Research Article

Absorption Characteristics of Combination Medication of Realgar and Indigo Naturalis: In Vitro Transport across MDCK-MDR1 Cells and In Vivo Pharmacokinetics in Mice after Oral Administration

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Realgar and indigo naturalis are clinically combined to treat varieties of leukemia. Exploring the drug-drug interactions might be beneficial to find active substances and develop new targeted drugs. This study aimed at exploring the change of arsenic concentration in mice and across MDCK-MDR1 cells and the cytotoxicity on K562 cells when realgar and indigo naturalis were combined. In the presence or absence of indigo naturalis, pharmacokinetics and cell-based permeability assays were used to evaluate the change of arsenic concentration, and K562 cell line was applied to evaluate the change of cytotoxicity. The drug concentration-time profiles exhibited that the combination medication group generated higher AUC, t½, and longer MRT for arsenic, compared with the single administration of realgar. The apparent permeability coefficients (Papp) of bidirectional transport in MDCK-MDR1 cell permeability experiments showed that arsenic permeability obviously went up when indigo naturalis was incubated together. The combination medication significantly decreased the cell viability of K562 cells when both the concentration of realgar and the concentration of indigo naturalis were nontoxic. The pharmacokinetic research, the MDCK-MDR1 based permeability study, and the K562 cytotoxicity study were united together to verify the combination medication of realgar and indigo naturalis enhanced the absorption and the permeability across cells for arsenic and effectively inhibited the proliferation of K562 cell line. The molecular binding of $\text{As}_4\text{S}_4$ and indirubin was analyzed by computational study. It is predicted that the formation of the complex $[\text{As}_4\text{S}_4\cdot\text{Indirubin}]$ involves noncovalent interaction that changes the concentration of arsenic.

1. Introduction

Arsenicals such as $\text{As}_2\text{O}_3$ and realgar ($\text{As}_4\text{S}_4$) have been used to treat hematological malignancy widely in both Eastern and Western world [1, 2]. One of the most famous arsenicals is the ATO injection ($\text{As}_2\text{O}_3$), Trisenox®, which shows remarkable effect against APL after being approved by FDA [3]. Another famous arsenical is realgar, which has been proved to be as effective as arsenic trioxide with less irritation according to the clinical trials and treatments [4, 5]. Nowadays, oral administration of realgar is utilized widely to treat APL and MDS in clinical therapeutics [5, 6]. In fact, arsenicals have long been used in TCM to treat a variety of diseases, including syphilis, malaria, skin problems, and several other parasitic infections for more than 1500 years [7]. TCM practitioners for millennia have accumulated some therapeutic experiences prescribing a combination of arsenicals with several other Chinese herbal medicines or
mineral medicines [8, 9]. Realgar and indigo naturalis are a common combination regimen therapeutically used in the treatment of different types of leukemia [8–10]. Leukemia is a group of malignant clonal diseases of hematopoietic stem cells [11]. The ineffective hematopoiesis results in the decrease of normal blood cells [12]. There are four main types of leukemia, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), as well as a number of less common types [5, 6, 13, 14].

Realgar, a mineral arsenical drug, contains more than 90% As$_2$S$_3$ and is regarded as the principle medicine to provide arsenic [15]. Indirubin, a 3,2'-bisindole isomer of indigo, was a stable and active ingredient of indigo naturalis [16, 17]. Zhou et al. (1981) clinically used realgar and indigo naturalis in a formula to treat patients with CML [18]. Subsequently, realgar-indigo naturalis formula (RIF) which contains realgar, indigo naturalis, and salvia miltiorrhiza was found effective against APL in some clinical trials [8]. An observation in their work was that the combination therapy of their main active ingredients, As$_2$S$_3$, indirubin, and tanshinone IIA can increase the cellular uptake of arsenic in NB4 cells through significantly inducing upregulation of AQP9 which is a transmembrane protein playing an essential role in arsenic uptake and determining cellular arsenic sensitivity, compared with As$_2$S$_3$ treatment alone. The upregulation of AQP9 was detected at 24 h after combination treatment, but some other events or interactions were not addressed in their paper that might also lead to the enhancement of arsenic action at the earlier stage. Here, the pharmacokinetic study in mice whole blood was monitored between 0 and 24 h after medication to compare realgar pharmacokinetic parameters with or without indigo naturalis. In order to further study the effects of indigo naturalis on the penetration of arsenic, the MDCK-MDRI based permeability assay was used to analyze the arsenic transport across cells. For proving the combination effect of realgar and indigo naturalis on treating CML, K562 cell line was incubated to evaluate the cytotoxicity.

