

Original Article

Covalent protein binding and tissue distribution of houttuynin in rats after intravenous administration of sodium houttuynifonate

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Aim: To investigate the potential of houttuynin to covalently bind to proteins *in vitro* and *in vivo* and to identify the adduct structures.

Methods: Male Sprague-Dawley rats were intravenously injected with sodium houttuynifonate (10 mg/kg). The concentrations of houttuynin in blood, plasma and five tissues tested were determined using an LC/MS/MS method. The covalent binding values of houttuynin with hemoglobin, plasma and tissue proteins were measured in rats after intravenous injection of [^{14}C]sodium houttuynifonate (10 mg/kg, 150 mCi/kg). Human serum albumin was used as model protein to identify the modification site(s) and structure(s) through enzymatic digestion and LC/MSⁿ analysis.

Results: The drug was widely distributed 10 min after intravenous injection. The lungs were the preferred site for disposition, followed by the heart and kidneys with significantly higher concentrations than that in the plasma. The extent of covalent binding was correlated with the respective concentrations in the tissues, ranging from 1137 nmol/g protein in lung to 266 nmol/g protein in liver. Houttuynin reacted primarily with arginine residues in human serum albumin to form a pyrimidine adduct at 1:1 molar ratio. The same adduct was detected in rat lungs digested by pronase E.

Conclusion: This study showed that the β -keto aldehyde moiety in houttuynin is strongly electrophilic and readily confers covalent binding to tissue proteins, especially lung proteins, by a Schiff's base mechanism. The findings explain partially the idiosyncratic reactions of houttuynin injection in clinical use.

Keywords: sodium houttuynifonate; houttuynin; arginine-specific modification; pyrimidine adduct; LC/MSⁿ analysis; tissue distribution; houttuynin preparations

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Introduction

Idiosyncratic drug reactions are a major source of concern because they can cause drug treatment limitations and even drug withdrawals from the market. The toxic mechanisms remain largely undefined, but accumulating data suggests that the covalent binding of drugs or their reactive metabolites/intermediates to cellular proteins is associated with idiosyncratic toxicity^[1–4].

Drug-induced idiosyncratic reactions cannot be predicted from the pharmacological mechanism(s) of drugs, as they usually have a low occurrence rate of 1 in 1000 or even 1 in 100 000 patients^[5]. To date, no available animal models can accurately predict the toxic interactions of drugs with the human immune system. Several experimental methods have been used to understand this toxic phenomenon^[2]. Using radiolabeled

drugs is the most common approach to assess their potential for covalent binding with biological macromolecules and to evaluate the idiosyncratic risk.

Houttuynin injection (or sodium new houttuynifonate injection) was once widely used in clinical practice for the treatment of chronic bronchitis, pneumonia, upper respiratory infection, and other infectious diseases in China^[6, 7]. Despite its clinical efficacy, occasionally serious and even fatal allergic reactions have been reported during an intravenous (iv) drop infusion of these injections^[8–10]. As a result, the intravenous injection has been suspended since 2006 (although its oral preparations are still used in the clinic), and its safety has drawn great public attention.

The major active ingredients in houttuynin preparations are a class of β -keto aldehyde compounds^[11], including houttuynin (decanoyl acetaldehyde). It has been reported that some aldehyde compounds, such as formaldehyde^[12, 13], acetaldehyde^[14, 15], acrolein^[16, 17], 2-nonenal^[18, 19], and aldehyde intermediates formed by metabolism^[20–22] might react with protein

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nucleophiles to form aldehyde-protein adducts, which are capable of specifically interfering with costimulatory signals on T cells and of inducing immunotoxic effects^[23]. Therefore, we hypothesized that the acute or idiosyncratic toxicities of houttuyniae injection might be caused by the covalent binding of β -keto aldehyde compounds to cellular proteins.

In this study, houttuynin is used as a representative compound to investigate the *in vivo* distribution of the β -keto aldehyde-type structural moieties existing in houttuyniae injection and oral preparations, and their potential to covalently bind to proteins *in vivo*. To improve the water solubility of houttuynin, an adduct with sodium bisulfite (sodium houttuuyfonate, see Figure 1) was used, which could liberate houttuynin under physiological conditions. Human serum albumin (HSA) was used as a model protein to investigate the covalent binding chemical mechanism of houttuynin to target proteins through enzymatic digestion and LC/MSⁿ analysis.

