Combined treatment with vitamin C and methotrexate inhibits triple-negative breast cancer cell growth by increasing H_2O_2 accumulation and activating caspase-3 and p38 pathways

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Abstract. Methotrexate (MTX) is widely used as both an anticancer and anti-rheumatoid arthritis drug. Although MTX has been used to inhibit the growth of many cancer cells, it cannot effectively inhibit growth of triple-negative breast cancer cells (TNBC cells). Vitamin C is an antioxidant that can prevent oxidative stress. In addition, vitamin C has been applied as adjunct treatment for growth inhibition of cancer cells. Recent studies indicated that combined treatment with vitamin C and MTX may inhibit MCF-7 and MDA-MB-231 breast cancer cell growth through G2/M elongation. However, the mechanisms remain unknown. The aim of the present study was to determine whether combined treatment with low-dose vitamin C and MTX inhibits TNBC cell growth and to investigate the mechanisms of vitamin C/MTX-induced cytotoxicity. Neither low-dose vitamin C alone nor MTX alone inhibited TNBC cell growth. However, combined low-dose vitamin C and MTX had synergistic anti-proliferative/cytotoxic effects on TNBC cells. In addition, co-treatment increased H_2O_2 levels and activated both caspase-3 and p38 cell death pathways.

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Introduction

Approximately 15-20% of breast cancer cells are triple-negative (TNBC cells) (1,2), lacking estrogen receptors (ERs), progesterone receptors (PRs) and epidermal growth factor receptor 2 (EGFR2). Expression of these receptors allows for treatment with endocrine or targeted therapies in clinical cases (3-5), which are not useful for clinical TNBC cell treatment (6-8). Therefore, it is important to develop new methods for suppressing TNBC cell growth and survival. Methotrexate (MTX) is a well-known antagonist of folic acid (9,10) and has been used widely for rheumatoid arthritis treatment (11,12). In addition, MTX has been applied for clinical cancer treatment (13,14). Previous studies demonstrated that MTX can inhibit the growth of various cancer cells, including hepatoma, leukemia, lymphoma and gastric cancer cells (15-17). Nevertheless, MTX alone is not effective for breast cancer treatment. In order to enhance the anticancer activities of MTX on breast cancer cells, combining MTX with other agents has been considered. Currently, combined chemotherapy with MTX and other anticancer drugs, such as mitomycin C, cyclophosphamide and 5-fluorouracil, is used to treat breast cancer (18-20). However, serious side-effects of these chemicals have been reported (21-25). Therefore, drugs that can promote the anticancer activities of MTX with reduced side-effects are urgently needed.

Vitamin C, a common nutrient, has anti-oxidative (26,27) and anticancer activities (28,29). Previous studies have also demonstrated that combined treatment with vitamin C and conventional anticancer agents can enhance anticancer activities (15,30,31). Currently, vitamin C supplements are being applied for clinical cancer therapy (32-34). However, vitamin C

actually inhibits tamoxifen-induced cell death in ER-positive breast cancer (35). Alternatively, high-dose vitamin C alone can inhibit cancer cell growth, though the mechanisms remain elusive (36-38). One study also showed that vitamin C can attenuate the incidence of ER-positive breast cancer cells (39). However, there is no evidence demonstrating that vitamin C alone is useful for TNBC treatment.

A recent study reported that vitamin C (30 μM to 4 mM) plus MTX can inhibit the growth of MCF-7 cells (an ER-positive breast cancer cell line) and MDA-MB-231 cells (a type of TNBC) through G2/M elongation and PI3K activation (30). However, the mechanisms of vitamin C/MTX-induced cytotoxicity on breast cancer cells are still unclear. Therefore, whether combined treatment with low-dose vitamin C (5 μM) and MTX can inhibit TNBC cell growth and the mechanisms of vitamin C/MTX-induced cytotoxicity were examined in the present study.

