Quantitative Evaluation of the Compatibility Effects of Aidi Injection on the Treatment of Hepatocellular Carcinoma Using Targeted Metabolomics: A New Strategy on the Mechanism Study of an Anticancer Compound in Traditional Chinese Medicine

Ran Liu¹, Lin-Lin Zhu¹, Chun-Yu Yu¹, Ya-Ping Shuai¹, Ling-Ling Sun², Kai-Shun Bi¹, Qing Li¹

¹School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China, ²Liaoning Institute of Drug Inspection and Testing, Shenyang 110030, China

Objective: Compound traditional Chinese medicine (CTCM) with the application of compatibility from multiple active ingredients with multiple-specific targets can achieve a synergistic effect on cancer therapy. This study is aimed to observe the compatibility effects of Aidi injection on the treatment of hepatocellular carcinoma and to explore the mechanism of CTCM. Methods: Aidi injection is a clinical compound prescription containing Mylabris, Ginseng, Astragalus, and Acanthopanax, which can inhibit tumor growth and induce apoptosis. In this study, the anticancer activity of Aidi injection, as well as its disassembled and combined compositions, had been evaluated by varying levels of polyamine biomarkers on human hepatoma Hep-G2 cells detected using ultrahigh-performance liquid chromatography-tandem mass spectrometry. Results: According to the different variations in polyamine levels, it was revealed that Mylabris and Ginseng had an antitumor effect, while Astragalus acted as an assistant and Acanthopanax had weak anticancer activity. The increased level of polyamines in Hep-G2 cells had been found in HL-7702 cells. On combining Mylabris and Ginseng, polyamine levels went close to the normal level, which was even more marked when Astragalus was added. Aidi injection acted like the combination of Mylabris, Ginseng, and Astragalus. Conclusions: This study established a quantitative evaluation of the compatibility effects of Aidi injection based on polyamine biomarkers and evaluated the consistency of its anticancer effect, providing a manner to research the efficacy evaluation of CTCM. Moreover, the correlation between polyamine metabolism and anticancer activity can be used in anticancer drug screening.

Keywords: Compatibility effects evaluation, compound traditional Chinese medicine, hepatocellular carcinoma, polyamine biomarker

Introduction

Hepatocellular carcinoma has always stood at the forefront of lethal cancers worldwide.¹⁻⁴ As part of the anticancer reagents, compound traditional Chinese medicine (CTCM) exhibits huge advantages in therapy for many kinds of cancer with plentiful cases of successful treatment and survival. At the same time, owing to the multiple-target characteristics of its various components, its multiple therapy approach, and its synergistic effect, prescription compatibility may be the core of CTCM, and it could become a critical issue in therapy evaluation on cancer management. The most important principles of CTCM compatibility are “monarch, minister, assistant, and messenger,” which can be used to reveal the essence of prescription compatibility. Several studies, from in vitro to in vivo, such as “serum pharmacology,” “Chinese herb serum,” and “bioactive constituents in Chinese herbal medicines”, have...
been involved in the research of the compatibility evaluation of CTCM.\textsuperscript{[5-9]}

For decades, with the development of metabolomics, especially the targeted metabolomics study combined with the quantitative analysis method has gained more attention in biomarker identification, pathogenesis, and effective drug mechanism discussions.\textsuperscript{[10-13]} Polyamines, which could be used as a form of biomarkers for monitoring cancers, were first indicated in the early 1970s.\textsuperscript{[14]} Thereafter, numerous studies describing elevated polyamine levels in biological samples that have potential in detecting cancer have been published.\textsuperscript{[15-19]} and increasing reports have described that polyamine catabolism may contribute to the development of various cancers.\textsuperscript{[16,17,20,21]} Based on the above study, carcinoma cell proliferation together with the administration of anticancer drugs may interfere with polyamine metabolism. However, the correlation between polyamine metabolism and anticancer activity has rarely been reported; to date, in our study, we have successfully associated polyamine metabolism with anticancer activity through the simultaneous determination of polyamines.\textsuperscript{[12,23]}

