Antitumor Effect of Skin of *Venenum Bufonis* in a NCI-H460 Tumor Regression Model

Jung-Sun Kim, Tae-Young Jeong, Chong Kwan Cho, Yeon-Weol Lee, Hwa Seung Yoo*

*East-West Cancer Center, Dunsan Oriental Medical Hospital, Daejeon University, Daejeon, Korea*

Received: Dec 16, 2009
Accepted: Jul 15, 2010

**KEY WORDS:**
antitumor; 
in *vivo*; 
lung cancer; 
NCI-H460; 
*venenum bufonis*

---

1. Introduction

Chan Su is a traditional Chinese medicine prepared from *venenum bufonis*, the dried white Secratio of the auricular and skin glands of toads, which has been used as an oriental drug for the treatment of a number of diseases, including cancer [1]. In several previous reports, Chan Su has been shown to be potent in the treatment of various cancer cells in *vivo* by induction of cell cycle arrest and apoptosis [2–9]. Chan Su has been reported to significantly induce apoptosis in human bladder carcinoma cells. This process was mediated by an increase in Bax expression, decrease in Bcl-2 expression and a proteolytic activation of caspase-3 and caspase-9 [10]. The *in vitro* apoptotic effect of *Venenum Bufonis* venom has also been reported in the A549...
cell line obtained from human lung carcinoma. In this study, SVB-induced-apoptosis was accompanied by modulation of the death receptor system, Bcl-2 family members, mitochondrial dysfunction and activation of caspases [11]. Based on these results, we decided to investigate the antitumor effect of SVB in an animal model. The present study attempts to evaluate the antitumor activity of SVB in a NCI-H460 human lung carcinoma cell, in vivo, nude mouse xenograft model.

2. Materials and Methods

2.1. Cell line, culture conditions and SVB treatment

NCI-H460 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. The SVB was obtained from Dunsan Oriental Hospital (Daejeon, South Korea). One hundred grams of SVB was washed with distilled water, and then boiled in 1 L of water at 80ºC for 2.5 hours. Solid particles and aggregates were removed by centrifugation at 3000 g for 30 minutes and supernatants were lyophilized. Finally, 21.7 g lyophilized SVB were obtained and used in this experiment. The lyophilized extract was stored at −20ºC until use.

2.2. Experimental animals

BALB/c nude mice (male, 9–11 weeks, n=25) weighing 21–25 g were purchased from Japan SLC, Inc (Shizuoka, Japan) and were housed under specific pathogen-free conditions according to the guidelines of Chungbuk National University Animal Care and Use Committee. The animal room was controlled for temperature (22ºC ± 2ºC), light (12-hour light/dark cycle) and humidity (50% ± 10%). All laboratory feed pellets and beddings were autoclaved.

2.3. Experimental design

The tumor regression model in nude mice has been successfully and commonly applied to evaluate antitumor activity. Therefore, this model was used to evaluate suppression of solid tumors following SVB administration. When the tumor volume reached 100mm³, the nude mice with xenografted tumor fragments were randomly distributed into four groups [positive control, oral SVB (0.25 mg/kg), intraperitoneal SVB (0.25 mg/kg), and intraperitoneal (0.5 mg/kg)]. Seven mice were allocated to each group; SVB was administered daily for 4 weeks.

2.4. Cell preparation

NCI-H460 cells were cultured in 260-mL tissue culture flasks in Eagle’s minimum essential medium containing 100U/mL penicillin and 10% heat inactivated fetal calf serum in an incubator with 95% air and 5% CO₂ at 37ºC. When the cells reached confluency they were washed twice with Hank’s balanced salt solution (HBSS), trypsinized with 0.25% trypsin, and washed twice with fresh culture medium.

2.5. Xenografts

Following cell preparation, NCI-H460 cells (1 × 10⁶ cells/mouse in 0.1 mL HBSS) were injected subcutaneously into the flank of mice using a 26-gauge needle. After 14–16 days of observation, a solid tumor mass was observed in three out of five mice inoculated with NCI-H460 cells. Tumor fragments were made to 27 mm³ (3 mm × 3 mm × 3 mm) with a knife, and xenografted into the flank of new mice using a trocar. The suppressive effect of anticancer agents on solid tumors was evaluated in a tumor-regression model. In brief, from the day tumor volume reached 100mm³, mice xenografted with a tumor fragment were orally or intraperitoneally administered SVB daily for 28 days.

