Inhibitory effects of four Chinese herbal medicines on oral squamous cancer cell lines

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We examined the inhibitory effect of Gypenoside (GP), Baicalia (BC), Salvia millitorrhiza (SM), and Erigeron breviscapus (EB) to determine whether Chinese herbal medicines are effective against oral cancer. Phenazine methosulfate-methyl thiazoly tetrazolium (PMS-MTT) assay was performed on the oral squamous cancer cell lines Tca-8113 and CAL-27. The ID 50 of the four Chinese herbal medicines was calculated. We found that BC and EB were the most effective. The ID 50 values for Tca-8113 and CAL-27 of BC were 128.63 μmol/L and 117.79 μmol/L, respectively. They were 223.18 μmol/L and 1010.98 μmol/L, respectively for EB. The ID 50 for the combined effect of BC and EB, was 27.131 μmol/L for Tca-8113 and 20.674 μmol/L for CAL-27. Thus BC and EB had an synergistic effect in inhibiting Tca-8113 and CAL-27 (CI<1). (J Osaka Dent Univ 2010; 44: 83–86)

Key words: Chinese herbal medicine; Oral squamous cancer cell lines; Median-effect principle; Combination index

INTRODUCTION

In recent years, drug treatment for tumors has developed from the traditional concept of conservative treatment alleviating symptoms to the new concept of chemopreventive treatment.¹ The current goal for prevention and treatment of lung cancer has shifted to the exploration and development of natural drugs that have fewer adverse effects, are more effective, and are suitable for long-term intake. Research has indicated that intragastric administration of GP and EB can reduce the incidence of cheek cancer in hamsters.² CAL-27 and Tca-8113 are the most popular cell lines for oral squamous cancer research and provide convenient prerequisites for the study of tongue cancer. In our study, we observed the in vitro inhibitory effect of four traditional Chinese medicines (GP, BC, SM and EB) on Tca-8113 and CAL-27. We then selected the two medicines that were most effective and observed their combined inhibitory effect on the cancer cell lines.

MATERIALS AND METHODS

Cell lines
We used the human tongue cancer cell line CAL-27 (ATCC CRL-2095; ATCC, Manassas, VA, USA). Tca-8113 was provided by the oral cancer research lab of Shanghai Ninth People’s Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The cultures for the two cell lines were maintained under standard conditions recommended by ATCC and our laboratory. The cells were grown in mono-layer culture in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum with 100 U/mL penicillin and 100 μg/mL streptomycin. They were seeded in the 10 cm culture capsules at 37°C in a humidified atmosphere comprising 95% air and 5% CO₂. Based on cell growth, the medium was replaced every two days. After the cells proliferated to approximately 75% of the area of the culture dish, they were passaged at a ratio of 1 : 3.
Drug selection
The active component of the traditional Chinese medicines Baicalin (BC, molecular weight 460, batch number 110715), Salvia miltiorrhiza (SM, molecular weight 470, batch number 111562), Erigeron breviscapus (EB, molecular weight 462, batch number 110842) were purchased from the Shanghai Drug Research Institute, Shanghai, China. Gypenoside (GP, molecular weight 708) was provided by the Drug Department of the 9th People’s Hospital, Shanghai, China.

Drug preparation and processing
After accurately weighing, each drug was dissolved in 50 μL dimethylsulfoxide (DMSO) to obtain stock solutions with different concentrations. The solutions were then passed through a 0.22 μm filter. They were diluted to final concentrations of 6, 20, 60, 200, 600 and 1000 μM, which were suitable for assaying and kept at 4°C. The highest concentration of DMSO was 0.041%, which was 300 μM. The concentrations of cis-Diaminedichloroplatinum (CDDP) were 0.0003, 0.003, 0.03 and 0.3 μM.

PMS-MTT assay
The PMS-MTT assay is used to detect drug effects. Cells are seeded at a density of $1 \times 10^6$/mL onto a 96-well dish for 48 hours before being treated with the drugs. Cell growth is then observed with a microscope. The medium is discarded before the drug is added, and 200 μL of solution that contains the drug at different concentrations is added to each well. Each concentration is replicated in at least three wells. After incubation for 72 hours, the supernatant is discarded and 100 μL DMSO is added to each well. After sufficiently succussing the culture capsule for 10 seconds, the supernatant is discarded and the OD value measured on a BP 800 (Biohit, Helsinki, Finland). Based on the above results, the two drugs among the four that had the best inhibitory effect were selected. The same assay was then used to determine the inhibition effect when the two drugs were combined.