Aidi injection has been formulated through the extraction of Mylabris (\textit{Mylabris phalerata} Pallas), Ginseng (\textit{Ginseng Radix} et Rhizoma), Astragalus (\textit{Astragali Radix}), and Acanthopanax (\textit{Acanthopanacis Senticosi Radix} et Rhizoma seu Caulis), clinical compounds with anticancer activity used for the treatment of several cancers including liver cancer, lung cancer, gastric cancer, and colorectal cancer. Several experiments demonstrated that Aidi injection has an antitumor function with the mechanism of inducing apoptosis, decreasing chemotherapy-related toxicity, and improving the patients immune system.\textsuperscript{[24-28]} Cantharidin, as the major component of the principal drug \textit{Mylabris}, showed a strong antitumor effect in different cancers.\textsuperscript{[29-31]} Ginseng Radix and Astragali Radix have been reported to have significant advantages in suppressing tumor progression, relieving surgery complications, increasing the sensitivity of chemo- and radiotherapeutics, improving the immune function, and lessening the damage caused by surgery.\textsuperscript{[32,34]} However, the antitumor effects of Acanthopanax are hardly known so far; neither is the coordinating effect of these four drugs.\textsuperscript{[35]} Currently, clinical research on Aidi injection has mainly focused on the comprehensive therapy of the entire group combined with chemotherapy and radiotherapy to reduce drug toxicity and enhance clinical efficacy. Nevertheless, the disassembled and combined compositions of Aidi injection for cancer treatment were rarely studied to reveal its compatibility effects and direct its clinical application.

Our previous research revealed that the significance of polyamines, including 1,3-diaminopropane, putrescine, cadaverine, spermidine, spermine, agmatine, N-acetylputrescine, N-acetylspermine, N-acetylspermidine, N\textsuperscript{1}, N\textsuperscript{12}-diacetylspermine, and N\textsuperscript{4}, N\textsuperscript{8}-diacetylspermidine, had been proven in liver cancer.\textsuperscript{[22,23]} In this study, research on Aidi injection as well as its disassembled and combined compositions was conducted to reveal its inhibitory effect on human Hep-G2 cells using these 11 polyamines as evaluation indicators. The results obtained from the evaluation of compatibility rationality of the Aidi injection could provide an effective way for CTCM evaluation and may provide a theoretical basis for the design and development of new anticancer drugs. Furthermore, it will contribute to the modernization and globalization of traditional Chinese medicine.

**Methods**

**Materials and medicines**

The reference standards – 1,3-diaminopropane, putrescine, cadaverine hydrochloride, spermidine hydrochloride, spermine, agmatine sulfate salt, N-acetylputrescine hydrochloride, N-acetylspermine trihydrochloride, N-acetylspermidine dihydrochloride, and 1,6-diaminohexane (used as an internal standard [IS]) – were all obtained from Sigma-Aldrich (St. Louis, MO). N\textsuperscript{3}, N\textsuperscript{12}-diacetylspermine n-hydrochloride and N\textsuperscript{4}, N\textsuperscript{8}-diacetylspermidine were obtained from Wako (Osaka, Japan). Methanol of high-performance liquid chromatography (HPLC) grade was purchased from Fisher Chemicals (Fair Lawn, NJ). Perfluoropentanoic acid was obtained from Sigma-Aldrich (St. Louis, MO).

Raw materials of \textit{Mylabris}, \textit{Ginseng Radix} et Rhizoma, \textit{Astragali Radix}, and \textit{Acanthopanacis Senticosi Radix} et Rhizoma Seu Caulis were purchased from Tianyitang Chinese Drugstore (Shenyang, China). These drugs were identified by a Professor of Pharmacognosy in Shenyang Pharmaceutical University (China). The Aidi injection was prepared according to the Ministerial Standards of Chinese Medicine. All other reagents were of analytical grade. Redistilled and deionized water was used throughout the study.

**Preparation of stock solutions and calibration standards**

Stock solutions of 11 analytes and IS were prepared in methanol and kept at 4°C. Further dilution with methanol was performed to obtain the calibration standard solutions. Calibration standard samples of analytes and IS were prepared by spiking the Hep-G2 cell pellets cultured in \textit{Mylabris} (100 cells, homogeneous mixture of cells from 70 culture dishes) with different concentration working standards. The quality control (QC) samples were prepared with the same Hep-G2 cell pellets at low, medium, and high concentration levels, which were used in developing the analytical method during the analytical run.

**Cell culture**

Human hepatic carcinoma cell lines (Hep-G2 cells) and human hepatic cell lines (HL-7702 cells) were purchased from CHI Scientific (ATCC, USA). High glucose – Dulbecco’s Modified Eagle Medium (H-DMEM) culture medium, RPMI1640 (Roswell Park Memorial Institute 1640) culture medium, fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from Gibco (Thermo Fisher Scientific). Methyl thiazolyl tetrazolium (MTT) was purchased from Genview Company (USA). Cell lifters and 10-cm polystyrene non-pyrogenic culture dishes were acquired from
Hep-G2 cells were cultured in H-DMEM medium-containing 10% (v/v) heat-inactivated FBS, 1% (v/v) glutamine, and 1% (v/v) penicillin and streptomycin, which were maintained in an incubator at 37°C with 5% CO₂ in a humidified atmosphere. The culture medium was replaced every day, and the cells at a logarithmic growth phase were used for the following experiments.