2.6. Changes in tumor volume

The changes in tumor mass were recorded twice a week following measurement with digital calipers. That is, the largest and smallest diameters were measured in each mouse, and the tumor volume (V) was estimated according to the formula

\[ V_{\text{mean}} = \frac{(A \times B^2)}{2} \]

where \( V \) is the tumor volume in mm³, and A and B are the largest and smallest tumor diameters in mm, respectively. Based on the regression of tumor volume, the antitumor activities of treatment were expressed by inhibition rate (IR).

\[ \text{IR (\%)} = \left(\frac{(C V - T V)}{T V}\right) \times 100 \]

where CV and TV are tumor volumes in control (water) and treatment groups, respectively. Tumor weights were also measured on the final day after animals were sacrificed and tumors removed.

2.7. Mean survival time and percent increase in life span

To compare the life span of mice xenografted with NCI-H460 tumor fragments, survival time was estimated from the day tumor volume reached 100mm³.
as described previously, and percent increase in life span (ILS) was calculated according to the equation:

\[ \text{ILS} \% = \left[ \frac{\text{T} - \text{C}}{\text{C}} \right] \times 100, \]

where C and T are mean survival days of mice in control and treatment groups, respectively.

2.8. Blood chemistry

Blood samples were centrifuged at 1400g at 4°C for 10 minutes. The supernatant (serum) were used to determine aspartate aminotransferase and alanine aminotransferase levels in an automatic analyzer (7170; Hitachi Ltd., Tokyo, Japan).

2.9. Statistical analysis

Data are presented as mean ± SD. Differences between the mean of control and treatment groups were analyzed using one-way analysis of variance followed by a Dunnett’s t test correction, paired t test, and linear regression analysis. Statistical significance was determined at the level of \( p < 0.05 \) or \( p < 0.01 \).

3. Results

3.1. Tumor size

Treatment with oral (0.25 mg/kg) and intraperitoneal (0.25 mg/kg and 0.5 mg/kg) SVB inhibited the growth of NCI-H460 cell-transplanted solid tumors compared with positive control (Figure 1). The mean tumor volume in the orally treated group (3980.16 mm³) was lower than that of the control group throughout the study period, with a value of 4492.22 mm³. Also the intraperitoneal treated group (0.5 mg/kg SVB) also showed a reduced tumor volume compared to control (3525.81 mm³).

3.2. Tumor volume

IR (%) of each group is shown in Table 1. From day 8 to day 28, each IR tended towards dose-dependency. For example at day 11, oral SVB (0.25 mg/kg; IR 90.79%) was less than intraperitoneal SVB (0.25 mg/kg; IR 85.96%), which in turn was less than intraperitoneal SVB (0.5 mg/kg; IR 53.11%).

3.3. Final tumor weight and volume

Final tumor weights and volumes of each group are shown in Table 2. Tumor weight and volume of the positive control group (NCI-H460 only) were 2.52 g ± 0.75 g and 4.67 cm³ ± 1.18 cm³ on the final day. Tumor weight and volume following oral administration of SVB (0.25 mg/kg) were 2.50 g ± 0.77 g and 5.38 cm³ ± 1.73 cm³. Tumor weight and volume of the intraperitoneal SVB (0.25 mg/kg) treatment group were 2.62 g ± 0.64 g and 5.61 cm³ ± 1.41 cm³, while tumor weight and volume of intraperitoneal SVB (0.5 mg/kg) treatment group was 2.45 g ± 1.21 g and 4.49 cm³ ± 1.63 cm³. Compared with the positive

Table 1  Inhibition rate (IR) on tumor volume in NCI-H460 tumor-bearing mice*

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>11</th>
<th>14</th>
<th>18</th>
<th>21</th>
<th>25</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460 cells alone</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oral SVB (0.25 mg/kg)</td>
<td>100.19</td>
<td>90.45</td>
<td>87.88</td>
<td>90.79</td>
<td>82.61</td>
<td>85.88</td>
<td>85.16</td>
<td>88.12</td>
<td>88.60</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.25 mg/kg)</td>
<td>106.96</td>
<td>91.95</td>
<td>86.25</td>
<td>85.96</td>
<td>86.27</td>
<td>86.43</td>
<td>80.56</td>
<td>94.38</td>
<td>93.76</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.5 mg/kg)</td>
<td>89.74</td>
<td>92.49</td>
<td>57.04</td>
<td>53.11</td>
<td>42.08</td>
<td>31.07</td>
<td>42.54</td>
<td>46.04</td>
<td>78.49</td>
</tr>
</tbody>
</table>

*All values expressed as percentages. SVB= skin of venenum bufonis.
control group, tumor weight and volume of SVB treatment groups were decreased.

