Aspirin and metformin exhibit antitumor activity in murine breast cancer

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Abstract. Studies have shown that aspirin and metformin play important roles in chemoprevention and repression of breast cancers, even though the exact mechanism remains unclear. Aspirin is capable of stimulating apoptosis through prostaglandin-dependent or prostaglandin-independent pathways. Metformin inhibits cell growth by enhancing the tumor suppressive function of transforming growth factor (TGF-β). In the present study, we report a new link between aspirin, metformin, TGF-β1 and murine breast cancer inhibition. Specifically, we showed that aspirin and metformin enhanced 4T1 cell apoptosis by inducing secretion of TGF-β1, whereas estradiol weakened the effect.

Introduction

Traditionally, patients with type 2 diabetes mellitus and cardio-vascular diseases are treated with aspirin and metformin (1-3). In recent years, both drugs have been reported to decrease the risk of several types of cancers, including breast cancer (4-10).

Aspirin, acetylsalicylic acid, has a wide range of uses, such as an analgesic, antipyretic and anti-inflammatory agent (11). As a nonsteroidal anti-inflammatory drug and cyclooxygenase (COX) inhibitor, aspirin prevents breast tumorigenesis in humans (12). The COX pathway plays an important role in cellular proliferation, migration and invasiveness (13,14). However, the precise mechanism accounting for a possible anti-neoplastic action of aspirin is not clear. A recent study showed that small interfering RNA-mediated inhibition of the Smad signaling pathways decreases transforming growth factor (TGF)-β1-induced COX-2 expression (15). Thus, there may be connection between aspirin and TGF-β.

Metformin (1,1-dimethylbiguanide) is the most widely prescribed drug to treat type 2 diabetes mellitus, notably in overweight or obese individuals (16,17). Recently, metformin was reported to limit proliferation of breast cancer cells by acting upon specific micro (mi)RNAs (18-20). Treatment with metformin inhibits growth by enhancing the tumor suppressive function of TGF-β. This occurs as a result of metformin disrupting the TGF-β/miRNA-181 signaling axis in cancer cells (21-23).

TGF-β plays a central role in tumor inhibition by both aspirin and metformin. Not surprisingly, metformin and aspirin have synergistic effects and share several underlying mechanisms for controlling cancer (24-26). In the present study, we investigated the antitumor activity of aspirin and metformin mediated by the TGF-β signaling pathway.

TGF-β is a versatile cytokine intimately involved in cell growth (27-29). Depending on the tumor type and tissue context, it may act both as a tumor suppressor or a promoter of migration, invasion and tumor survival (30). Furthermore, TGF-β can be regulated by estrogen (mainly estradiol) *in vivo* (31). Estrogen contributes to the inhibition of TGF-β/Smad signaling by promoting R-Smad (Smad2 and Smad3) degradation (31-34).

4T1 is a p53-deficient breast cancer cell line (35,36). Triple-negative breast cancer cell lines [i.e., lacking the estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2)] are less affected by estrogen than traditional cell lines (37,38). Accordingly, estrogen suppression treatment is usually not recommended in such cases, including in 4T1 cells. In the present study, we hypothesized that the lack of estrogen inhibition in triple-negative breast cancer cells may change the effect of aspirin and metformin on tumor growth inhibition *in vivo* by regulating TGF-β activity. We also discuss the link between aspirin, metformin, TGF-β1 and estradiol in murine breast cancer inhibition.

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Materials and methods

Cell culture and treatment. The mouse breast carcinoma cell line 4T1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and maintained at 37°C in a humidified condition of 95% air and 5% CO_2. Cells were cultured in 75 cm² flasks or 6-well plates with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Bedford, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. Before addition of aspirin, metformin, estradiol or LY364947, which is one of inhibitors of TGFβ R-I, cells are allowed to attach to the substrate for 24 h. Aspirin, metformin, estradiol and LY364947 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For TGF-β1 treatment, 0.01 μg/ml to 1x10⁴ 4T1 cells of human recombinant TGF-β1 (BioLegend, Inc., San Diego, CA, USA) were used, and 0.01 μg/ml to 1x10⁴ 4T1 cells PBS as for the control group. For drug treatment group, nine groups were divided: i) 5 μM aspirin; ii) 10 μM metformin; iii) 5 μM aspirin plus 10 μM metformin; iv) 5 μM aspirin with 1 μM LY364947; v) 10 μM metformin with 1 μM LY364947; vi) combination of 5 μM aspirin and 10 μM metformin with 1 μM LY364947; vii) 5 μM aspirin plus 10 nM estradiol; viii) 10 μM metformin plus 10 nM estradiol; and ix) combination of 5 μM aspirin and 10 μM metformin plus 10 nM estradiol, the same amount of dimethyl sulfoxide (DMSO) as control group. Each group (n=5) was treated for 24, 36 and 48 h before harvested for further study. The N-values of cell experiments are three, respectively. The dose was based on literature (26,39) and our earlier study, then we identified a roughly dose range. From the range in our results from the MTT assay, we chose the final dose.

