Antitumor Effect of Hang-Am-Dan Non-boiled Water Extracts on NCI-H460 Tumor Regression Model

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Objective: This experimental study was performed to examine if Hang-Am-Dan non-boiled water extracts (HAD-N) induce apoptosis in human lung carcinoma NCI-H460 cells in vitro and inhibits the growth of NCI-H460 cell-transplanted solid tumor in vivo.

Materials and Methods: We cultured NCI-H460 cell lines and xenografted them to nude mice. The mice were divided into 3 groups, NCI-H460 cell alone, NCI-H460 + 90 mg/kg HAD-N treated group, and NCI-H460 + 180 mg/kg HAD-N treated group, with seven mice per group. HAD-N was orally administrated every day for four weeks. We checked their body weight and tumor weight and volumes two times a week and their absolute organ weight and biochemical blood analysis at the final day by sacrificing them. We also calculated their tumor inhibition rate (IR), mean survival time and percent increase in life span (% ILS).

Results: In this study, we observed that all of the HAD-N treated mice got smaller tumors. The more doses of HAD-N used, the less IR showed at the 8th day after starting this experiment. Tumor weight and volume of HAD-N treatment groups also decreased. Mean survival time and percent increase in life span (% ILS) in the high-dose HAD-N treatment groups were higher than those of other groups. The test substances in the blood level UN results showed reduction in the significance in both HAD-N 90 mg/kg and HAD-N 180 mg/kg (p <0.01). The blood level phosphatase results in HAD-N 90 mg/kg group compared to NCI-H460 cell alone group showed a reduction in significance (p<0.05). AST levels HAD-N 180 mg/kg group compared to NCI-H460 cell alone group significance as well (p<0.05).

Conclusion: We suggest that the results of the in vivo study showed that HAD-N may have potential as a growth inhibitor of tumor-induced NCI-H460 of nude mice in spite of the shortcomings of this study. More studies to overcome those shortcomings and to find out significant antitumor mechanism will be needed.

Key Words: non-boiled, lung cancer, apoptosis, NCI-H460, nude mouse

Introduction

Lung cancer is the most frequent cause of cancer-related deaths for both men and women throughout the world. Clinical trials have demonstrated that the benefit of combination chemotherapy among the fittest patients with advanced non-small cell lung carcinoma (NSCLC) is marginal\(^1\,\text{-}\,^2\). That is why novel treatment strategies are urgently needed to improve the clinical management of this disease.

Recently targeted apoptosis, a programmed cell death, has been promoted as a good strategy for cancer prevention and treatment, since many anticancer drugs induce apoptosis in cancer cells including NSCLC cells\(^3\).

Hang-Am-Dan (HAD) is a medication produced...
by grinding 9 herbal ingredients (Coix lachryma Semen, Panax notoginseng Radix, Hippocampus kelloggi, Cordyceps militaris, Cremastra appendiculata Tuber, Panax ginseng Radix, Bos taurus Calculus, Pteria martensii, and Moschus moschiferus) and mixing them into powdered form for oral administration. First developed at the East-West Cancer Center (EWCC), HAD has been routinely used for treating solid tumor patients at EWCC for decades, especially for patients with lung cancer. Of the herbal gradients, Panax notoginseng and Cordyceps militaris have been proven effective on apoptosis of human lung carcinoma cells in vitro\(^4\)\(^5\). EWCC also reported a persuasive case of the effect of HAD on lung cancer in the Best Case Series Program of U.S National Cancer Institute (NCI) in 2007\(^6\).

In the screening process, different decocting temperatures were tested to if they are a significant factor for antitumor experiments. The effect of the water extract of HAD-boiled water extract (HAD-B) and HAD-non boiled water extract (HAD-N) on human lung carcinoma cells and the underlying intracellular signal transduction pathways involved in regulating apoptosis were to be elucidated in this experiment. The anti-tumor activity of HAD-N was also evaluated, which was more effective in vitro, for a NCI-H460 tumor regression model.

