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Inhibitory effects of salicylic acid on A549 human lung adenocarcinoma cell viability

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Abstract: Antiinflammatory drugs, such as salicylic acid and its metabolite acetylsalicylic acid, have been singled out as apoptosis-inducing agents with antitumoral activity. In addition, they reduce the risk of some cancers via an unclear mechanism. In this study, we have investigated the apoptotic, cytotoxic, and growth-inhibiting effects of salicylic acid on A549, the human lung adenocarcinoma, alveolar epithelial cells. Cytotoxicity was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay. We observed the ultrastructural and morphological changes in A549 cells by using transmission electron microscopy (TEM) and confocal microscopy. Cell viability was decreased by the optimal concentration (IC50) of 6.0 mM salicylic acid. Our TEM results showed cell structure damage and ultrastructural changes, concretely indicating apoptosis. With these results, the cytotoxic and potent antiproliferative effects of salicylic acid can be concluded to induce apoptosis in A549 cells.

Key words: Salicylic acid, A549, apoptosis, cancer

1. Introduction

Salicylates, including salicylic acid, were isolated from extracts of various plants in the 19th century. Subsequently, salicylic acid was chemically synthesized and became a widely used medication (Klessing and Malamy, 1994). Acetylsalicylic acid is an effective antiinflammatory, nonsteroidal analgesic and antipyretic drug. Acetylsalicylic acid and salicylic acid are considered antiinflammatory drugs with antitumoral efficacy and induce programmed cell death, known as apoptosis (Lee et al., 2000). In addition, acetylsalicylic acid, a metabolite of salicylic acid, is a component of many analgesics and has been shown to be effective in reducing the risk of some cancers, but the mechanism of its action is not yet clear (Giardiana et al., 1999). Lung cancer is one of the most common cancers resulting in death in many countries in the world (Duan and Zhang, 2006). Despite many cancers responding to chemical treatment with classical chemotherapeutics effective on tumor cell viability, such as vincristine (Orosz et al., 1997), vinblastine (Glass-Marmor and Beitner, 1999), and paclitaxel (Spitz, 2009), resistance to such drugs is frequently reported (Liu, 2004; Spitz, 2009; Hsieh, 2010; Obradovic et al, 2013). Therefore, current research is focused on finding new effective drugs for the prevention or treatment of cancers of different types (Mao et al., 2003; Duan and Zhang, 2006). In this respect, novel effective drugs are required. In a study performed by Özkan and Erdoğan (2013), eugenol, eucalyptol, terpinen-4-ol, and camphor were investigated in parental and drug-resistant human lung cancer cell lines in order to understand the effects of these natural agents on cell membrane and DNA damage or protection, and they were reported to be effective. Results of recent biomedical research demonstrated the potent efficacy of nonsteroidal antiinflammatory drugs and acetylsalicylic acid in cancer prevention or treatment (Antunes et al., 2007). In a number of studies on nonsteroidal antiinflammatory drugs, primarily aspirin, it was found that they decreased the risk of cancers, including breast, lung, ovary, prostate, and skin, as well as those of the gastrointestinal system (Schreinemachers and Everson, 1994; Gupta and DuBois, 1998; Akre et al., 2001; Thun et al., 2002; Schildkraut et al., 2006; Urlich et al., 2006; Jacobs et al., 2007). In addition, these agents showed anticancer effects in animal cancer cells by modulating important regulatory enzymes (Loll et al., 1995; Garcia-Heredia et al., 2008).

Here we aimed to evaluate the cytotoxic, growth-inhibiting, and apoptotic effects of salicylic acid on A549 cells. Using transmission electron microscopy (TEM) and confocal microscopy, we detected ultrastructural and morphological changes in the treated cells, leading us to conclude that salicylic acid exhibited potent antiproliferative effects, inducing apoptosis, in a dose-dependent manner on A549 cells.

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2. Materials and methods

2.1. Materials

Salicylic acid, fetal bovine serum, penicillin-streptomycin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA), and Roswell Park Memorial Institute medium (RPMI-1640) was obtained from GIBCO (USA). A549 cells were from the American Type Culture Collection.