The in vivo model. Five-week-old female BALB/c mice (Beijing HFK Bioscience Co., Ltd., Beijing, China) were used for the *in vivo* animal experiments. The animals were housed in constant laboratory conditions with a 12-h light/dark cycle and fed with water and food *ad libitum*. All animal care followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee of Sichuan University. Mice were subcutaneously inoculated into the right-back with 1x10⁶ 4T1 cells in 100 μl PBS. For the treatment, the tumor-bearing mice were divided into groups, respectively: i) 100 μl normal saline; ii) tamoxifen (100 mg/kg/24 h); iii) aspirin (60 mg/kg/24 h); iv) metformin (160 mg/kg/24 h); v) aspirin and metformin; vi) aspirin with tamoxifen; vii) metformin with tamoxifen; viii) aspirin and metformin with tamoxifen. Each group had 5 mice. Metformin and tamoxifen were dissolved in normal saline through intragastric administration for 15 days. Aspirin was dissolved in ultrapure water with 4% ethanol as cosolvent through intraperitoneal injection for the same days. The mice with a tumor (0.5-1.0 cm wide, and 0.5-1.0 cm long) were randomized and injected (n=5/group). The tumor volume [0.5 x [major axis] x [minor axis]²] of every mouse was monitored every 2 days. Mice were sacrificed at day 15, and samples were analyzed as previously described (40). All procedures regarding the care and use of animals followed the guidelines of and were approved by the Animal Ethics Committee of Sichuan University.

Cell proliferation assay. Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well plates and cultured for 24, 36 and 48 h following by addition of MTT solution to the cells for 4 h. After removing the medium, the remaining MTT formazan crystals were solubilized in DMSO and measured at 560 nm using a microplate reader (Benchmark Electronics, Angleton, TX, USA).

ELISA. 4T1 tumors were collected and then homogenized in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 150 mM NaCl and 50 mM Tris-HCl), followed by centrifugation at 13,300 rpm for 30 min at 4°C. DEAB assay was used to test the protein concentration of samples. The prepared samples were stored at -80°C until used. Levels of TGF-β1 in the samples were assessed by mouse ELISA kits (eBioscience or R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, and the colorimetric reaction was measured at 450 nm, the color absorbance was recorded at 450 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The amount of TGF-β1 secreted into the supernatant of 4T1 cells was quantified using the same ELISA kits.

Blood samples were collected from the eye socket and placed at room temperature for 3 h to obtain the serum. The serum levels of estradiol detected using ELISA kits (Yan Hui Biological Technology, Shanghai, China).

Flow cytometric analysis of apoptosis. 4T1 cells were treated as described above and then harvested, washed in cold phosphate-buffered saline (PBS), double-stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) and analyzed by flow cytometry (FACSAria SORP; BD Biosciences, Erembodegem, Belgium). Apoptosis assays were performed with FITC/PI as FITC⁺/PI⁻ and FITC⁺/PI⁺ to measure early and late apoptosis, respectively. PI is a cell viability marker and FITC an apoptosis marker.

Western blot analysis. 4T1 cells were harvested, lysed and total protein was quantified with Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). Total protein (10 μg) from each sample was separated by electrophoresis using 12% SDS-PAGE gels, transferred onto polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA), blocked with 5% skim milk, and incubated using the primary antibodies (1:1,000) against Mcl-1, Bax, Bcl-2, caspase-8, TGF-β1, Smad2/3, pSmad2, pSmad3, Smad4 and β-actin overnight (16 h) at 4°C. β-actin was used as a loading control. All primary antibodies were from Abcam Science Company (Cambridge, UK). Blots were then incubated with the corresponding secondary antibodies (1:10,000; Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. After exposed to ECL reagent (Merck Millipore) advanced luminescence, signals were developed on X-ray film (Kodak, Rochester, NY, USA), and performed as previously described (41).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. For each sample, 5-mm sections
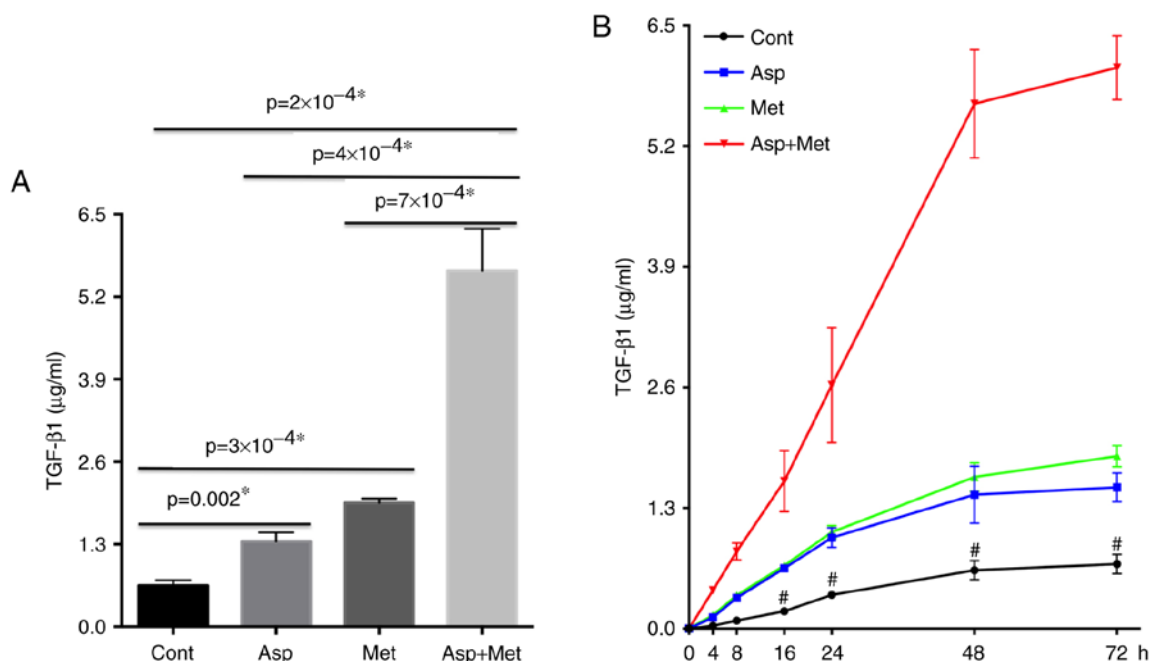
Figure 1. The combination of metformin (Met) and aspirin (Asp) promotes transforming growth factor (TGF)-β1 in the supernatant fluid of 4T1 cells. The amounts of TGF-β1 after a 48 h treatment and at different time points are shown; [#]P<0.05 vs. Asp, Met and the Asp+Met groups at the same time point. Cont, control.