**Table 1. Prescription of HAD**

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Relative amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coix lachryma Semen</td>
<td>259.0</td>
</tr>
<tr>
<td>Panax notoginseng Radix</td>
<td>86.0</td>
</tr>
<tr>
<td>Hippocampus kelloggi</td>
<td>26.0</td>
</tr>
<tr>
<td>Cordyceps Militaris</td>
<td>26.0</td>
</tr>
<tr>
<td>Santsigu Tuber</td>
<td>26.0</td>
</tr>
<tr>
<td>Ginseng Radix</td>
<td>26.0</td>
</tr>
<tr>
<td>Bovis Calculus</td>
<td>17.0</td>
</tr>
<tr>
<td>Margarita</td>
<td>17.0</td>
</tr>
<tr>
<td>Moschus</td>
<td>17.0</td>
</tr>
<tr>
<td><strong>Total amount (1 capsule)</strong></td>
<td><strong>500.0</strong></td>
</tr>
</tbody>
</table>

**Materials and Methods**

1. *In vitro* study

1) Material

HAD is a medication produced by grinding 9 herbal ingredients and mixing them in powdered form (Table 1). HAD was supplied by Daejeon University Dunsan Oriental Hospital, Daejeon, Korea. One hundred mg of HAD was measured using a balance and melted into D.D.W. to be 1 ml of volume, which becomes 100 mg/ml.

HAD-B was decocted in a double boiler at 100 °C, and HAD-N at 60 °C for 30 minutes, then insoluble impurities eliminated by 0.8 um syringe filter. Solid particles and aggregates were removed by centrifugation at 3,000 rpm for 10 min and the supernatants were lyophilized. The lyophilized extract was dissolved in distilled water to produce 100 g/L of CLL extract. Both HAD-B and HAD-N were kept refrigerated at 4 °C until used. Cells were treated by filtered solution at proper concentrations.

2) Cell cultures and cell viability study

The human lung carcinoma cell lines NCI-H460 were obtained from the KCLB (Korean Cell Line Bank, Korea) and cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-
streptomycin in a 37°C incubator with 5% CO2. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. For the morphological study, the cells were treated with HAD-N or HAD-B for 24 hrs and photographed directly using an inverted microscope (Carl Zeiss, Germany).

3) Nuclear staining with DAPI
After treating the cells with HAD-N or HAD-B for 48 hours, the cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed two more times with PBS and analyzed via a fluorescence microscope (Carl Zeiss).

4) Protein extraction and Western blot analysis
Cell lysates were lysed in extraction buffer as previously described7. The protein concentrations were measured using a Bio-Rad protein assay (BioRad Lab., Hercules, CA, USA) according to the manufacturer’s instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The blots were probed with the desired antibodies for 1 hour, incubated with the diluted enzyme-linked secondary antibody, and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., Arlington Heights, IL, USA). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp.

2. In vivo study
1) Material
HAD-N was orally administered at 90 and 180 mg/kg. HAD-N was prepared in the same method as in vitro. In summary, HAD was decocted in a double boiler at 60°C for 30 minutes and the insoluble impurities in materials were eliminated by 0.8 um syringe filter. This material was administered orally, once a day, seven times a week, for 4 weeks in total.

2) Cell line and culture conditions
Human lung cancer (NCI-H460) cells were from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in the RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin8.

3) Experimental animal
BALB/c nude mice (male, 9-11 weeks n=25) weighing 21-25 g were purchased from Japan SLC, Inc (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 (22 ± 2°C), light (12 hrs light/dark cycle) and humidity (50 ± 10%). All laboratory feed pellets and beddings were autoclaved.

4) Experimental design
The tumor regression model in nude mouse has been successfully applied to evaluate anti-tumor activity, therefore was used to evaluate suppression of solid tumors by HAD-N. When the tumor volume reached 100 mm³, the nude mice, xenografted with tumor fragments, were randomly divided into three groups: NCI-H460 cells alone, NCI-H460 + 90 mg/kg HAD-N treated, and NCI-H460 + 180 mg/kg HAD-N treated groups, seven mice per group. HAD-N was
orally administrated every day for four weeks.

