Translational Oncology
www.transonc.com

Volume 13 Number 2 February 2020 pp. 401–409 401

Enhanced Anticancer Effect of Adding Magnesium to Vitamin C Therapy: Inhibition of Hormetic Response by SVCT-2 Activation

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Abstract

L-Ascorbic acid (vitamin C, AA) is known as an antioxidant, but at high concentrations, AA can kill cancer cells through a prooxidant property. Sodium-dependent vitamin C transporter family-2 (SVCT-2) determines the cellular uptake of AA, and the activity of SVCT-2 is directly related to the anticancer activity of AA. Cancer cells that showed high SVCT-2 expression levels were more sensitive to AA treatment than cancer cells with low SVCT-2 expression levels. Cells with low SVCT-2 expression showed a hormetic response to a low dose of AA. Magnesium ions, which are known to activate SVCT-2, could increase the V_{max} value of SVCT-2, so we investigated whether providing magnesium supplements to cancer cells with low SVCT-2 expression that had shown a hormetic response to AA would elevate the V_{max} value of SVCT-2, allowing more AA to accumulate. To evaluate the effects of magnesium on cancer cells, $MgSO_4$ and $MgCl_2$ were screened as magnesium supplements; both forms showed synergistic anticancer effects with AA. Taken together, the results of this study suggest that magnesium supplementation enhanced the anticancer effect of AA by inhibiting the hormetic response at a low dose. This study has also demonstrated that AA treatment with magnesium supplementation provided more effective anticancer therapy than AA treatment alone.

Translational Oncology (2020) 13, 401–409

Introduction

High-dose L-ascorbic acid (vitamin C, AA) cancer therapy was introduced by Linus Pauling and Ewan Cameron [1–3]. Clinical demonstration results by Pauling and Cameron showed that intravenous injection of 10 g/day of vitamin C extended the survival time of terminal cancer patients by about 4.2 times. However, results from the Mayo Clinic showed that the survival time of vitamin C–treated patients was even shorter than that of the placebo group patients [4]. A significant difference between those two research groups was the route of AA administration: intravenous injection and oral administration, respectively. To understand the mechanism of AA's anticancer activity, many research groups have treated colon, prostate, leukemia, lymphoma, brain, and stomach cancer cells and chemically or genetically transformed cancer cells with AA and showed cancer growth inhibition and even cancer cell death through hydrogen peroxide–mediated reactive oxygen species (ROS) generation [5–11]. In most cases, the pharmacological concentration of vitamin C required for anticancer effects (EC_{50} value of 1–10 mM)

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Received 3 May 2019; Revised 24 October 2019; Accepted 29 October 2019

1936-5233/19
https://doi.org/10.1016/j.tranon.2019.10.017

could only be achieved by intravenous administration [12]. Thus, to apply [13] vitamin C as an anticancer therapy, a high intracellular concentration in cancer cells is critically important.

In our previous study, we investigated the hormetic proliferation response of cancer cell lines with low cellular expression levels of sodium-dependent vitamin C transporter family-2 (SVCT-2). When a low dose of vitamin C was delivered into such cancer cells, increased proliferation activity was observed [13]. Because hormetic proliferation of cancer cells occurred on low-dose treatment with vitamin C in cell lines with low SVCT-2 expression, we need to develop a more effective approach for vitamin C anticancer therapy in cells that express little SVCT-2. The close correlation between SVCT-2 expression and the anticancer effects of vitamin C therapy suggests that SVCT-2, which is a key transporter for vitamin C uptake [14,15], could be a potent biomarker for high-dose vitamin C cancer therapy [16]. In breast and colon cancer cell lines, the anticancer effect of vitamin C showed a positive correlation between the SVCT-2 expression of the cancer cells and intracellular vitamin C concentration [16].

To prevent a hormetic response during vitamin C treatment and induce vitamin C cancer therapy more effectively, magnesium ion supplementation was recommended to increase the vitamin C uptake activity of SVCT-2 [17]. Myer's cocktail, which contains vitamins and mineral mixtures (including magnesium ions), was developed for use with intravenous vitamin C injections [18—20], and it has been broadly used in clinics [21,22].

When Myer's cocktail was first introduced, no information was available about the relationship between magnesium ions and SVCT-2 activity. Therefore, we have focused on using magnesium ion supplementation with vitamin C cancer therapy to prevent the hormetic response in cancer cell lines with low SVCT-2 levels. In this study, we demonstrated that adding magnesium ions to the vitamin C solution enhanced the anticancer effects of vitamin C by increasing the vitamin C transport activity of SVCT-2 in cancer cell lines with both high and low levels of SVCT-2. Moreover, our results show that adding magnesium supplementation to vitamin C cancer therapy could provide more effective cancer therapy and prevent the hormetic response in cancer cells with low SVCT-2 levels.

Materials and Methods

Cell Culture and Reagents

Mouse colorectal cancer cells from the CT26 cell line and human breast cancer cells from the SK-BR-3 and MCF-7 cell lines were purchased from ATCC. Human breast cancer cells were cultured in 10% fetal bovine serum (Pan Biotech, Aidenbach, Germany) and 1% Pen-strep (Pan Biotech) added to Dulbecco Modified Eagle Medium (DMEM) (Gilbco, Cergy Pontoise, France) at 37 °C in a humidified incubator with 5% CO_2. L-Ascorbic acid was purchased from BCWorld Pharm. Co. (Seoul, Korea). Magnesium chloride ($MgCl_2$) and magnesium sulfate ($MgSO_4$) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell Viability Assay

Cells were stained and counted with a cell counting kit-8 (Dojindo's Molecular Technologies, Tokyo, Japan). SK-BR-3 and MCF-7 cells were plated on 96-well plates (1×10^4 cells/well). Twenty-four hours after plating, the cells were treated with vitamin C in serum-free DMEM. After 4 h, the cells were washed with phosphate buffered saline (PBS) and cultured with DMEM (plus 10% fetal bovine serum with 1% Pen-strep) for 20 h. To test the cytotoxicity of the magnesium supplements, $MgSO_4$ and $MgCl_2$ were mixed with DMEM and added to the cells. After a 24-h incubation, cell viability was tested.

