Enhanced Anti-cancer Efficacy in MCF-7 Breast Cancer Cells by Combined Drugs of Metformin and Sodium Salicylate

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Metformin or sodium salicylate is known to induce apoptosis and G0/G1 phase arrest in a variety of cancer cells. However, the anti-cancer effects of the combined treatments for these drugs-induced apoptosis are yet unclear. Here, we found that the combined treatment of metformin and sodium salicylate increased the efficacy of chemotherapeutics against breast cancer cells. These combined drugs significantly inhibited cellular proliferation and induced apoptosis at an earlier stage in human MCF-7 breast cancer cells. Also, co-treatments of metformin and sodium salicylate induced G1 cell cycle arrest in MCF-7 cells more effectively than either agent alone. Taken together, these results demonstrate that dual metformin/sodium salicylate treatment prevents proliferation of MCF-7 cells by inducing apoptosis and G1 cell cycle arrest.

Key Words: Breast cancer cell, Combination treatment, Metformin, Sodium salicylate

Breast cancer is the most common cause of cancer death in women worldwide (Mandy et al., 2012). The treatment approach in breast cancer, with antitumor agents in combination chemotherapy, is used routinely due to widespread evidence that multi-chemotherapy offers a survival advantage compared with single-drug therapy (Aapro, 2001; Hu et al., 2012). Thus, it is important to provide evidence of the effectiveness of new combined drugs for the control of proliferation, invasive and metastatic capacity of breast cancer (Conforti and Menichini, 2011). Currently, metformin is an oral antihyperglycemic drug applied for treatment of type 2 diabetes. Recent studies have demonstrated that metformin inhibits proliferation and induces cell cycle arrest and apoptosis in breast cancer (Evans et al., 2005; Bowker et al., 2006). Other nonsteroidal anti-inflammatory drugs (NSAIDs) such as sodium salicylate are utilized as agents for the treat-

ment of inflammation (Weissmann, 1991). Especially, sodium salicylate is known to induce apoptosis and arrest cell cycle in a variety of cancer cells (Bellosillo et al., 1998; Klampfer et al., 1999; Law et al., 2000; Marra et al., 2000; Lee et al., 2003; Dikshit et al., 2006). However, the combined effects of metformin and sodium salicylate on cancer inhibition are not known. Therefore, in this study, we identified the anti-cancer effects of combined metformin and sodium salicylate treatment leading to apoptosis induction, cell cycle arrest, and inhibition of cell proliferation in MCF-7 human breast cancer cells.

To evaluate the combination effect of metformin and sodium salicylate on cancer cell inhibition, we first tested WST-1 cell proliferation assay using different doses of metformin or sodium salicylate. MCF-7 cells were seeded into 96-well plates at 40,000 cells per well and were cultured

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in EMEM (ATCC, Manassas, VA, USA) for 24 h. After treatment with IC_{50} values of metformin or/and sodium salicylate for 48 h, the cell viability was estimated using EZ-CyTox reagents (Daeil Lab Service, Seoul, South Korea) at 450 nm by VersaMax ELISA microplate reader. We observed that the combination of metformin (Fig. 1A, IC_{50} value = 300 mM) and sodium salicylate (Fig. 1B, IC_{50} value = 60 mM) caused a synergistic inhibition of cell viability in both MCF-7 and 293T (control; human embryonic kidney) cells (Fig. 1C). Compared with the control 293T cells, the cell viability of MCF-7 cells showed greater cytotoxicity by the combined treatment [IC_{50} value = IC_{25} of metformin (150 mM) + IC_{25} of sodium salicylate (30 mM)].

To verify the combination therapeutic effect of metformin and sodium salicylate, MCF-7 and 293T cells were treated

with the drugs individually or in combination and assayed by Annexin V-FITC/PI staining (Kim and Lee, 2016). MCF-7 and 293T cells were seeded into 6-well plates (5×10^5 cells/well) and treated with IC_{50} values of metformin, sodium salicylate and combination of these drugs for 48 h. Cells were washed in DPBS and resuspended in 100 μ L 1X Annexin-binding buffer at 10^6 cells/mL. Cells were incubated with Annexin V Alexa Fluor 488 for 20 min at room temperature in the dark. After centrifugation, the supernatant was removed and cells were resuspended in 100 μ L Annexin binding buffer. Finally, cells were incubated with PI for 5 min in the absence of light and then stained cells were analyzed by a NovoCyte Flow Cytometer (ACEA Biosciences Inc, San Diego, CA, USA). In MCF-7 cells, metformin or sodium salicylate treatment induced early apoptosis by