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

6) Xenografts
NCI-H460 cells were washed twice with Hank’s balanced salt solution (HBSS), trypsinized with 0.25% trypsin, and washed twice with fresh culture medium. 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’ observation, apparent solid tumor mass was removed from 3 out of 5 mice inoculated with NCI-H460 cells. Tumor fragments (3 × 3 × 3 mm) were made by trimming with a knife, and xenografted into the flank of new mice using a trocar.

The suppressive effect of anticancer agents on solid tumor was evaluated in a tumor-regression model. In brief, from the day tumor volume reached 100 mm³, HAD-N (water or 90 mg or 180 mg/kg) were administrated orally to the mice xenografted with a tumor fragment, every day, for 28 days.

7) Changes in tumor volume
The changes in the size of tumor mass were recorded twice a week using digital calipers. The largest and smallest diameters were measured in each mouse, and the tumor volume was estimated according to the following formula:

\[ V = \frac{(A \times B^2)}{2} \]

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 a tumor volume, the antitumor activities of treatment were expressed by the inhibition rate,

\[ IR (inhibition rate)(\%) = \frac{[(CV-TV)/TV] \times 100}{CV = Control group tumor volume, TV = Treatment group tumor volume} \]

where CV and TV are tumor volumes in control (water) and treatment groups, respectively. Also, the tumor weights were measured on the final day after sacrifice of animals and removal of the tumor mass.

8) 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 when the tumor volume reached 1,500 mm³ as described previously, and the increased percentage of life span (%ILS) were calculated according to the equation of:

\[ %ILS (increase in life span) = \frac{[(T-C)/C] \times 100}{T and C are mean survival days of mice in control and treatment groups, respectively.} \]

9) Blood chemistry levels
Blood samples were centrifuged at 1,400 ×g at 4°C for 10 min. The supernatants (serum) were used for the determination of alanine phosphatase (ALP), creatinine (CA), phosphatase (PHOS), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) with an automatic analyzer (7170, Hitachi, Tokyo, Japan).

10) Statistical analysis
The results are presented as mean ± S.D., and the significance of the difference between the mean of control and treatment groups was analyzed using one-way analysis of variance (ANOVA), 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.
Results

1. *In vitro* study

1) Inhibition of cell viability and induction of apoptosis by HAD-B/HAD-N

In order to determine if HAD-B or HAD-N decrease lung cancer cell viability, NCI-H460 cells were stimulated with various concentrations of HAD-B or HAD-N for 24 hours, and the cell viability was measured using an MTT assay. As shown in Fig. 1, HAD-N treatment significantly inhibited the cell viability of both cell lines in a concentration-dependent manner (after 24 hrs treatment at 50 μg/ml, HAD-N decreased the NCI-H460 cell viability by approximately 41.89% and 63.97%, respectively, compared to the controls). In particular, cell shrinkage, cytoplasm condensation, and formation of cytoplasmic filaments appeared after 50 μg/ml HAD-N treatment for 24 hours. Further experiments using fluorescence microscope analyses were carried out to determine if the anti-proliferative effect of HAD-N is the result of apoptotic cell death. Morphological analysis with DAPI staining showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with HAD-B, HAD-N in a concentration-dependent manner. In contrast, only a few were observed in the control culture (Fig. 2). These results indicate that the cytotoxic effects observed in response to HAD-B/HAD-N are associated with the induction of apoptosis.

