P-gp transport activity after *Salmonella* infection in tumor cells, the intracellular accumulation of Rho-123 was assessed. The P-gp functionality was measured by detecting the intracellular accumulation of fluorescent (Rho-123). As shown in Fig. 1A, Rho-123 was detected modestly at the nuclear periphery and in the cytoplasm in control cells, whereas it was detected exclusively in the cytoplasm in the cells treated with *Salmonella*. We also observed the similar phenomenon in B16F10 cells (Fig. 1A). By measuring the fluorescence values, the accumulation of Rho-123 was significantly more in the *Salmonella*-treated cells than that in the control cells (Fig. 1B). These results show that *Salmonella* increases the accumulation of Rho-123, indicating that *Salmonella* suppresses P-gp transport activity.

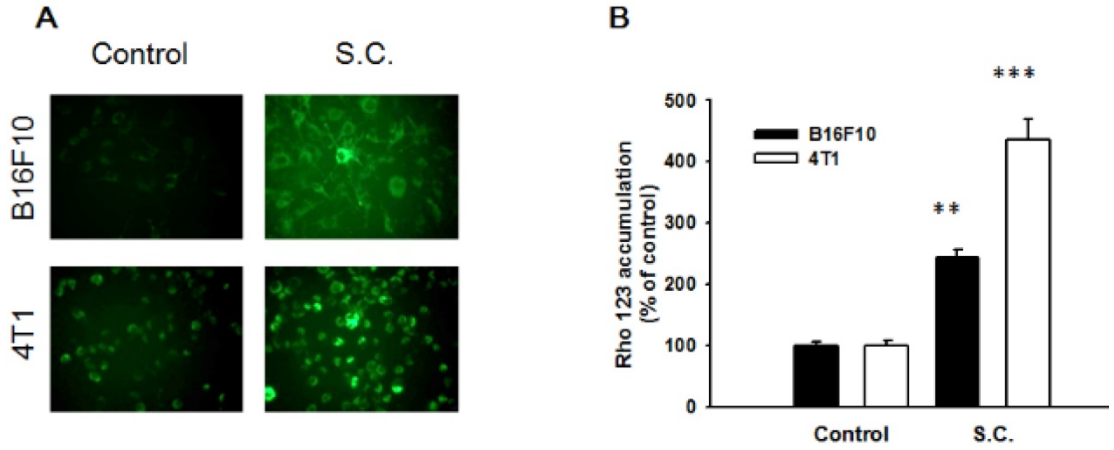

Figure 1. Rhodamine 123 (Rho-123) accumulation in B16F10 and 4T1 cells treated with *Salmonella* (S.C.). B16F10 and 4T1 cells were infected with S.C. for 4 h, washed and incubated with culture medium for 2 h prior to addition of Rho-123. (A) The accumulation of Rho-123 in cells was analyzed using a fluorescence microscope. (B) The Rho-123 were determined from the fluorescence by using spectrofluorometer. ** $P < 0.01$; *** $P < 0.001$.

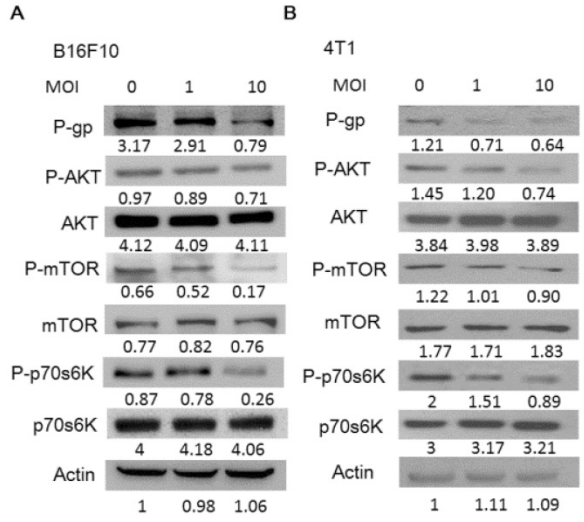

Figure 2. *Salmonella* (S.C.) regulated the expression of P-glycoprotein (P-gp), and AKT/mTOR signaling pathway. The expression of P-gp, AKT, mTOR, p70s6K was measured in B16F10 (A) and 4T1 (B) cells by Western blotting analysis. The β-actin expression served as loading controls for and total protein. Each experiment was repeated three times with similar results.