RESULTS
Effect of the four drugs on the cell lines
According to the median-effect principle, a drug’s inhibitory effect (i.e., its inhibition ratio), $fa$, can be expressed as

$$fa = \frac{\text{Mean OD of the experimental group (fu)}}{\text{Mean OD of the control group}}$$

According to the median-effect principle equation

$$fa = \frac{D}{Dm}$$

where D is the drug concentration, and $Dm = 10^{-\text{m}}$. The variable $Dm$ represents the median-effect concentration for each drug on each cell line. The variable m is a parameter determined from the logarithmic transformation $Y = \log(\text{fa}) + X$, where $Y = log(\text{fa/fu})$ and $X = \log D$. Thus m is as the slope of the linear equation. After this we can plot the concentration-effect charts. The logarithmic transformation of this equation becomes $Y = \text{mX} + a$, where $Y = \log(\text{fa/fu})$ and $X = \log D$. With these equations we can plot the concentration-effect charts. When two drugs are combined, the combination index is

$$CI = \frac{D_1 + D_2 + \alpha D_1 D_2}{DX_1 + DX_2}$$

where $D_1$ and $D_2$ represent the required concentration for each drug in order to get X effect when the two drugs are combined. $DX_1$ and $DX_2$ represent the required concentration for each drug in order to get X effect when they are used separately. $\alpha$ is a coefficient that represents the degree to which the two drugs impact each other when used together. When $\alpha = 0$, the two drugs are antagonistic, and when $\alpha = 1$, they are not antagonistic. A CI index of less than 1.0 means the two drugs are synergistic, a CI index equal to 1.0 means they are additive, and CI index greater than 1.0 means they are antagonistic.

CDDP had a significantly greater inhibitory effect on both cell lines than the four drugs we tested ($p < 0.01$). For both of the human tongue squamous cancer cell lines, squared difference analysis showed statistically significant differences among the in vitro inhibition effect of the four Chinese herbal medicines ($p < 0.01$) (Figs. 1 and 2). Compared with the individual use of BC or EB, the two drug combination was significantly more effective ($p < 0.01$) (Figs. 3 and 4). We calculated the median-effect principle, ID50, slope rate m, and correlation
Inhibition rate of four drugs on the tongue squamous cancer cell line Tca-8113. There were significant differences among the in vitro inhibitory effect of the four Chinese herbal medicines on the Tca-8113 tongue squamous cancer cell line. BC was the most effective.

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID$_{50}$ (mM)</th>
<th>m</th>
<th>r</th>
<th>ID$_{30}$ (mM)</th>
<th>m</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>0.1</td>
<td>1.5</td>
<td>1.5</td>
<td>0.1</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>GP</td>
<td>2610</td>
<td>0.9</td>
<td>0.9</td>
<td>1920</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>BC</td>
<td>129</td>
<td>1.3</td>
<td>0.8</td>
<td>118</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>EB</td>
<td>223</td>
<td>0.4</td>
<td>0.9</td>
<td>1011</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>SM</td>
<td>1700</td>
<td>0.9</td>
<td>0.9</td>
<td>2900</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>BC + EB</td>
<td>27.1</td>
<td>0.8</td>
<td>0.9</td>
<td>20.7</td>
<td>0.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Coefficient r for each drug. The combination index (CI) for two combined drugs at various concentrations indicated that the two had a synergetic effect (CI < 1).

DISCUSSION

Traditional Chinese medicine has specific theories on the treatment of tumors. Based on its clinical practice and research over many years, a complete theory on the pathogenesis and treatment for tumors has been developed. The basic treatments are reducing heat, removing toxins, activating blood circulation, softening hard lumps and eliminating nodes.3

GP, BC, SM and EB have been well studied in recent years. Clinical research has indicated that drugs activating blood circulation have multiple functions including improved microcirculation, anti-coagulation, inhibition of the growth and metastasis
of tumor cells, and enhancement of the body's immunity. Clinical practice has also proven that treating tumors with drugs that activate blood circulation improves results. The public has become aware of the anticancer effect of these drugs, which have become a topic of research. As we know, BC and SM are traditional drugs that enhance circulation, reduce heat and eliminate toxins.

We selected four active components of traditional medicines and explored their in vitro inhibitory effect on tongue squamous cancer cell lines. We found that for the two human tongue squamous cancer cell lines Tca-8113 and CAL-27, CDDP had a dose dependent inhibitory effect. The effect of CDDP was significantly better than that of the other four medicines (p < 0.01). All of the four Chinese herbal medicines (GP, BC, SM and EB) had inhibitory effects on the two human tongue squamous cancer cell lines Tca-8113 and CAL-27. Using squared difference analysis, there was a statistically significant (p < 0.01) difference among the inhibitory effect of the four drugs, and the effect increased as the drug concentration increased (p < 0.01). In addition, although BC and EB were the most effective, there was no significant difference between the two cell lines (p < 0.01). BC and EB synergistically inhibited the two cancer cell lines in a dose dependent manner. Combining the two drugs was more effective than using them independently and the drugs concentration was lower (p < 0.01).

This study provided the basis for further research on clinical anti-cancer compound traditional Chinese medicine for treating cancers. In addition, we found that GP and SM were both effective at alleviating internal heat, removing toxins, and tonifying Qi, which is the most essential substance in the body and maintains life activities. All vital substances in the body are transformed by constant motion and changes in Qi. The viscera, the meridians, the five sensory organs, the nine orifices and the body itself are formed by the motion, transformation and accumulation of Qi. Qi, did not show inhibitory effects to the extent expected. We conjecture that the reason for this has to do with the overall balance of the human body's physiological systems. This topic deserves further exploration.

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REFERENCES