2) Modulation of the expression of Bcl-2 family proteins and cleaved caspase-3 by HAD-N

To elucidate the mechanisms underlying HAD-N-induced apoptosis, the levels of Bcl-2 family proteins, cleaved caspase-3 were examined using Western blotting. When NCI-H460 cells were treated with HAD-N, a clear decrease in anti-apoptotic Bcl-2 protein expression was observed in a concentration-dependent manner. In the case of the pro-apoptotic protein Bax, there was a concentration-dependent upregulation observed in NCI-H460 cells treated with HAD-N. The expression levels and activities of cleaved caspase-3 in cells that had been exposed to HAD-N were measured in order to determine if HAD-N-induced apoptosis is associated with the

![Graph](image1.png)

**Fig. 1.** Inhibitory effects of sample on the growth of NCI-H460 cancer cells

Cancer cells were treated with different concentrations (0, 3.125, 6.25, 12.5, 25, 50 μg/ml) of sample at 37°C for 24 hours and Inhibitory effects were estimated by the MTT-assay. Each bar represents the mean ± S.D.

*: Significant difference from positive control (NCI-H460 cell alone) group at p<0.05.

**: Significant difference from positive control (NCI-H460 cell alone) group at p<0.01.
activation of caspases. As seen in Fig. 3, Western blot data showed that the levels of cleaved caspase-3 proteins were increased in a concentration-dependent manner in HAD-N-treated NCI-H460 cells.

2. In vivo study

1) Changes in body weights

Changes in body weights of each group were shown in Fig. 4. The mean body weights of HAD-N treatment group were slightly increased over those of the positive control group (25.33-26.64 g), with value of 24.81-26.14 g in HAD-N 90 mg/kg treated and 25.88-27.38 g in HAD-N 180 mg/kg treated. However, no significant inter-group (HAD-N treated groups and positive control group) differences were observed.

2) Changes in tumor volume (measurement with calipers)

Treatment with HAD-N (90-180 mg/kg) inhibited the growth of NCI-H460 cell-transplanted solid tumors compared with the value of the positive

Fig. 2. Morphological changes of NCI-H460 human lung cancer cells due to HAD-B, HAD-N treatment

The cells were treated with various concentrations of HAD-B or HAD-N for 24 hours. The cells were sampled and examined using an inverted microscope. The cells grown under the same conditions were sampled, fixed, and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter (magnification, x100, x400).

Fig. 3. Effects of HAD-N on cleaved caspase-3, Bcl-2, and Bax in NCI-H460 cells

NCI-H460 cells were treated with HAD-N (0, 25, 50, 100, 200 µg) for 24 hours. Cell lysates were subjected to Western blotting with an antibody against cleaved caspase-3, Bcl-2, and Bax. Photographs of chemiluminescent detection of the blots, which were representative of 4 independent experiments, are shown.
control group (Fig. 5). On the 29th day, the mean tumor volume of the HAD-N 180 mg/kg treatment group was lower than that of the positive control group throughout the study period, with value of 4492.22 mm$^3$ - 2845.87 mm$^3$.

3) Inhibition rate (IR) on tumor volume
IR (%) of each group is shown in Table 2. From the eighth day to the final day (29th day), each IR (%) indicated dose-dependent tendency (HAD-N 90 mg/kg IR 85.66% < HAD-N 180 mg/kg IR 56.76% at 15 day).

4) Tumor weights and volume (plethysmography)
Final tumor weights and volume of each group are shown in Table 3. Tumor weight and volume of positive control group (NCI-H460 only) were 2.52 ± 0.75 g and 4.67 ± 1.18 cm$^3$, respectively, on the final day. Tumor weight and volume of HAD-N 90 mg/kg were 2.24 ± 0.78 g and 4.03 ± 1.28 cm$^3$.

![Fig. 4. Changes in body weights in nude mice bearing NCI-H460 cell solid tumors](image)

After nude mice with NCI-H460 cell-transplanted tumor were treated with daily dose of HAD-N (90-180 mg/kg) for 29 days, the body weights of nude mice in the positive control, NCI-H460 cell alone (□, n=7), HAD-N 90 mg/kg (●, n=7), and HAD-N 180 mg/kg (▲ n=7), were measured two times a week.