2. Methods

2.1. Materials and Reagents. Realgar was provided by Department of Hematology, Xiyuan Hospital, China Academy of Chinese Medical Sciences (Beijing, China). Indigo naturalis was purchased from Beijing Bencaofangyuan Co. Ltd. (Beijing, China). Standard solutions of As were prepared by using the stock solution which was purchased from National Institute of Metrology, China. Realgar was provided by Department of Hematology, Xiyuan Hospital, China Academy of Chinese Medical Sciences (Beijing, China). Realgar was provided by Department of Hematology, Xiyuan Hospital, China Academy of Chinese Medical Sciences (Beijing, China). Realgar was provided by Department of Hematology, Xiyuan Hospital, China Academy of Chinese Medical Sciences (Beijing, China).


| Time | Temperature | Power |
|------|-------------|-------|
| 0–3 min | 0°C → 120°C | 1600 W |
| 3–6 min | 120°C | 1600 W |
| 6–9 min | 120°C → 150°C | 1600 W |
| 9–12 min | 150°C | 1600 W |
| 12–17 min | 50°C → 180°C | 1600 W |
| 17–27 min | 180°C | 1600 W |
| 27–60 min | 180°C → 25°C | 0 W |

from Biotopped Life Sciences (Beijing, China). Polyester transwells (3460) were purchased from Costar (Cambridge, MA). Male ICR mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were housed in a temperature and humidity controlled environment and maintained in a 12-hour light/dark cycle for three days with free access to food and water. Electrical resistor (Millicell® ERS-2) was purchased from Millipore (USA). The human CML cell line K562 was obtained from the Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China).

2.2. Apparatus. An Agilent 7700 inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Palo Alto, CA, USA) was used to detect and quantify arsenic. 1.0 μg/L of mass spectrometer turned liquid and 1.0 μg/L of online internal standard solution were used to optimize the conditions of ICP-MS. The radio frequency power was set as 1500 W; the plasma gas flow rate was 15.0 L/min; the auxiliary flow rate was 1.0 L/min; the carrier gas flow was 1.0 L/min; the sampling deepness was 7.5 mm; the aperture of sampling cone was 1.0 mm; the aperture of skimmer cone was 0.4 mm; the temperature in spray chamber was 2°C; the peristaltic pump speed was 0.1 rps. The analysis room vacuum degree was ranging from 1 × 10$^{-5}$ Torr to 2 × 10$^{-5}$ Torr. The measurement mode adopted peak jumping type. The data collection repetition times were set as three times, sampling points for each peak were three. The increasing rate of test solution was 1.0 ml/min.

Microwave digestion system (MARS 6 MARSXpress, CEM Corporation, Matthews, NC, USA) was used to digest samples. Samples were digested in seven steps shown in Table 1.

2.3. Oral Administration Methods. Researchers found that rats had a longer retention time of arsenic in their blood than that in humans mainly because of the retention in RBCs. Hemoglobin in RBCs has been regarded as a target protein of arsenic. Arsenic can significantly accumulate in rat RBCs because of the binding of the reactive arsenic metabolite, dimethylarsinous acid (DMA$_3^+$), to a highly reactive cysteine residue in rat hemoglobin (cys-13 in the alpha chain) [19]. Therefore, it is suggested that rats are not appropriate for model animals of arsenic pharmacokinetic studies. All animal experimental procedures were followed by the National Institutes of Health Guide for the Care and Use of Laboratory
Animals were approved by the committee of Animal Care and Utilities.

2.3.1. Pharmacokinetic Method. Realgar (R) and the combination of realgar and *indigo naturalis* (RI) were both mixed in 0.5% CMC-Na solution. Mice were orally dosed with R (606 mg/kg) and RI (606 mg/kg of R, 1212 mg/kg of I), respectively. Whole blood samples (about 0.1 ml) were drawn from ocular fundus veins of mice at 0, 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, and 24 h after dosing and treated with nitric acid and hydrogen peroxide for microwave digestion. Using Ge and Rh as internal standard, samples were detected by ICP-MS to determine the concentration of arsenic. The pharmacokinetic parameters of As were calculated by Kinetica 4.4 software (Thermo Scientific, USA).

2.3.2. Multiple Oral Administration Method. Realgar (R) and the combination of realgar and *indigo naturalis* (RI) were both mixed in 0.5% CMC-Na solution. Mice were orally dosed with R (606 mg/kg) and RI (606 mg/kg of R, 1212 mg/kg of I), respectively, for seven days. Whole blood samples (about 0.1 ml) were drawn from ocular fundus veins of mice after the administration for seven days and treated with nitric acid and hydrogen peroxide for microwave digestion. Using Ge and Rh as internal standard, samples were detected by ICP-MS to determine the concentration of arsenic.