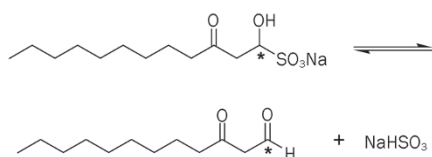


Figure 1. Reversible balance of sodium houttuuyfonate and houttuynin in aqueous solution. (*) denotes the radiolabeled carbon.

Materials and methods

Chemicals and reagents

Sodium houttuuyfonate (98.9% purity) was obtained from Qingping Pharmaceutical Co Ltd (Shanghai, China). [1-¹⁴C] Sodium houttuuyfonate (specific activity, 4.98 Ci/mol) was synthesized in our laboratory. HSA (Fraction V) was purchased from Sigma-Aldrich (St Louis, MO, USA). Pronase E was obtained from Roche Diagnostics GmbH (Mannheim, Germany). HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (St Louis, MO, USA) and HPLC-grade formic acid from Tedia (Fairfield, OH, USA). Other chemicals that were used were all of analytical reagent grade and commercially available.

Animals and treatment

The experiments were performed according to procedures approved by the Animal Care and Use Committee of the Shanghai Institute of Materia Medica (Shanghai, China). Fifteen male Sprague-Dawley rats (200±20 g) were divided randomly into five groups (three rats per group). In group 1, the rats received an iv administration of [1-¹⁴C]sodium houttuuyfonate at a target dose of 10 mg/kg (radioactivity: 150 μ Ci/kg) dissolved in normal saline solution at 2 mg/mL, and the animals were euthanized at 10 min after the injection; in groups 2–5, the rats were given non-radiolabeled sodium houttuuyfonate at the same chemical dose and administration route, and the animals were euthanized at 10 min, 1 h, 5 h,

and 24 h after dosing, respectively. For each group, blood was collected into heparinized tubes via exsanguinations. A portion of blood was centrifuged at 2000 \times g for 10 min to isolate plasma. The obtained plasma and the remaining blood were removed for the covalent binding and liquid chromatography tandem mass spectrometry (LC/MS/MS) analyses. Heart, liver, spleen, lung and kidney tissues were collected and stored at -80°C until analysis.

Quantification of houttuynin in rat blood, plasma, and tissues

Houttuynin concentrations in rat blood, plasma, and the five tested tissues were determined by LC/MS/MS employing pre-column derivatization, which was reported previously by our laboratory^[24]. Briefly, tissue samples of approximately 250 mg were homogenized with 1 mL of methanol. After centrifugation at 16000 \times g for 5 min, the supernatants were used to determine houttuynin concentrations in tissues. To 100 μ L of blood, plasma and tissue homogenate samples in glass tube, 50 μ L of benzophenone (2.0 μ g/mL, internal standard, IS) and 100 μ L of 2,4-dinitrophenylhydrazone solution were added. Houttuynin and IS were derivatized and subjected to LC/MS/MS analysis. Standard curves were fitted to the equations by a linearly weighed ($1/x^2$) least squares regression method in the concentration range of 10.0–20000 ng/mL for blood and plasma samples and 40.0–80000 ng/g for tissues.

In vivo covalent protein binding assay

Aliquots of samples (150 μ L of blood, 150 μ L of plasma, and 500 μ L of tissue homogenates) were placed in test tubes, and 1.5 mL of acetonitrile was added to each tube to precipitate protein. The mixtures were sonicated, vortexed for 10 min and were then placed at -20°C for 30 min. Samples were centrifuged at 2000 \times g for 10 min, and the precipitate was washed several times with 1.5 mL of acetonitrile to remove any unbound radioactivity. Aliquots of the supernatant were checked for radioactivity, and no more washing was performed until the radioactivity level was within two-fold of that of the control samples. The resultant protein pellets were then dissolved in 4 mL of 100 mmol/L sodium hydroxide. Subsequently, 50 μ L of the sample was transferred to a 1.5-mL Eppendorf tube, and 500 μ L scintillation fluid (AIM Research Co, Hockessin, DE, USA) was added. The sample was vortexed for 1 min. The radioactivity was measured by a PE 1450 liquid scintillation analyzer (Perkin-Elmer, Waltham, MA, USA), and the protein concentration was determined by Bradford assay using a standard kit (Bio-Rad)^[25]. Covalent binding to proteins was expressed as micromole equivalents per gram of protein.