HL-7702 cells were cultured in RPMI1640 medium containing 10% (v/v) heat-inactivated FBS, 1% (v/v) glutamine, and 1% (v/v) penicillin and streptomycin, which were maintained in an incubator at 37°C with 5% CO₂ in a humidified atmosphere. The culture medium was replaced every day, and the cells at the logarithmic growth phase were used for the following experiments.

Hep-G2 cells were seeded in 96-well culture plates at 5 × 10^3 cells/well. After overnight incubation, the culture medium was replaced and added with 8 mg/mL, 16 mg/mL, 32 mg/mL, 64 mg/mL, and 128 mg/mL of Aidi injection, and the concentrations of the disassembled (Mylabris, Ginseng, Astragalus, and Acanthopanax) and combination groups (Mylabris and Ginseng as Group A+B, Mylabris, Ginseng, and Astragalus as Group A+B+C) were converted from the ingredient proportion of Aidi injection.

A blank group added with PBS only, and a control group, added with Hep-G2 cells, were set up with six duplicate wells in each group.

**Detection of cell proliferation**

Cell viability was measured by the MTT assay. Cell proliferation was detected by the addition of 20 μL MTT (5 g/L) into each well at 37°C for 4 h. After the culture medium was removed, DMSO (150 μL) was added and shaken, protected from light to completely dissolve the formazan crystals for 10 min. Absorbance (A) was measured at 490 nm with a Multiskan GO microplate reader (Thermo Scientific). For each group, the experiment was repeated thrice with six duplicate wells. The cell viability was calculated as follows:

\[
\text{Cell viability} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})/(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})}{100}
\]

Cells were harvested by PBS from a culture dish to one tube. The cell suspension was centrifuged at 1500 rpm and 4°C for 5 min, the PBS was removed, and the cell pellet was suspended in PBS again and counted; approximately 10⁶ cells were prepared in 1.5-mL Eppendorf tubes. PBS was removed by centrifugation (1500 rpm and 4°C for 5 min), and quenching and deproteinization agents were immediately added to cell pellets and stored at −80°C until analysis.

**Detection of polyamine**

All samples, standards, and QC samples were prepared similarly by adding IS solution, to compensate for any possible bias in accuracy originating from the sample preparation process. An aliquot of 20 μL IS (100 ng/mL) was added to the aforementioned Eppendorf tube containing approximately 10⁶ Hep-G2 cell pellet and vortex mixed with 20 μL methanol for 30 s, sequentially adding deproteinization agents of methanol (containing 5% trifluoroacetate [TFA]) water (containing 5% TFA) (80:20, v/v), and immediately frozen at −80°C. Thawing was carried out in an ultrasonic bath at 50 Hz. Then cells were frozen at −80°C again. Three cycles of freezing/thawing were performed. Vortex mixed for 3 min, cell debris was separated from intracellular metabolic content by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was transferred to another Eppendorf microtube, followed by evaporating to dryness at 30°C under a stream of nitrogen. The residue was dissolved in 100 μL methanol (containing 0.0125% perfluoropentanoic acid)-water (containing 0.0125% perfluoropentanoic acid) (25:75, v/v), and an aliquot of 5 μL of the supernatant was injected into the ultra HPLC tandem mass spectrometry (UHPLC-MS/MS) system, which performs a Shimadzu LC-20A UFLC XR system (Japan) and one AB SCIEX 4000 QTRAP™ linear ion trap triple-stage quadrupole tandem mass spectrometer (USA). All operations involved in the acquisition and analysis of data were controlled by the Analyst software (version 1.6, AB SCIEX, USA).

**Method validation**

To evaluate linearity, seven calibration standard samples were assayed on three different days. Because of the presence of endogenous analyts in the cell, blank values of polyamines should be subtracted from each calibration point. The linearity was then evaluated by linear least-squares regression of the analyts-to-IS peak area ratios subtracted to the blank samples (y) versus the normalized standard concentration (x) with a weighted (1/x²) factor at three different analytical batches. Limit of quantitation (LOQ) was determined as the lowest concentration measurable with a signal-to-noise (S/N) ratio above ten. The LOQ acceptable accuracy was relative error (RE) within ±20% deviation, and precision was relative standard deviation (RSD) within 20%.

Accuracy was calculated by comparing the mean experimental concentrations of QC samples with their nominal values, and percentage values were used as the index. Intra-day precision was determined by six replicate analyses in 1 day, while inter-day precision was determined by QC samples on three consecutive days. Accuracy was defined as RE (%), and precision was defined as the RSD (%).

The extraction recovery was calculated based on QC samples of six duplicates, i.e., A_y/A_x, where A_y is the response of the extracted analytes or IS subtracted to the blank samples and A_x is the response of the post-extracted blank samples spiked with analytes subtracted to the blank samples.