2.2. Cell culture

In this study, A549 cells were used as the test cell line. Initially, A549 cells were cultured in RPMI medium without L-glutamine, containing penicillin-streptomycin (100 units/mL) and 10% fetal bovine serum (v/v) at 37 °C under 5% CO_2 humidified incubator conditions. For continuity of the proliferated cells, the medium in the culture flasks was replaced with fresh RPMI every third day. Confluent cells on the bottom of the culture flasks were washed with phosphate buffered saline (PBS) and harvested by treatment with trypsin solution (0.25% trypsin, 1 mM EDTA) for 3 min at 37 °C. For neutralizing the effect of trypsin, 2 mL of fresh culture medium was added before the mixture was centrifuged at 1200 rpm at room temperature. The pellets were resuspended with fresh RPMI and reseeded in sterile culture flasks in a laminar flow cabinet. Subcultures were then incubated at 37 °C.

2.3. Cytotoxicity test (MTT assay)

For cytotoxicity evaluation, a stock solution of salicylic acid (20 mM) was prepared in RPMI. Further dilutions were made with fresh RPMI. The viability of harvested A549 cells was determined through staining with trypan blue, and the cells were counted using a hemocytometer. Counted cells were plated at 10^5 cell/mL per well into 96-well plates and were exposed to salicylic acid at concentrations of 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 mM for 24 h. After incubation at 37 °C in a 5% CO_2 humidified incubator, 20 µL of MTT solution (5 mg/mL in distilled water) was added to each well and the plates were incubated for 3 h at 37 °C. The medium in each well was removed and 200 µL of DMSO was added per well (Mosmann, 1983). After mixing at room temperature, the plates were read with an ELISA reader (EL × 808) at 540 nm wavelength (n = 3). Each experimental and control group was repeated in triplicate. The percentage of viability was calculated by the following formula:

$$\text{Cell proliferation (\%)} = [\text{OD sample}] \times 100 / [\text{OD control}],$$

where OD is optical density.

2.4. Evaluation via confocal microscopy

A549 cells were plated onto sterilized coverslips in a petri dish and exposed to the IC_{50} concentration of salicylic acid for 24 h at 37 °C. After 24 h, the cells were washed in PBS and then stained with acridine orange and Alexa Fluor 488 Phalloidin. The preparations were observed based on changes in nuclei and structures using a Leica TCS-SP5 confocal microscope.

2.5. Evaluation using TEM

The human lung carcinoma epithelial cell line treated with the IC_{50} concentration of salicylic acid for 24 h was further investigated by TEM for structural and ultrastructural changes. Harvested cells were centrifuged at 1200 rpm for 5 min before fixing with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). Fixed pellets were left overnight at 4 °C in the same fixative. After this period, the pellets were embedded in agar and then fixed once more, but this time in osmium tetroxide (2%). At the end of the second fixation, samples were dehydrated in graded ethyl alcohol (70%, 90%, 96%) and then in pure ethanol (100%). Dehydrated cells were embedded in EPON 812 epoxy resin, sectioned with an ultramicrotome (LEICA EM UC6), and subsequently thin-sectioned using a diamond knife to a 100-nm thickness. The sections were stained with lead citrate and uranyl acetate before taking TEM images.

2.6. Statistical analysis

Statistical comparison of the groups was carried out by one-way analysis of variance for multiple comparisons using SPSS 11.5 for Windows. The data were expressed as means ± SDs to detect differences at a 0.05 level of significance.

3. Results

3.1. Cytotoxicity of salicylic acid on A549 cells

A549 cells were treated with various concentrations of salicylic acid in the range of 1.5–9.5 mM. According to the cytotoxicity test results, salicylic acid inhibited cell viability in a concentration-dependent manner. The IC_{50} value of the agent for 24 h of exposure was detected as 6.0 mM. The mean ± SD values of cell viability beginning from 1.5 mM were 0.0031, 0.0023, 0.0014, 0.0008, 0.0033, 0.001, 0.0051, 0.0043, and 0.0008, respectively, as shown in the Table. Cell viability percentage decreased as the applied concentration increased. The sharpest decrease was detected at the highest concentration.