of root tips were fixed using 4% paraformaldehyde (#18814; Polysciences, Inc., Warminster, PA, USA) in PBS for 45 min at room temperature. The fixation step was followed by a permeabilization step with 0.25% Triton X-100 in PBS for 20 min at room temperature. Next, terminal deoxynucleotidyl transferase-mediated dUTP (2'-deoxyuridine, 5'-triphosphate) nick end-labeling (TUNEL) assay was performed following the manufacturer's instructions (Click-iT® TUNEL Alexa Fluor® Imaging Assay Protocol) and the nuclei were stained for 3 min with 0.3 mg/ml 4',6-diamidino-2-phenylindole (DAPI). Finally, the cells were mounted in Vectashield embedding medium (Vector Laboratories, Burlingame, CA, USA). All images were recorded at exactly the same time of integration using an AxioCam ERc5s CCD camera and AxioVision 4.8 software (both from Zeiss, Jena, Germany). Image processing was carried out in Adobe Photoshop 7.0 Adobe Systems, Inc., San Jose, CA, USA). Three samples worked each mouse and 10 visual fields were analyzed for each sample.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). Analysis of variation (ANOVA) were used for the statistical analysis, and P<0.05 was considered statistically significant. All statistics were performed using SAS 9.2.

Results

Aspirin combined with metformin increases secretion of TGF-β1 by 4T1 cells. Following treatment with aspirin and metformin for at least 48 h, we detected increased TGF-β1 secretion and consequent tumor growth inhibition in 4T1 cells. Next, we used ELISA to measure the amount of TGF-β1 in the supernatant of 4T1 cells after the different treatments (Fig. 1A). TGF-β1 levels were maximal following combined aspirin and

metformin treatment. Additionally, TGF-β1 secretion was proportional to the length of the treatment, in spite of slower growth after 48 h (Fig. 1B). This led us to conclude that aspirin and metformin stimulated the secretion of TGF-β1. More importantly, the combination of aspirin and metformin had a synergistic effect on TGF-β1 secretion.

TGF-β1 reduces cell viability and induces apoptosis in 4T1 cells. To evaluate the effect of TGF-β1 on proliferation and apoptosis in 4T1 cells, we used the MTT assay with different concentrations of TGF-β1 (Fig. 2A). We observed that, within a certain range, TGF-β1 reduced 4T1 cell viability. We then used flow cytometry to assess apoptosis of 4T1 cells following treatment with 100 ng/ml TGF-β1, 1 μM LY364947 (a TGF-β type I receptor inhibitor), or 10 nM estradiol (Fig. 2B). The results indicated that, depending on the concentration, TGF-β1 induced both early and late apoptosis in 4T1 cells. PI is a cell viability marker and FITC an apoptosis marker, of early apoptosis is PI[-]/FITC[+] and late apoptosis PI[+]/FITC[+].