The matrix effect had been measured by “IS-normalized matrix factor (MF)” and evaluated as follows: for each analyte and IS, the MF was calculated as the ratio of the peak area with the matrix (measured by analyzing the blank matrix spike after extraction of analytes at three concentration levels subtracted by the blank samples) divided by the IS and peak area without
the matrix (pure standard solutions of the analytes at three concentration levels) divided by the IS.

Stability tests of the analytes were assessed using triplicate spiked samples at low and high QC levels under different conditions: 4 h at room temperature and reconstituted extract at 4°C for 7 h.

**Statistical test**

Polyamine concentrations were deduced from the calibration curves and expressed as mean ± SD. The results were presented as mean ± SD and analyzed using statistical Student’s t-test and nonparametric Mann–Whitney test with the assistance of SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). The threshold of significance was set at $P < 0.05$. The decreasing ratios (DR) were calculated using the following equation: $\text{DR} \% = \left( \frac{C_{\text{model}} - C_{\text{sample}}}{C_{\text{model}}} \right) \times 100$, where $C_{\text{sample}}$ is the average concentration of polyamine biomarkers from administration groups and $C_{\text{model}}$ is the average concentration of the corresponding polyamine biomarkers from cancer model groups.

**RESULTS**

Confirming the Aidi injection dosage affecting Hep-G2 cells according to the methyl thiazolyl tetrazolium study

Hep-G2 cells were greatly affected by the dosage of the Aidi injection, and its disassembled and combined groups were investigated by the MTT method. Figure 1 illustrates the cell viability of Hep-G2 cells at 24-h cultivation by 8, 16, 32, 64, and 128 mg/mL Aidi injection as well as its disassembled and combined compositions. The low-dose medication groups exhibited a little effect on the cells, while the inhibitory effect of the high-dose medication groups was dose-dependent. Results showed that 16 mg/mL of Aidi injection may inhibit Hep-G2 cells, which was not high enough to induce cell apoptosis. Therefore, the concentration of a single herb and disassembled and combined compositions of Aidi injection had been converted according to the ratio of Aidi injection prescription.

Besides, it was observed that Mylabris had the strongest inhibitory effect on Hep-G2 cells. Subsequently, a higher inhibitory effect of Aidi injection had been presented in other prescriptions. Mylabris, a monarch drug in Aidi injections, can induce apoptosis and inhibit the proliferation, migration, and invasion of cancer cells; however, it also induces systemic toxicity. In a previous study, Astragalus, Acanthopanax, and Ginseng had been shown to have antitumor activity and immune regulation functions. Through a rational combination of CTCM, the inhibitory effect of Mylabris might be mitigated by other herbs.

Level of polyamine metabolome in Hep-G2 cells

Optimization of ultra-high-performance liquid chromatography tandem mass spectrometry conditions

Consideration of the highly polar properties of the analytes, a long-chain carboxylic acid used as the ion pair agent had been added to the mobile phase to obtain a satisfactory retention behavior and better chromatographic separation. In this study, 0.05% perfluoropentanoic acid had been used as an ion-pairing agent, and all compounds were perfectly detected with a sharp peak shape and high sensitivity within 8 min. The typical chromatograms of the analytes are shown in Figure 2.

Method validation

The present method was validated with Hep-G2 cells cultured in Mylabris. The LOQ of this method had a S/N ratio >10, which was sufficient in determining all samples. All standard calibration curves were linear, and the calibration regression coefficients ranged from 0.9900 to 0.9982 for all analytes. The intra- and inter-day precisions with RSD were all ≤15%, and the RE values were all <15%. The extraction recoveries of 11 analytes were not lower than 80%, and the extraction recovery of IS was 99.1% ± 2.5% (1,6-diaminohexane). Moreover, the internal normalization matrix effect and stability study, including cell samples stored at room temperature within 4 h, and reconstituted extract analytes at 4°C for 7 h, had all been found to be excellent with RSD <15%.

Results of polyamine metabolome in Hep-G2 cells

The established UHPLC-MS/MS method had been applied to the analysis of 11 polyamines in HL-7702 cells and Hep-G2 cells that were added to the Aidi injection and its disassembled and combined compositions. The levels of 11 polyamines in HL-7702 cells and Hep-G2 cells are shown in Table 1.