Western Blotting

Proteins were extracted from cells with an RIPA buffer containing a protease inhibitor cocktail (iNtRON, Seongnam, Korea). Protein concentrations were measured using the Bradford assay (Bio-Rad, Munich, Germany). A total of 20 μg of protein were denatured in sample buffer for 5 min at 95 °C. The samples were loaded onto 12% SDS-polyacrylamide gels and transferred to nitrocellulose blotting membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline at room temperature for 30 min. After three washes in Tris-buffered saline-0.10% Tween 20, the membranes were incubated with anti-SVCT-2 (1:2500; NB2-13319; Novus Biologics, Littleton, CO, USA), anti—beta-actin (1:5000; NB600-501; Novus Biologics), anti-p21 (1:2500; bs-10129R; Bioss, Beijing, China), and anticaspase-3 (1:2500; #9662S; Cell Signaling Technology, Danvers, MA, USA) antibodies at 4 °C overnight. After four washes in Tris-buffered saline-0.10% Tween 20 for 20 min, the membranes were incubated with secondary antirabbit antibodies for 2 h at room temperature. After additional washing, immune-reactive bands were detected using ECL substrate (Pierce, Rockford, IL, USA) and exposed to X-ray film (Agfa-Gevaert N.V., Septestraat, Mortsel, Belgium).

Immunocytochemistry

After vitamin C treatment, the cells were stained with amine-reactive fluorescent dye in PBS (1:500; 423111; Biolegend, San Diego, CA). Then, the cells were fixed with 4% paraformaldehyde for 10 min and washed 3 times with PBS for 5 min. After 3 washes, the cells were mounted on slide glass with mounting medium containing DAPI (H-1200, Vector Laboratories, Burlingame, CA). The cells were observed using an LSM 700 laser scanning confocal microscope (Zeiss, Berlin, Germany) with a C-apochromat 40 × /1.2 water immersion objective. Stained cells were observed by confocal microscopy, and images were processed by the Zen black edition program (Zeiss).

Annexin V and Propodium Iodine Analysis

About 2×10^5 cells were seeded in 6-well plates and incubated for 24 h. Six hours after treatment with vitamin C and a magnesium supplement, the cells were stained with annexin V and propodium iodine according to the manufacturer's protocol (640914, Biolegend). After staining, cells were analyzed on a Guava EasyCyte mini instrument using Cytosoft software version 4.2.1 (Merck Millipore, Billerica, MA, USA).

Detection of ROS Generation

Cells were incubated with 20 μM 2′,7′-dichlorofluorescin diacetate (Sigma) in the culture medium for 20 min, detached with trypsin, and collected in 1 mL of PBS. Cells were washed two times with 500 μL of PBS and analyzed on a Guava EasyCyte mini instrument using Cytosoft software version 4.2.1 (Merck Millipore).

Vitamin C Uptake

Cells were harvested after a 2 h incubation with 1 mM vitamin C and washed with PBS. The magnesium cotreatment group was incubated

with 1 mM vitamin C and 5 mM $MgCl_2$ and $MgSO_4$. The cells were resuspended in 1 mL of PBS with 10% metaphosphoric acid (MPA) solution and lysed three times by freeze-thaw cycles in a −80 °C deep freezer. In addition, frozen mouse liver tissue was homogenized with a 10% MPA solution (2 g/mL). Animal and cell lysates were centrifuged at 16,000 rpm at 4 °C for 5 min, and the supernatant was harvested. Next, 100 μL of sample was mixed with 100 μL of precipitation reagents from vitamin C diagnostic kits (Chromsystems, Gräfelfing, Germany) and incubated for 10 min at 4 °C. The mixture was then centrifuged at 13,000 rpm for 5 min, and the supernatant was analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Tokyo, Japan) equipped with a Shim-pack CLC-ODS column (6 mm × 15 cm) connected to a Shim-pack G-ODS guard column (4 mm × 1 cm) (Shimadzu). The mobile phase was provided by Chromsystems, and the experiment was performed according to the instruction manual. The concentration of vitamin C in cells was determined by manual calculation: $C_{Analyte,\ Sample}(\mathrm{mg/l}) = \frac{A_{Sample} \times IS_{Standard}}{A_{Standard} \times IS_{Sample}} \times C_{Standard}$. The following instrument settings were used: injection volume 20 μL, run time 10 min, flow rate 1 mL/min, column temperature 25 °C, and UV detector wavelength 245 nm.

Animal Experiments

Eight-week-old male BALB/c mice were purchased from DBL. All the mice were housed in 12 h day/night conditions at 24 °C in a pathogen-free facility. This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Sungkyunkwan University School of Medicine (SUSM). SUSM facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all experimental procedures performed here are in accordance with the guidelines of the Institute of Laboratory Animal Resources. This study was also approved by the Administrative Panel of the Laboratory Animal Research Center of Sungkyunkwan University (approval number: 12−37).

About 1×10^6 of CT26 cells were used to create a subcutaneous xenograft tumor on each mouse's back. The tumor volume was measured using calipers and calculated as volume = (length) × (width)^2 × 0.5. When the tumor volume reached 20−30 mm^3, vitamin C (4 g/kg) and magnesium supplements were prepared in 200 μL of PBS according to a published protocol [23]. The vitamin C and magnesium supplement mixture was injected intraperitoneally every 2 days. Thirteen days after the first injection, the mice were sacrificed, and the livers were extracted for a vitamin C uptake analysis.

Results

Magnesium Supplementation—Enhanced Anticancer Effect of Vitamin C

To determine a concentration of the magnesium supplements that did not show cytotoxicity in SK-BR-3 and MCF-7 cells, the cells were cultured in DMEM supplied with 1−10 mM of two types of magnesium ($MgSO_4$ and $MgCl_2$). Twenty-four hours after treatment, cell viability was measured (Figure 1*A* and *B*). The cells showed no cytotoxicity at any of the tested concentrations. Next, SK-BR-3 (Figure 1*C*) and MCF-7 cells (Figure 1*D*) were cotreated with gradient concentrations (5 mM) of $MgSO_4$ and $MgCl_2$ and 1 mM vitamin C. In both SK-BR-3 and MCF-7 cells, the cell viability and anticancer effect of vitamin C increased from 5 to 41 percent, depending on the concentration of $MgSO_4$ and $MgCl_2$.