![Fig. 5. Time-course of increase in tumor volumes in NCI-H460 cell-bearing mice treated with HAD-N](image)
The mice xenografted with tumor fragments were treated with the anticancer agents from the day when tumor mass reached 100 mm$^3$. The length and width of solid tumor in the positive control, NCI-H460 cell alone (□, n=7), HAD-N 90 mg/kg (●, n=7), and HAD-N 180 mg/kg (▲ n=7) were measured two times a week and tumor volume was evaluated.
Antitumor Effect of Hang-Am-Dan Non-boiled Water Extracts on NCI-H460 Tumor Regression Model

Tumor weight and volume of HAD-N 180 mg/kg treatment groups were 2.17 ± 0.60 g and 3.79 ± 0.85 cm³. Compared with the positive control group, tumor weights and volumes of HAD-N treatment groups decreased.

5) Mean survival time and percent increase in life span (% ILS)

Mean survival time and percent increase in life span are shown in Table 4. and Fig. 6. The positive control group (NCI-H460 only) survived 18.29 ± 3.59 days. Mean survival time of the low HAD-N treatment group (90 mg/kg) was extended to 18.71 ± 2.12 days and 2.29 % ILS. Also, the mean survival time of the high HAD-N treatment group (180 mg/kg) was 24.40 ± 1.92 days and 33.40 % ILS. Each percent increased in a dose dependent manner. Especially, the high HAD-N treatment group (180 mg/kg) showed significant difference compared to the positive control (NCI-H460 cell alone) group.

6) Histopathological findings (light microscopy) and organ weights

Absolute organ weights of kidneys, liver, spleen, heart and lungs are shown in Table 5. Especially HAD-N (90-180 mg/kg) treatment group resulted in a remarkable decrease in liver weights (p<0.05). Light microscopic histopathological examination of liver tissue of mice treated with HAD-N (180 mg/kg) did not show any specific lesions compared with liver tissue of NCI-H460 cells-bearing mice in the positive control (Fig. 6).

Table 2. Inhibition Rate (IR) on Tumor Volume of NCI-H460 Tumor-bearing Mice (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
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<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460 cell only</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>HAD-N 90 mg/kg</td>
<td>99.94</td>
<td>98.13</td>
<td>87.66</td>
<td>89.92</td>
<td>85.66</td>
<td>88.84</td>
<td>79.17</td>
<td>80.1</td>
<td>78.31</td>
</tr>
<tr>
<td>HAD-N 180 mg/kg</td>
<td>88.1</td>
<td>62.35</td>
<td>53.99</td>
<td>56.8</td>
<td>56.76</td>
<td>59.78</td>
<td>54.06</td>
<td>63.69</td>
<td>63.35</td>
</tr>
</tbody>
</table>

Results are shown as mean ± S.D. (n=5-7)

Table 3. Tumor Weights in Mice Xenografted with NCI-H460 Cells on the Final Day (%)

<table>
<thead>
<tr>
<th></th>
<th>NCI-H460 tumor alone</th>
<th>HAD-N 90 mg/kg</th>
<th>HAD-N 180 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor weight (g)</td>
<td>2.52 ± 0.75</td>
<td>2.24 ± 0.78</td>
<td>2.17 ± 0.60</td>
</tr>
<tr>
<td>Tumor volume (cm³)</td>
<td>4.67 ± 1.18</td>
<td>4.03 ± 1.28</td>
<td>3.79 ± 0.85</td>
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</tbody>
</table>

Results are shown as mean ± S. D. (n=5-7)

Table 4. Percent Increase in Life Span (% ILS) of NCI-H460 Tumor-bearing Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean survival time (day)</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460 tumor only</td>
<td>18.29 ± 3.59</td>
<td>0</td>
</tr>
<tr>
<td>HAD-N 90 mg/kg</td>
<td>18.71 ± 2.12</td>
<td>2.29</td>
</tr>
<tr>
<td>HAD-N 180 mg/kg</td>
<td>24.40 ± 1.92</td>
<td>33.40</td>
</tr>
</tbody>
</table>

