ANTIVIRAL AND ANTI-TUMOR AGENTS

The present invention relates to polypeptide-containing compound(s), composition(s) comprising such compound(s) along with divalent metal ions and/or a carbohydrate moiety, their antiviral and anti-hepatoma activities, and the enhancement of activity of antiviral polypeptide-containing agents by their combination with a carbohydrate and at least one divalent metal ion. Preferably, the carbohydrate is a polysaccharide of arabinose and galactose and, independently, the at least one divalent metal ion(s) include magnesium and zinc. The carbohydrate and divalent metal ion can be present in the form of a complex. The composition has been isolated and purified from the root tubers of the Chinese plant, Zei-Bai.
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ANTIVIRAL AND ANTITUMOR AGENTS

The present invention relates to polypeptide-containing compound(s) (hereinafter "PROTEINA"), composition(s) comprising such compound(s) along with divalent metal ions and/or a carbohydrate moiety (hereinafter "ALICIN"), their antiviral and anti-hepatoma activities, and the enhancement of activity of antiviral polypeptide-containing agents by their combination with a carbohydrate and at least one divalent metal ion. Preferably, the carbohydrate is a polysaccharide that consists essentially of arabinose and galactose and, independently, the at least one divalent metal ion consists essentially of magnesium and zinc. The carbohydrate and divalent metal ion can be present in the form of a complex.

Thus, in one principal aspect, the invention relates to a composition comprising an amino acid-containing compound having the formula:

R-Thr-R-Gly-Asn-Tyr-R-Arg-Leu-R-Ala-Gly-R-Leu-
Arg-Glu-Asn-Ile-R-Leu-Gly-R-Leu-R-Ala-Ile-R-Leu-
R-Tyr-Tyr-R-Ile-Gln-R-Ser-Glu-Ala-Ala-Arg-R-Ile-
Glu-R-Arg-R-Ile-R-Asn-R-Gly-R-Phe-R-Ser-Pro-R-Leu-R
wherein each R is independently at least one amino acid or analog thereof. Preferably, the compound has the formula:

Xaa-Xaa-Xaa-Ala-Gly-Xaa-Leu-Arg-Glu-Asn-Ile-Xaa-Leu-Gly-
Xaa-Xaa-Xaa-Leu-Xaa-Xaa-Ala-Ile-Xaa-Leu-Xaa-Tyr-Tyr-
Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Ile-Gln-
Xaa-Xaa-Ser-Glu-Ala-Ala-Arg-Xaa-Xaa-Xaa-Ile-Glu-Xaa-Xaa-
Xaa-Xaa-Ile-Xaa-Xaa-Xaa-Asn-Xaa-Gly-Xaa-Phe-Xaa-Ser-
Pro-Xaa-Xaa-Leu-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-

wherein each xaa is independently a substantially homologous spacer or linker formed of at least one amino acid or analog thereof. Usually, each R is independently one or more amino acids. In a particularly preferred embodiment, the amino acid-containing compound has the formula:

Arg-Lys-Val-Thr-Leu-Pro-Tyr-Ser-Gly-Asn-Tyr-Glu-Arg-Leu-
Gln-Thr-Ala-Ala-Gly-Gly-Leu-Arg-Glu-Asn-Ile-Pro-Leu-Gly-
Leu-Pro-Ala-Leu-Asp-Ser-Ala-Ile-Thr-Arg-Leu-Phe-Tyr-Tyr-
Asn-Ala-Asn-Ser-Ala-Ala-Ser-Ala-Leu-His-Val-Leu-Ile-Gln-
Ser-Thr-Ser-Glu-Ala-Ala-Arg-Tyr-Lys-Phe-Ile-Glu-Gln-Gln-
Ile-Gly-Ser-Arg-Val-Asp-Lys-Thr-Phe-Leu-Pro-Ser-Leu-Ala-
Ile-Ile-Ser-Leu-Glu-Asn-Ser-Leu-Trp-Leu-Ala-Leu-Ser-Lys-
Gln-Ile-Gln-Ile-Ala-Ser-Thr-Asn-Asn-Gly-Thr-Phe-Glu-Ser-
Pro-Val-Val-Leu-Ile-Asn-Ala-Gln-Asn-Gin-Arg-Asn-Asn-His

In another aspect, the above composition(s) further include a carbohydrate. The carbohydrate can be a monosaccharide, i.e. (CH₂O), where n is at least 3, preferably hexoses such as glucose, mannose, galactose and pentoses such as arabinose and the like or one or more of the known disaccharides. The carbohydrate can also be a homo- or hetero- polysaccharide. When the carbohydrate is a polysaccharide, it is preferably a homo- or hetero- polymer of glucose, mannose, arabinose, galactose or monosaccharide
amine. A particularly preferred polysaccharide is one that consists essentially of arabinose and galactose, particularly in a weight ratio of about 1:0.05, respectively.