Better Anticancer Effect of Vitamin C with Magnesium Supplement in Cancer Cells with High SVCT-2 Expression

The SVCT-2 expression of two cell lines was investigated using western blot analysis (Figure 2*A*). SVCT-2 expression in MCF-7 cells

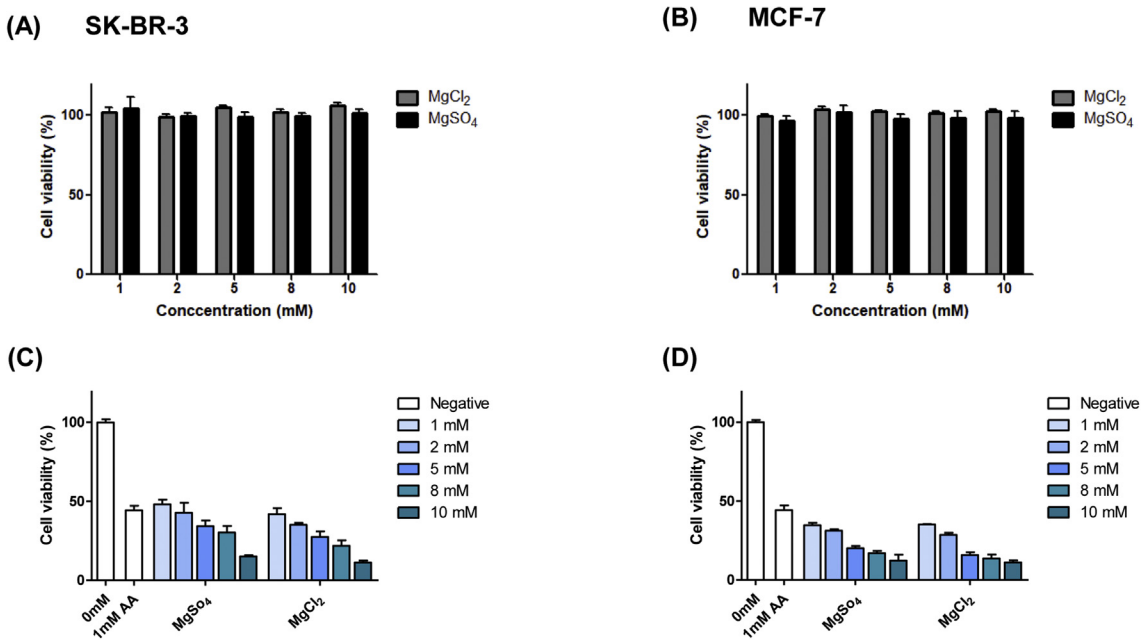

Figure 1. Cytotoxicity test of $MgCl_2$ and $MgSO_4$ and the synergistic anticancer effects of vitamin C with gradient concentrations of $MgCl_2$ and $MgSO_4$. Noncytotoxic concentrations of $MgCl_2$ and $MgSO_4$ were determined using cell viability assays. **A, B.** The cytotoxicity of $MgCl_2$ and $MgSO_4$ was tested in MCF-7 and SK-BR-3 cells. A noncytotoxic concentration of $MgCl_2$ and $MgSO_4$ was added to 1 mM vitamin C. **C, D.** Cell viability assay results from the cotreatment of 1 mM vitamin C with gradient concentrations of $MgCl_2$ and $MgSO_4$. Data are presented as means ± SEMs.

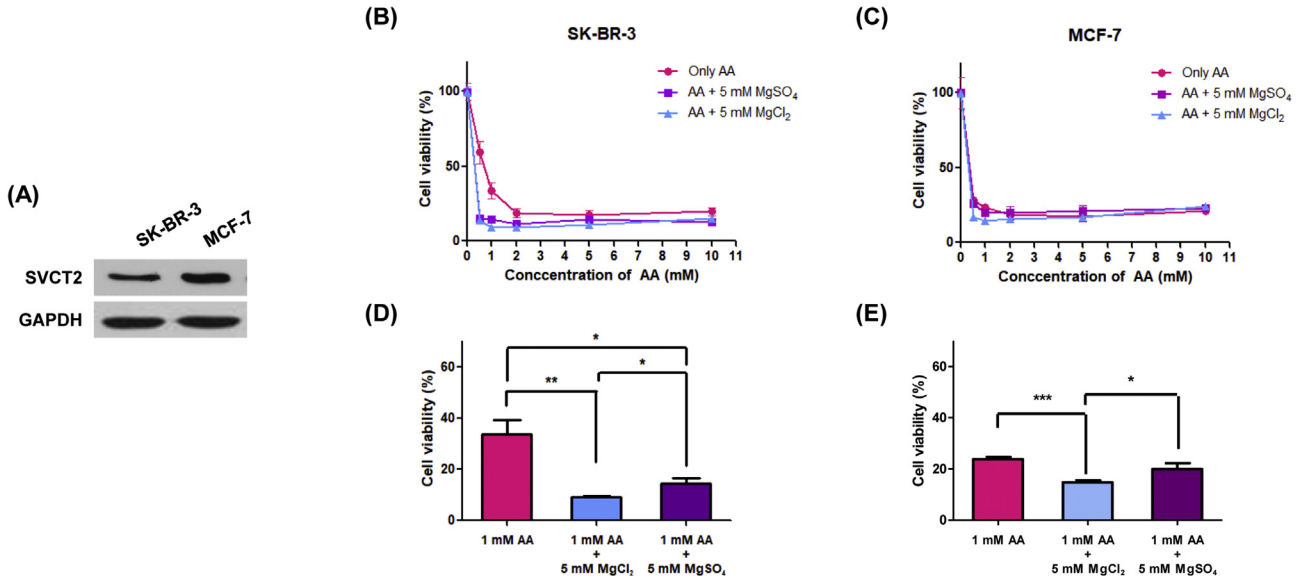

Figure 2. Cotreatment with vitamin C and a magnesium supplement showed more effective anticancer activity in cells with low SVCT-2 expression than in cells with high SVCT-2 expression. A. The expression of SVCT-2 in cancer cells was analyzed using a western blot analysis. **B, C.** Cell viability results of cotreatment with a gradient concentration of vitamin C and 5 mM $MgCl_2$ and $MgSO_4$. **D, E.** 5 mM magnesium supplementation enhanced the anticancer effects of vitamin C. Data are presented as means ± SEMs. $^*P < 0.05$, $^{**}P < 0.005$ $^{***}P < 0.001$.