In vitro covalent binding of [1-¹⁴C]houttuynin with HSA

HSA (0.05 μ mol, 0.50 mmol/L) was dissolved in 100 μ L of 100 mmol/L phosphate-buffered saline (PBS, pH 7.4) at 37°C. The reaction was started by adding [1-¹⁴C]sodium houttuuyfonate aqueous solution (684 nCi/ μ mol, 1.0 mmol/L) in a total incubation volume of 0.2 mL. Reactions were terminated at the pre-determined time points (0 min, 1 min, 10 min, 1 h, 3 h, 8 h,

and 24 h) by adding 600 μL of acetonitrile. The reaction samples were treated as described above. The total radioactivity and protein concentration of the incubation samples at each time point were measured as described above.

To further evaluate the covalent adduction of HSA by houttuynin, the washed protein from the 1 min to 24 h incubation periods was dissolved in 100 mmol/L PBS (100 μL , pH 7.4), and the protein concentrations were determined by the Bradford assay. The sample was treated with SDS-loading buffer and boiled for 5 min to denature HSA. The resulting HSA sample was then loaded on 8% SDS-polyacrylamide gels^[26]. After the first electrophoresis, the target protein band was stained with Coomassie and was cut and transferred into dialysis tubing. The band was electrophoresed for 1 h in 150 V to recovery the protein^[27]. After the second electrophoresis, 50 μL of running buffer solution containing [^{14}C]houttuynin-modified HSA in dialysis tubing was dissolved in 500 μL scintillation fluid, and the radioactivity was compared with the control sample by liquid scintillation counting.

Pronase E digestion

The washed protein pellets from rat blood, plasma, tissue homogenates and houttuynin-modified HSA were resuspended in 400 μL of water. Pronase E was dissolved in 100 mmol/L Tris buffer (pH 7.5) and 0.5% SDS solution^[28], giving a final concentration of 1.0 mg/mL. This solution was added to the protein suspensions (1:10, *w/w*). The mixture was incubated for 72 h at 37°C, then loaded onto a Waters Oasis[®] HLB C₁₈ cartridge (30 mg, 1 mL; Milford, MA, USA). The cartridge was pretreated sequentially with 2 \times 1 mL methanol and 2 \times 1 mL water. After loading the sample, the cartridge was washed with 1 mL water. The modified amino acids were eluted with 1 mL methanol. The eluate was evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μL acetonitrile/water (50:50, *v/v*). An aliquot of 20 μL was injected into the LC/MSⁿ system.

Instrumentation and analytical methods

An API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with a Turbo Ionspray (ESI) source and an Agilent 1100 HPLC system consisting of a G1322A vacuum degasser, a G1311A quaternary pump, and a G1316A column oven (Agilent Technologies, Waldbronn, Germany) was employed to determine houttuynin in blood, plasma and tissue samples. The chromatographic separation and MS conditions were the same as in the reference^[24].

LC/MSⁿ experiments for identifying the modification sites and structures of adducts were carried out on an Agilent 1200 HPLC system coupled with a 6330 MSD Trap XCT Ultra (Agilent Technologies, Waldbronn, Germany). The mass spectrometer was equipped with an ESI source. The ionization mode was positive. The interface and MS parameters were as follows: nebulizer pressure, 40 psi (N_2); dry gas, 12 L/min (N_2); dry gas temperature, 350°C; spray capillary voltage, 3500 V; skimmer voltage, 40 V; ion transfer capillary exit,

124 V; scan range, *m/z* 200–2000; spectra average, 3; and target 200000. For MSⁿ spectra, the fragmentation amplitude varied between 0.8 and 1.0 V. Data acquisition was performed in full-scan MS and MSⁿ modes (*n*=2 or 3). All data acquired were processed through Agilent Chemstation Rev B 01.03 software (Agilent, Palo Alto, CA, USA). Chromatographic separation was performed with a Capcell Pak C₁₈ UG 120 column (100 \times 4.6 mm id, 5 μm ; Shiseido, Tokyo, Japan) protected by a Security-Guard C₁₈ column (4.0 \times 3.0 mm id, 5 μm ; Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile with 0.1% formic acid (solvent A) and 0.1% aqueous formic acid (solvent B) running at a constant flow rate of 0.6 mL/min. The solvent gradient was initially held at 10% A for 2 min and then increased linearly to 60% A over another 13 min. It was kept at 60% A for an additional 1 min, then increased to 90% A in 0.5 min. Afterwards, it was kept at 90% A for 8.5 min and was finally immediately decreased to 10% A in 0.5 min, where it was held constant at 10% A for 4.5 min before the next sample injection.