Aspirin and metformin reduce cell viability and induce apoptosis in 4T1 cells. To evaluate whether different concentrations of aspirin and metformin had a synergistic effect on the proliferation of 4T1 cells, we performed the MTT assay. Combined treatment with these drugs led to a synergistic inhibition of cell viability, notably at 5 μM aspirin and 10 μM metformin (P=0.002) (Fig. 3A). Next, we assessed the expression of apoptosis-related proteins. Western blotting revealed increased levels of Bcl-2 and caspase-8 (p18), and decreased levels of Bax and Mcl-1 following a 48-h treatment with aspirin and/or metformin. Changes were notable after combined treatment (Fig. 3B). To determine whether apoptotic cell death occurred, we evaluated cells by flow cytometry using co-staining with FITC and PI (Fig. 3C). The results
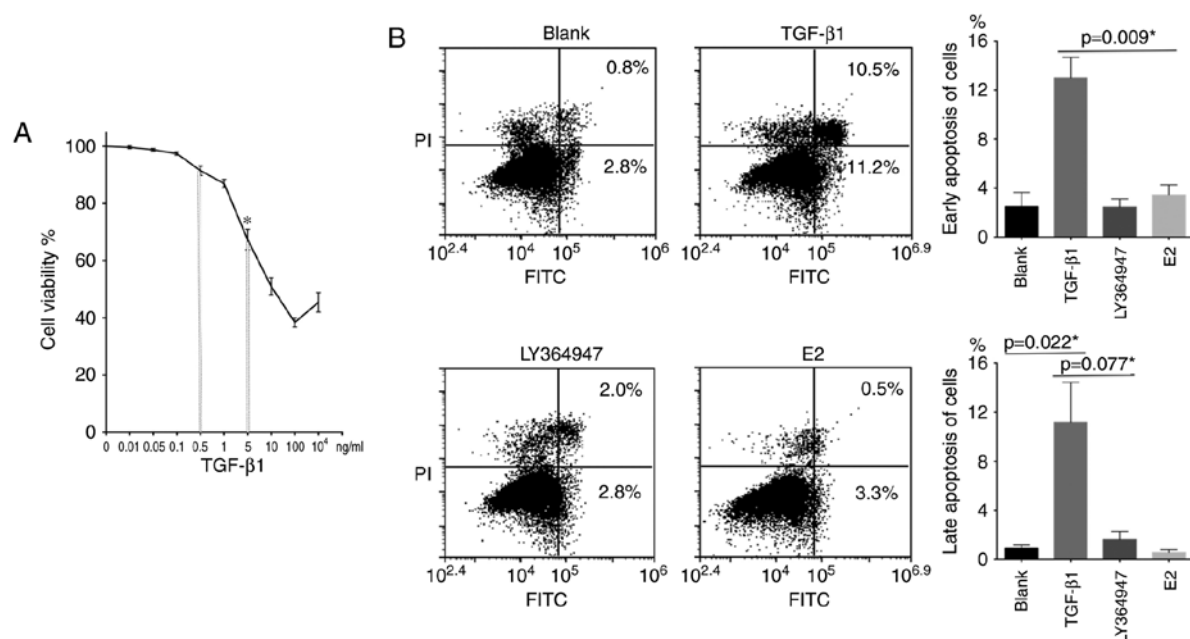
Figure 2. Effects of transforming growth factor (TGF)-β1 on 4T1 cell viability. (A) 4T1 cells were exposed to different concentrations of TGF-β1 for 48 h (P<0.05 for 0.5 vs. 5 µg/ml). (B) Apoptosis was assessed in 4T1 cells after treatment with 100 ng/ml TGF-β1, 1 µM LY364947 or 10 nM estradiol for 48 h.
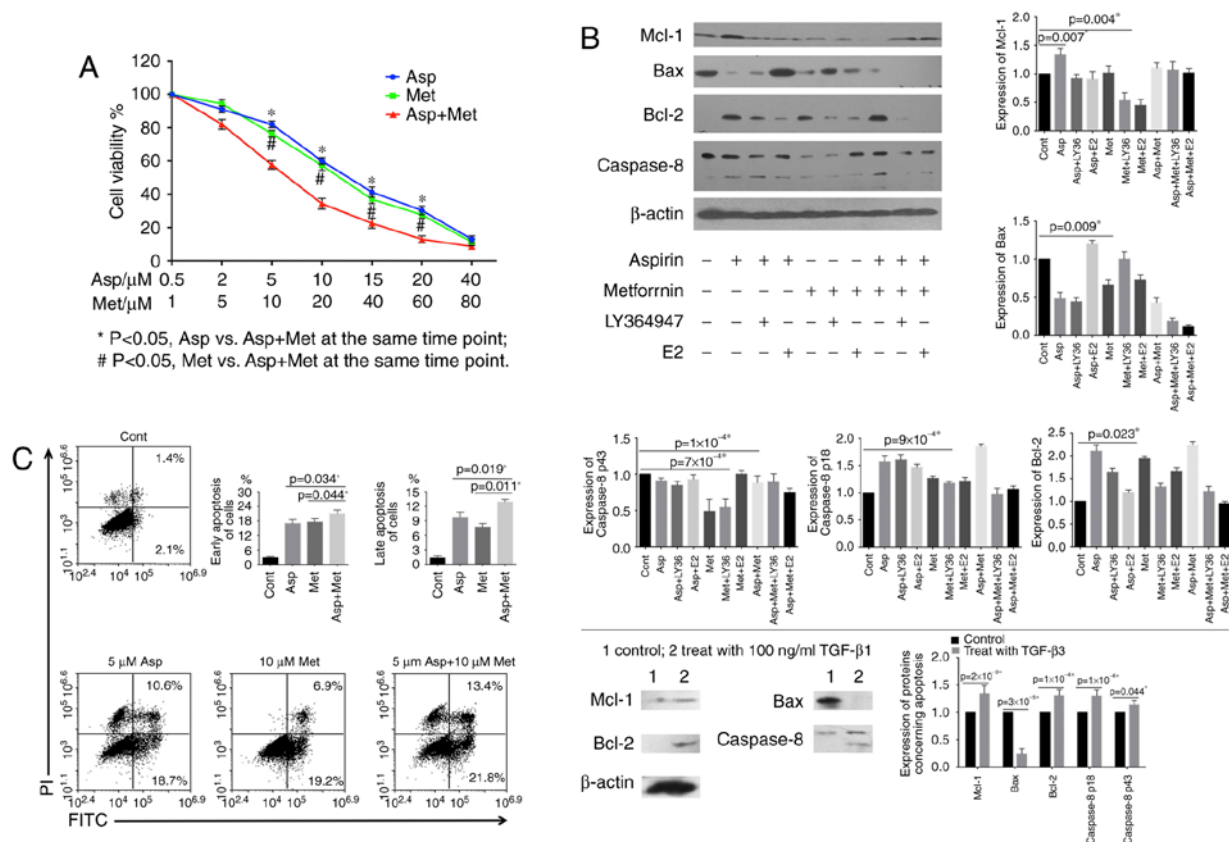