Materials and methods

Materials. Vitamin C, Luminol, Lucigenin and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-tubulin (1:1,000; cat. no. BS1699), anti-p38 (1:400; cat. no. BS3567) and anti-p-p38 (1:400; cat. no. BS4766) primary rabbit polyclonal antibodies were acquired from Bioworld Technology, Inc., (Louis Park, MN, USA). Anti-cleaved PARP (1:2000; cat. no. 9544) and anti-caspase-3 (1:1000; cat. no. 9965) primary rabbit polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2,000, cat. no. 7074) were from Cell Signaling Technology (Danvers, MA, USA). Tarceva (Erlotinib) was purchased from Roche Ltd. (Kaiseraugst, Switzerland). An MTT assay kit was obtained from Bio Basic Inc. (Markham, ON, Canada). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, L-glutamine and penicillin/streptomycin were obtained from Gibco-BRL (Invitrogen Life Technologies, Carlsbad, CA, USA).

Cell lines and culture. Triple-negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468) were purchased from the Bioresource Collection and Research Center (Hsin-chu, Taiwan). Tarceva-resistant MDA-MB-231 cells (MDA-MB-231 TR) were kindly provided by Dr Yung-Luen Yu (Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan). These cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C and cultured with DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin. In addition, 100 μM tarceva was added to the media for MDA-MB-231 TR culture.

Determination of cell viability. Cell viability was measured by the MTT assay described in previous studies (40,41). Briefly, cells were cultured into 96-well plates ($5x10^3$ cells/well). Every 24 h, the control and experimental groups were treated with MTT. After incubation for 3 h at 37˚C, the formazan product was dissolved and absorbance measured at 570 nm (A570) using a Multiskan™ FC microplate photometer (Molecular Devices, Sunnyvale, CA, USA). The viable cell count (%) was calculated as (A570 experimental group)/(A570 control group) x 100%.

Measurements of intracellular H_2O_2 and O_2^-. Intracellular H_2O_2 and O_2^- were measured using the lucigenin-amplified chemiluminescence method (40,42). The samples (200 μl) were added to 0.2 mmol/ml of luminol solution (100 μl) for H_2O_2 measurement or to 0.1 mmol/ml lucigenin solution (500 μl) for O_2^- measurement. Next, all samples were analyzed using a chemiluminescence analyzing system (CLA-FSI; Tohoku Electronic Industrial, Co., Ltd., Sendai, Japan). The H_2O_2 and O_2^- were observed and incubated for 5 min.

Observation of DNA fragmentation and nuclear condensation. Nuclear condensation and DNA fragmentation, cardinal characteristics of apoptotic cells, were observed using Hoechst 33342 nuclear staining (40,41). Control and experimental (MTX and/or vitamin C-treated) cells were incubated in Hoechst 33342 (10 μg/ml) for 5 min. DNA fragmentation and nuclear condensation were observed under an Olympus DP71 fluorescence microscope (excitation, 352 nm; emission, 450 nm; Olympus Corp., Tokyo, Japan).

SDS electrophoresis and western blotting. Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (cat. no. 20-188; EMD Millipore, Billerica, MA, USA). Proteins were collected from the supernatant layer after centrifugation (16,000 x g; 4˚C) for 20 min. The protein concentration was measured using a protein assay kit (cat. no. 23200; Thermo Fischer Scientific, Inc., Waltham, MA, USA). Equal quantities (40 μg) of protein were separated by SDS-PAGE using 13.3% gels (80 volts) and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk at room temperature for 2 h then washed with phosphate-buffered saline (PBS). After the incubation with primary antibodies for 4 h, the membranes were washed with PBS and treated with anti-rabbit HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the immunolabeled proteins were treated with Western Lightning® chemiluminescence Plus reagent (Perkin-Elmer, Inc., Waltham, MA, USA) and observed with a Luminescence Image Analysis system (LAS-4000; FujiFilm Electronic Materials Taiwan, Co., Ltd., Tainan, Taiwan).

Statistical analysis. All data were obtained from four independent experiments and presented as the mean ± SE. Means were compared by Student's t-test using Microsoft Excel (http://microsoft-excel-2010.updatestar.com/zh-tw). A $P<0.05$ was considered statistically significant.