Salmonella reduced P-gp expression through P-AKT/ P-mTOR pathway

To gain insights into the role of P-gp on *Salmonella* infection, we used Western blotting analysis to study the correlation between P-gp and *Salmonella* infection. Fig. 2 shows that the protein levels of P-gp were decreased in *Salmonella*-treated tumor cells. Furthermore, the AKT /mTOR signaling pathway can promote P-gp protein synthesis through phosphorylation of p70s6K [17, 18]. In dose dependent manner, *Salmonella* significantly reduced the phosphorylation of AKT, mTOR, and p70s6K in B16F10 and 4T1 cells (Fig. 2). These results suggest that reduction of P-gp expression in tumor cells was associated with inhibition AKT/mTOR/p70s6K signaling pathway during *Salmonella* infection.

Salmonella increased susceptibility of tumor cells to 5-FU

Furthermore, we examined the downregulation of P-gp expression by *Salmonella* infection upon the sensitivity of the tumor cells to killing by 5-FU that induce cell death. Herein, the proliferation of tumor cell was measured after 5-FU, *Salmonella* or *Salmonella* combined with 5-FU treatment. The results show that the amounts of tumor cells were lowest when tumor cells were treated with *Salmonella* combined 5-FU (40 μM) compared with the control group (Fig. 3A and C). Treatment with 5-FU significantly enhanced cell death in *Salmonella*-infected cells as compared to uninfected cells. Meanwhile, we also observed that *Salmonella* and 5-FU increased the apoptosis marker (cleaved caspase 3) (Fig. 3B and D). Collectively, these results indicate that *Salmonella* may inhibit the proliferation of tumor cells through the enhancing susceptibility of tumor cells to 5-FU.

Salmonella suppressed P-gp expression via an inhibition AKT signaling pathway

Consequently, we next examined the extent to which changes in P-gp expression contributed to the *Salmonella*-inhibited AKT/mTOR/p70s6K signaling pathway. By transfecting constitutively active AKT plasmid, the AKT/mTOR/p70s6K signaling pathway can be reversed [16]. *Salmonella* did not inhibit the constitutively active AKT. As shown in Fig. 4, *Salmonella* lost the ability to suppress the expression of the P-gp through downregulation AKT/mTOR/ p70s6K signaling pathway after transfecting constitutively active AKT plasmid. The similar results were also observed in the expression of cleaved-caspase 3 in the B16F10 and 4T1 cells treated

with 5-FU in combination with *Salmonella*. After transfecting constitutively active AKT plasmid in two tumor cells, the expression of cleaved-caspase 3 was reduced after the combo therapy (Fig. 5). The results demonstrate that downregulation AKT/mTOR signaling pathway is necessary for *Salmonella*-reduced P-gp expression in tumor cells.