was about 1.5 times higher than the expression in SK-BR-3 cells. Both $MgSO_4$ and $MgCl_2$ supplementation (5 mM) enhanced the anticancer effect of vitamin C in both MCF-7 and SK-BR-3 cells (Figure 2*B* and *C*). However, the cytotoxicity of vitamin C in the two cancer cell lines depended on their SVCT-2 expression levels; higher anticancer effects were shown in MCF-7 cells than in SK-BR-3 cells, with a positive correlation between SVCT-2 expression levels and the anticancer effects in both cell lines. In SK-BR-3 cells, both $MgSO_4$ and $MgCl_2$ significantly increased the cell deaths caused by vitamin C treatment (Figure 2*D*). In the MCF-7 cells, both $MgSO_4$ and $MgCl_2$ statistically increased cell viability compared with vitamin C treatment alone, but the anticancer effect of the $MgCl_2$ supplement was higher than that of the $MgSO_4$ supplement (Figure 2*E*).

We used amine-reactive green fluorescent dye to observe the enhanced anticancer activity of vitamin C according to magnesium supplementation (Figure 3*A* and *B*). The amine-reactive fluorescent dye could not enter live cells but could pass into dead cells. In both cell lines, cells treated with vitamin C plus a magnesium supplement showed more green fluorescence than those that received only vitamin C. But the green fluorescence intensity and the number of stained cells were both higher in MCF-7 cells, which express more SVCT-2 than SK-BR-3 cells.

To analyze the enhanced apoptosis caused by co-treatments of vitamin C and magnesium, annexin V and propodium iodine staining were performed by flow cytometry (Figure 4*A* and *B*). In the culture media supplemented with 5 mM $MgSO_4$ and $MgCl_2$, the numbers of

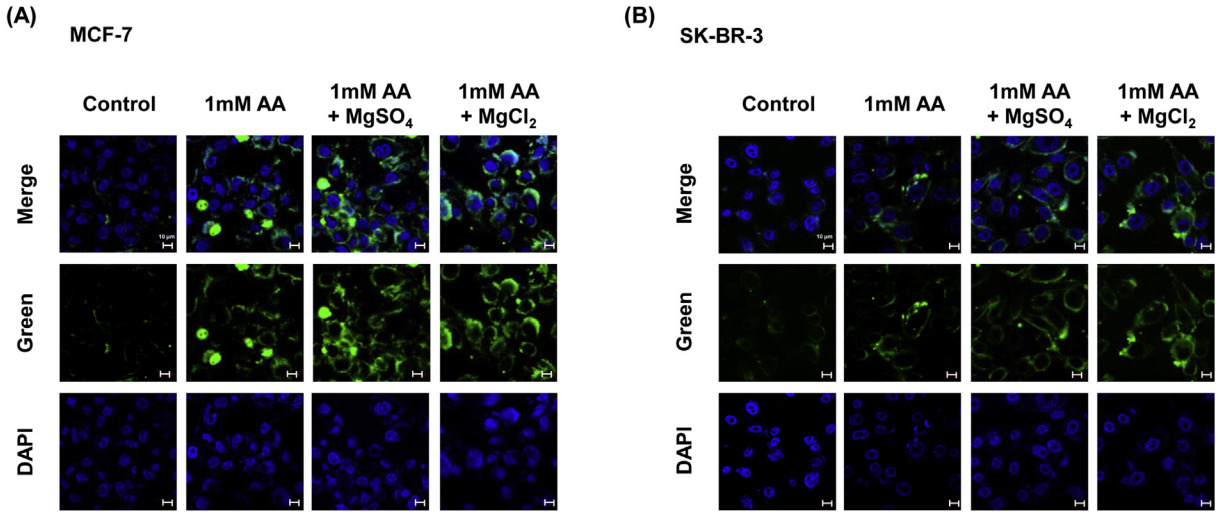

Figure 3. Amine-reactive fluorescent dye staining results for live/dead cell determination. A, B. Confocal microscopy image of MCF-7 and SK-BR-3 cells treated with vitamin C and magnesium. Dead cells were stained with green fluorescent dye localized to the cytosol. The cells were observed with a C-apochromat 40 × /1.2 water immersion objective.