2.3.3. Microwave Digestion for Blood Samples. Under the setting programs (shown in Table 1), whole blood samples were digested by microwave digestion system with 8 mL of nitric acid and 2 mL of hydrogen peroxide in Teflon vessels, followed by electric heating plate to volatilize spare liquid. After being cooled, the remaining droplets were poured out from Teflon vessels and diluted with ultrapure water to certain volumes.

2.4. Transmembrane Transport across MDCK-MDR1 Cells

2.4.1. Cell Culture. MDCK-MDR1 cells (1 × 10^6 cells/mL) were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and streptomycin at 37°C in a tissue culture incubator with 5% CO₂ and 95% air for 2-3 days to reach 80% confluence. Cells (4 × 10⁵ cells/mL, 0.5 mL) were then seeded onto transwells and incubated for 4-5 days to reach cell monolayers. The integrity of cell monolayers was assessed by measuring the transepithelial electrical resistance (TEER). 250-350 Ω·cm⁻² of TEER presented cells connected tightly and cell monolayer was integrated. The medium was replaced with fresh medium every day.

2.4.2. Bidirectional Permeability Measurements. Medium from all apical inserts and basolateral wells was replaced with HBSS solution for 30 min at 37°C in incubator. Compound permeability was assayed by adding drugs to an apical insert for the apical to basolateral (A to B) transport and to a basolateral well for the basolateral to apical (B to A) transport. For A to B transport, 0.5 mL of drug solution was added in A side and 1.5 mL of HBSS in B side to measure A-B transporter, and 600 μL of uptake solution samples was collected from the B side and diluted with ultrapure water. For B to A transport, 1.5 mL of drug solution was added in B side and 0.5 mL of HBSS in A side to measure B-A transporter, and 200 μL of efflux solution was collected from the A side and diluted with ultrapure water.

Realgar (R) was suspended in PBS; *indigo naturalis* (I) was suspended in DMSO. The cells were treated with R (35 μg/mL of R) and RI (35 μg/mL of R and 70 μg/mL of I), respectively. The proportion of realgar and *indigo naturalis* was followed with the proportion in pharmacokinetic study. The treated cells were then returned to the incubator at 37°C. The samples were collected at 30 min, 60 min, 90 min, 120 min, 150 min, and 180 min from both apical inserts and basolateral wells. Using Ge and Rh as internal standard, samples were detected by ICP-MS to determine the concentration of arsenic.

2.5. Cytotoxic Effects on K562 Cell Line. K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) in a humidified 37°C chamber with 5% CO₂ [20]. The cytotoxic effects of realgar (R), *indigo naturalis* (I), and realgar and *indigo naturalis* (RI) on K562 cells were evaluated by MTT assays. For MTT assays, K562 cells were seeded in 96-well flat-bottom microtiter plates at a density of 1 × 10⁴ cells/mL with 100 μL of cell solution.

Realgar (R) was suspended in PBS; *indigo naturalis* (I) was suspended in DMSO. K562 cells were, respectively, treated with various concentrations of R (0, 3.5, 7, 14, and 28 μg/mL), I (0, 7, 14, 28, and 56 μg/mL), and RI (0, 3.5, 7, 14, and 28 μg/mL of R and 0, 7, 14, 28, and 56 μg/mL of I). The proportion of realgar and *indigo naturalis* was followed with the proportion in pharmacokinetic study. PBS and DMSO were used as the vehicle control. The treated cells were incubated for 24 h in a humidified 37°C chamber with 5% CO₂. Then the medicated cells were treated with MTT working solution (0.5 mg/mL) for 3-4 h at 37°C. The plates were centrifuged at 800 g for 5 min, and the supernatant was subsequently removed from all the wells. Then, 150 mL of DMSO was added to dissolve the formazan crystals precipitated at the bottom. The absorbance of the formazan solution was measured at 570 nm by a microplate reader.

2.6. Apoptosis Assay. K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) in a humidified 37°C chamber with 5% CO₂. Realgar (R) was suspended in PBS, *indigo naturalis* (I) was suspended in DMSO. K562 cells were seeded in six-well plates at a density of 4 × 10⁵ cells per well. The cells were, respectively, incubated with R (0, 28 μg/mL) and RI (0, 28 μg/mL of R and 0, 56 μg/mL of I). The treated cells were incubated in a humidified 37°C chamber with 5% CO₂ for 24 h. PBS and DMSO were used as the vehicle control. The apoptotic K562 cells were resuspended in 295 μL of binding buffer and stained with 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI). Next, the cells were incubated for 20 min in the dark. The viable, apoptotic, and necrotic cell numbers were determined by flow cytometry.
The stability of total arsenic under various storage conditions was tested. Stability studies showed that total arsenic in samples was stable at the condition of 4°C for 24 h and the condition of three freeze-thaw cycles. However, the concentrations of total arsenic significantly decreased after being stored at −20°C for one month.