3.2. Effects of salicylic acid on A549 cell morphology using confocal microscopy

The morphological changes on A549 cells caused by 24 h of exposure to the IC_{50} concentration of salicylic acid are shown in Figures 1A and 1B. Condensations of the nuclei and DNA were the most significant morphological changes. Changes in cell membranes and vacuolization in the cells were observed by confocal microscopy. In addition, a decrease in cell volume was recorded, whereas control cells remained normal (Figures 1C and 1D). Owing to these

Table. Percentages of viable A549 cells treated with various salicylic acid concentrations (1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 mM) for 24 h. The viability of the cells decreased 50% at a 6.0 mM concentration of salicylic acid. This concentration was recorded as the IC_{50} value.

Salicylic acid doses (mM)	Viability (%)	Standard deviation (±)
1.5	91.87	0.0031
2.5	84.5	0.0023
3.5	65,4	0.0014
4.5	56.8	0.0008
5.5	50.8	0.0033
6.0	50.00	0.0134
6.5	49.9	0.0010
7.5	41.00	0.0051
8.5	36.00	0.0043
9.5	5.8	0.0008

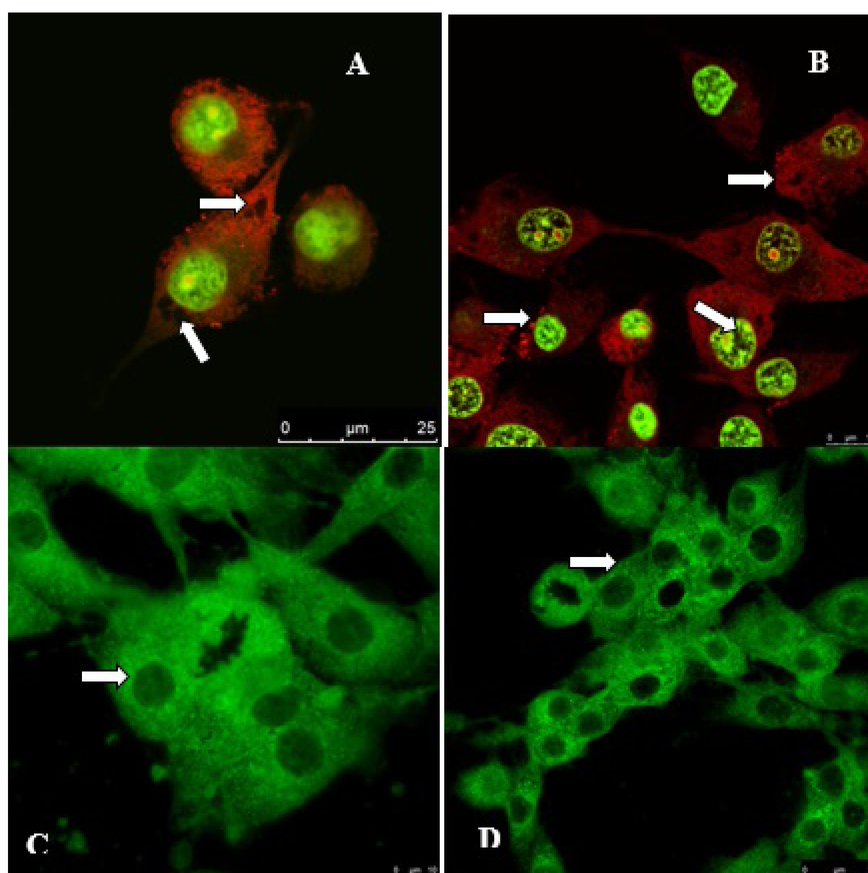

Figure 1. A and **B:** A549 cells exposed to IC_{50} concentration of salicylic acid for 24 h, double-stained with acridine orange and Alexa Fluor 488 Phalloidin; **C** and **D:** untreated A549 cells stained with acridine orange.