3.4. Survival time and percent ILS

Mean survival time and percent increase in life span are shown in Table 3 and Figure 2. Mean survival time following administration of oral, low SVB (0.25 mg/kg) was extended to 20.71 days ± 2.61 days with a 4.66% increase in ILS. ILS was in fact increased in a dose-dependent manner. High intraperitoneal SVB (0.5 mg/kg) treatment was significantly different from the positive control (NCI-H460 cell alone) group.

3.5. Body weight and spleen weight

Absolute weight of the kidney, liver, spleen, heart and lung are shown Table 4. Treatment with intraperitoneal SVB (0.5 mg/kg) caused a significant decrease in total body weight and spleen weight (p < 0.05).

3.6. Histological examination of liver tissue

Light microscopic histopathological examination of liver tissue in mice treated with SVB did not show any specific lesions compared with liver tissue from the control, NCI-H460 cell-bearing mice (Figure 3).

3.7. Blood chemistry

The results of blood analyses are outlined in Table 5. Biochemical blood analysis revealed no significant changes in alkaline phosphatase, creatinine and aspartate transaminase levels. Phosphatase and urea nitrogen levels in blood were significantly reduced following oral SVB (0.25 mg/kg; p < 0.01). The blood levels of CA and ALT were also significantly decreased following administration of intraperitoneal SVB (0.5 mg/kg; p < 0.05 and p < 0.01, respectively).

4. Discussion

The aim of this study was to examine whether SVB demonstrated antitumor effects by inhibiting the growth of tumors in NCI-H460 cell-xenografted mice. Human tumor xenografts in immunodeficient animal models provide a means to evaluate potential antitumor drugs in preclinical studies and are applicable for many different types of human malignancies [12].

Venenum bufonis has been used in traditional Chinese medicine for many years. Its main components include bufalin, cinobufagin, resibufogenin, cinobufotoxin, cinobufotalin and cinobufotalidin [13]. Reported pharmacological effects of venenum bufonis include regional anesthesia and analgesia, cardiotonic and pressor effects, as well as anti-inflammatory, antitumor, and anti-asthmatic actions.
### Table 4  Weight of organs in NCI-H460 tumor-bearing mice on final day of study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absolute organ weights (g)</th>
<th>Body weight</th>
<th>Liver</th>
<th>Spleen</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460 cells alone</td>
<td>26.64±1.61</td>
<td>1.76±0.07</td>
<td>0.33±0.04</td>
<td>0.15±0.01</td>
<td></td>
</tr>
<tr>
<td>Oral SVB (0.25 mg/kg)</td>
<td>27.62±2.34</td>
<td>1.72±0.13</td>
<td>0.41±0.07</td>
<td>0.16±0.02</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.25 mg/kg)</td>
<td>27.94±1.62</td>
<td>1.68±0.09</td>
<td>0.46±0.08</td>
<td>0.17±0.02</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.50 mg/kg)</td>
<td>31.18±2.02*</td>
<td>1.55±0.22</td>
<td>0.19±0.22*</td>
<td>0.17±0.01</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absolute organ weights (g)</th>
<th>Kidney</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>NCI-H460 cells alone</td>
<td>0.22±0.06</td>
<td>0.21±0.05</td>
<td>0.11±0.06</td>
</tr>
<tr>
<td>Oral SVB (0.25 mg/kg)</td>
<td>0.24±0.02</td>
<td>0.23±0.02</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.25 mg/kg)</td>
<td>0.25±0.04</td>
<td>0.24±0.03</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.50 mg/kg)</td>
<td>0.27±0.02</td>
<td>0.26±0.02</td>
<td>0.09±0.01</td>
</tr>
</tbody>
</table>

*Compared to positive control group (NCI-H460 cells alone), p<0.05. SVB = skin of *venenum bufonis*.

---

**Figure 3**  Gross liver structure from NCI-H460 cell-bearing mice. (A) NCI-H460 cells alone (positive control). (B) Oral skin of *venenum bufonis* (SVB) (0.25 mg/kg). (C) Intraperitoneal SVB (0.25 mg/kg). (D) Intraperitoneal SVB (0.5 mg/kg). No significant differences in liver tissue were observed. (100×, scale bar = 100 μm)
Moreover recent studies have shown that *venenum bufonis* has antitumor effects in several tumor cells [2−9,13,15]. Based on these findings, we expected it to also have an antitumor effect in the in vivo model used in this study.