3.2. Combination Medication of Realgar and Indigo Naturalis Enhances Arsenic Absorption in Mice Whole Blood. This study focused on the changes of arsenic concentration in whole blood after the administration of realgar with or without indigo naturalis.

The PK parameters of R can be found mostly lower than that of RI. Arsenic whole blood concentration after the administration of R reached the maximum at 45 min with an average C_{max} of 2472.41 μg/L. The area under the curve AUC_{(0−24)} was 18992.00 (h μg/L), AUC_{(0−∞)} was 22039.2 (h μg/L), half-life was 8.58 h, and mean residence time (MRT) was 12.97 h, whereas arsenic whole blood concentration after the combination medication reached the maximum at 45 min with an average C_{max} of 1681.32 μg/L. AUC_{(0−24)} grew to 24057.60 (h μg/L), AUC_{(0−∞)} changed to 30340.00 (h μg/L), half-life prolonged to 9.91 h, and MRT extended to 15.57 h. In general, the addition of indigo naturalis increased AUC, half-life, and MRT in spite of decreasing C_{max}. AUC_{(0−∞)} reflects the actual body exposure to drug after administration of a dose of the drug from time zero to infinite time, corresponding to the total amount of the dose absorbed in the systematic circulation. The half-life t_{1/2} of the drug is the time to eliminate 50% of the drug blood concentration in the body. Increased t_{1/2} implied the elimination rate became slow. To discuss MRT, it is essential to change the idea to the residence time of individual molecules in the body. Each molecule spends a different period of time in the body, with some molecules lasting a very short amount of time and others lasting longer. MRT reflects overall behaviors of individuals. Therefore, the higher PK parameters of the AUC, t_{1/2}, and MRT presented after arsenic medication, the bigger amount of arsenic was absorbed by the bloodstream, the longer time was spent in the systematic circulation, and the slower decline in the blood arsenic concentration. The pharmacokinetic profile (mean whole blood concentration of arsenic versus time (n=6)) was shown in Figure 1, and the main pharmacokinetic parameters derived from these data are summarized in Table 2.

After consecutive oral administration of R and RI in mice for seven days, the arsenic concentration of R can be found lower than that of RI. Arsenic whole blood concentration
Table 2: Pharmacokinetic parameters of arsenic after oral administration of realgar and realgar-indigo naturalis.

| Parameters          | Unit     | realgar          | realgar+ indigo naturalis |
|---------------------|----------|------------------|---------------------------|
| T<sub>max</sub>     | h        | 0.75             | 0.75                      |
| C<sub>max</sub>     | µg L<sup>-1</sup> | 2472.41         | 1681.32                   |
| AUC<sub>(0-24)</sub> | h µg L<sup>-1</sup> | 18992.00       | 24057.60                  |
| AUC<sub>(0-∞)</sub> | h µg L<sup>-1</sup> | 22039.20       | 30343.00                  |
| t<sub>half</sub>    | h        | 8.58             | 9.91                      |
| MRT                 | h        | 12.97            | 15.57                     |

C<sub>max</sub> R/RI 1.47
AUC<sub>(0-24)</sub> R/RI 0.79
AUC<sub>(0-∞)</sub> R/RI 0.73

![Figure 2](image_url)

**Figure 2:** Cumulative amount of transported arsenic in R and RI across MDCK-MDR1 cells.

After the accumulative administration of R reached the 3361.34 µg/L. Arsenic whole blood concentration after the accumulative administration of RI reached 4239.15 µg/L.

3.3. Combination Medication of Realgar and Indigo Naturalis Increases Arsenic Transport on MDCK-MDR1 Cell Line. The apparent permeability coefficients (P<sub>app</sub>) were calculated according to (1): P<sub>app</sub> = (dQ / dt) / A C, where dQ / dt is the apparent appearance rate of arsenic in the receiver side calculated using linear regression of amounts in the receiver chamber versus time, A is the surface area of the polyester membrane of Transwell (cm<sup>2</sup>), and C is the arsenic concentration incubated in donor side. The efflux ratio (ER) was calculated according to (2):

ER = P<sub>app</sub> (B-A) / P<sub>app</sub> (A-B).