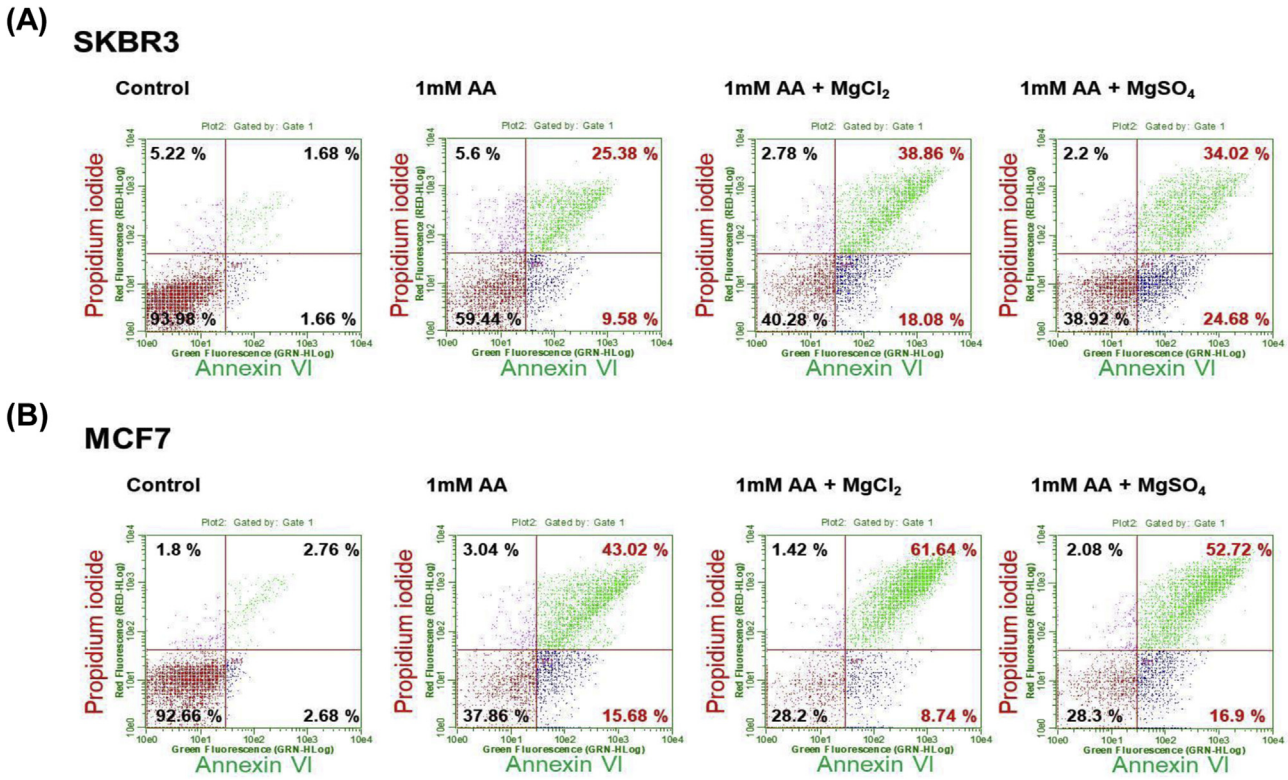

Figure 4. Cotreatment with vitamin C and magnesium supplement increased late apoptotic response. A. Annexin V and pI staining results from SK-BR-3 cells treated with 1 mM vitamin C or 1 mM vitamin C with 5 mM $MgCl_2$ and $MgSO_4$. **B.** Annexin V and pI staining results from MCF-7 cells treated with 1 mM vitamin C or 1 mM vitamin C with 5 mM $MgCl_2$ and $MgSO_4$. Data are presented as means ± SEMs.

dead cells were higher than in the medium containing cells treated only with vitamin C. $MgCl_2$ supplementation induced more apoptosis at the late phase (double positive of annexin V and propodium iodine) than $MgSO_4$ supplementation, but the difference in the number of dead cells between $MgCl_2$ and $MgSO_4$ supplementation was higher in MCF-7 cells than in SK-BR-3 cells. Taken together with the immunocytochemistry and flow cytometry results, these results show that vitamin C—mediated cell deaths were

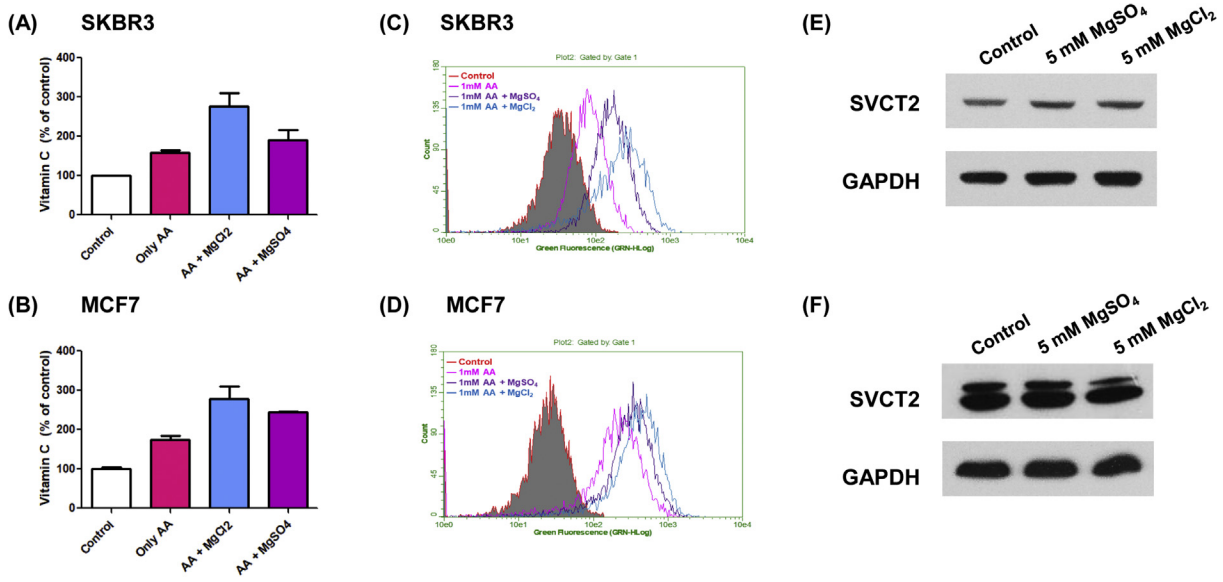

Figure 5. Magnesium supplementation increased cellular uptake of vitamin C and ROS generation. A, B. HPLC results from SK-BR-3 and MCF-7 cells. The uptake of vitamin C increased when cells were cotreated with $MgCl_2$ and $MgSO_4$. **C, D.** ROS generation analysis in SK-BR-3 and MCF-7 cells. DCF-Da staining was performed for ROS detection. More ROS was generated in cells treated with vitamin C and $MgCl_2$ and $MgSO_4$ than in cells that received only vitamin C. **E, F.** Western blot analysis of SVCT-2 expression in SK-BR-3 and MCF-7 cells. SVCT-2 expression was not changed by treatment with 5 mM $MgCl_2$ and $MgSO_4$. Data are presented as means ± SEMs.