In another aspect, the composition further comprises at least one divalent metal ion. Preferably, the divalent metal ion is selected from the group consisting of magnesium and zinc. It is particularly preferred that they be present in a weight percent of about 4 - 20 and 2 - 10, respectively, of the total composition. Range of ratios between Mg and Zn is generally 1:1 to 3:1, preferably 2:1, and their over all weight percentage in the composition is 6 - 30 weight percent of the composition.

Another aspect of the invention relates to a method for treating a viral infection in an individual in need thereof which comprises administering a therapeutically effective amount of the composition(s). Particularly contemplated viral pathogens intended for treatment include Hepatitis A virus, Epstein-Barr and Influenza A viruses.

Another aspect of the invention relates to a method for treating a hepatoma in an individual in need thereof which comprises administering a therapeutically effective amount of the composition(s).

Another aspect of the invention relates to a composition which comprises the composition of an antiviral polypeptide-containing agent, such as one or more of the interferons, trichosanthin and others, in combination with a carbohydrate, particularly a carbohydrate such as those described above, and at least one divalent metal ion, particularly such as those described above. Preferably, the carbohydrate is a polysaccharide that consists essentially of

-3-
arabinose and galactose and, independently, the at least one
divalent metal ion consists essentially of magnesium and zinc.

As used herein, the term "substantially homologous" means
that a particular subject sequence, for example, a sequence of
amino acid analogs, varies from a reference sequence by one or
more substitutions, deletions, or additions, the net effect of
which does not result in an adverse functional dissimilarity
between reference and subject sequences. For purposes of the
present invention, sequences having greater than 90 percent
homology, equivalent biological activity are considered
substantially homologous. Sequences having lesser degrees of
homology, but comparable bioactivity, are considered
equivalents.

The PROTEINA polypeptide-containing portion of the ALICIN
composition(s), its fragments or other derivatives, or analogs
thereof, or cells expressing them can be used as an immunogen
to produce antibodies thereto. These antibodies can be, for
example, polyclonal, monoclonal, chimeric, single chain, Fab
fragments, or an Fab expression library. Various procedures
known in the art may be used for the production of polyclonal
antibodies.

For preparation of monoclonal antibodies, any technique
which provides antibodies produced by continuous cell line
cultures can be used. Examples include the hybridoma
technique (Kohler and Milstein, 1975, Nature 256:495-497), the
trioma technique, the human B-cell hybridoma technique (Kozbor
et al., 1983, Immunology Today 4:72), and the EBV-hybridoma
technique to produce human monoclonal antibodies (Cole et al.,
1985, in Monoclonal Antibodies and Cancer Therapy, Alan R.
Liss, Inc., pp. 77-96). Techniques described for the
production of single chain antibodies (U.S. Patent 4,946,778)
can be adapted to produce specific single chain antibodies.
The antibodies can be used in methods relating to the localization and activity of the protein sequences of the invention, e.g., for imaging these proteins, measuring levels thereof in appropriate physiological samples and the like.

**Therapeutic Administration and Compositions**

Modes of administration of the compound(s) and composition(s) include but are not limited to intravenous, intramuscular and subcutaneous routes as well as by suppository. The compounds may be administered by any convenient route, for example by infusion or bolus injection and may be administered together with other biologically active agents. Administration is preferably systemic.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of the, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ameliorate any pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it
can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

Pharmaceutical preparations can also often be administered rectally, using suppositories, particularly for sustained-release administration or administration to very young, old, infirm or those for whom other routes of administration present unusual obstacles. Suppository formulations include an appropriate amount of the compound(s)/composition(s) of the invention in a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, gelatin rectal capsules can be used whose base or excipient includes liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

The therapeutics of the invention can be formulated as neutral or salt forms. Pharmacologically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The compound and composition are used in at least 5 \( \mu g/kg \) body weight and most generally need not be more than 500 \( \mu g/kg \). Preferably, it is at least about 20 \( \mu g/kg \) and usually need not be more than about 100 \( \mu g/kg \). The compound is typically administered for a period of at least about 7 days but generally not to exceed 30 days, with a typical therapeutic treatment period of 7 to 14 days. It will
preferably be administered rectally by suppository, one to three times per day, and will be adjusted to meet optimal efficacy and pharmacological dosing.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.
Example 1
Isolation/Purification of Glycoprotein ALICIN