In order to study the effects of indigo naturalis on arsenic transport, the permeability of arsenic was assessed in the presence or absence of indigo naturalis across MDCK-MDR1. The cumulative amount and P<sub>app</sub> of transported arsenic were calculated. Results were expressed as mean±SD, n=3, shown in Figure 2. The apparent permeability coefficients (×10<sup>-6</sup> cm/s) of realgar alone and the combination were listed in Table 3 and Figure 3. All of the apparent permeability coefficients were greater than 2 × 10<sup>-6</sup> and lower than 20 × 10<sup>-6</sup> in MDCK-MDR1 cells, which proved that arsenic should be classified to medium permeability drugs absorbed by small intestine [21]. In addition, the efflux ratios (ER) of arsenic in R and RI groups were between 0.5 and 2 in MDCK-MDR1 cells, which indicated that arsenic entered into cells through nonactive transport [22]. Moreover, the P<sub>app</sub> A-B or P<sub>app</sub> B-A values increased when cells were treated with realgar and indigo naturalis, compared to the treatment with realgar alone. Therefore, the results manifested that indigo naturalis could enhance the arsenic penetration amount and permeability rate. The MDCK-MDR1 based permeability results were consistent with the pharmacokinetic study. Drug-drug interaction between realgar and indigo naturalis occurred mainly in the absorption process.

3.4. Combination Medication of Realgar and Indigo Naturalis Increases the Cytotoxic Effect of K562 Cells. In order to study the effects of combination therapy on K562 cell line, K562 cells were exposed to R, I, and RI. MTT assays were
used to measure the cell viability of K562 cells after 24 h of drug treatment. Results were expressed as mean±SD, n=3, shown in Figure 4. The results showed that the cell viability effectively decreased when R and I were combined, while the cell viability was not decreased after the single medication at the same single concentration. In general, the combined medication group showed strong cytotoxicity and significantly decreased K562 cells proliferation and viability compared to that of the vehicle controls. (shown in Figure 4).

3.5. Combination Medication of Realgar and Indigo Naturalis Increases the Apoptotic Effect of K562 Cells. After K562 cells were treated with R and RI for 24 h, the proportion of viable cells was substantially reduced from 91.50% ± 2.51% to 56.30%± 7.83%. In addition, the proportion of early apoptotic cells increased from 6.20%± 0.51% to 28.60%± 11.58%, along with a strong increase in necrotic and late apoptotic cells from 2.40% ± 1.44% to 15.10%± 2.98%. These data indicated that R and RI reduce the viability of K562 cells through inducing K562 cell apoptosis (shown in Figure 5).

4. Discussions
Both of the oral administration study and the cell-based permeability study manifested a phenomenon that the additive *indigo naturalis* facilitated the arsenic absorption. And the cytotoxicity study indicated that the combination medication efficiently inhibited the proliferation of K562 cell line when neither the concentration of realgar nor the concentration of *indigo naturalis* was lethal. In order to further discuss the reason why *indigo naturalis* enhanced the arsenic absorption and permeability, we conducted the inference with the help of computational methods. It is regarded that *indigo naturalis* contains two main active ingredients, indirubin and indigo. Indigo is very unstable and easy to be reduced to indigo white [23]. Therefore, indirubin was taken to proceed with the further study.

In this research, geometries and harmonic vibrational frequencies of the \( \text{As}_4S_4 \), indirubin, and complex of
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Control RI

0 10^3 10^4 10^5
-357 PI-A

FITC-A

102 103 104 105

(a)

Viable cells

Necrosis cells

Apoptotic cells

(b)

Figure 5: Effects of R and RI on apoptosis in K562 cells. (a) Quantification of viable, apoptotic and necrotic cells by flow cytometry with FITC-Annexin V/PI double staining after treatment with R (0, 28 µg/mL) and RI (0, 28 µg/mL of R and 0, 56 µg/mL of I) for 24 h. (b) Percentages of viable, apoptotic and necrotic cells after treatment with R (0, 3.5 µM) and RI (0, 3.5 µM of R and 0, 7 µM of I) for 24 h. Data are represented as the mean ± SD from three independent experiments (* p < 0.05 versus control).

[As₄S₄−Indirubin] that being formed by the former two compounds were fully optimized by using Hartree-Fock (HF) [24] and density functional theory (DFT) [25] methods, where the DFT method using Beck’s three-parameter nonlocal exchange functional along with the Lee-Yang-Parr nonlocal correlation functional (B3LYP) [26, 27] and the wB97XD functional including a long-range correction and accounting for dispersive forces from Chai and Head-Gordon[28]. The standard 6-31G* basis set of double-zeta contraction quality plus six-d-like polarization functions [29] and the 6-31+G* basis set with diffuse function were performed in the present study. In addition, the correction of the basis set superposition error (BSSE) by the counterpoise method [30] of complex [As₄S₄−Indirubin] has been investigated at the wB97XD /6-31+G* level of theory. Throughout this paper, bond lengths are given in Å angstroms, total energies and BSSE correction in zero-point energies, and binding energies in kcal/mol. All calculations are performed with Gaussian 09 program package [31] and the Gaussian View program.