All analytes were applied to the clustering analysis to distinguish the differential efficacy of the Aidi injection and its disassembled and combined compositions, and the dendrograms are shown in Figure 3. All analyte levels were reduced in the Aidi injection, except for putrescine, compared with that in the control group. The levels of cadaverine and 1,3-diaminopropane were below normal in HL-7702 cells, and the levels of spermine decreased below the normal level. The levels of other analytes were close to normal in the Aidi injection. All analytes were downregulated, except for putrescine and N-acetylputrescine, and the levels of putrescine increased in the Mylabris and Ginseng groups compared with that in the control group. The cadaverine and 1,3-diaminopropane levels

![Figure 1: Cell viability of Hep-G2 cells cultured in Aidi Injection and its disassembled and combined group (mean ± standard deviation of three independent experiments). (ADI for given Aidi Injection, A + B for given Mylabris and Ginseng, A + B + C for given Mylabris, Ginseng and Astragalus)](image-url)
were below normal in the Mylabris group and returned to normal in the Ginseng group. The levels of other analytes were close to normal in the Mylabris and Ginseng groups. Agmatine, cadaverine, 1,3-diaminopropane, N-acetylputrescine, spermidine, and spermine were downregulated, and the N₁, N₈-diacetylspermidine and N-acetylspermidine levels increased in the Astragalus group compared with that in the control group. Only putrescine was upregulated, while agmatine, cadaverine, 1,3-diaminopropane, N-acetylputrescine, spermidine, spermine, N-acetyl spermidine, N-acetyl spermine, and N₁, N₈-diacylspermidine levels were downregulated in the Eleutherococcus senticosus group compared with that in the control group. The cadaverine and 1,3-diaminopropane levels returned to normal, and the downregulation of other analytes were close to normal in the Astragalus and Acanthopanax groups. The variation of polyamine levels in the Aidi injection was similar to its combination groups. The cadaverine and 1,3-diaminopropane levels were below normal in HL-7702 cells in the two combined, groups and the levels of spermine returned to normal in the combination group containing Mylabris and Ginseng. The downregulation of other analytes was close to normal levels in the combination groups. The polyamine levels were close to or below normal levels after delivery within 24 h. The degree of downregulation of polyamine levels was more significant and had a better treatment effect, presumably with prolonged treatment. The putrescine level was increased in the Mylabris, Ginseng, and Acanthopanax groups, while it weakly reduced in the Aidi injection and its combination groups compared with that in the control group. This means that the efficacy of the combination groups was better than the individual herbs, and Aidi injection had the best efficacy compared with other medication groups. Aidi injection and its disassembled and combination groups were distinguished from the control group based on the polyamine levels by clustering analysis [Figure 3]. It was shown that the mechanism of action of Astragalus and Acanthopanax was different from other medication groups, and the variation of polyamine levels was not remarkable in the Astragalus and Acanthopanax groups. The significant variation of polyamine levels was carried out by the medication group containing Mylabris and Ginseng. Ginseng provided a little effect on Hep-G2 cells, as shown in Figure 3. In summary of the t-test and clustering analysis results, the variation of polyamine levels in vitro might be a useful method to test the efficacy of potential antitumor drugs.

**Discussion**

We have previously reported the UHPLC-MS/MS method in determining the polyamine levels using 0.05% heptfluorobutyric acid[20] as an ion-pairing agent. In this study, we slightly modified the liquid phase conditions with reference to the previous experimental conditions, and all analytes exhibited satisfactory detection. The results of the polyamine metabolome levels in the Hep-G2 cells demonstrated that the polyamine level was reduced in different degrees by the Aidi injection and its disassembled and combination groups. The polyamines were catalyzed by spermidine/spermine...

![Figure 2: The representative MRM chromatograms obtained from Hep-G2 cells by ultra high-performance liquid chromatography-tandem mass spectrometry using the optimized method.](image-url)
Table 1: Amounts of analytes in HL-7702 cell, Hep-G2 cells and the Hep-G2 cells which were replenished with Aidi injection and its disassembled and combined compositions, (mean ± standard deviation, ng/10^6 Cell).