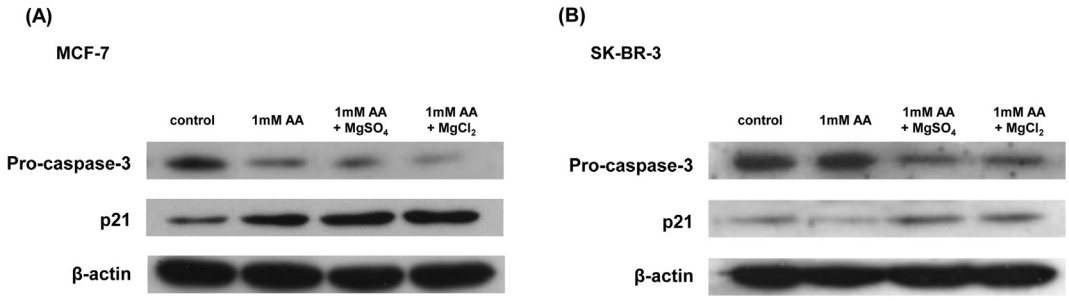

Figure 6. Western blot analysis for apoptosis response to vitamin C treatment and cotreatment with magnesium ions and vitamin C. A, B. Expression of caspase-3 and p21 was analyzed with western blot analyses in MCF-7 and SK-BR-3 cells. Both vitamin C treatment and cotreatment with magnesium ions and vitamin C increased the expression of p21 and the cleavage of caspase-3. However, cotreatment with magnesium ions and vitamin C showed larger increases than vitamin C treatment alone.

induced more in cells with high SVCT-2 expression (MCF-7 cells) than in cells with low SVCT-2 expression (SK-BR-3 cell) after Mg supplementation.

More ROS Generation Was Caused by Enhanced Vitamin C Uptake after Magnesium Supplementation

To investigate the enhanced anticancer effects of vitamin C seen with magnesium supplementation, the amount of vitamin C in the cancer cells was measured by HPLC. Adding $MgCl_2$ and $MgSO_4$ to the culture medium containing vitamin C increased the cellular uptake of vitamin C by 1.5−2 times compared with vitamin C−only treatment in both the SK-BR-3 and MCF-7 cell lines (Figure 5*A* and *B*). To investigate ROS generation, we performed DCF-Da staining and analysis with flow cytometry (Figure 5*C* and *D*). Those results show that both the cellular uptake of vitamin C and ROS generation increased with magnesium supplementation. Magnesium supplementation enhanced the uptake of vitamin C into cells, which in turn caused more ROS generation. To determine whether the increased uptake of vitamin C was caused by *de novo* expression levels of SVCT-2, we used a western blot analysis. In both SK-BR-3 (Figure 5*C*) and MCF-7 cells (Figure 5*F*), the SVCT-2 expression levels in the Mg supplemented cells did not change compared with control cells. The western blot analysis also showed that magnesium supplementation enhanced the anticancer effects of vitamin C by inducing more expression of p21 and procaspase-3, which are apoptotic marker proteins (Figure 6*A* and *B*).

Hormetic Response of Breast Cancer Cells Caused by Low Uptake of Vitamin C Was Prevented by Magnesium Supplementation

Magnesium supplementation enhanced the anticancer effects of vitamin C therapy (Figure 2*B* and *C*; 0.5 mM >). We treated cancer cells with low-dose vitamin C previously shown to induce a hormetic response (0.5 mM <), so that we could determine whether magnesium supplementation could prevent that hormetic response. MCF-7 cells, which have high SVCT-2 expression levels (Figure 2*A*), did not show a hormetic response with vitamin C alone or with $MgSO_4$ and $MgCl_2$ supplementation (Figure 7*A*). SK-BR-3 cells clearly showed hormetic proliferation responses on treatment with 10 μM of vitamin C (Figure 7*B*). However, when vitamin C−treated cells received a supplement of 5 mM magnesium, the hormetic proliferation response was prevented, even when a very low amount of vitamin C (10 μM) was used. Therefore, the magnesium supplement activated SVCT-2 and enhanced the low expression level of SVCT-2 enough to increase the vitamin C uptake into cells and kill cancer cells through ROS generation.

Magnesium-Supplemented Vitamin C Therapy Enhanced the Anticancer Effect in an In Vivo Xenograft Mouse Model

All of our *in vitro* data demonstrated that magnesium-supplemented vitamin C treatment prevented the hormetic response and killed cancer cells more effectively than vitamin C treatment alone. Therefore, we extended and applied our findings to an *in vivo*

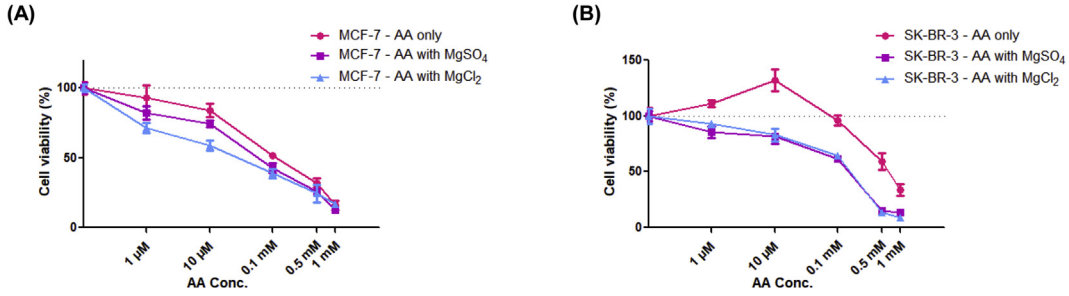

Figure 7. Hormetic proliferation response to low-dose vitamin C was inhibited by magnesium supplementation. Cell viability assay results from treatment with low-dose vitamin C and magnesium. **A.** SK-BR-3 showed a hormetic proliferation response to low-dose (<10 μM) vitamin C treatment. However, with 5 mM $MgCl_2$ and $MgSO_4$, the anticancer activity of vitamin C was increased, and the hormetic proliferation response was inhibited. **B.** MCF-7 did not show a hormetic proliferation response with low-dose (<10 μM) vitamin C, but cotreatment with vitamin C and 5 mM $MgCl_2$ and $MgSO_4$ nonetheless enhanced the anticancer effects compared with vitamin C treatment alone.