High-resolution mass spectral analysis was performed using a quadrupole time-of-flight hybrid mass spectrometer (Synapt Q-TOF MS, Waters, Manchester, UK) operated in (+)ESI mode. The mass resolution was adjusted to 10 000 (FWHM). A leucine enkephalin solution (*m/z* 556.2771, Waters) was used as the internal calibration standard. The capillary and cone voltages were set at 2.7 kV and 20 V, respectively. The nebulization gas (N_2) was set to 700 L/h at a desolvation temperature of 300°C and a source temperature of 120°C.

Statistical analysis

The ORIGIN 6.1 software package (OriginLab, Northampton, MA, USA) was used for statistical analysis and data display. The data were expressed as the mean \pm standard deviation.

Results

Tissue distribution of houttuynin in rats

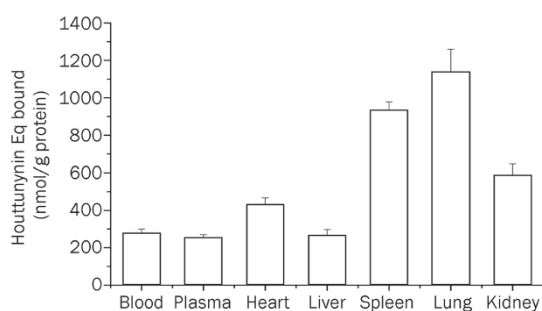
Houttuynin was rapidly distributed into tissues after an iv dose of 10 mg/kg, achieving peak concentrations within 10 min postdose. In the assayed rat tissues, houttuynin concentrations were highest in lung tissue, ranging from 68878 \pm 2839 ng/g to 625 \pm 229 ng/g from 10 min to 24 h, followed by heart, kidney, blood, spleen and plasma; they were lowest in liver tissue (Table 1). The ratios of tissue to the plasma concentration of houttuynin at 10 min were greater than the reference value of 1.0 except for liver tissue (ratio 0.38), demonstrating that houttuynin was distributed into tissues preferentially to plasma. Moreover, high ratios of blood to plasma (>1.0) at each sampling time from 10 min to 24 h suggested that houttuynin was not only distributed into plasma but also into erythrocytes, which might lead it to conjugate with hemoglobin in the erythrocytes. The concentrations of houttuynin in the blood, plasma and liver at 24 h were higher 6% of the peak concentrations.

The covalent binding levels of houttuynin to hemoglobin, plasma and tissue proteins at 10 min were high, ranging from 254 to 1137 nmol equivalents/g protein. Among them, the

Table 1. Tissue distribution of houttuynin in Sprague-Dawley rats following a 10 mg/kg iv dose of sodium houttuynifonate ($n=3$). Mean \pm SD.

	Concentration ($\mu\text{g/L}$; ng/g)			
	10 min	1 h	5 h	24 h
Blood	11 794 \pm 86.2	7 309 \pm 2819	2 693 \pm 504	803 \pm 254
Plasma	4 148 \pm 708	3 054 \pm 155	1 756 \pm 88.8	511 \pm 28.3
Heart	19 500 \pm 3962	8 315 \pm 1911	698 \pm 55.6	310 \pm 85.9
Liver	1 573 \pm 244	1 397 \pm 192	237 \pm 37.2	155 \pm 80.8
Spleen	5 259 \pm 758	2 207 \pm 211	317 \pm 41.1	311 \pm 142
Lung	68 878 \pm 2839	21 141 \pm 8364	1 430 \pm 298	625 \pm 229
Kidney	14 004 \pm 5188	9 816 \pm 4697	1 084 \pm 407	324 \pm 155

highest radioactivity existed in lung tissue, and the lowest radioactivity was found in plasma and liver (Figure 2).

**Figure 2.** Covalent binding assay of houttuynin with proteins of plasma, blood and five tissues after a 10 mg/kg iv dose of [$1\text{-}^{14}\text{C}$]sodium houttuynifonate to rats ($n=3$).