Results are shown as mean ± S.D. (n=5-7)

*: Significant difference from positive control (NCI-H460 cell only) group at p<0.05.
Table 5. Organ Weights, on the Final Day, of NCI-H460 Tumor-bearing Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absolute organ weights</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Liver (g)</td>
<td>Spleen (g)</td>
<td>Heart (g)</td>
</tr>
<tr>
<td>NCI-H460 cell alone</td>
<td>26.67 ± 1.60</td>
<td>1.76 ± 0.07</td>
<td>0.34 ± 0.04</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>HAD-N 90 mg/kg</td>
<td>26.14 ± 1.55</td>
<td>1.60 ± 0.09 *</td>
<td>0.35 ± 0.05</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>HAD-N 180 mg/kg</td>
<td>27.86 ± 1.52</td>
<td>1.76 ± 0.07</td>
<td>0.37 ± 0.07</td>
<td>0.16 ± 0.01</td>
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</table>

<table>
<thead>
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<th>Treatment</th>
<th>Absolute organ weights</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney (g)</td>
<td>Testis (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H460 cell alone</td>
<td>0.22 ± 0.06</td>
<td>0.21 ± 0.05</td>
<td>0.11 ± 0.05</td>
<td>0.11 ± 0.05</td>
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<tr>
<td>HAD-N 90 mg/kg</td>
<td>0.22 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.09 ± 0.009</td>
<td>0.09 ± 0.007</td>
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<tr>
<td>HAD-N 180 mg/kg</td>
<td>0.24 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.09 ± 0.01</td>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative organ weights (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Liver (g)</td>
<td>Spleen (g)</td>
<td>Heart (g)</td>
</tr>
<tr>
<td>NCI-H460 cell alone</td>
<td>26.64 ± 1.60</td>
<td>6.61 ± 0.22</td>
<td>1.27 ± 0.21</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>HAD-N 90 mg/kg</td>
<td>26.14 ± 1.55</td>
<td>6.11 ± 0.20 *</td>
<td>1.38 ± 0.24</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>HAD-N 180 mg/kg</td>
<td>27.86 ± 1.52</td>
<td>6.20 ± 0.24 *</td>
<td>1.34 ± 0.27</td>
<td>0.59 ± 0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative organ weights (%)</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney (g)</td>
<td>Testis (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H460 cell alone</td>
<td>0.82 ± 0.22</td>
<td>0.80 ± 0.18</td>
<td>0.42 ± 0.20</td>
<td>0.42 ± 0.20</td>
</tr>
<tr>
<td>HAD-N 90 mg/kg</td>
<td>0.83 ± 0.05</td>
<td>0.84 ± 0.09</td>
<td>0.35 ± 0.03</td>
<td>0.36 ± 0.93</td>
</tr>
<tr>
<td>HAD-N 180 mg/kg</td>
<td>0.86 ± 0.03</td>
<td>0.85 ± 0.05</td>
<td>0.32 ± 0.03</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Results are shown as mean ± S.D. (n=5-7)

*: Significant difference from positive control (NCI-H460 cell only) group at p<0.05.

Fig. 6. Gross finding of liver of NCI-H460 cells-bearing mice with positive control, NCI-H460 cell only (A), HAD-N 90 mg/kg (B), HAD-N 180 mg/kg (C)

Light microscopic histopathological examination showing liver tissue of mice treated with HAD-N did not show any specific lesion compared with control.
Antitumor Effect of Hang-Am-Dan Non-boiled Water Extracts on NCI-H460 Tumor Regression Model

7) Blood chemistry levels on the final day

Biochemical blood analysis ALP, CA, CRE, ALT levels did not indicate significant results in test. The test substances in the blood level UN results showed reduction in both the significance (HAD-N 90 mg/kg, HAD-N 180 mg/kg, p <0.01). The blood level phosphatase results showed HAD-N 90 mg/kg group 7.98 ± 0.92 mg/dl, NCI-H460 cell alone group 9.26 ± 0.98 mg/dl compared to a reduction in significance (p<0.05). AST levels HAD-N 180 mg/kg group(18.66 ± 2.16 IU/L) compared to NCI-H460 cell alone group (25.75 ± 4.62 IU/L) showed significance as well (p<0.05).