Results

Combined treatment with low-dose vitamin C and MTX effectively inhibits TNBC cell proliferation and viability. We first examined the effects of various concentrations of MTX on TNBC cell (MDA-MB-231) growth and survival. Low-dose MTX alone (0.1 and 0.01 μM) did not inhibit TNBC cell growth after 96-h treatment, and cell viability as measured by MIT assay was maintained at ~75-100% of control from 24 to 96 h (Fig. 1). Only 10 μM MTX reliably inhibited

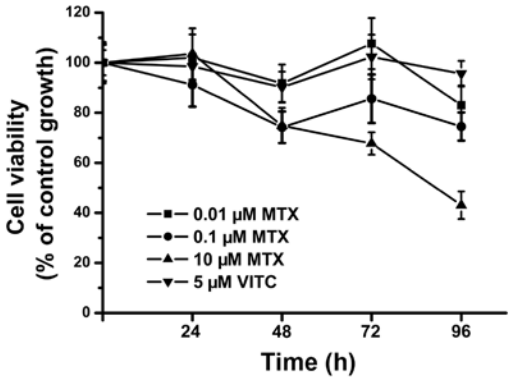

Figure 1. Effects of MTX alone on TNBC cell viability. MDA-MB-231 cells were treated with 0.1, 0.1 or 10 μM MTX for 96 h and viability measured by MTT assay. Cell viability was calculated as A570 experimental group/A570 control group x 100%. Data are from four independent experiments and presented as mean ± SD. The *P<0.05, compared to 0.01 μM MTX-treated group.

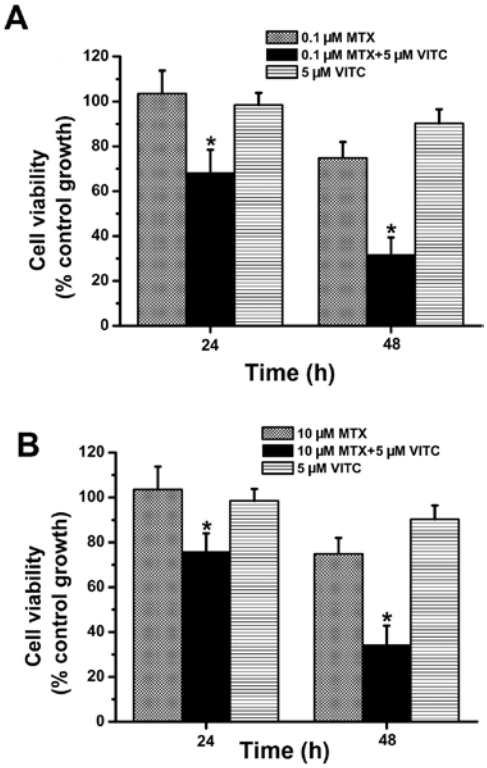

Figure 2. Effects of MTX plus low-dose vitamin C on TNBC cell viability. (A) MDA-MB-231 cells were treated with 0.1 μM MTX, 5 μM vitamin C, or 0.1 μM MTX plus 5 μM vitamin C. (B) MDA-MB-231 cells were treated with 10 μM MTX, 5 μM vitamin C, or 10 μM MTX plus 5 μM vitamin C. Cell viability was measured with MTT. Data are from four independent experiments and presented as mean ± SD. *P<0.05, compared to MTX alone group.