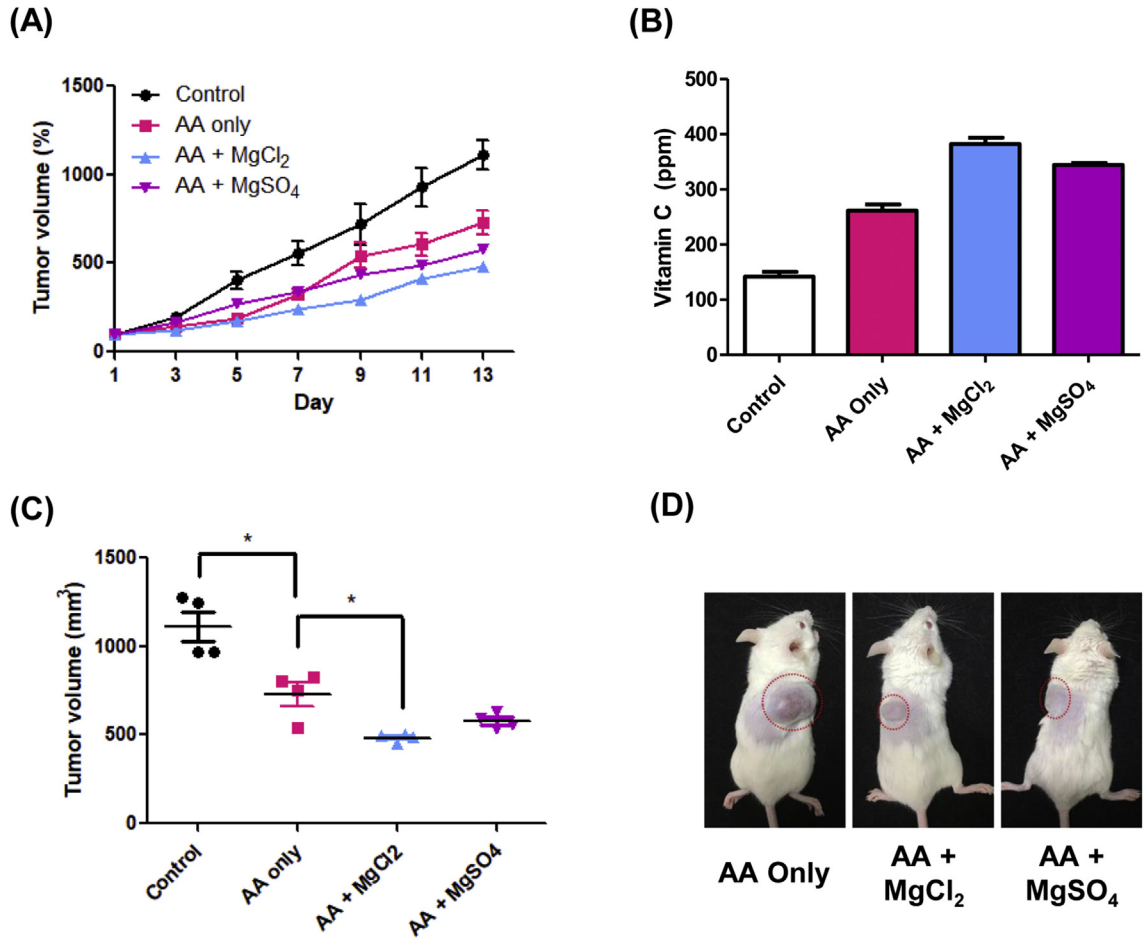

Figure 8. Cotreatment with vitamin C and $MgCl_2$ and $MgSO_4$ enhanced the anticancer effects of vitamin C in an *in vivo* xenograft mouse model system. The synergistic anticancer effects of vitamin C and $MgCl_2$ and $MgSO_4$ were tested in a xenograft mouse model. **A.** Relative tumor volume of xenograft mouse. Cotreatment with vitamin C and $MgCl_2$ and $MgSO_4$ showed enhanced anticancer effects in the *in vivo* system. **B.** Vitamin C in liver tissue was analyzed by HPLC. Vitamin C uptake in the tissue was increased in the $MgCl_2$ and $MgSO_4$ cotreatment group mice. **C.** The tumor volume of the mice was measured as mm^3. The data are presented as means ± SEMs. *$P < 0.05$. **D.** Each representative subcutaneous xenograft tumor mouse demonstrated a treatment response: AA only, AA with $MgCl_2$, and AA with $MgSO_4$.

xenograft mouse model. Mice that had received a subcutaneously injected CT26 xenograft were prepared and divided into 4 groups, with tumor volumes measured every two days for up to 14 days ($n = 4$). The results show that cotreating with vitamin C and magnesium ions inhibited tumor growth more effectively than treating with only vitamin C (Figure 8*A*).

To confirm those results, the vitamin C content of the mouse livers was measured by HPLC (Figure 8*B*). The vitamin C uptake in the mouse livers was increased by cotreatment with $MgCl_2$ or $MgSO_4$, confirming the *in vitro* cell system results. Furthermore, the anticancer effects of the treatment were greater when mice received $MgCl_2$ than when they received $MgSO_4$ (Figure 8*C*). Figure 8*D* shows that each mouse with a xenograft tumor (AA only, AA with $MgCl_2$, and AA with $MgSO_4$) had a treatment response. The tumor size of AA-only–treated mice was bigger than that of the mice treated with AA and $MgCl_2$ or $MgSO_4$.