Discussion and Conclusion

Apoptosis, which is programmed cell death, plays an important role in developmental processes, maintenance of homeostasis, and elimination of damaged cells. Apoptosis is a tightly regulated process characterized by a series of distinct morphological and biochemical alterations to cells such as DNA fragmentation, chromatin condensation, cell shrinkage, and plasma membrane blebbing. Thus, the regulation of apoptosis has become an area of extensive study in cancer research and has been considered an ideal way of eliminating precancerous and/or cancerous cells. Several gene products have been demonstrated to be critical in the regulation of apoptosis. For instance, caspases, a group of cysteine proteases, play a key role in apoptosis. Caspases are synthesized as proenzymes, which are activated by the cleaving of the prodomain at a specific aspartic acid cleaving site. Caspase activation is often regulated by various cellular factors, including members of the Bcl-2 family and/or the death receptor-related gene products. However, most cancer cells can block apoptosis, which allows them to survive despite the genetic and morphologic transformations. Therefore, the induction of apoptotic cell death is an important mechanism in many anti-cancer drugs.

The major aim of this study was to examine if HAD-N induces apoptosis in human lung carcinoma NCI-H460 cells in vitro and inhibits the growth of NCI-H460 cell-transplanted solid tumors in vivo.

The results of MTT assay indicated that HAD-N treatment results in a significant inhibition of cell viability in NCI-H460 cells (Fig. 1, 2). Interestingly, HAD-N showed much stronger effects than HAD-B. In the screening step, we wanted to know whether decocting temperature was a significant factor for antitumor experiments or not, so we tried to elucidate the effect of the water extracts of HAD-B and HAD-N.
HAD-N on human lung carcinoma cells. HAD is a medication produced by grinding 9 herbal ingredients and mixing them into powdered form. HAD-B was decocted in a double boiler at 100 °C, and HAD-N at 60 °C for 30 minutes.

Based on these results, we selected HAD-N as a candidate material for mechanism study. HAD-N treatment was shown to induce apoptosis in lung carcinoma cells through modulation of the Bcl-2 family proteins and activation of cleaved caspase-3. It has recently been proposed that mitochondria are possible targets for anti-cancer drug-induced apoptosis in the death receptor (extrinsic) pathway as well as the mitochondrial (intrinsic) pathway. Since the discovery of Bcl-2, several mechanisms for anti-apoptotic properties of this protein have been proposed. The anti-apoptotic function of Bcl-2 against pro-apoptotic Bax may be explained by its ability to control several key steps involved in death signaling. The Bcl-2 family significantly regulates apoptosis either as an activator (Bax) or as an inhibitor (Bcl-2); therefore, it has been suggested that Bax/Bcl-2 ratio is a key factor in the regulation of the apoptotic process.

To elucidate the mechanisms underlying HAD-N-induced apoptosis, the levels of Bcl-2 family proteins and cleaved caspase-3 were examined using Western blotting. NCI-H460 cells were treated with HAD-N, a clear decrease in anti-apoptotic Bcl-2 protein expression was observed in a concentration-dependent manner. In the case of the pro-apoptotic protein Bax, there was a concentration-dependent upregulation observed in NCI-H460 cells treated with HAD-N. The expression levels and activities of cleaved caspase-3 in cells that had been exposed to HAD-N were measured in order to determine if HAD-N-induced apoptosis is associated with the activation of caspases. As seen in Fig. 3, Western blot data showed that the levels of cleaved caspase-3 proteins were increased in a concentration-dependent manner in HAD-N-treated NCI-H460 cells.