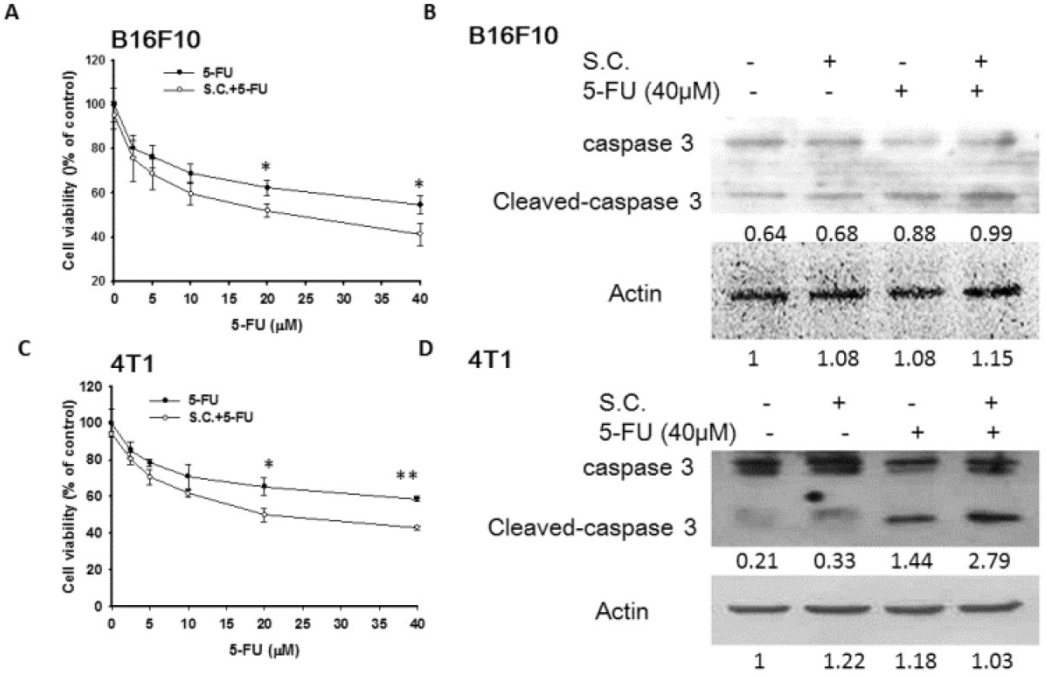

Figure 3. *Salmonella* (S.C.)-regulated P-glycoprotein (P-gp) expression in conjunction with 5-FU exerted cytotoxic effects on B16F10 and 4T1 cells. *Salmonella*-treated (MOI=10), or control cells were exposed to 5-FU (0-40 μM) for 48 h followed by determination of their viability by the WST-8 assay in B16F10 (A) and 4T1 (C) cells. Data are expressed as mean ± SD of hexaplicate determinations. *Salmonella*-treated (MOI=10), or control cells were exposed to 5-FU (40 μM) for 48 h followed by determination of caspase 3 expression by the Western blotting analysis in B16F10 (B) and 4T1 (D) cells. Multiplicity of infection (MOI). * $P<0.05$.

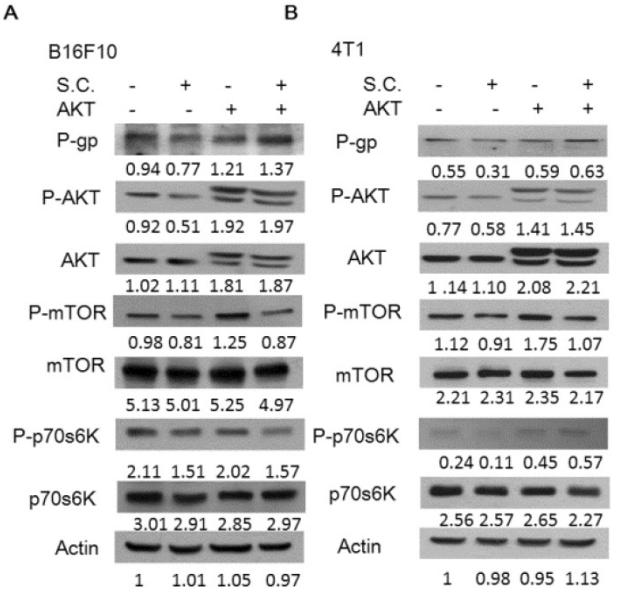

Figure 4. Constitutively active-AKT reduced the effects of *Salmonella* (S.C.). The B16F10 and 4T1 cells were transfected with constitutively active AKT plasmid (5 μg) for 16 h prior to treatment with *Salmonella* (MOI = 10) or not for 4 h. The expression of P-glycoprotein (P-gp), AKT, mTOR, p70s6K protein in B16F10 (A) and 4T1 cells (B) was determined. The β-actin expression served as loading controls for and total protein. Each experiment was repeated three times with similar results.