We conducted an in vivo experiment where nude mice carried a NCI-H460 cell-xenografted tumor for 28 days. These mice were treated with oral or intraperitoneal SVB (0.25 mg/kg), or with a higher intraperitoneal dose (0.5 mg/kg). Time-dependence changes of tumor volume were measured using digital calipers, and the removed tumor volume was measured using a plethysmometer in the final day of experiments. We observed that all of the SVB treated mice had smaller tumors. For example, the higher the dose of SVB, the lower the IR seen on day 11 of the experiment. Furthermore, tumor weight and volume in mice treated with the highest dose of SVB were the smallest in size. This may imply that SVB reduced tumor size at a higher dose, although given that results were not identical, a direct causal relationship cannot be established at this stage.

Mice with cancer usually lose weight due to cachexia. In this study, high-dose, intraperitoneal SVB-treated mice significantly gained body weight compared with untreated mice. Inhibition of tumor growth may have caused weight gain in this circumstance, but further investigation is required into the mechanism by which this occurred.

Usually a larger spleen is seen when a malfunctioning liver is present. Histopathological studies, however, showed no remarkable changes in liver tissues in this study. Interestingly, spleen size was reduced in animals treated with intraperitoneal SVB (0.5 mg/kg), but the mechanism by which this reduction in organ size occurred is unknown. Therefore further studies are required to fully explain this observation.

Mean survival time and ILS were higher in the low-dose SVB intraperitoneal treatment group compared with the other treatment groups. Considering previous results, we would expect that mean survival time and ILS would increase in the high-dose SVB treatment group, but this was not observed. High-dose SVB may have influenced other physiologic functions involved in longevity and therefore may have influenced mean survival time and ILS. This possibility may have in fact been the case, given the results of biochemical blood analysis.

Biochemical blood analysis revealed significant decrease in phosphatase and urea nitrogen levels in animals treated with oral SVB (0.25 mg/kg; p<0.01). Additionally, calcium and ALT levels were significantly decreased in blood from animals in the intraperitoneal SVB (0.5 mg/kg) group (p<0.05). Excluding these results, no other significant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urea nitrogen mg/dL</th>
<th>Phosphatase mg/dL</th>
<th>AST IU/L</th>
<th>ALT IU/L</th>
<th>Creatinine mg/dL</th>
<th>Calcium mg/dL</th>
<th>ALP IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460 cells alone</td>
<td>29.38±3.68</td>
<td>9.27±0.99</td>
<td>20.26±2.39</td>
<td>7.48±0.79</td>
<td>0.35±0.05</td>
<td>10.18±0.44</td>
<td>67.62±7.27</td>
</tr>
<tr>
<td>Oral SVB (0.25 mg/kg)</td>
<td>26.58±3.87</td>
<td>7.15±1.16</td>
<td>8.35±1.42</td>
<td>9.27±0.99</td>
<td>0.32±0.08</td>
<td>9.96±0.27</td>
<td>62.10±5.79</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.25 mg/kg)</td>
<td>25.75±4.62</td>
<td>9.15±0.78</td>
<td>8.15±1.36</td>
<td>7.48±0.79</td>
<td>0.37±0.05</td>
<td>16.58±5.68</td>
<td>64.35±8.56</td>
</tr>
<tr>
<td>Intraperitoneal SVB (0.50 mg/kg)</td>
<td>25.24±6.67</td>
<td>8.15±1.36</td>
<td>8.35±1.42</td>
<td>7.48±0.79</td>
<td>0.30±0.05</td>
<td>10.03±0.95</td>
<td>69.35±8.85</td>
</tr>
</tbody>
</table>

*Results are presented as mean±SD; †p<0.05 compared to positive control group (NCI-H460 cells alone); ‡p<0.01 compared to positive control group (NCI-H460 cells alone), p<0.01.

SVB = skin of *venenum bufonis*; ALP = alkaline phosphatase; AST = aspartate transaminase.
changes in blood chemistry were observed. These findings demonstrate that SVB treatment did not cause hepatic malfunction and indicate that other physiological process may be affected by SVB treatment.

Calcium levels were significantly decreased following intraperitoneal administration of 0.5 mg/kg SVB ($p<0.05$). However, calcium could be elevated with tumor growth, so decreased calcium level could indicate that tumor activity was decreased.

Though the results of this study may show that SVB has an antitumor effect, similar result patterns were not observed in every experiment. SVB treatment may also prevent liver malfunction but no dose-dependent action of SVB was seen and histopathological tests showed no visible differences between control and treated groups. The cause of decreased spleen size also needs further investigation.

The results of this in vivo study showed that SVB may have potential as a tumor growth inhibitor in NCI-H460 tumor induced nude mice. Further studies to overcome the limitations in the present work are needed, as are further investigations into the antitumor mechanism of SVB.

Acknowledgments

This work was supported by a Korean Pharmacopuncture Institute (KPI) grant.

References