Fresh root tubers (1-0 kg) of the Zei-Bai plant (ZB) was obtained from local vendors of produce and farmers in the He-Nan Province, PRC. After the skin of the tuber was removed, its juice (approximately 200 ml) was extracted using a Panasonic Juice Extractor. The extract were chilled to 4° C on an ice bath and adjusted to pH 4 by dropwise addition of 2M hydrochloric acid. While precipitates occurred, slowly added 0.8 volume of the iced acetone to the chilled extracts. Centrifuge and discard the precipitates using Beckman Centrifuge at 4° C and 3000 rpm for 20 minutes. Collect the supernantant and add slowly 80 ml of ice acetone to the supernatant until the precipitates was complete. Centrifuge and collect the precipitates (same protocol described above). The precipitates were dissolved in 10 ml double deionized (d.d.) water and then dialyzed against 10 volumes of d.d. water for 48 hours at 4° C (3 Changes in buffer). The undissolved precipitate was removed and the supernantant was collected by centrifugation as above. The protein solution was lyophilized (Labonco, Germany) to a dry powdered glycoprotein (1.2 ± 0.3 g).

SDS-Page revealed one large protein band (85%) and three other minor bands. The large band has a molecular weight approximately 21 kD.

The above prepared lyophilisate was reconstituted to about 200 ml and maintained at 4° C overnight observed precipitate was removed by centrifugation at 3000 rpm for 30 minutes at 4° C. The supernatant was loaded over a Ricinus communis Agglutinin affinity column (Sepharose 4B, 2.5x58 cm) which was prepared following the procedures of Dulaney (Mol. Cell. Biochem. 21, 43-63, 1978). The glycoprotein was
displaced from the column with a carbohydrate buffer (0.1 M phosphate and 0.2 M galactose, pH 7.2). After lyophilization, about 1.0 g of the glycoprotein was obtained.

Both cellulose acetate membrane and SDS-PAGE showed that the glycoprotein prepared from this affinity column has a purity of > 95%. The molecular weight observed was approximately 21 kD.

**Example 2**

**Purification of Polypeptide Protein PROTEINA**

ALICIN was dissociated into the polypeptide component, PROTEINA, as follows. ALICIN (1-0 g) prepared as described above was dissolved in 20 ml solution composed of 8M urea and 0.1M phosphate buffer (pH 7.2). Centrifuge and discard the undissolved solid with the Beckman Centrifuge at 4° C, 3000 rpm for 20 minutes. Pass the supernatant through a CM Sepharose C-50 column (5 x 53 cm), eluted the protein by applying 2L buffer of 8M urea and 0.1M phosphate at pH 7.2 and followed at pH 9 respectively. The flow rate was 36 ml/hr with each collected fraction of 1ml per tube. The protein content was detected by 280 nm absorption, one large peak of the Protein was observed. The protein solution from each tube was pooled and dialyzed against double distilled water for 48 hours at 4° C with several buffer changes.

After removing precipitate by centrifugation at 3000 rpm for 20 min, the supernatant was dialized against barbital buffer (pH 8.6) at 4° C for 24 hours with several buffer changes. The pure protein was obtained by crystalization with the barbital buffer. About 500 mg of the PROTEINA was obtained. SDS-PAGE showed one pure protein band (>98%) with a molecular weight approximately 15 kDa.
Example 3
Amino Acid Sequence Analysis of PROTEINA

PROTEINA (20 mg), prepared as described in Example 2, was dissolved in 70% formic acid (1 ml). An excess amount (50 times) of cyanogen bromide solution was added and mixed continuously with the protein solution at 20° C in the dark for 24 hours. During this time, nitrogen was also continuously added to the mixture. At the end of this protocol, the protein was broken down to constituent fractions. The hydrolyzed peptide fractions were diluted with ten volumes of d.d. water and freeze-dried.