Briefly, the data also showed that HAD-N-induced apoptosis was related to upregulation of the Bax protein, downregulation of Bcl-2 and upregulation of cleaved caspase-3 in HAD-N-treated cells (Fig. 3), which indicates that HAD-N increased the Bax/Bcl-2 ratio and induced mitochondrial dysfunction, leading to apoptosis in human lung carcinoma cells.

The caspase family of aspartate-specific cysteine proteases also plays a critical role in regulating apoptosis. Caspase signaling is initiated and propagated by proteolytic autocatalysis and the cleavage of downstream caspases and substrate proteins. Caspase-3 is the most important executioner of apoptosis. These data suggested that HAD-N-induced apoptosis was caused by caspase-3-dependent cell death.

So, in vivo experiments of nude mice with NCI-H460 cell-transplanted tumors were also conducted over 29 days, which were treated with HAD-N (90-180 mg/kg). For example, time-dependent changes of tumor volume were measured by digital calipers and results of removed tumor volumes were measured by a plethysmometer on the final day. All tumor volume results were suppressed with dose-dependent tendency.

The results showed that HAD-N inhibited dose-dependently the growth of NCI-H460 cell-transplanted solid tumor compared with the control group. On the 29th day, numerical values of solid tumors were 2845.87 mm³ (180 mg/kg), 3517.70 mm³ (90 mg/kg) and 4492.22 mm³ (control group) respectively (Fig. 5). Compared with the control group, the experimental groups showed tumor growth inhibition from the 8th to 28th day (HAD-N 90 mg/kg I.R. 85.66% < HAD-N 180 mg/kg I.R 56.76%) in a dose-dependent manner, although not significantly (Table 2). On the final day, tumor weights showed 2.17 ± 0.60 g (180 mg/kg), 2.24 ± 0.78 g (90 mg/kg) and 2.52 ± 0.75 g (control group) (Table 3).

Mean survival time and the rate of increasing lifespan of the low dosage HAD-N (90 mg/kg) treatment group was 18.71 ± 2.12 days and 2.29 %
ILS. The high dosage HAD-N (180 mg/kg) treatment group showed 24.40 ± 1.92 days and 33.40 % ILS (p<0.05). The control group (NCI-H460 tumor alone) survived only 18.29 ± 3.59 days (Table 4). Thus the high HAD-N treatment group (180 mg/kg) was significantly different from the positive control (NCI-H460 tumor alone) group (p<0.05).

Absolute organ weights, especially for the HAD-N (90-180 mg/kg) treatment groups, showed a marked decrease in liver weights (p<0.05). But light microscopic histopathological examination showed that liver tissue of mice treated with HAD-N (180 mg/kg) had no specific lesions compared with liver tissue of NCI-H460 cells-bearing mice with positive control (Table 5, Fig. 6).

Biochemical blood analysis of ALP, CA, CRE, and ALT levels did not show any significant results in test. The test substances in the blood level of UN results showed reduction in both treatment groups (HAD-N 90 mg/kg, HAD-N 180 mg/kg, p <0.01). The blood level of phosphatase had a significant result in the HAD-N 90 mg/kg group (p<0.05), and AST levels of HAD-N 180 mg/kg group were significantly reduced, as well (p<0.05).

The results of the in vivo study showed that HAD-N may have potential as a growth inhibitor of solid tumors induced by NCI-H460 without remarkable side effects. These results suggested that HAD-N may be a useful anticancer agent for treating human lung cancer.

In summary, these studies demonstrated HAD-N exposure induced apoptosis in human lung carcinoma cells. The apoptotic response was associated with the increase of Bax, caspase-3 activation and the decrease of Bcl-2. Such result has been proven by experiments using nude mice with human lung cancer. These results provide new information on the possible mechanisms for the anti-cancer activity of HAD-N. However, it is still unclear if HAD-N can induce apoptosis through other pathways, such as death receptor pathway or endoplasmic reticulum pathway. Therefore, more research is necessary to examine the mechanisms for activation of multiple apoptosis-related proteins in HAD-N-induced apoptosis.

References