Figure 3. Effects of aspirin (Asp) and metformin (Met) on 4T1 cells. (A) 4T1 cells were treated with aspirin and metformin at the indicated concentrations for 48 h. Cell viability was evaluated by the MTT assay. *P<0.05 vs. the Asp and Met groups at the same concentrations; #P<0.05 vs. the Asp and Met groups at the same concentrations. (B) Regulation of proteins affecting apoptosis. Protein levels were detected by western blotting. β-Actin was used as the loading control. (C) 4T1 cells were stained with Annexin V-FITC and propidium iodide following treatment with or without 10 µM metformin or 5 µM aspirin for 48 h. Apoptosis was determined by flow cytometry. Cont, control.

showed that aspirin and metformin decreased cell viability and induced apoptosis in 4T1 cells, with the combined treatment having the strongest effect.

Aspirin and metformin enhance the TGF-β-dependent pathway to promote suppression of 4T1 cells, whereas estradiol weakens the effect. Given that secretion of TGF-β1
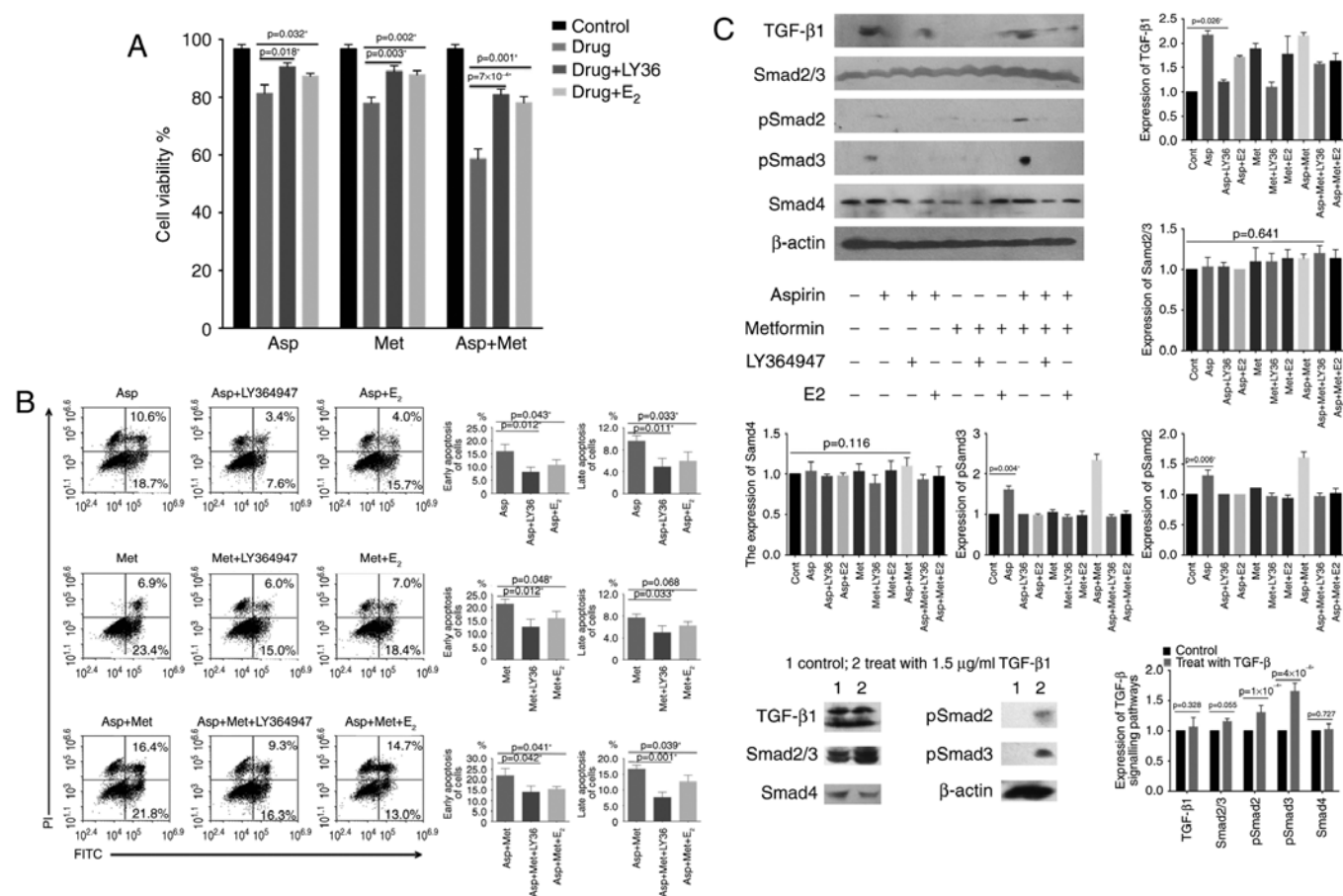
Figure 4. Aspirin and metformin enhance transforming growth factor (TGF)-β dependent pathways to suppress 4T1 cells, but estradiol weakens the suppression. (A) 4T1 cells were treated with different concentrations of aspirin (Asp) and metformin (Met) for 48 h. Cell viability was evaluated by the MTT assay. (B) 4T1 cells were stained with Annexin V-FITC and propidium iodide following treatment with or without 10 μM Met, 5 μM Asp, 1 μM LY364947 (Ly36), or 10 nM estradiol (E2) for 48 h. Apoptosis was determined by flow cytometry. (C) Regulation of the TGF-β signaling pathway. Levels of TGF-β signaling pathway proteins were detected by western blotting. β-Actin was used as the loading control. Cont, control.