The hydrolyzed peptide fractions (5 mg) were dissolved together in 0.3 ml of elution solution (8 M urea and 10% HoAc) and passed through a Sephadex G 25 column to achieve homogeneity and remove cyanogen bromide. The column flow rate was 4 ML/h. The peptide fractions (0.7 ml/tube) were pooled and processed for amino acid sequence analysis using a Beckman 890C amino acid sequence autoanalyzer. The results obtained by computer analysis are shown in Table 1.
Table 1

Minimal Amino Acid Sequence of PROTEINA (126 amino acids)
H-Arg-Lys-Val-Thr-Leu-Pro-Tyr-Ser-Gly-Asn-Tyr-
Glu-Arg-Leu-Gln-Thr-Ala-Ala-Gly-Gly-Leu-Arg-Glu-
Asn-Ile-Pro-Leu-Gly-Leu-Pro-Ala-Leu-Asp-Ser-Ala-
Ile-Thr-Thr-Leu-Phe-Tyr-Tyr-Asn-Ala-Asn-Ser-Ala-
Ala-Ser-Ala-Leu-His-Val-Leu-Ile-Gln-Ser-Thr-Ser-
Glu-Ala-Ala-Arg-Tyr-Lys-Phe-Ile-Glu-Gln-Gln-Ile-
Gly-Ser-Arg-Val-Asp-Lys-Thr-Phe-Leu-Pro-Ser-Leu-
Ala-Ile-Ile-Ser-Leu-Glu-Asn-Ser-Leu-Trp-Leu-Ala-
Leu-Ser-Lys-Gln-Ile-Gln-Ile-Ala-Ser-Thr-Asn-Asn-
Gly-Thr-Phe-Glu-Ser-Pro-Val-Val-Leu-Ile-Asn-Ala-
Gln-Asn-Gln-Arg-Asn-Asn-His-OH

This protein showed a negative content of carbohydrate based on the periodic acid test. The atomic emission analysis of the protein indicated the absence of Mg and Zn (< 0.001%).

Example 4

Isolation and Characterization of ALICIN Carbohydrate Moiety

ALICIN (1.0 g), purified as described in Example 1 (>95%) in double distilled water (10 ml). The resulting protein solution was boiled on a steam bath for 20 minutes to denature the protein. Carbohydrate was separated from the denatured protein by the method of Sevag (Staub, A.M., Removal of Proteins from polysaccaridies, Methods in Carbohydrate Chem. 1965, 5:5). Approximately 217 mg of polysaccharide was detected and determined.

A portion of the polysaccharide (100 mg) from step 1 was further purified using Sephadex G-75 column (2 x 100 cm) and eluted with d.d. water. Fractions were collected and showed a positive absorption at 620 nm when reacted with Anthrone Reagent. Purified polysaccharide (about 55 mg) was obtained.
A portion of the purified Polysacchride (10 mg) from step 2 was hydrolysed to monosaccharides by mixture with 2 ml concentrated sulfuric acid (2 ml) at 100°C for 3 hours. Saturated Ba(OH)_2, was added to neutralize the acid solution. BASO, was removed by centrifugation (3000 rpm for 20 minutes). The supernantant was vacuum dried by rotary evaporator. Hydrolysed monosaccharides (about 9 mg) were obtained.

A portion of the hydrolyzed monosaccharides (5 mg) from step 3 was analyzed for sugar content by gas chromatography (Gas Chromatogram-model 103, Shanghai Analytical Instruments Corp.). DC-200 column (i.d. 3 mm, length 2 m) and FID detector were used for analysis. Arabinose and galactose were identified as the sugar components present, and were observed in a weight ratio of 1:0.05.

A small amount of the unhydrolyzed polysaccharide from step 2 was analyzed using a Beckman IR Spectrometer (PE-685). A strong absorption band at 895-905 cm\(^{-1}\) was observed, indicating the existence of a B-D-pyranose-glycoside linkage.

Example 5

Isolation and Characterization of Divalent Metal Ions from ALICIN

ALICIN (500 mg), purified as described in Example 1 (>95%) was dissolved in d.d water (10 ml) and dialyzed in 20 volume of d.d. (20 volumes) water at 4°C for 24 hours with several water changes. Collect the protein solution inside the dialysis bag, and the dialysate solution outside the bag separately and evaporating each to 1 ml volume. Add 1 ml concentrated acid solution (nitric acid:hydrochloric acid = 1:1) to each of the tube to hydrolyze the protein. Dilute each of the solution with d.d. water to 6 ml. Atomic Emission Method (Beckman Jaeerl-Ash 96-975 Division Plasma Spectrum
Analyzer) was used to analyze the Zn and Mg contents. The result showed that the glycoprotein contains magnesium and zinc in a weight percent of 7.3-8.6% and 2.7-4.3%, respectively.