Fig. 1. Combined treatment of metformin and sodium salicylate synergistically inhibited the viability of cancer cells. Cells were exposed to the indicated concentrations of (A) metformin (100, 200 and 300 mM) and (B) sodium salicylate (40, 80 and 160 mM) in MCF-7 cells for 48 h. (C) MCF-7 and 293T cells were then treated with metformin, sodium salicylate and their combinations (IC_{25} of metformin (150 mM) + IC_{25} of sodium salicylate (30 mM)) for 48 h. Cell viability was each monitored by WST assay. Data are represented as the mean $\pm$ standard deviation (n = 3). ** $P < 0.01$ compared to various concentrations treated with control. ## $P < 0.01$ compared to 293T cells with MCF-7 cells.

Fig. 2. Combination treatment of metformin and sodium salicylate greatly induced apoptosis in MCF-7 cells. MCF-7 and 293T cells were treated with IC_{50} values of metformin or sodium salicylate, respectively and combined metformin and sodium salicylate for 48 h. Apoptosis was determined by Annexin-V/PI staining and measured by cell cytometer. The mean was calculated from three different measurements. (A) Representative flow cytometry scatter plots of propidium iodide (PI) (Y axis) vs Annexin-fluorescein isothiocyanate (FITC) (X axis). (B) Bar charts showed average quantitative data of three independent flow cytometry experiments for early apoptosis in MCF-7 and 293T cells. $**P < 0.01$ compared metformin or sodium salicylate, respectively with combination in early apoptosis.

51.98% and 51.94%, respectively. Combination of metformin and sodium salicylate induced early apoptosis by 70.12%. However, 293T cells did not undergo apoptosis induction by metformin and sodium salicylate (Fig. 2A and B). The results suggest that the pro-apoptotic ability of combination treatment was more pronounced than that of each treatment alone.

To elucidate the mechanism of inhibition of MCF-7 cell growth by a function of cell cycle arrest of metformin and sodium salicylate, cell cycle arrest was analyzed in the treated

MCF-7 cells by Tali™ Image-based Cytometer (Invitrogen, Carlsbad, CA, USA). MCF-7 cells were treated with IC_{50} values of metformin, sodium salicylate and combination of metformin and sodium salicylate for 48 h. After washing with DPBS, the harvested cells were fixed with ice-cold 70% ethanol in distilled water at 10^6 cells/well and kept at 20 °C overnight. After centrifugation, the supernatant was removed and cells were resuspended in 200 μ L Tali® cell cycle solution (Invitrogen, Carlsbad, CA, USA). Finally, these cells were incubated at room temperature for 30 min in dark and then

Fig. 3. Dual drugs of metformin and sodium salicylate effectively induced G1 phase cell cycle arrest in MCF-7 cells. (A) Representative histograms depicting cell cycle distribution of MCF-7 cells treated with IC₅₀ values of metformin or sodium salicylate, respectively and combined metformin and sodium salicylate for 48 h. (B) The cell cycle distributions were presented as cumulative proportions of cells within each of four cell cycle compartments (Sub1, G1, S and G2/M); Data were presented as means $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01 compared to metformin or sodium salicylate, respectively with combination in G1 phase.

stained cells were analyzed by Tali[®] image-based cytometry. We observed that the combined treatment of metformin and sodium salicylate further increased the population of cells in the G0/G1 phase than treatments with either metformin or sodium salicylate alone (Fig. 3A and B).

The *in vitro* findings of our study demonstrated that combination treatment of metformin and sodium salicylate synergistically increased synergistic cytotoxicity in MCF-7 breast cancer cells and inhibited the proliferation of cancer cells by inducing apoptotic cell death and G0/G1 phase arrest. These studies, together with our study, collectively suggest that the combined effect by dual chemotherapeutic drugs on cancer cells is a universal phenomenon and hold a promise

for future cancer treatments. However, many of the therapeutic compounds such as metformin and sodium salicylate have the disadvantage of short circulation half-life, poor solubility and instability *in vivo* (Hu et al., 2012). To overcome these obstacles and improve therapeutic efficiency *in vivo*, further studies are required to develop nanocarrier systems using liposomes to enhance drug loading capacity, increased solubility, and minimized toxic effect to normal cells.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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