by 4T1 cells was a major finding of the present study, we decided to design an appropriate treatment. Results from the MTT assay (Fig. 3A) indicated that both drugs led to growth inhibition, as determined by a decrease in optical absorbance with 5 μM aspirin and 10 μM metformin. We used LY364947 to block the TGF-β1 receptor and observed an increase in optical density. A similar effect was seen with estradiol (Fig. 4A). Expression of apoptosis-related proteins decreased in 4T1 cells following addition of LY364947 and estradiol, in contrast to the pro-apoptotic effect of aspirin and metformin (Fig. 3B). These findings were confirmed by flow cytometry (Fig. 4B).

Once cells were no longer stimulated by TGF-β1, growth inhibition was relieved, suggesting that the inhibition caused by aspirin was indeed mediated by TGF-β1. To further determine whether the TGF-β-dependent pathway was involved in the induction of apoptosis by metformin and aspirin, we examined the effect of the two drugs on downstream targets of TGF-β1 (Smad2, Smad3 and Smad4). Treatment with 100 ng/ml TGF-β1 (Fig. 4C) was used for comparison. The phosphorylation of Smad2 and Smad3 was significantly stronger in cells treated with a combination of metformin and aspirin whereas treatment with aspirin or metformin alone had only a moderate or small effect, respectively. Accordingly,

specific bands corresponding to phosphorylated Smad2 or Smad3 were barely detectable once TGF-β1 induction was suspended.

Aspirin and metformin inhibit growth of 4T1 tumors in BALB/c mice. A combination of metformin and aspirin caused a strong inhibitory effect on tumor growth *in vivo*, whereas aspirin or metformin alone had only a mild inhibitory effect. It should be noted that administration of tamoxifen with aspirin and metformin also had a significant inhibitory effect on tumor growth *in vivo* (Fig. 5A); tumor size in the group treated with all three agents was the smallest while the control group showed the largest tumor size. Tumor size in the aspirin plus metformin group was smaller than that in the aspirin or metformin alone groups.