Total energies with zero-point energy (ZPE) of As₄S₄, indirubin and complex of [As₄S₄−Indirubin], and BSSE of complex [As₄S₄−Indirubin] at the HF/6-31G*, B3LYP/6-31G*, and wB97XD/6-31+G* levels of theory are shown in Table 4. The geometry and harmonic vibrational frequency analyses show that the complex of [As₄S₄−Indirubin] is a local minimum with all real frequencies. As shown in Figure 6, the present calculated As-O distance d(As-27O) is 3.105, 2.934, and 2.892 Å at the HF/6-31G*, B3LYP/6-31G*, and wB97XD/6-31+G* levels of theory, respectively, which is larger than the sum of the covalent radii of the As and O atoms (1.84 Å), but shorter than the sum of the van der Waals radii of the As and O atoms (3.37 Å), indicating that the α-As₄S₄ and indirubin (C₁₆H₁₀N₂O₂) may form a complex of [As₄S₄−Indirubin] by the noncovalent interaction. The predicted binding energy with ZPE and BSSE corrections is 0.3, 0.3, and -5.8 kcal/mol at the HF/6-31G*, B3LYP/6-31G*, and wB97XD/6-31+G* levels of theory, respectively, listed in Table 5. Considering the importance of using larger basis sets as well as adequately correlated methods to compute
This represented the fact that the arsenic of combination medication of realgar and indigo naturalis enhanced the arsenic concentration in blood. The MDCK-MDR1 based permeability experiments verified the transport rate and penetration amount of arsenic increased when two drugs were used together in both A-B and B-A pathways. Indigo naturalis enhanced the arsenic penetration amount and permeability rate. The MDCK-MDRI based permeability results were consistent with the pharmacokinetic study. Cyto-toxicity study proved that the combination therapy efficiently decreased the survival of K562 cell line, while the cell viability was not decreased during the single medication at the same single concentration. These experiments suggested that the combination administration of realgar and indigo naturalis could produce synergistic action. An apoptotic study found that R and RI reduce the viability of K562 cells through inducing cells apoptosis and the combination medication of realgar and indigo naturalis increases the apoptotic effect of K562 cells. A theoretical result was provided by the computational work that the formation of the complex [As₄S₄⁻ - Indirubin] which involves noncovalent interaction might be another reason that promotes the arsenic concentration.

Realgar and indigo naturalis are both traditional oral drugs for treating various types of leukemia, commonly being combination medication. Starting with the combinatorial modes in traditional medicines to find mechanisms has advantages of sufficient clinical experiences accumulated in hundreds of years [32]. United applications of pharmacokinetics, cell transport, and computational quantum chemistry explore the drug-drug interactions, possibly offering a novel line for multicomponents analysis [33].

5. Conclusion

The phenomenon which occurred in the pharmacokinetic study illustrated that the combination medication of realgar and indigo naturalis enhanced the arsenic AUC in blood. This represented the fact that the arsenic of combination group had higher bioavailability in mice bodies. Realgar had higher C max while it had shorter t ½ and MRT, which meant realgar reached the peak concentration in a short time and it was eliminated in a relatively short period. Multiple oral administration study future illustrated that the combination medication of realgar and indigo naturalis enhanced the arsenic concentration in blood. The MDCK-MDRI based permeability experiments verified the transport properties of noncovalent interactions, the predicted binding energy of -5.8 kcal/mol at the wB97XD/6-31+G* level of theory is more reasonable than the other two.

Computational study shows that As₄S₄ and indirubin form a noncovalent interaction through linking As and O atoms. It is predicted that the noncovalent interaction between As₄S₄ and indirubin raises the solubility and improves the releasing degree into bloodstream of As₄S₄ owing to the organic characteristics of indirubin.

Table 4: Total energies (a.u.) with ZPE [kcal/mol] in parentheses of As₄S₄, indirubin, complex of [As₄S₄⁻ - Indirubin], and BSSE of complex [As₄S₄⁻ - Indirubin] at the HF/6-31G*, B3LYP/6-31G*, and wB97XD/6-31+G* levels of theory.

| Structures                              | HF/6-31G*         | B3LYP/6-31G*       | wB97XD/6-31+G*    |
|-----------------------------------------|-------------------|-------------------|-------------------|
| As₄S₄                                   | -10518.24526      | -10528.05290      | -10528.08202      |
| (Dₙ)                                    | (8.0)             | (7.0)             | (7.4)             |
| Indirubin C₁₆H₁₀N₂O₂                    | -870.37812        | -875.71055        | -875.44319        |
| (C₁)                                    | (152.6)           | (142.1)           | (143.8)           |
| complex                                 | -11388.64324      | -11403.78579      | -11403.57410      |
| (C₁)                                    | (161.5)           | (149.9)           | (152.2)           |
| BSSE energy                             | 0.02180           | 0.02400           | 0.04130           |