For isolation of the divalent metal ions from glycoprotein, one mg of Glycoprotein was heated to ashes in oven at 800 oC. One ml of d.d. water was added to dissolve the white ashes. Another 9 ml of d.d. water was used to further dilute the solution. Atomic Emission analysis showed the content of magnesium and zinc are 2.7% and 7.3% and 2.7%, respectively.
Example 6
Anti-Heptitis A Virus (HAV) Activity

1) Preparation of HAV samples:
HAV suspension was purchased from the Center of China Preventive medicine in Shanghai. It was diluted with Hank's solution (Sigma, St. Louis, MO) to give a final concentration of 200TCD50 per 0.1 ml.

2) Preparation of human serum samples containing HAV:
Prepare six samples of 0.5 ml heat (56°C) inactivated healthy human serum. Each sample has the following dilutions with the Hank's solution: 1:4, 1:8, 1:16, 1:32, 1:64, 1:128. Add 0.5 ml of HAV suspension from step 1 to each sample and vortex thoroughly. Incubate the sample mixtures at 37°C for 1 hour.

3) Preparation of samples containing hepatic cell, HAV and serum:
Hepatic cell line (BEL-7405) was purchased from Shanghai Cell Biology Institute. Prepare 24 samples of 0.8 ml cell suspension (BEL-7405), each sample contains a cell concentration of 3x10^6. Add 0.2 ml each of the six HAV-serum samples to 6 BEL-7405 cell suspensions respectively. Repeat this protocol 4 times. A group of four identical samples composed of HAV-serum-hepatic cell were thus prepared at each serum dilution.

4) Incubate all 24 samples from step 3 in an incubator at 37°C until ready for experiments. Examine and count the number of infected cells in each tube daily for ten days. Determine the serum dilution
which leads to a 50% cell infection. This serum dilution was found to be 1:45 and was used as the control sample and for all other samples prepared below.

5) Prepare 48 samples containing HAV-serum-hepatic cells according to the protocols of step 3 except that the serum dilution used is determined at step 4 (i.e. 1:45). The samples were divided into 6 groups, and each group had 8 identical tubes. The six groups are defined below:

Group 1: control samples.
Group 2: control samples added with 400 ug of 85% pure ALICIN.
Group 3: control samples added with 400 ug of pure PROTEINA.
Group 4: control samples added with 400 ug of combination (200 ug pure PROTEINA, 104 ug polysacchrides (prepared in example 4) and 96 ug divalent metals prepared in example 5).
Group 5: control samples added with 96 ug of divalent metals.
Group 6: control samples added with 104 ug of polysacchrides.

6) Incubate all 48 samples in an incubator (5% CO2) at 37°C for 10 days. Examine and count infected hepatic cells of each tube using Olympus phase contrast microscope daily. At the end of tenth day, the experiments were stopped.

Results of each group are compared to the control group as shown in Table 2.
### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubes infected</th>
<th>% Infection</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/8</td>
<td>72.5</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>1/8</td>
<td>12.5</td>
<td>83%</td>
</tr>
<tr>
<td>3</td>
<td>2/8</td>
<td>25.0</td>
<td>66%</td>
</tr>
<tr>
<td>4</td>
<td>0/8</td>
<td>0.0</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>3/8</td>
<td>37.5</td>
<td>48%</td>
</tr>
<tr>
<td>6</td>
<td>4/8</td>
<td>50.0</td>
<td>31%</td>
</tr>
</tbody>
</table>

Table 2 clearly shows that all 5 formulations inhibit the growth of HAV. Especially, PROTEINA combination formula can irreversibly neutralize the virus completely in-vitro at 400 ug. The ALICIN (85%) at the same concentration can neutralize virus up to 83% and the PROTEINA at the same dosage can neutralize virus up to 66%. These results lead us to the choice of the combination formula for further study.

### Example 7

**Anti-Epstein-Barr Virus Activity**

1) Preparation of lymphocyte cells:
Collect ten tubes of 8 ml human cord blood and add equal volume of Hank’s solution (pH7.5) for dilution. White blood cells were separated from red blood cells by sucrose density method.

After washing the separated white cells twice with Hank’s solution, the white cell suspension was transfered to a glass dish and incubated on water bath at 37°C for 40 minutes. Most of the monocytes were adhered to the glass wall. Remove lymphocyte
cells. Twenty test tubes containing lymphocyte cells were prepared. Each tube had 1 ml of cell solution and a cell concentration of $2 \times 10^6$ /ml.

2) Preparation of lymphocyte samples containing Epstein-Barr Virus (EBV):

EBV suspension (200 TCD50) was purchased from Center of China Preventive Medicine. 0.1 ml of EBV suspension was added to the above prepared 20 lymphocyte tubes (step 1).