The TUNEL assay was used to detect apoptosis in subcutaneously transplanted tumors in mice. This experiment revealed that the triple drug combination caused the most significant increase in apoptosis (Fig. 5B). After euthanasia, the amount of estradiol in the blood was measured (Fig. 5C). Based on these results, a lower estradiol content could mediate the strong inhibitory effect caused by a combination of aspirin and metformin. The amount of TGF-β1 was greatest in subcutaneously transplanted tumors of mice subjected to a combined drug treatment (Fig. 5D).
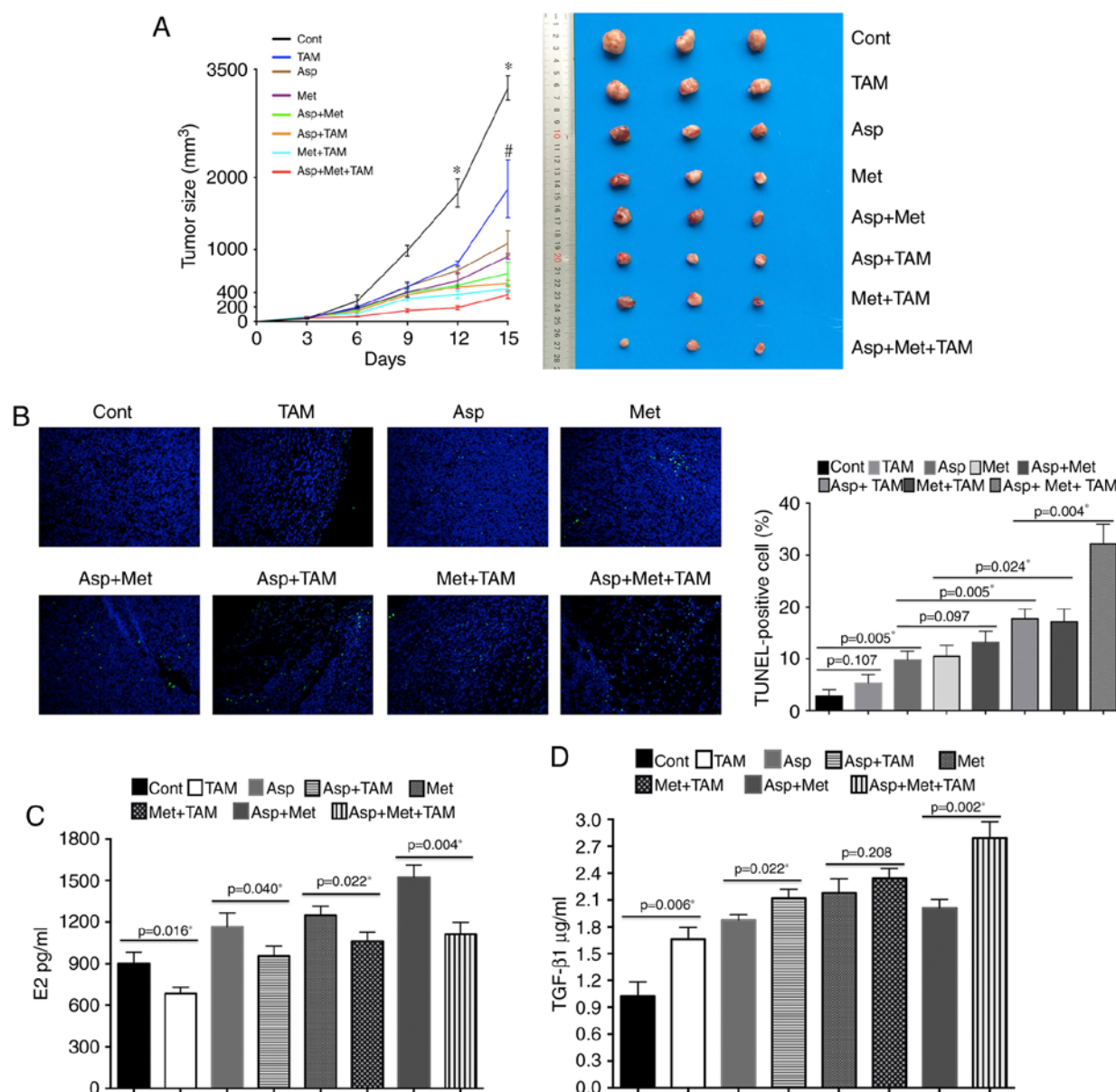
Figure 5. Tumor sizes after treatment of BALB/c mice with aspirin (Asp), metformin (Met) and tamoxifen (TAM). (A) The administration of TAM with Asp and Met significantly inhibited 4T1 tumor growth. Tumor size in the Asp + Met + TAM group is the smallest while that in control animals is the largest. Tumor size in the Asp + Met group is smaller than in the Asp and Met alone groups. The left panel shows changes of tumor volume in each group, and the right panel shows representative images of the tumors. Values are expressed as means ± SE, n=5; *P<0.05 vs. the TAM, Asp, Met, Asp + Met, Asp + TAM, Met + TAM and Asp + Met + TAM groups at the same time point; #P<0.05 vs. the Asp, Met, Asp + Met, Asp + TAM, Met + TAM and Asp + Met + TAM groups at the same time point. (B) Apoptosis assayed with TUNEL staining (magnification, x200). TUNEL (green) and DAPI (blue) merge. The control shows the least apoptosis while the Asp + Met + TAM group shows the most. (C) Serum levels of estradiol in 4T1 tumor-bearing mice. After 15 days of treatment, blood samples were collected from the eye socket and placed at room temperature for 3 h to obtain serum. Estradiol was detected using ELISA kits. (D) Asp + Met + TAM treatment increased transforming growth factor (TGF-β1) levels in tumors. The DEAB assay was used to measure the protein concentration of samples. Levels of TGF-β1 in the samples were assessed by ELISA kits. Cont, control.

Discussion

In the present study, we evaluated the effect of combining low doses of aspirin and metformin on the growth of 4T1 breast cancer cells *in vitro* and *in vivo*. We also highlighted the link between TGF-β and estradiol in tumor apoptosis. A combination of aspirin and metformin showed synergistic cytotoxicity in 4T1 cells and a significant inhibitory effect on *in vivo* tumor growth through regulation of important apoptosis-related proteins, such as Bcl-2, Mcl-1, Bax, and caspase-8, and consequent cell death. A combination of the two drugs was notably

effective at increasing TGF-β1 levels in the supernatant fluid of 4T1 cells and in the blood of 4T1 tumor-bearing mice. Estradiol in 4T1 tumor-bearing mice weakened the antitumor effect of aspirin and metformin by downregulating TGF-β1 and promoting Smad2 and Smad3 degradation *in vivo*.

To the best of our knowledge, this is the first study to show that the TGF-β signaling pathway mediates the inhibitory effect of combined aspirin and metformin treatment on tumors. We reported previously that apoptosis of tumor tissue was induced and micro-vessel density was decreased after high-dose aspirin treatment, without any severe damage to the

stomach, small intestine, liver and spleen (40). Epidemiological evidence has shown a consistent prophylactic effect of aspirin on breast cancer (42,43). After 20 years of follow-up, overall cancer mortality has been shown to be decreased by ~20% in people taking aspirin, with the greatest benefit for adenocarcinomas (36% reduction) in randomized prevention trials (44,45). In particular, aspirin has a significant effect on preventing colorectal cancer (46) and risk reduction (47).