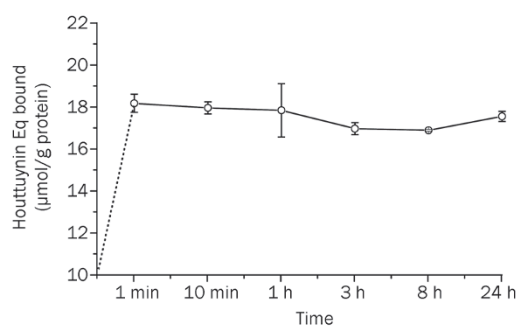
In vitro protein covalent binding assay

When [$1\text{-}^{14}\text{C}$]sodium houttuynifonate (1.0 mmol/L) was incubated with HSA, covalent adducts with HSA rapidly formed, reaching 18.2 ± 0.4 μmol equivalents/g protein after only 1 min of incubation and maintaining the binding level after 60 min of incubation (Figure 3). This yield was not statistically significant from that measured at the 24-h time point. The covalent binding of houttuynin to HSA was high and rapid, and multiple molecules of houttuynin were adducted to HSA.

SDS-PAGE analysis of the washed protein pellets obtained from the [$1\text{-}^{14}\text{C}$] sodium houttuynifonate and HSA incubation experiment also revealed the presence of one radiolabeled protein band with a molecular mass of 66.5 kDa. By liquid scintillation counting, the radioactivity of protein-bound [$1\text{-}^{14}\text{C}$]houttuynin was found to be 10 times higher than that of the control sample in the 1 min to 24 h incubation samples, which further established that houttuynin could readily covalently bind with HSA.

In vitro identification of pronase E-digested houttuynin-HSA adducts

To break down the modified HSA into individual amino acids,

**Figure 3.** Covalent binding assay of [$1\text{-}^{14}\text{C}$]sodium houttuynifonate (1.0 mmol/L) with HSA (0.25 mmol/L) following incubation.

native and houttuynin-treated HSA that had been washed were digested with pronase E and then analyzed by LC/MSⁿ. In Figure 4, peaks for five adducted amino acid residues designated as P1–P5 were observed at 14.0, 15.0, 17.7, 18.5, and 19.6 min, respectively.

High-resolution MS analysis revealed that the pronase E enzymatic product P1 contained four nitrogen atoms ($\text{C}_{18}\text{H}_{32}\text{N}_4\text{O}_2$), indicating that P1 corresponded to an arginine adduct. The protonated P1 molecule at m/z 337 was 162 Da higher than that of the arginine standard, indicating that the adduct was formed by a condensation reaction (1:1) of houttuynin with an arginine residue accompanied by the loss of two water molecules (Figure 5A). Taken this possible reaction pathway into account^[29], a reasonable mechanism for this condensation reaction is proposed in Figure 6. The initial reaction of the primary N^{α} -amino group of arginine occurred with the carbonyl carbon of houttuynin and resulted in the formation of a carbinolamine intermediate; next, one water molecule was eliminated in the reaction to form a Schiff's base. Subsequently, an intramolecular nucleophilic addition of an $\text{N}^{\alpha-1}$ amine to the remaining carbonyl carbon of the original houttuynin occurred. This resulted in the formation of a cyclic carbinolamine intermediate. Further rearrangement and dehydration of the exocyclic imine to a conjugated system formed a stable pyrimidine derivative. In the MS/MS spectrum (Figure 5A), P1 showed typical losses of 18 Da (H_2O), 35 Da (H_2O and NH_3), and 63 Da (NH_3 and HCOOH) from the $[\text{M}+\text{H}]^+$ ion. Collectively, the data suggested that the structure of P1 was a pyrimidine-type adduct in houttuynin and arginine residues^[30].

P2 exhibited the protonated molecule $[\text{M}+\text{H}]^+$ at m/z 327, which was 180 Da higher than that of the lysine standard. This indicated that the addition of one molecule of houttuynin to lysine residues was accompanied by the loss of one water molecule. In the MS/MS spectrum of P2 (Figure 5B), major fragment ions at m/z 310 and 264 were also 180 Da higher than those in the MS/MS of lysine, corresponding to a neutral loss of ammonia (NH_3 , loss of 17 Da) as well as formic acid (NH_3 and HCOOH , loss of 63 Da). The adduct could be easily reduced by NaBH_4 , resulting in a novel and stable adduct with an expected mass increment of 4 Da produced by the