TNBC cell growth at 96 h, with cell viability <50% (Fig. 1). Next, combined treatment with low-dose vitamin C (5 μM) and MTX was examined. As shown in Fig. 2, compared to MTX-treated and vitamin C-treated groups, the MTX plus vitamin C-treated group exhibited significantly lower cell viability at 24 and 48 h. Cell viability was >70% in all MTX-treated and vitamin C-treated groups (Fig. 2), but <40% at 48 h in the 0.1 μM MTX plus vitamin C-treated group and

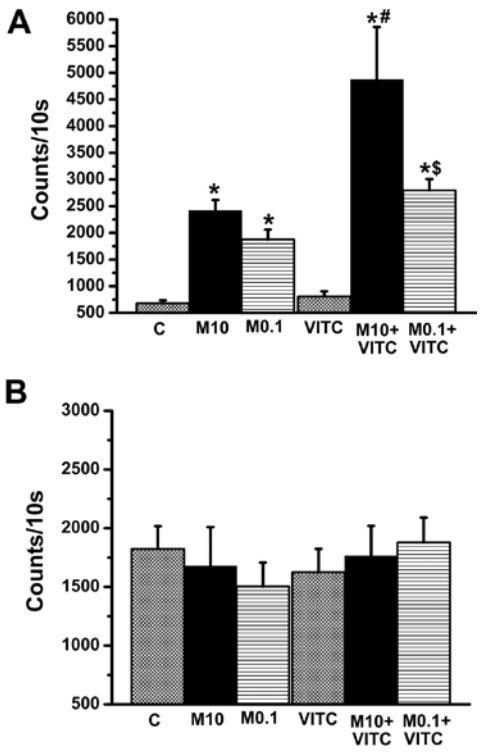

Figure 3. Effects of MTX plus low-dose vitamin C on intracellular H_2O_2 and O_2^-. (A) H_2O_2 fluorescence emission counts and (B) O_2^- counts in the control group (Con), 10 μM MTX group (M10), 0.1 μM MTX group (0.1M), 5 μM vitamin C group (VITC), 10 μM MTX plus 5 μM vitamin C group (M10 + VITC), and 0.1 μM MTX plus 5 μM vitamin C group (M0.1 + VITC). Data are from four independent experiments and presented as mean ± SD. *P<0.05, compared to control group. $^{\#}$P<0.05, compared to 10 μM MTX group. $^{\$}$P<0.05, compared to 0.1 μM MTX group.

10 μM MTX plus vitamin C-treated group (Fig. 2). Overall, these data demonstrate that combined treatment with low-dose vitamin C and MTX effectively inhibits TNBC cell proliferation and survival.

Vitamin C enhances MTX-induced intracellular H_2O_2 accumulation. MTX can increase reactive oxygen species (ROS) accumulation in cells with ensuing cytotoxicity (15,43). In contrast, vitamin C is an anti-oxidant against ROS increase (26,27). Both H_2O_2 and O_2^- are major ROS species in cells. Intracellular H_2O_2 and O_2^- were compared among the control group, MTX-treated group, vitamin C-treated group, and MTX plus vitamin C-treated group (Fig. 3A). Intracellular H_2O_2 levels were increased in 0.1 and 10 μM MTX groups, in accordance with a previous study (15). Surprisingly, vitamin C did not reduce H_2O_2 levels in MTX-treated groups. Compared to MTX-treated groups, H_2O_2 levels were increased significantly in the vitamin C plus MTX-treated group. In contrast, O_2^- levels did not differ among treatment groups (Fig. 3B). Our data suggest that increased intracellular H_2O_2 may contribute to the decrease in cell viability induced by MTX plus vitamin co-treatment.

MTX-treatment and combined MTX plus vitamin C treatment induce apoptosis and caspase-3 activation. Induction of apoptosis by these treatments was assessed by nuclear staining. As shown in Fig. 4, nuclear condensation and