Table 5: Binding energies (in kcal/mol) with BSSE correction values in parentheses of complex [As₄S₄⁻ - Indirubin] relative to separated As₄S₄ and indirubin (C₁₆H₁₀N₂O₂) at the HF/6-31G*, B3LYP/6-31G*, and wB97XD/6-31+G* levels of theory.

| Structures                              | HF/6-31G*         | B3LYP/6-31G*       | wB97XD/6-31+G*    |
|-----------------------------------------|-------------------|-------------------|-------------------|
| [As₄S₄⁻ C₁₆H₁₀N₂O₂]                     | -13.4             | -14.8             | -31.7             |
|                                         | (0.3)             | (0.3)             | (-5.8)            |

The binding energy for the complex was evaluated according to the following definition given in the following equation: $E_{\text{binding}} = E_{\text{complex}} - E_{\text{AS4S4}} - E_{\text{indirubin}} + \text{BSSE}$.

Abbreviations

As: Arsenic
O: Oxide
As₄S₄: Tetraarsenic tetrasulfide
Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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References

[1] D. Douer and M. S. Tollman, "Arsenic trioxide: New clinical experience with an old medication in hematologic malignancies," Journal of Clinical Oncology, vol. 23, no. 10, pp. 2396–2410, 2005.

[2] J. Liu, Y. Lu, Q. Wu, R. A. Goyer, and M. P. Waalkes, "Mineral arsenicals in traditional medicines: Orpiment, realgar, and arsenolite," The Journal of Pharmacology and Experimental Therapeutics, vol. 326, no. 2, pp. 363–368, 2008.

[3] J. T. Barbey, J. W. Singer, D. Unnikrishnan et al., "Cardiac toxicity of arsenic trioxide [4] (multiple letters)," Blood, vol. 98, no. 5, pp. 1632–1634, 2001.

[4] D.-P. Lu, J.-Y. Qiu, B. Jiang et al., "Tetra-arsenic tetra-sulfide for the treatment of acute promyelocytic leukemia: A pilot report," Blood, vol. 99, no. 9, pp. 3136–3143, 2002.

[5] D. Lu and Q. Wang, "Current study of APL treatment in China," International Journal of Hematology, vol. 76, no. S1, pp. 316–318, 2002.

[6] H.-H. Zhu, D.-P. Wu, J. Jin et al., "Oral tetra-arsenic tetra-sulfide formula versus intravenous arsenic trioxide as first-line treatment of acute promyelocytic leukemia: a multicenter randomized controlled trial," Journal of Clinical Oncology, vol. 31, no. 33, pp. 4212–4223, 2013.

[7] J. Wu, Y. Shao, J. Liu, G. Chen, and P. C. Ho, "The medicinal use of realgar (As₄S₈) and its recent development as an anticancer agent," Journal of Ethnopharmacology, vol. 135, no. 3, pp. 595–602, 2011.

[8] L. Wang, G.-B. Zhou, P. Liu et al., "Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia," Proceedings of the National Academy of Sciences of the United States of America, vol. 105, no. 12, pp. 4826–4831, 2008.

[9] S. Xu, R. Ma, X.-M. Hu et al., "Clinical observation of the treatment of myelodysplastic syndrome mainly with Qinghuang Powder," Chinese Journal of Integrative Medicine, vol. 17, no. 11, pp. 834–839, 2011.

[10] X.-M. Hu, F. Liu, and R. Ma, "Application and assessment of Chinese arsenic drugs in treating malignant hematopathy in China," Chinese Journal of Integrative Medicine, vol. 16, no. 4, pp. 368–377, 2010.

[11] A. List, M. Beran, J. DiPersio et al., "Opportunities for Trisenox (arsenic trioxide) in the treatment of myelodysplastic syndromes," Leukemia, vol. 17, no. 8, pp. 1499–1507, 2003.

[12] R. Ma, "Treatment of bone marrow failure syndrome with integrated traditional and western medicine," Chinese Journal of Integrative Medicine, vol. 13, no. 2, pp. 85–88, 2007.

[13] E. Gorshein, S. Kalathil, and M. Gharib, "Prolonged Survival of Acute Lymphoblastic Leukemia with Intrathecal Treatments for Isolated Central Nervous System Relapse," Case Reports in Hematology, vol. 2018, Article ID 876285, 3 pages, 2018.