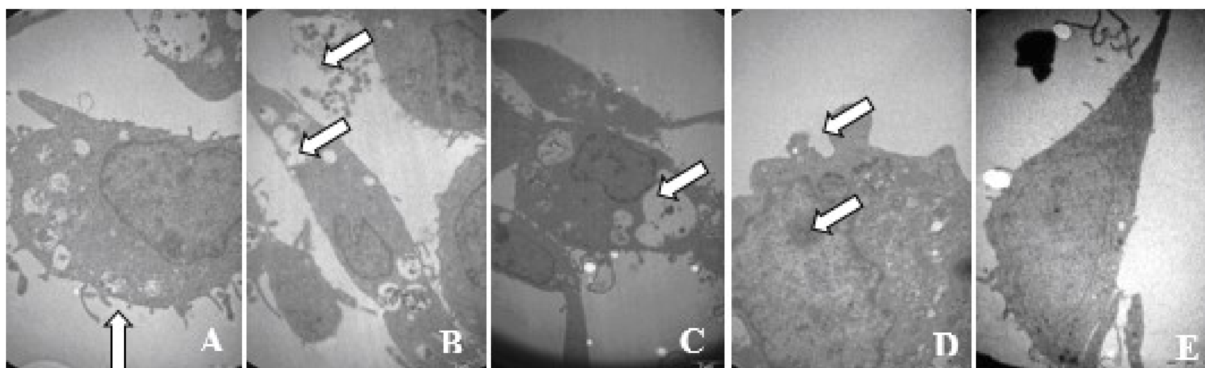

Figure 2. Ultrastructure of the A549 cells treated with the IC_{50} concentration of salicylic acid for 24 h. **A** (11,500×): membrane blebbing; **B** (8200×): holes and apoptotic bodies; **C** (6000×): vacuolization; **D** (16500×): membrane blebbing and nucleus condensation; **E** (8200×): the ultrastructure of an untreated A549 cell.

changes, we suggest that A549 cells treated with salicylic acid were led to programmed cell death, i.e. apoptosis.

3.3. TEM analysis results

A549 cells exposed to the IC_{50} concentration (6.0 mM) of salicylic acid for 24 h were observed under TEM with the aim of determining ultrastructural changes in the cells. Ultrastructural changes found in A549 cells were recorded as blebbing on the cell membrane (Figure 2A), formation of apoptotic bodies and holes (Figure 2B), vacuolization (Figure 2C), and nucleus condensation and reduction and fragmentation in the nucleus (Figure 2D). In contrast, control cells had normal cell ultrastructure (Figure 2E).

4. Discussion

The induction of programmed cell death in cancer cells has been a predominant research topic as of late (Ghobrial et al., 2005). Nonsteroidal antiinflammatory drugs are reported to induce apoptosis and necrosis in cancer cells (Tedeger et al., 2005; Jana, 2008). These agents also have the ability to inhibit tumor growth (Suzuki et al., 2010). The present study evaluates the apoptotic, growth-inhibiting, and cytotoxic effects of salicylic acid on A549 cells. The results of our study support the above effects of salicylic acid on A549 cells in a concentration-dependent manner.

As shown in the Table, the viability of the A549 cells exposed to different salicylic acid concentrations for 24 h decreased remarkably in a dose-dependent manner. A significant decrease was recorded at the IC_{50} value (6.0 mM) of salicylic acid for 24 h.

The morphological evaluation results showed that A549 cells treated with the IC_{50} concentration of salicylic acid for 24 h displayed signs of apoptosis such as damaged cell membranes, condensed chromatin, and increased number of vacuoles into the cells (Figures 1A and 1B). Untreated A549 cells are shown in Figures 1C and 1D.

Ultrastructural changes indicating apoptosis in A549 cells treated with the IC_{50} value (6.0 mM) of salicylic acid were demonstrated using TEM. Ultrastructural changes such as blebbing on cell membranes, condensation of nuclei, reduction and fragmentation in the nucleus, vacuolization, hole formation, and apoptotic bodies in the cells were observed in the TEM images (Figures 2A–2D). In conclusion, salicylic acid showed cytotoxic, growth-inhibiting, and apoptotic effects in the A549 cell line. Therefore, we recommend salicylic acid to be studied further as a potential agent for cancer treatment and prevention. Further in-depth investigations are required for other in vivo effects and elucidation of the mechanism of action.

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References

Akre K, Ekstrom AM, Signorello LB, Hansson LE, Nyren O (2001). Aspirin and risk for gastric cancer: a population-based case-control study in Sweden. Br J Cancer 84: 965–968.

Antunes LMG, Barros R, Bueno L, Dias FL, Bianchi ML (2007). Acetylsalicylic acid exhibits anticlastogenic effects on cultured human lymphocytes exposed to doxorubicin. Mutat Res 626: 155–161.