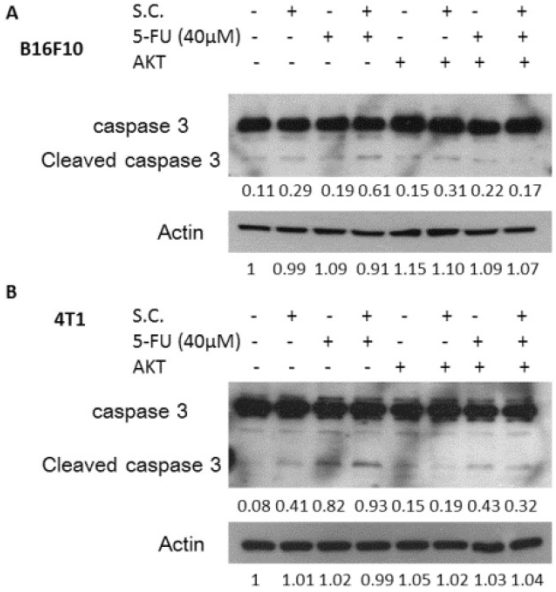

Figure 5. Constitutively active-AKT decreased the effects of *Salmonella* (S.C.) in combination with 5-FU. The B16F10 and 4T1 cells were transfected with constitutively active AKT plasmid (5 μg) for 16 h prior to treatment with *Salmonella* (MOI = 10) or not for 4 h. *Salmonella*-treated (MOI=10), or control cells were exposed to 5-FU (40 μM) for 48 h followed by determination of caspase 3 expression by the Western blot analysis in B16F10 (A) and 4T1 (B) cells. Multiplicity of infection (MOI).

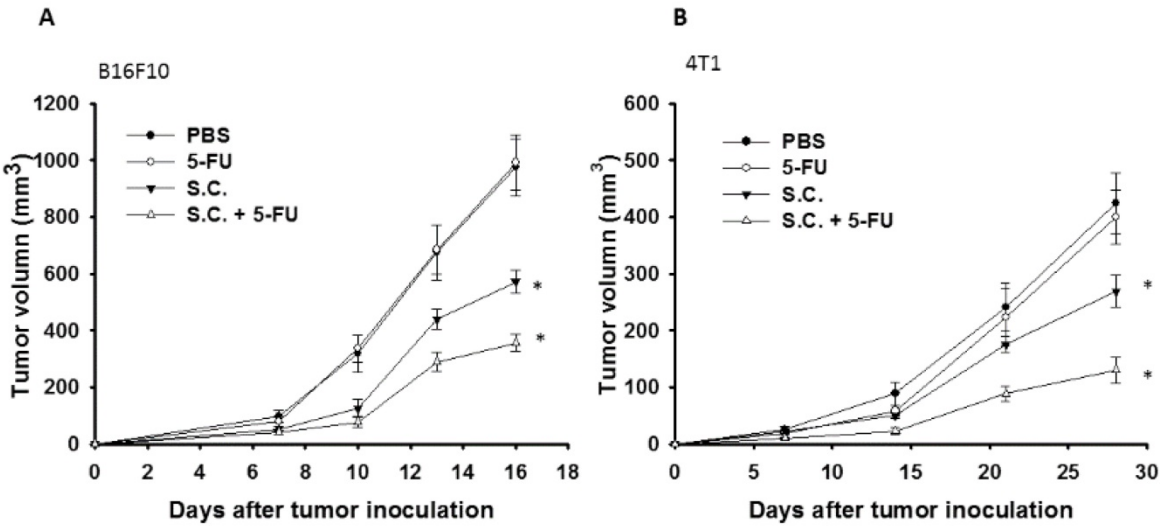

Figure 6. Additive antitumor effects of *Salmonella* (S.C.) in combination with 5-FU on subcutaneous B16F10 and 4T1 tumors. Groups of 8 mice that had been inoculated s.c. with B16F10 (a) and 4T1cells (b) at day 0 were treated i.p. with *Salmonella* (2 × 10^6 cfu) at day 7 followed by 5-FU (40 mg/kg) treatment at days 9, 11, and 13, or with either treatment alone. Vehicle control mice received PBS. Tumor volumes (mean ± SEM, n = 8) among different treatment groups were compared in mice bearing tumors. * $P<0.05$.

Salmonella in combination with 5-FU enhanced the antitumor activity

The tumor growth of the mice bearing B16F10 or 4T1 tumors was evaluated the antitumor effects of *Salmonella*. As shown in Fig. 6 A and B, *Salmonella* significantly inhibited tumor growth in comparison with that in PBS-treated control mice. The low-dose 5-FU did not show significant antitumor activity. As expected, the combination therapy of *Salmonella* and 5-FU enhanced the antitumor activity. In B16F10 and 4T1 tumor model, the tumor volume of mice treated with the combination therapy reduced by 37.7% and 51.67% compared with those in the *Salmonella*-treated group, respectively. Taken together, *Salmonella* alone could reduce tumor growth in murine tumor models. Furthermore, the combination therapy had the additive antitumor effects.