[14] U. Ibrahim, S. Parryo, S. Kedia, S. Hussein, and J. P. Atallah, "Large Granular Lymphocytic Leukemia: A Report of Response to Rituximab," in Case Reports in Hematology, vol. 4, pp. 1–3, 2017.

[15] Chinese Pharmacopoeia Commission, Pharmacopoeia of the People’s Republic of China, Part I, Realgar, 2015.

[16] G. Eisenbrand, F. Hippe, S. Jakobs, and S. Muehlbeyer, "Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phyto medicine," Journal of Cancer Research and Clinical Oncology, vol. 130, no. 11, pp. 627–635, 2004.

[17] R. Hoessel, S. Leclerc, J. A. Endicott et al., "Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases," Nature Cell Biology, vol. 1, no. 1, pp. 60–67, 1999.

[18] A. X. Zhou, B. S. Yao, and J. F. Deng, "Therapeutic efficacy of QingHuangSan in the treatment of Chronic myeloid leukemia, 25 cases," Chinese Journal of Integrative Medicine, pp. 16–18, 1981 (Chinese).

[19] M. Lu, H. Wang, X. F. Li, L. L. Arnold, S. M. Cohen, and X. C. Le, "Binding of dimethylarsinous acid to cys-13a of rat hemoglobin is responsible for the retention of arsenic in rat blood," Chemical Research in Toxicology, vol. 20, pp. 27–37, 2007.

[20] S. Lin, L. Tian, H. Shen et al., "DDX5 is a positive regulator of oncogenic NOTCH1 signaling in T cell acute lymphoblastic leukemia," Oncogene, vol. 32, no. 40, pp. 4845–4853, 2013.

[21] X. Jin, T.-L. Luong, N. Reese et al., "Comparison of MDCK-MDRI and Caco-2 cell based permeability assays for anti-malarial drug screening and drug investigations," Journal of Pharmaceutical and Toxicological Methods, vol. 70, no. 2, pp. 188–194, 2014.

[22] R. Cecchelli, B. Debouch, L. Descamps et al., "In vitro model for evaluating drug transport across the blood-brain barrier," Advanced Drug Delivery Reviews, vol. 36, no. 2-3, pp. 165–178, 1999.

[23] S. M. Jones and W. Spaans, "Rapid volumetric determination of indigo," Industrial and Engineering Chemistry, vol. 8, no. 11, pp. 1001-1002, 1916.

[24] G. D. Purvis and R. J. Bartlett, "A full coupled-cluster singles and doubles model: the inclusion of disconnected triples," The Journal of Chemical Physics, vol. 76, no. 4, pp. 1910–1918, 1982.

[25] E. Runge and E. K. U. Gross, "Density-functional theory for time-dependent systems," Physical Review Letters, vol. 52, pp. 997–1000, 1984.
[26] A. D. Becke, “A new mixing of Hartree-Fock and local density-functional theories,” *The Journal of Chemical Physics*, vol. 98, no. 2, pp. 1372–1377, 1993.

[27] C. Lee, W. Yang, and R. G. Parr, “Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density,” *Physical Review B: Condensed Matter and Materials Physics*, vol. 37, no. 2, pp. 785–789, 1988.

[28] J. D. Chai and M. Head-Gordon, “Long-range corrected hybrid density functionals with damped atom-atom dispersion corrections,” *Physical Chemistry Chemical Physics*, vol. 10, no. 44, pp. 6615–6620, 2008.

[29] J. S. Binkley and J. A. Pople, “Møller–Plesset theory for atomic ground state energies,” *International Journal of Quantum Chemistry*, vol. 9, no. 2, pp. 229–236, 1975.

[30] S. F. Boys and F. Bernardi, “The calculation of small molecular interactions by the differences of separate total energies. Some procedures with reduced errors,” *Molecular Physics*, vol. 19, no. 4, pp. 553–566, 1970.

[31] M. J. Frisch, *Gaussian*, Gaussian 09, Revision A.1., Inc, Wallingford CT, 2009.

[32] A. Zhang, H. Sun, and X. Wang, “Potentiating therapeutic effects by enhancing synergism based on active constituents from traditional medicine,” *Phytotherapy Research*, vol. 28, no. 4, pp. 526–533, 2014.

[33] L. Zazueta-Beltrán, L. Medina-Aymerich, N. Estela Díaz-Triste, A. E. Chávez-Piña, G. Castañeda-Hernández, and L. Cruz-Antonio, “Evidence against the participation of a pharmacokineti-netic interaction in the protective effect of single-dose curcumin against gastrointestinal damage induced by indomethacin in rats,” *Journal of Integrative Medicine*, vol. 15, no. 2, pp. 151–157, 2017.