Duan W, Zhang L (2006). Cyclooxygenase inhibitors not inhibit resting lung cancer A549 cell proliferation. Prostaglandins Leukot Essent Fatty Acids 74: 317–321.

Garcia-Heredia J, Hervas M, De la Rosa M, Navarro J (2008). Acetylsalicylic acid induces programmed cell death in *Arabidopsis* cell cultures. Planta 228: 89–97.

Ghobrial IM, Witzig TE, Adjei AA (2005). Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 55: 178–194.

Giardiana C, Bourares H, Inan MS (1999). NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF-α and Fas ligation: the role of reactive oxygen species. Biochim Biophys Acta 3: 425–438.

Glass-Marmor L, Beitner R (1999). Taxol (paclitaxel) induces a detachment of phosphofructokinase from cytoskeleton of melanoma cells and decreases the levels of glucose 1,6-bisphosphate and ATP. Eur J Pharmacol 370: 195–199.

Gupta RA, DuBois RN (1998). Aspirin, NSAIDS, and colon cancer prevention: mechanisms? Gastroenterology 114: 1095–1098.

Hsieh CC, Hernandez-Ledesma B, de Lumen BO (2010). Lunasin, a novel peptide, sensitizes human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induced apoptosis. Chem Biol Interact 186: 127–134.

Jacobs EJ, Thun MJ, Bain EB, Rodriguez C, Henley SJ, Calle EE (2007). A large cohort study of long-term daily use of adult-strength aspirin and cancer incidence. J Natl Cancer I 99: 608–615.

Jana NR (2008). NSAIDs and apoptosis. Cell Mol Life Sci 65: 1295–1301.

Klessing DF, Malamy J (1994). The salicylic acid signal in plants. Plant Mol Biol 26: 1439–1458.

Lee I, Kolata A, Gewirtz A, Stolgen K (2000). Antitumor efficacy of the cytotoxic RNase, ranpirnase, on A549 human lung cancer xenografts of nude mice. Anticancer Res 27: 299–308.

Liu RH (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 134: 3479–3485.

Loll PJ, Picot D, Garavito RM (1995). The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. Nat Struct Biol 2: 637–643.

Mao JT, Roth MD, Serio KJ, Baratelli F, Zhu L, Holmes EC, Strieter RM, Dubinett SM (2003). Celecoxib modulates the capacity for prostaglandin E2 and interleukin-10 production in alveolar macrophages from active smokers. Clin Cancer Res 9: 5835–5841.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Obradovic A, Zizic J, Trisovic N, Bozic J, Uscumlic G, Bozic B, Markovic S (2013). Evaluation of antioxidative effects of twelve 3-substituted-5,5-diphenylhydantoins on human colon cancer cell line HCT-116. Turk J Biol 37: 741–747.

Orosz F, Vertessy BG, Salerno C, Crifo C, Capuozzo E, Ovadi J (1997). The interaction of a new anti-tumor drug, KAR-2 with calmodulin. Brit J Pharmacol 121: 955–962.

Özkan A, Erdoğan A (2013). Membrane and DNA damaging/protective effects of eugenol, eucalyptol, terpinen-4-ol, and camphor at various concentrations on parental and drug-resistant H1299 cells. Turk J Biol 37: 405–413.

Schildkraut JM, Moorman PG, Halabi S, Calingaert B, Marks JR, Berchuck A (2006). Analgesic drug use and risk of ovarian cancer. Epidemiology 17: 104–107.

Schreinemachers DM, Everson RB (1994). Aspirin use and lung, colon and breast cancer incidence in a prospective study. Epidemiology 5: 138–146.

Spitz GA, Furtado, CM, Sola-Penno M, Zancan P (2009). Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-phosphofructo-1-kinase structure and activity. Biochem Pharmacol 77: 46–53.

Suzuki Y, Inoue T, Ra C (2010). NSAIDs, mitochondria and calcium signaling: special focus on aspirin/salicylates. Pharmaceuticals 3: 1594–1613.

Tedeger I, Pfeilschifter J, Geisslinger G (2005). Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 15: 2057–2072.

Thun MJ, Henley SJ, Patrono C (2002). Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic and clinical issues. J Natl Cancer I 94: 252–266.

Urlich CM, Bigler J, Potter JD (2006). Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer 6: 130–140.