| N1,N8-diacetylspermidine | N-acetylspermidine | N-acetylspermine | Putrescine | Agmatine | Cadaverine | 1,3-Diaminopropane | N-acetylputrescine | Spermidine | Spermine |
|--------------------------|-------------------|-----------------|------------|----------|-----------|-------------------|-----------------|-----------|----------|
| HL-7702                  | Mylabris + Ginseng | Mylabris + Astragalus | Mylabris + Ginseng + Astragalus | Aidi injection | Aidi injection + Mylabris | Aidi injection + Ginseng | Aidi injection + Astragalus | Aidi injection + Ginseng + Astragalus |
| 3.61±0.531              | 2.77±0.73±5*      | 2.27±0.74±5*    | 2.27±0.74±5* | 2.27±0.74±5* | 2.27±0.74±5* | 2.27±0.74±5* | 2.27±0.74±5* | 2.27±0.74±5* | 2.27±0.74±5* |
| 486.5±101±7             | 486.5±101±7       | 486.5±101±7     | 486.5±101±7 | 486.5±101±7 | 486.5±101±7 | 486.5±101±7 | 486.5±101±7 | 486.5±101±7 | 486.5±101±7 |
| 100.4±25±5              | 100.4±25±5        | 100.4±25±5      | 100.4±25±5 | 100.4±25±5 | 100.4±25±5 | 100.4±25±5 | 100.4±25±5 | 100.4±25±5 | 100.4±25±5 |
| 60.1±15±5               | 60.1±15±5         | 60.1±15±5       | 60.1±15±5 | 60.1±15±5 | 60.1±15±5 | 60.1±15±5 | 60.1±15±5 | 60.1±15±5 | 60.1±15±5 |
| 61.46±18±5*             | 61.46±18±5*       | 61.46±18±5*     | 61.46±18±5* | 61.46±18±5* | 61.46±18±5* | 61.46±18±5* | 61.46±18±5* | 61.46±18±5* | 61.46±18±5* |
| 8.94±5.0±5              | 8.94±5.0±5        | 8.94±5.0±5      | 8.94±5.0±5 | 8.94±5.0±5 | 8.94±5.0±5 | 8.94±5.0±5 | 8.94±5.0±5 | 8.94±5.0±5 | 8.94±5.0±5 |
| 3.19±1.2±5*             | 3.19±1.2±5*       | 3.19±1.2±5*     | 3.19±1.2±5* | 3.19±1.2±5* | 3.19±1.2±5* | 3.19±1.2±5* | 3.19±1.2±5* | 3.19±1.2±5* | 3.19±1.2±5* |
| 13.75±6.1±6             | 13.75±6.1±6       | 13.75±6.1±6     | 13.75±6.1±6 | 13.75±6.1±6 | 13.75±6.1±6 | 13.75±6.1±6 | 13.75±6.1±6 | 13.75±6.1±6 | 13.75±6.1±6 |
| 44.20±11±5              | 44.20±11±5        | 44.20±11±5      | 44.20±11±5 | 44.20±11±5 | 44.20±11±5 | 44.20±11±5 | 44.20±11±5 | 44.20±11±5 | 44.20±11±5 |
| 2.25±0.7±5              | 2.25±0.7±5        | 2.25±0.7±5      | 2.25±0.7±5 | 2.25±0.7±5 | 2.25±0.7±5 | 2.25±0.7±5 | 2.25±0.7±5 | 2.25±0.7±5 | 2.25±0.7±5 |

According to the combination theory of traditional Chinese medicine, a compound prescription usually contains four categories of drugs that are, by virtue of their functions in the therapy, labeled as principal, subordinate, adjuvant, and guide drugs. The principal drug is the drug that undertakes the overwhelming capacity against diseases. The subordinate drug is the drug that is used in combination with the principal drug to reinforce mutual action besides its individual therapeutic action. The adjuvant drug is the drug that enhances the therapeutic effect or alleviates the potential toxicity. The guide drug is the drug that leads the active ingredients to the target. In this study, Mylabris displayed a significant anticancer activity, while Ginseng also showed an anticancer activity subordinate to that of Mylabris. Astragalus and Acanthopanax did not exhibit anticancer activity significantly. Polyamine levels had been found to be higher in Hep-G2 cells compared to that in HL-7702 cells. When Mylabris and Ginseng were combined, the decrease in the level of polyamine biomarkers was lower than that of either individual drug. The decreasing effect was even more marked when Astragalus was added. These results are consistent with the combination principle of this prescription, wherein Mylabris is the principal drug, Ginseng is the subordinate drug, and Astragalus is the adjuvant drug.

To the best of our knowledge, this is the first study to explore the correlation of polyamine metabolism and anticancer activity; further, this is the first to conduct a compatibility study.
of anticancer drugs based on this correlation. The conclusion can be applied to the compatibility study of other anticancer drugs and anticancer drug screening.

**Conclusions**

In this study, the UHPLC-MS/MS method was applied to detect the polyamine levels in HL-7702 cells and Hep-G2 cells and to verify the anticancer effects of Aidi injection and its disassembled and combination compositions. Results showed that Aidi injection displayed the strongest inhibitory effect on Hep-G2 cells and altered polyamine levels. Ginseng can act as an assistant to Mylabris for cancer treatment. A relatively weak anticancer effect of Astragalus and Acanthopanax was observed, although they possess some synergies to Mylabris and Ginseng. In conclusion, this study could contribute to a new method of evaluating the efficacy of anticancer medication. In addition, it might provide new ideas about the mechanism of Aidi injection for the treatment of cancers.

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**Conflicts of interest**

There are no conflicts of interest.
REFERENCES

1. Siegel RL, Miller KD, Ahrendt Jemal DV. Cancer statistics. CA Cancer J Clin 2020;70:7-30.

2. Citores MJ, Luengo JL, de la Fuente S, Cuervas-Mons V. Serum biomarkers and risk of hepatocellular carcinoma recurrence after liver transplantation. World J Gastroenterol 2019;11:50-64.