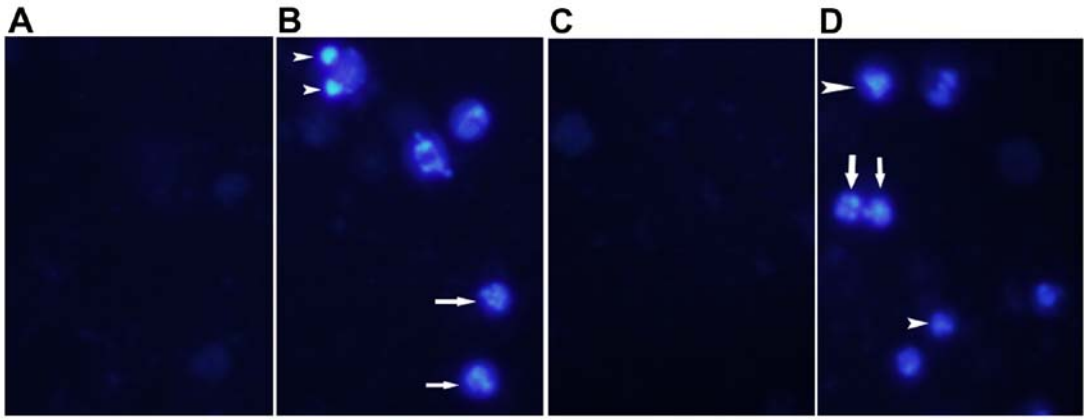

Figure 4. Induction of nuclear condensation and DNA fragmentation by MTX plus low-dose vitamin C. (A) Control cells, (B) 10 μM MTX-treated cells, (C) 5 μM vitamin C-treated cells, (D) 10 μM MTX plus 5 μM vitamin C-treated cells. MDA-MB-231 cells were treated for 72 h. Nuclear condensation and DNA fragmentation were observed by Hoechst 33342 staining. Nuclear condensation is indicated by arrow heads and DNA fragmentation by arrows.

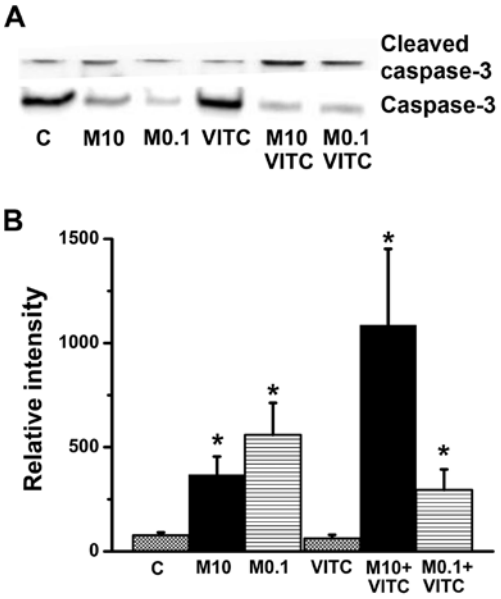

Figure 5. Caspase-3 activation by MTX plus low-dose vitamin C. (A) Western blot analysis and (B) cleaved caspase-3/caspase-3 intensity ratio were determined after 72 h in the control group (C), 10 μM MTX group (M10), 0.1 μM MTX group (0.1M), 5 μM vitamin C group (VITC), 10 μM MTX plus 5 μM vitamin C group (M10 + VITC), and 0.1 μM MTX plus 5 μM vitamin C group (M0.1 + VITC). Data from four independent experiments are presented as mean ± SD. *P<0.05, compared to control group.

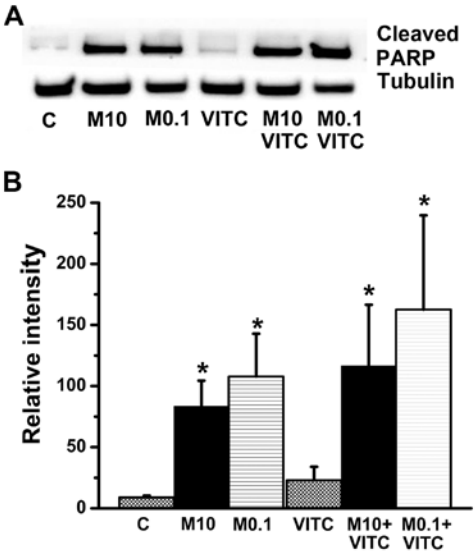

Figure 6. PARP cleavage by MTX plus low-dose vitamin C. (A) Western blot analysis and (B) cleaved PARP/tubulin intensity ratio determined after 72 h in the control group (C), 10 μM MTX group (M10), 0.1 μM MTX group (0.1M), 5 μM vitamin C group (VITC), 10 μM MTX plus 5 μM vitamin C group (M10 + VITC) and 0.1 μM MTX plus 5 μM vitamin C group (M0.1 + VITC). Data from four independent experiments are presented as mean ± SD. *P<0.05, compared to control group.

