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% CO₂ 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. A549 cells was determined through staining with trypan blue, and the cells were counted using a hemocytometer.

2.4. Evaluation via confocal microscopy

A549 cells remained normal (Figures 1C and 1D). Owing to these 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.

2.5. Evaluation using TEM
The human lung carcinoma epithelial cell line treated with the IC₅₀ 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.

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 IC₅₀ 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 IC₅₀ 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<sub>50</sub> value.

<table>
<thead>
<tr>
<th>Salicylic acid doses (mM)</th>
<th>Viability (%)</th>
<th>Standard deviation (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>91.87</td>
<td>0.0031</td>
</tr>
<tr>
<td>2.5</td>
<td>84.5</td>
<td>0.0023</td>
</tr>
<tr>
<td>3.5</td>
<td>65.4</td>
<td>0.0014</td>
</tr>
<tr>
<td>4.5</td>
<td>56.8</td>
<td>0.0008</td>
</tr>
<tr>
<td>5.5</td>
<td>50.8</td>
<td>0.0033</td>
</tr>
<tr>
<td>6.0</td>
<td>50.00</td>
<td>0.0134</td>
</tr>
<tr>
<td>6.5</td>
<td>49.9</td>
<td>0.0010</td>
</tr>
<tr>
<td>7.5</td>
<td>41.00</td>
<td>0.0051</td>
</tr>
<tr>
<td>8.5</td>
<td>36.00</td>
<td>0.0043</td>
</tr>
<tr>
<td>9.5</td>
<td>5.8</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

Figure 1. A and B: A549 cells exposed to IC<sub>50</sub> 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.
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