Metformin therapy weakens the risk of glioma-initiating cells (48), and inhibits ovarian cancer by increasing sensitivity to cisplatin (49,50), endometrial cancer through changes in Ki-67 proliferation (51-53), and breast cancer (54,55) and non-small cell lung carcinomas (56,57). Laboratory studies on breast cancer have shown that metformin increases the mean life span by 8% and mammary adenocarcinoma latency by 13.2% (P<0.05) in HER2/neu mice (58). In retrospective studies, long-term use of ≥40 prescriptions (>5 years) of metformin is associated with an improved adjusted odds ratio of developing breast cancer compared with no use (59).

In the present study, we observed that a combination of aspirin and metformin had a stronger inhibitory effect on 4T1 cell proliferation than either drug alone. This effect depend on TGF-β1, in which levels increased following aspirin and metformin treatment. Western blot results showed markedly increased pSmad2 and pSmad3 levels in the recombinant TGF-β1 group, and the combined aspirin and metformin group, but only a marginal increase in the aspirin alone group. Moreover, we showed that metformin combined with aspirin regulated apoptosis-related proteins, mimicking the effect of recombinant TGF-β1 treatment. This resulted in decreased Mcl-1 and Bax, and increased Bcl-2 and caspase-8 levels. No obvious increase in caspase-8 (p18) was detected following metformin treatment alone. Moreover, an increase in early and late apoptosis following TGF-β addition was consistent with the above data.

4T1 cells were injected subcutaneously into BALB/c mice *in vivo*. Compared to aspirin or metformin alone, mice subjected to a combination of the two drugs showed the highest TGF-β1 content, smallest tumor size, and highest degree of tumor cell apoptosis. These results suggested that a combination of aspirin and metformin could significantly inhibit 4T1 cell growth *in vitro* and *in vivo* by promoting autocrine/paracrine TGF-β1 to regulate apoptosis-related proteins.

TGF-β shows suppressive effects at the early stage of tumorigenesis, whereas tumor cells in advanced stages can avoid the antiproliferative effect and undergo tumorigenic progression in response to TGF-β (60,61). In the present study, we report that aspirin and/or metformin stimulated TGF-β1, which could then suppress survival of breast cancer cells and phosphorylation of Smad2 and Smad3. *In vivo* experiments revealed that, in tumor-bearing mice treated for a maximum of 72 h with aspirin and/or metformin, and sacrificed 15 days later when the tumor did not develop to an advanced stage, smaller sized tumors contained the most TGF-β1. Whether TGF-β secretion can also be induced by aspirin and/or metformin in advanced cancer, and what the ensuing effect may be, will be addressed in future studies.

It was reported earlier that treatment of an adenoma cell line with TGF-β triggered an increase in COX-2, which led to growth inhibition and apoptosis-mediated cell death (62-64).

As a nonsteroidal anti-inflammatory drug and COX inhibitor, aspirin prevents breast tumorigenesis in humans (12). Inhibition of the Smad signaling pathways attenuates TGF-β1-induced COX-2 expression (15). Thus, it is possible that the Smad signaling pathway mediates TGF-β-induced COX-2 by producing feedback inhibitory effects. There are some reports that metformin weakens the effect of TGF-β1 or inhibits the TGF-β pathway in normal cells (65,66) or some metastatic tumor cells (67,68) that differ from 4T1 cells. TGF-β plays different roles in different tumor stages (27-30). Thus, the different states of experimental cells may cause these different findings. It also has been shown that metformin increases nuclear p53 and TGF-β1 levels in human breast cancer cell lines (69) and the metformin-mediated stimulation of TGF-β1 secretion by mesangial cells is dose-dependent (70).

Tamoxifen, the mainstay of endocrine therapy for breast cancer, acts as a competitive inhibitor of the ER (71). It blocks the feedback loop of TGF-β signaling (72,73) by recruiting the N-CoR-histone deacetylase complex to the promoter (74-76). Binding of estrogen to the ER promotes formation of a multi-protein complex including N-CoR-histone deacetylase that removes acetyl groups and turns off transcription (77-79). Estrogen can reduce the expression of more than two-thirds of the genes induced by TGF-β treatment (33). Consistent with this, we believe that estradiol from female BALB/c mice could attenuate the growth inhibition induced by aspirin and metformin by downregulating TGF-β1. *In vitro*, we observed that, during early and late apoptosis, the levels of Bcl-2 and caspase-8 were further decreased by simultaneous treatment with estradiol and aspirin or metformin, compared to aspirin or metformin alone. Inhibitors of the TGF-β receptor produced a similar outcome. *In vivo,* the combination of aspirin, metformin, and tamoxifen led to the highest level of TGF-β1, smallest tumor size, greatest degree of apoptosis in tumors, and the least estradiol in peripheral blood.

In conclusion, a combination of aspirin and metformin exhibits a synergetic cytotoxic effect in 4T1 breast cancer cells *in vitro*, and a significant inhibitory effect on 4T1 tumor growth *in vivo*. In addition, inhibition of estrogen further maximizes antitumor activity of the combined drug treatment *in vivo*. However, according to the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology (80), patients with triple-negative breast cancer are not recommended for estrogen suppression treatment. The present study provides a rationale for clinical trials that combine aspirin with metformin, and brings attention to the link between estrogen levels and the outcome in triple-negative breast cancer patients.

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