Discussion

The effects of the combinational treatment of 5-FU and *Salmonella in vitro* and *in vivo* were determined by using mouse melanoma and breast tumor models. The combination of *Salmonella* and 5-FU inhibits tumor growth and tumor size compared to 5-FU alone, suggesting that *Salmonella* can increase the efficacy of 5-FU. However, the detailed mechanism of the combinational therapy remained uncertain. In this study, we used mouse tumor models to determine the mechanisms of *Salmonella*-induced signaling and the consequences of the combination therapy. *Salmonella* was demonstrated the ability to decrease the expression of P-gp through posttranscriptionally regulated [19]. Some studies also demonstrated downregulation of p-gp expression via inhibition of AKT/mTOR pathway [17]. There is a growing evidence that an increase in chemotherapy-mediated response to P-gp downregulation [20]. There is extensive literature of tumor-targeting *Salmonella* increasing the efficacy of chemotherapy [21-24]. *Salmonella* recruited peripheral immune cells to the tumor by upregulating interferon γ and T cells are also involved in the modulation of *Salmonella*-induced host immunity [25, 26]. Thus, some studies may suggest that tumor necrosis factor α is response for main cells infiltrating *Salmonella*-treated tumors [27]. *Salmonella* has been used as an antitumor agent that specifically proliferates within tumors and inhibits their growth [28]. In addition, *Salmonella* cannot disperse throughout the tumor and this is the disadvantage for its use as an anticancer agent [28]. Our previous studies suggested that *Salmonella* disperses within tumors and inhibits tumor growth when combined with chemotherapy [7, 8]. There is a significant increase of apoptosis in 5-FU-treated cell. Cell proliferation analysis and Western blotting assay showed a significant increase in cell death of *Salmonella* combined with 5-FU treated tumors compared to control. Downregulation of P-gp may allow high intracellular cytotoxic drug concentrations in tumor cells. The P-gp expression in tumor cells may influence the clinical response to chemotherapy. To show the results, *Salmonella* manipulate P-gp in conjunction with 5-FU treatment may provide new insights for tumor therapy. The 5-FU is one of the most commonly used drugs to treat cancer including breast, colon and some skin cancers. Therefore, the major limiting factor in the use of 5-FU

is the side effects in normal tissue including diarrhea, month ulcers, and hair thinning. This would allow the use of lower 5-FU concentration, thus decreasing the side effects. *Salmonella* provides to accelerate cell death in tumor cells while decreasing P-gp expression. Recent studies revealed that resveratrol decreases in P-gp expression is regulated by AKT/mTOR signaling pathways [29]. Previously, we demonstrated that AKT/mTOR signaling is involved in the cell-defense elimination of *Salmonella*. The AKT/mTOR signaling pathway negatively regulates some protein syntheses that are critical for tumor cell survival [11, 16]. We determined that the levels of phosphorylated AKT, mTOR, and p70s6K were significantly decreased in *Salmonella*-treated cancer cells. Herein, we investigated the effect of *Salmonella* on P-gp expression and function. We found that *Salmonella*-inhibited activation of AKT/mTOR pathway and are thought to play an important role in the downregulation of P-gp. By using of the cytoxicity effect of 5-FU and the pleiotropic activities of *Salmonella*, we suggest that *Salmonella* combining with 5-FU appears to aim at reversing cancer drug resistance.

Abbreviations

P-gp: P-glycoprotein; P-AKT: phosph-protein kinase B; P-mTOR: phosph-mammalian targets of rapamycin; P-p70s6K: phosph-p70 ribosomal s6 kinase; 5-FU: 5-Fluorouracil; Rho-123: Rhodamine 123: MDR1: multidrug resistance protein 1; ABCB1: ATP-binding cassette, sub-family B member 1; S.C.: *Salmonella enterica* serovar choleraesuis (*S.* Choleraesuis).

Acknowledgements

This work was supported by Ministry of Science and Technology, Taiwan (MOST 104-2320-B039-042-MY3)

Author Contributions

C.J.Y. and C.H.L conceived and designed the experiments; C.J.Y, W.W.C, S.T.L. and M.C.C. performed the experiments; C.J.Y. and C.H.L analyzed the data; C.H.L contributed reagents/materials/analysis tools; C.J.Y. and C.H.L wrote the paper.

Competing Interests

The authors have declared that no competing interest exists.

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