3) Divide the above 20 lymphocyte tubes (prepared in step 2) into 4 groups, each group has 5 identical samples. The four groups are defined below:

- **group 1**: Control group, samples prepared in step 2.
- **group 2**: Irradiation of EBV with X-ray (4000 R) for 1 minute before added to lymphocyte tubes.
- **group 3**: 200 ug of the combined formula of PROTEINA + polysaccharide + divalent metals was added to lymphocyte tubes.
- **group 4**: 400 ug of the combined formulation of PROTEINA + polysaccharide + divalent metals was added to lymphocyte tubes.

All tubes from group 1 to 4 were incubated in a cell incubator (5% CO$_2$) at 37°C until approximately 50% lymphocytes in the control group showed infection counted by Olympus phase contrast microscope. At this point, stop cell incubation and
record the results of all tubes. They are summarized in Table 3.

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Average infection(%)</th>
<th>Efficacy(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51.8 ± 3.6</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.4 ± 1.8</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>13.6 ± 3.2</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>1.2 ± 1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

These results demonstrate that the PROTEINA combination formula completely neutralizes EBV at 400 ug. Its efficacy is comparable to that of X-ray.

**Example 8**

**Anti-Hepatoma (Human) Activity**

Human hepatoma cell line (BEL-7402) was purchased from Shanghai Cell Biology Institute. Cells were removed from the medium in which they were provided, suspended in solution A (2.0 ml; 0.25% trypsin, 0.02% EDTA, 8 g/L of NaCl, 0.2 g/L KCl, 1.56 g/L Na,HPO,,-H,O, 0.2 g/L KH,PO,), maintained at room temperature for 1 minute, transferred to culture solution B, mixed thoroughly for 2 minutes and adjusted as necessary to suspension concentration of 104 cells/ml. The composition of culture solution B was as follows: DMEM cell culture solution (Sigma, St. Louis, MO), 15% fetal calf serum, 50 units of streptomycin, 50 units of penicillin. This suspension was used as the control.

2) Divide 125 ml BEL-7402 cell suspension from step 1 into 25 bottles and 5 groups. Each group consists of 5 bottles (5ml/bottle).
group 1  control solution of BEL-7402 cell suspension

group 2  control solution added with 10 ug PROTEINA combination agent (see example 6)

group 3  control solution added with 40 ug PROTEINA combination agent.

group 4  control solution added with 80 ug PROTEINA combination agent.

group 5  control solution added with 100 ug PROTEINA combination agent.

3) Incubate all bottles in a cell incubator (5% CO₂, 37°C) for 3 days. Examine and count cell numbers at day 1, 2 and 3. The results observed are set forth in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>0</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5 ± 0.5</td>
<td>8.6 ± 0.9</td>
<td>20.0 ± 1.1</td>
<td>28.5 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.5 ± 0.5</td>
<td>8.3 ± 0.9</td>
<td>19.5 ± 1.4</td>
<td>28.0 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.5 ± 0.5</td>
<td>6.6 ± 0.6</td>
<td>12.5 ± 1.0</td>
<td>19.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.5 ± 0.5</td>
<td>5.8 ± 0.5</td>
<td>10.0 ± 0.9</td>
<td>12.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.5 ± 0.5</td>
<td>no data</td>
<td>9.8 ± 1.1</td>
<td>11.3 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>

It is clear that the PROTEINA combination formula can effectively inhibit the growth of hepatoma. The inhibition activity increases with the concentration, reaching saturation at 100ug.
Example 9
Comparison of Anti-HAV Activities of ALICIN (>95% pure), PROTEINA and Trichosanthin

The protein trichosanthin has abortifacient, antitumor, ribosome inactivation, anti-HIV, immunomodulatory and insulin-like activities. Trichosanthin is a nonmetallo- and nonglyco- protein of 234 amino acids with an N-terminal aspartate and an carboxy terminal alanine- In arriving at the present invention it has been observed by the inventor that a selected partial amino acid sequence of trichosanthin starting from 108 (arginine) to 158 (leucine) and from 160 (valine) to 232 (asparagine) are homologous to the major part of PROTEINA: from amino acid 1 (arginine) to 51 (leucine) and 53 (valine) to 125 (asparagine).

Seventy samples containing HAV-serum-hepatic cells (BEL-7405) were prepared according to the protocol of Example 7. They were divided into 7 groups of 10 samples each as follows.