Discussion

Our previous study demonstrated a hormetic proliferation response to low-dose vitamin C in cancer cell lines with low SVCT-2 expression [13]. Therefore, we screened the approaches observed to prevent that hormetic response in previous work [13]. One potent approach was treatment with magnesium ions and vitamin C together because magnesium had already been reported as an activator of SVCT-2, which is a vitamin C transporter [17]. Godoy et al. (2006) demonstrated that Ca^{2+} and Mg^{2+} supplementation switched the inactive form of SVCT-2 into the active form of SVCT-2 by increasing the V_{max} value of SVCT-2 itself. Therefore, we applied magnesium ion supplementation to vitamin C cancer therapy. In this study, we found that magnesium supplementation (both $MgSO_4$ and $MgCl_2$) increased the cellular uptake of vitamin C in cancer cells via activation of SVCT-2. Moreover, ROS generation via dihydrogen peroxide [12,24,25] also increased because more vitamin C accumulated inside of cancer cells when magnesium was added to vitamin C treatment. This prooxidant activity of vitamin C led to the breakage of cellular DNA, which interrupted the redox balance and eventually altered the cellular metabolism of cancer cells, such as energy metabolism through NAD depletion [26,27]. Collectively, the strong correlation between this anticancer mechanism of vitamin C and the hormetic response of cancer cells to vitamin C indicates that the amount of cellular uptake of vitamin C might be an important check in the application of vitamin C to cancer therapy.

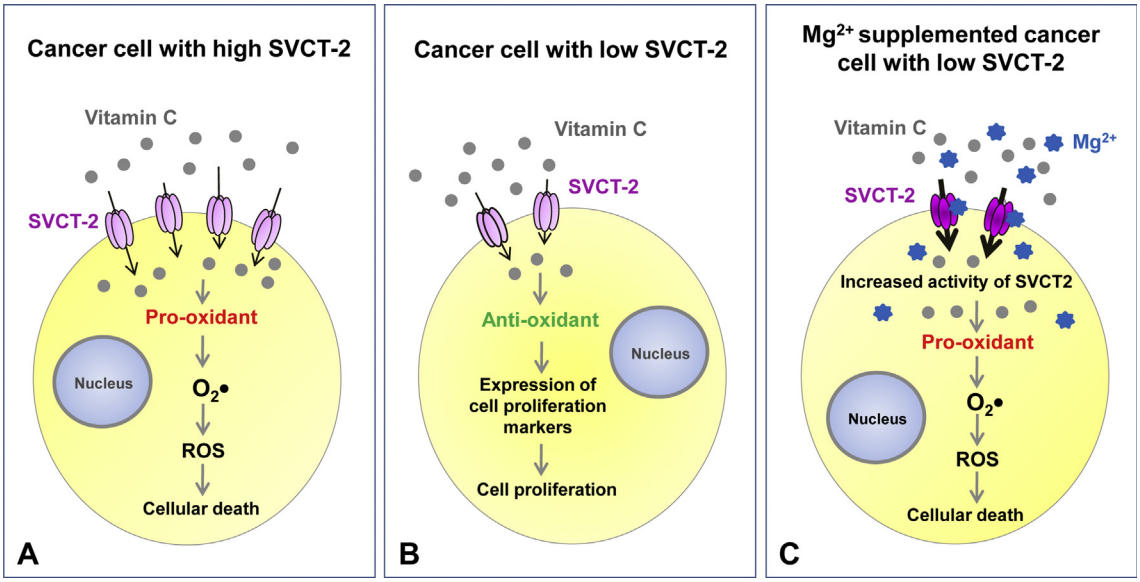

Figure 9. Proposed role of magnesium ions with vitamin C in enhanced cancer killing mechanism via SVCT-2 activation.

Magnesium ion supplementation increased the cellular uptake of vitamin C and enhanced the anticancer effects of vitamin C in both *in vitro* and *in vivo* systems (Figures 2 and 8). Furthermore, the hormetic proliferation response was inhibited when a magnesium supplement was added to vitamin C treatment in the SK-BR-3 cell line, which has low SVCT-2 expression (Figure 7). Both $MgSO_4$ and $MgCl_2$ showed an enhanced anticancer effect when added to vitamin C treatment, but $MgCl_2$ showed slightly better effects than $MgSO_4$ both *in vitro* and in the xenograft. Perhaps, $MgCl_2$ is taken into cells better than $MgSO_4$ [28,29]. Other studies have revealed that $MgCl_2$ interacts with all the exchangers in the cell membrane, whereas $MgSO_4$ affects only paracellular components [30–32]. Therefore, we suggest that more magnesium ions fluxed into cells via increased SVCT-2 activity when $MgCl_2$ was used than when $MgSO_4$ was used.

Myers′ cocktail, which includes $MgCl_2$ and vitamin C, has been used as an auxiliary to high-dose vitamin C cancer therapy [18–20]. However, the effect of each ingredient (magnesium chloride, calcium gluconate, hydroxocobalamin, pyridoxine hydrochloride, dexpanthenol B complex) of Myers′ cocktail on cancer cells has not been fully investigated. Therefore, we are here the first to reveal that the magnesium ions in Myers′ cocktail are a synergistic anticancer agent with vitamin C treatment.

Various chemotherapeutic agents with vitamin C have been tested as cancer therapy [33,34]. In many reports, vitamin C alleviated the side effects of and provided synergistic effects with anticancer drugs [35–39]. However, some reports have claimed that vitamin C is noneffective as an anticancer therapy [4,40] or even that it has adverse effects on patients [35,41]. Among several possibilities for the negative effects caused by vitamin C anticancer therapy in patients, we focused on the hormetic proliferation response caused by low cellular uptake of vitamin C even when high doses of vitamin C were given. In our previous work [13], we demonstrated that a low expression level of SVCT-2 could cause a hormetic response to vitamin C anticancer therapy. Here, we suggest that the activation of SVCT-2 by magnesium supplementation given along with vitamin C, without *de novo* induction of SVCT-2, could prevent the hormetic response shown in cancer cells with low expression levels of SVCT-2. Although magnesium ions have been widely used in vitamin C therapy as a component of Myers′ cocktail, the synergistic anticancer effects of vitamin C and magnesium ions are first reported by this research to result from the activation of SVCT-2. Our results also suggest that magnesium ion supplementation, such as $MgCl_2$ or $MgSO_4$, is an attractive cofactor that could increase the anticancer effects of vitamin C therapy (Figure 9).

Competing Interests

The authors declare no competing interests.

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