DNA fragmentation were observed in MTX-treated and MTX plus vitamin C-treated groups. Apoptosis can be initiated by caspase-dependent and caspase-independent pathways (44,45). Therefore, caspase-3 activation was examined by western blotting. As shown in Fig. 5, compared to the control group, the ratio of cleaved (activated) caspase-3 to native caspase-3 was increased significantly in both the MTX-treated and MTX plus vitamin C-treated group. PARP is a downstream substrate of caspase-3, thus, cleaved PARP is a sign of caspase-3 activation. Indeed, cleaved PARP level was also increased in both MTX-treated and MTX plus vitamin C-treated groups. Taken together, these results indicate that combined MTX/vitamin C induces caspase-3-dependent apoptosis in TNBC cells.

p38 phosphorylation in MTX-treated and MTX plus vitamin C-treated cells. The MAPK family kinases ERK, JNK and p38 are involved in cell death, cell differentiation, and cell proliferation (46-48). In the present study, expression levels of EKR, JNK, p38 and their phosphorylated (activated) forms (p-ERK, p-JNK and p-p38) were estimated by western blotting. The ratio of p-p38 to p38 was significantly increased in both MTX-treated and MTX plus vitamin C-treated groups (Fig. 7), while ERK and JNK expression levels did not differ significantly among groups (data not show). Thus, MTX/vitamin C-induced cytotoxicity of TNBC cells is associated with p38 activation.

Combined treatment with vitamin C and MTX inhibits growth of tarceva-sensitive, but not tarceva-resistant TNBC cells. As

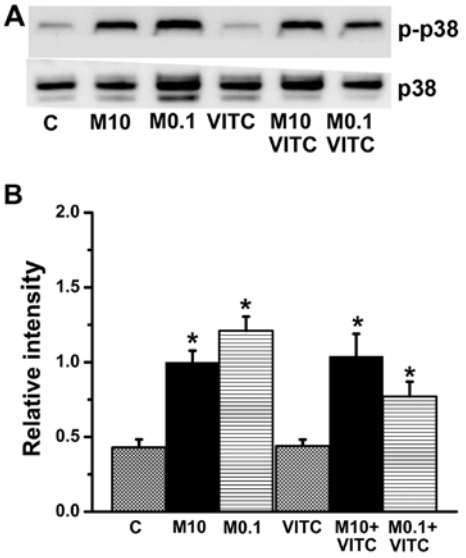

Figure 7. Phospho-activation of p38 by MTX plus low-dose vitamin C. (A) Western blot analysis and (B) phosphorylated p38/p38 intensity ratio determined at 30 min in the control group (C), 10 μM MTX group (M10), 0.1 μM MTX group (0.1M), 5 μM vitamin C group (VITC), 10 μM MTX plus 5 μM vitamin C group (M10 + VITC), and 0.1 μM MTX plus 5 μM vitamin C group (M0.1 + VITC). Data from four independent experiments are presented as mean ± SD. *P<0.05, compared to control group.