Group 1: control group (untreated cells)
Group 2: sample plus 300 ug of ALICIN (>95%);
Group 3: sample plus 200-300 ug of PROTEINA (>99%);
Group 4: sample plus 200-300 ug of trichosanthin;
Group 5: sample plus 100-300 ug of zinc glucose;
Group 6: sample plus 200 ug of PROTEINA and 100 ug of zinc glucose;
Group 7: sample plus 200 ug of trichosanthin and 100 ug of zinc glucose;

Following the same protocols of example 7, the incubation and microscopic examination were carried out for 10 days. The results are summarized in Table 5.
Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubes infected</th>
<th>% Infection</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/10</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0/10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>2/10</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>3/10</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>3/10</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>1/10</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>1/10</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

These data show that pure ALICIN has similar potency as that of the PROTEINA combination formula. Each of them irreversibly neutralizes the HAV. PROTEINA alone, rather than in combination with the carbohydrate and divalent metal, is less potent than ALICIN, but more potent than trichosanthin. However, the combination of PROTEINA with zinc glucose provided a composition of enhanced antiviral activity.

Similarly, enhancement in potency of trichosanthin was observed when combined with zinc glucose. Since zinc glucose contains both the carbohydrate and divalent metal, these results clearly indicate that adding sugar and divalent metal to the antiviral protein solution significantly enhances the antiviral activity of the proteins which do not contain sugar or metal or both. These results are in agreement with the data demonstrated in example 6.
Example 10

In-vivo anti-Influenza A3 Virus (IAV) activities

A total of 90 Kun-Min white mice (body weight 20 ±1), with 50% male and female each, were divided into 9 groups. Each group had 10 identical mice. They are defined below:

Group 1: control group, abdominal injection of 0.5 ml of saline solution (0.9% NaCl) 2 hours prior to tail vein injection of IAV;

Group 2: same as 1, except 4 hour prior to injection of IAV.

Group 3: treated group, abdominal injection of 0.5 ml of the Proteina Combination Formula (400 ug) 2 hours prior to tail vein injection of IAV.

Group 4: Same as 3, except 4 hours prior to injection of IAV.

Group 5: Same as 3, except 6 hours prior to injection of IAV.

Group 6: Same as 3, except 10 hours prior to injection of IAV.

Group 7: Same as 3, except 18 hours prior to injection of IAV.

Group 8: Same as 3, except 24 hours prior to injection of IAV.

Group 9: Same as 3, except 48 hours prior to injection of IAV.

2) Preparation of Influenza A3 Virus solution:

Influenza A3 Virus (Jinfong-75-39) was purchased from Center of China Preventive Medicine. It was multiplied in chick embryo (10 days old) at 37°C for 40 hours. The
allantoic fluid containing 10-0 titer of the virus was harvested from the chick embryo.

The obtained allantoic fluid was centrifuged at 4000 rpm for 30 minutes to remove large particulates. The supernatant was mixed with a final concentration of 3.5% chick red blood cells (prefixed by formaldehyde) and stored at 4°C overnight. After centrifugation at 2000 rpm for 10 minutes, the precipitated RBCs and viruses were washed twice with cold (0°C) saline solution and centrifuged. Appropriate amount (1/5 volume of the original allantoic fluid) of 0.01 M phosphate buffer (pH 7.8) was added to RBCs and viruses and incubated at 37°C water bath for 3 hours before centrifugation (2000 rpm for 10 min). Save the supernatant which contained the viruses. Added 100 volume (of the original allantoic fluid) of phosphate buffer to RBCs and incubated at 37°C for 2 hours. Centrifuged again. Combined the two supernatant which contained viruses and recentrifuged at 4000 rpm for 30 minutes to remove residual RBCs. Applied 20 ml of the supernatant to a Sephadex G200 column (2.5 cm x 60 cm, flow rate 40 ml/hr) preequilibrated with the phosphate buffer (0.01 M). Centrifuged the virus solution (collected from the column) at 2800 rpm for 60 minutes. Discarded the supernatant. Added 2 ml of the phosphate buffer to the virus precipitate and dispersed the virus suspension. Added more phosphate buffer until the volume of the virus suspension was 1/300 volume of the original allantoic fluid. Homogenized the suspension by sonication for 1-2 minutes. The virus solution was ready for use.
3) vein injection of 0.25 ml of the virus solution to each tail of mice following the protocol of step 1.

4) Observed and recorded the number of mice died in 5 days after the virus injection.