3. Lim J, Singa AG. Surveillance and diagnosis of hepatocellular carcinoma. Clin Liver Dis (Hoboken) 2019;13:2-5.

4. Zhang Y, Wang C, Xu H, Xiao P, Gao Y. Hepatocellular carcinoma in the noncirrhotic liver: A literature review. Eur J Gastroenterol Hepatol 2019;31:743-8.

5. Luan X, Zhang LJ, Li QX, Rahman K, Zhang H, Chen HZ, et al. Compound-based Chinese medicine formula: From discovery to compatibility mechanism. J Ethnopharmacol 2020;254:112687.

6. Cao H, Zhang X, Zhang H, Sun H, Wang X. The application of metabolomics in traditional Chinese medicine opens up a dialogue between Chinese and Western medicine. Phytother Res 2015;29:159-66.

7. Lou JS, Yao P, Tsim KW. Cancer Treatment by Using Traditional Chinese Medicine: Probing Active Compounds in Anti-multidrug Resistance During Drug Therapy. Curr Med Chem 2018;25:5128-41.

8. Sun JN, Sun WY, Dong SF. Discussion on efficacy evaluation thought and method for innovation medicine of Chinese herbal compound formula based on clinical application characteristics. Zhongguo Zhong Yao Za Zhi 2017;42:852-5.

9. Qi F, Zhao L, Zhou A, Zhang B, Li A, Wang Z, et al. The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer. Biosci Trends 2015;9:16-34.

10. Wang X, Sun H, Zhang A, Sun W, Wang P, Wang Z. Potential role of metabolomics approaches in the area of traditional Chinese medicine: As pillars of the bridge between Chinese and Western medicine. J Pharm Biomed Anal 2011;55:859-68.

11. Zhang A, Sun H, Wang Z, Sun W, Wang P, Wang X. Metabolomics: Towards understanding traditional Chinese medicine. Planta Med 2010;76:2026-35.

12. Hu CX, Xu GW. Metabolomics and traditional Chinese medicine. Trac Trends Analytical Chem 2014;61:207-14.

13. Jiang X, Oh IH, Lee SG, Choi HK. The application of metabolomics to processed traditional Chinese medicine. J Korean Soc Appl Biol Chem 2013;56:475-81.

14. Russell DH. Increased polyamine concentrations in the urine of human cancer patients. Nat New Biol 1971;233:144-5.

15. Morselli E, Mariho G, Benetti MV, Eisenberg T, Megalou E, Schroeder S, et al. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. J Cell Biol 2011;192:615-29.

16. Umemori Y, Sato Y, Kuribayashi K, Tsuji N, Nishidate T, Kameshima H, et al. Evaluating the utility of N1, N2-diacylserotonin and N1, N8-diacylsermidine in urine as tumor markers for breast and colorectal cancers. Clin Chim Acta 2010;411:1894-9.

17. Nakayama Y, Toriioe T, Minagawa N, Yamaguchi K. The clinical usefulness of urinary N1, N2-diacylserotonin (DiAcSpn) levels as a tumor marker in patients with colorectal cancer. Oncol Lett 2012;3:970-4.

18. Casero RA Jr, Pegg AE. Polyamine catabolism and disease. Biochem Soc Trans 2009;41:323-38.

19. Moinard C, Cynober L, de Bandt JP. Polyamines: Metabolism and implications in human disease. Clin Nutr 2005;24:184-97.

20. Agostinelli E, Marques MP, Calheiros R, Gil FP, Tempera G, Viceconte N, et al. Polyamines: Fundamental characters in chemistry and biology. Amino Acids 2010;38:393-403.

21. Samejima K, Hiramatsu K, Takahashi K, Kawakita M, Kobayashi M, Tsumoto H, et al. Identification and determination of urinary acetylpolyamines in cancer patients by electrospray ionization and time-of-flight mass spectrometry. Anal Chem 2010;82:22-9.

22. Yu C, Liu R, Xie C, Zhang Q, Yin Y, Bi K, et al. Quantification of free polyamines and their metabolites in biofluids and liver tissue by UHPLC-MS/MS: Application to identify the potential biomarkers of hepatocellular carcinoma. Anal Bioanal Chem 2015;407:6891-7.

23. Liu R, Li Q, Ma R, Lin X, Xu H, Bi K. Determination of polyamine metabolome in plasma and urine by ultrahigh performance liquid chromatography-tandem mass spectrometry method: Application to identify potential markers for human hepatic cancer. Analytica Chimica Acta 2013;791:36-45.

24. Xu HX, Huang XE, Li Y, Li CG, Tang JH. A clinical study on safety and efficacy of Aidi injection combined with chemotherapy. Asian Pac J Cancer Prev 2011;12:2233-6.

25. Zhang MM, Liu YL, Chen Z, Li XR, Xu QM, Yang SL. A new triterpenoid saponin from Aidi injection. Chin Herbal Med 2014;4:84-6.