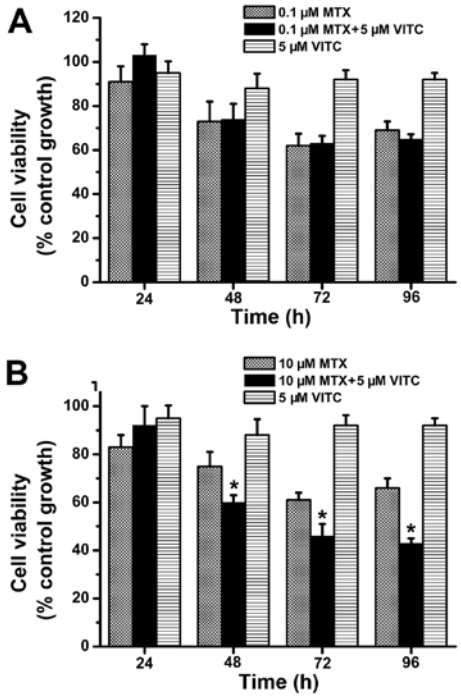

Figure 8. Effects of MTX plus low-dose vitamin C on other TNBC cell lines. (A) MDA-MB-468 cells were treated with 0.1 μM MTX, 5 μM vitamin C, and 0.1 μM MTX plus 5 μM vitamin C. (B) MDA-MB-468 cells were treated with 10 μM MTX, 5 μM vitamin C, and 10 μM MTX plus 5 μM vitamin C. Cell viability were measured with MTT assays. Data from four independent experiments are presented as mean ± SD. *P<0.05, compared to MTX alone group.

shown in Fig. 2, combined treatment with vitamin C and MTX effectively inhibited MDA-MB-231 cell growth. However, it is unclear whether MTX plus vitamin C is useful against other

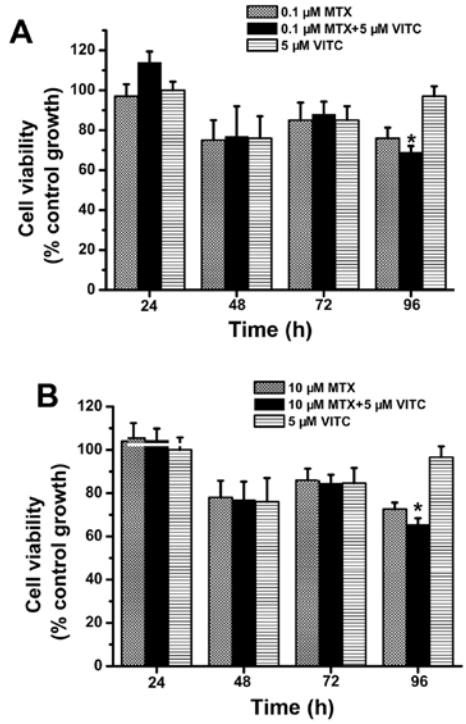

Figure 9. Effects of MTX plus low-dose vitamin C on tarceva-resistant TNBC cells. (A) MDA-MB-231 TR cells were treated with 0.1 μM MTX, 5 μM vitamin C, and 0.1 μM MTX plus 5 μM vitamin C. (B) MDA-MB-231 TR cells were treated with 10 μM MTX, 5 μM vitamin C, and 10 μM MTX plus 5 μM vitamin C. Cell viability measured with MTT assays. Data from four independent experiments are presented as mean ± SD. *P<0.05, compared to MTX alone group.

TNBC cells, thus, we examined the cell viability of TNBC cell lines MDA-MB-468 and MDA-MB-231 TR during MTX, vitamin C and combined treatment. Compared to the 10 μM MTX-treated group, the combined treatment group exhibited lower viability at 48, 72 and 96 h (Fig. 8B). Importantly, cell viability was <50% at 72 and 96 h in the 10 μM MTX plus vitamin C-treated group (Fig. 8B). Alternatively, cell viability did not differ among the control, 0.1 μM MTX-treated, and 0.1 μM MTX plus vitamin C-treated groups (Fig. 8A). These data indicate that 1 μM MTX plus vitamin C can effectively inhibit MDA-MB-468 proliferation/survival compared to MTX treatment. However, in similar assays of tarceva-resistant MDA-MB-231 TR cells, the MTX plus vitamin C-treated group exhibited significantly lower cell viability only at 96 h and was >60% for all other groups (Fig. 9). That is, neither MTX alone nor MTX plus vitamin C inhibited the growth of tarceva-resistant TNBC cells as effectively as tarceva-sensitive TNBC cells. Taken together, these findings (Figs. 2, 8 and 9) suggest that MTX plus vitamin C treatment can inhibit the growth of tarceva-responsive, but not tarceva-resistant TNBC cells.