The results are summarized in Table 6:

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample #</th>
<th># Death</th>
<th>% Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>9</td>
<td>90</td>
</tr>
</tbody>
</table>

The results indicate that the combination agent can effectively reduce the infectiousness of the Influenza Virus A3. In view of the seriousness of the i.v. infection of the virus, it is encouraging to see that the mortality rate can be reduced at least to half if the invention composition was injected to mice at least 4 hours in advance.
What Is Claimed Is:

1. A composition comprising an amino acid-containing compound having the formula:

   H-R-Thr-R-Gly-Asn-Tyr-R-Arg-Leu-R-Ala-Gly-R-Leu-
   Arg-Glu-Asn-Ile-R-Leu-Gly-R-Leu-R-Ala-Ile-R-Leu-
   R-Tyr-Tyr-R-Ile-Gln-R-Ser-Glu-Ala-Ala-Arg-R-Ile-
   Glu-R-Arg-R-Ile-R-Asn-R-Gly-R-Phe-R-Ser-Pro-R-Leu-R-OH

   wherein each R is independently at least one amino acid or analog thereof.

2. The composition of claim 1 wherein the amino acid-containing compound has the formula:

   Xaa-Xaa-Xaa-Thr-Xaa-Xaa-Xaa-Xaa-Gly-Asn-Tyr-Xaa-Arg-Leu-
   Xaa-Xaa-Xaa-Ala-Gly-Xaa-Leu-Arg-Glu-Asn-Ile-Xaa-Leu-Gly-
   Xaa-Xaa-Leu-Xaa-Xaa-Ala-Ile-Xaa-Xaa-Leu-Xaa-Tyr-Tyr-
   Xaa-Xaa-Ser-Glu-Ala-Ala-Arg-Xaa-Xaa-Ile-Glu-Xaa-Xaa-
   Xaa-Xaa-Ile-Xaa-Xaa-Xaa-Asn-Xaa-Gly-Xaa-Phe-Xaa-Ser-

   wherein each Xaa is independently at least one amino acid or analog thereof.

3. The composition of claim 1 wherein each R is independently one or more amino acids.

4. The composition of claim 2 wherein the amino acid-containing compound has the formula:

   Arg-Lys-Val-Thr-Leu-Pro-Tyr-Ser-Gly-Asn-Tyr-Glu-Arg-Leu-

5. The composition of claim 1 which further comprises a carbohydrate.

6. The composition of claim 5 wherein the carbohydrate is a polysaccharide.

7. The composition of claim 6 wherein the polysaccharide consists essentially of arabinose and galactose.

8. The composition of claim 7 wherein the arabinose and galactose are present in a weight ratio of about 1:0.05.

9. The composition of claim 1 which further comprises at least one divalent metal ion.

10. The composition of claim 9 wherein the divalent metal ion is selected from the group consisting of magnesium and zinc.

11. The composition of claim 10 wherein the magnesium and zinc are present in the composition in a weight percent of about 4 - 20 and 2 - 10, respectively.

12. The composition of claim 5 which further comprises at least one divalent metal ion.

13. A method for treating a viral infection in an individual in need thereof which comprises administering a
therapeutically effective amount of the composition of claim 1.

14. A method for treating a viral infection in an individual in need thereof which comprises administering a therapeutically effective amount of the composition of claim 5.

15. A method for treating a viral infection in an individual in need thereof which comprises administering a therapeutically effective amount of the composition of claim 12.

16. A method for treating hepatoma in an individual in need thereof which comprises administering a therapeutically effective amount of the composition of claim 1.

17. A method for treating hepatoma in an individual in need thereof which comprises administering a therapeutically effective amount of the composition of claim 5.

18. A method for treating hepatoma in an individual in need thereof which comprises administering a therapeutically effective amount of the composition of claim 12.

19. A composition which comprises an antiviral polypeptide-containing agent in combination with a carbohydrate and at least one divalent metal ion.

20. The composition of claim 19 wherein the carbohydrate is a poly saccharide that consists essentially of arabinose and galactose and the at least one divalent metal ion consists essentially of magnesium and zinc.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : AG1K 38/16, 31/70
US CL : 514/8, 23; 530/395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/8, 23; 530/395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>US 5,417,979 A (FAN et al.) 23 May 1995, see whole document.</td>
<td>1-20</td>
</tr>
</tbody>
</table>

* Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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  "A" document member of the same patent family

Date of the actual completion of the international search
09 SEPTEMBER 1997

Date of mailing of the international search report
28 OCT 1997

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INTERNATIONAL SEARCH REPORT

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, EMBASE, WPIDS

search terms: zei-bai, tubers, potato, hepatoma, glycoprotein, anti-viral, arabinose, galactose.