26. Lou HZ, Pan HM, Jin W. Clinical study on treatment of primary liver cancer by Aidi injection combined with cool-tip radiofrequency ablation. Zhongguo Zhong Yi Yi Jie He Za Zhi 2007;27:393-5.

27. Wang D, Chen Y, Ren J, Cai Y, Liu M, Zhan Q. A randomized clinical study on efficacy of Aidi injection combined with chemotherapy in the treatment of advanced non-small cell lung cancer. Zhongguo Fei Ai Za Zhi 2004;7:247-9.

28. Xu XT, Song Y, Qin S, Wang L, Zhou JY. Radio-sensitivity of SHG44 glioma cells by Aidi injection in vitro. Mol Med Rep 2012;5:1415-8.

29. Zhu M, Shi X, Gong Z, Su Q, Yu R, Wang B, et al. Cantharidin treatment inhibits hepatocellular carcinoma development by regulating the JAK2/STAT3 and PI3K/Akt pathways in an EphB4-dependent manner. Pharmaco Res 2020;81:108468.

30. Zhang W, Ma YZ, Song L, Wang CH, Qi TG, Shao GR. Effect of cantharidins in chemotherapy for hepatoma: A retrospective cohort study. Am J Chin Med 2014;42:561-7.

31. Wang GS. Medical uses of mylarias in ancient China and recent studies. J Ethnopharmacol 1989;26:147-62.

32. Yang H, Lee DY, Kang KB, Kim JY, Kim SO, Yoo YH, et al. Identification of ginsenoside markers from dry purified extract of Panax ginseng by a dereplication approach and UPLC-QTOF/MS analysis. J Pharm Biomed Anal 2015;109:91-104.

33. Sun L, Wu D, Ning X, Yang G, Lin ZH, Tian MH, Zhou YF. α-amylase-assisted extraction of polysaccharides from panax ginseng. Int J Biol Macromol 2015;75:152-7.

34. Thwe AA, Mai NT, Li X, Kim Y, Kim YB, Uddin R, et al. Production of astragaloside and flavonoids from adventitious root cultures of Astragalus membranaceus var. mongholicus. Plant Oomics 2012;5:376-80.

35. Guan S, Ma J, Chu X, Gao Y, Zhang Y, Zhang X, et al. Effects of total flavones from Acanthopanax senticosus on L-type calcium channels, calcium transient and contractility in rat ventricular myocytes. Phytother Res 2015;29:533-9.

36. Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. Biochem J 2003;376:1-4.

37. Pegg AE. Spermidine/spermine-N1-acetyltransferase: A key metabolic regulator. Am J Physiol Endocrinol Metab 2008;294:995-1010.

38. Vujicic S, Diegelman P, Bacchi CJ, Kramer DL, Porter CW. Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem J 2002;367:665-75.

39. Linsalata M, Cavallini A, Di Leo A. Polyamine oxidase activity and polyamine levels in human colorectal cancer and in normal surrounding mucosa. Anticancer Res 1997;17:3757-60.

40. Quash G, Keoluangkhot T, Gazzolo L, Ripoll H, Saez S. Diamine oxidase and polyamine oxidase activities in normal and transformed cells. Biochem J 1979;177:275-82.

41. Wallace HM, Duthie J, Evans DM, Lamond SJ, Nicoll K, Heys SD. Alterations in polyamine catalytic enzymes in human breast cancer tissue. Clin. Cancer Res 2000;6:3657-61.

42. Takenoshita S, Matsuzaki S, Nakano G, Kimura H, Hoshi H, Shoda Y, et al. Selective elevation of the N1-acetylspermidine level in human colorectal adenocarcinomas. Cancer Res 1984;44:845-7.

43. Gramziniski RA, Parchment RE, Pierce GB. Evidence linking programmed cell death in the blastocyst to polyamine oxidation. Differentiation 1990;43:59-65.

44. Thomas T, Thomas TJ. Polyamines in cell growth and cell death: Molecular mechanisms and therapeutic applications. Cellular Molecular Life Sci 2001;58:244-58.

45. Raul F, Grosse F, Galluser M, Hasselmann M, Seiler N. Functional and metabolic changes in intestine mucosa of rats after enteral administration of ornithine alphaketo glutarate salt. J Parenter Enteral
46. Seiler N. Catabolism of polyamines. Amino Acids 2004;26:217-33.
47. Mennigen R, Kusche J, Krakamp B, Elbers A, Mnoei B, Kessebohm M, Sommer H. Large bowel tumors and diamine oxidase (DAO) activity in patients: A new approach for risk group identification.
48. Zhang M, Gong Y, Assy N, Minuk GY. Increased GABAergic activity inhibits alpha-fetoprotein mRNA expression and the proliferative activity of the HepG2 human hepatocellular carcinoma cell line. J Hepatol 2000;32:85-91.