Discussion

MTX alone is not useful for breast cancer treatment, but a recent study found that high-dose vitamin C (30 μM to 4 mM) enhanced the anticancer activities of MTX on breast cancer cells, including MCF-7 cells (ER-positive breast cancer)

and MDA-MB-231 cells (30). That study also found G2/M elongation and PI3K pathway activation associated with vitamin C/MTX-induced cytotoxicity. The present study found that MTX had anti-proliferative/cytotoxic effects on TNBC cells only when combined with low-dose vitamin C (5 μM). The present study further suggests that increased intracellular H_2O_2 levels and activation of caspase-3 and p38 pathways are involved in vitamin C/MTX-induced cytotoxicity. In addition, combined treatment with low-dose vitamin C and MTX inhibited cell growth not only of MDA-MB-231 cells but also of MDA-MB-468 cells. Therefore, this study suggests that vitamin C plus MTX treatment may be effective for clinical suppression of TNBC cell growth.

Although a previous study (30) and the present study (Figs. 2 and 8) demonstrated that vitamin C plus MTX effectively inhibits TNBC cell growth, vitamin C plus MTX treatment did not effectively inhibit tarceva-resistant TNBC cells (Fig. 9). Tarceva (erlotinib) is an EGFR tyrosine kinase inhibitor (49,50) and has been applied for clinical treatment of lung and breast cancers (51-54). These findings suggest that EGFR signaling may be involved in vitamin C/MTX-induced cytotoxicity.

Apoptosis can be induced via caspase-dependent and caspase-independent pathways (44,45). Vitamin C treatment can induce apoptosis of breast cancer cells and lung cancer cells via the caspase-independent pathway (36,55,56). On the other hand, vitamin C treatment can induce apoptosis of melanoma cells and hepatoma cells via the caspase-dependent pathway (15,57). In the present study, vitamin C plus MTX treatment activated caspase-3 in TNBC cells. Thus, whether apoptosis occurs via caspase-dependent or caspase-independent pathways may depend on the specific cancer cell type.

At low doses, vitamin C has anti-oxidant activities (26,27), and many studies have demonstrated that vitamin C supplements can decrease oxidative stress (15,58,59). However, high-dose (millimolar) vitamin C treatment can increase oxidative stress (60-62), and previous studies showed that high-dose vitamin C can enhance intracellular H_2O_2, resulting in cancer cell death (37,63,64). Surprisingly, another study reported that low-dose (micromolar) vitamin C attenuated H_2O_2 levels but enhanced the anticancer activities of MTX on hepatoma cells (15). In addition, vitamin C/MTX only enhances H_2O_2 levels but not O_2^- levels in TNBC cells. These results indicated vitamin C/MTX does not inhibit the function of superoxide dismutase, while vitamin C/MTX may influence GSH levels or activity of glutathione peroxidase.

As shown in Fig. 9, the cell viability was not significantly different before 72 h between MTX alone group and MTX plus vitamin C group. The cell viability was significant different only at 96 h between MTX alone group and MTX plus vitamin C group. The results indicated that vitamin C is not efficient in enhancing MTX-induced cytotoxicity in tarceva-resistant TNBC cells. Previously, many studies showed that tarceva-resistant cells have EGFR gene mutations or additional bypass signaling pathways to activate downstream of EGFR (65,66). These mutation genes and bypass pathways are important factors for cell proliferation with EGFR inhibitor treatment. These factors may cause the vitamin C/MTX inefficiency in inducing cytotoxicity in tarceva-resistant cells. However, many bypass signals are related to tarceva-resistant cells, such as SOS1, NF-κB and Fas receptor, signals of which influence vitamin C/MTX-induced cytotoxicity, however, this remains to be studied in the future.

Collectively, we found that combined treatment with low-dose vitamin C and MTX enhanced intracellular H_2O_2 accumulation and suppressed TNBC cell growth. Therefore, we suggest that both vitamin C dose and cell type may influence cellular H_2O_2 levels during treatment.

Acknowledgements

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