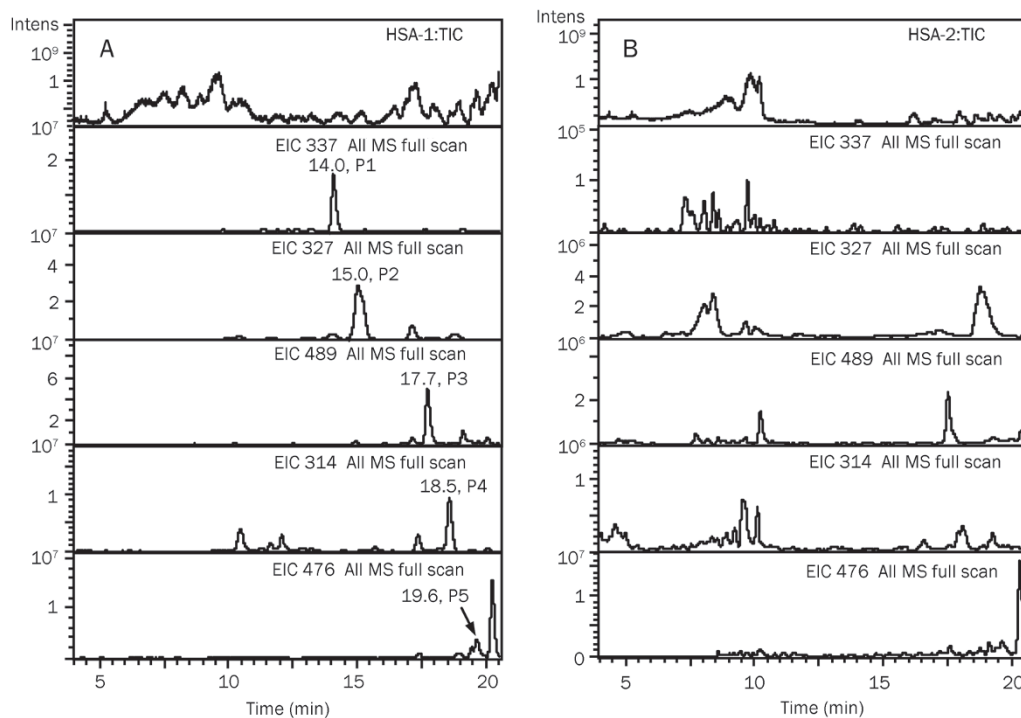


Figure 4. LC/MSⁿ analysis of the pronase E digest of HSA with houttuynin (A) and without houttuynin (B).

elimination of hydrogen. Based on the data, the P2 structure was proposed as a houttuynin-lysine Schiff's base. Theoretically, by a nucleophilic addition reaction, both aldehyde and ketone groups of houttuynin can react with the primary amine of lysine to form imine derivatives.

A reduction product of the Schiff's base adduct of houttuynin and N^α-t-BOC-L-lysine was synthesized and purified. The obtained authentic compound was characterized by MS and NMR. Compared with the NMR data of N^α-t-BOC-L-lysine, the synthesized adduct displayed an oxygenated methine proton at δ 3.68 (1H, m, H-3), corresponding to a carbon signal at δ 71.1 in the DEPT spectrum. This implied that the C-1 of the aldehyde group in houttuynin reacted with the primary amino group of BOC-lysine to form a Schiff's base adduct.

Data from multi-stage and high-resolution MS analyses also showed that the corresponding structure of P2 was a Schiff's base adduct of lysine and the aldehyde group, not lysine and the ketone group of houttuynin. In the MS/MS spectrum of P2, the elemental compositions of two fragment ions at m/z 173 and 156 were assigned as C₈H₁₇N₂O₂ and C₈H₁₄NO₂, respectively, by accurate mass measurement indicating that lysine structure existed. They were formed by C_α-C_β bond cleavage in houttuynin. Their mass fragment patterns are presented in Figure 5B. If the two ions were derived from the ketone adduct of houttuynin and lysine, their formation should be involved in the cleavage of two chemical bonds: one is via C_α-C_β bond cleavage, and another is via C-C cleavage in the houttuynin aliphatic chain, which requires high collision energies. This is difficult under an atmospheric pressure ionization mode. If a ketone adduct was formed, a fragment ion produced by acetaldehyde loss (-44 Da) should be observed in

its MS/MS spectrum. Based on the foregoing analysis, P2 was finally assigned as the reaction product of the aldehyde group of houttuynin with lysine. The result indicated that the aldehyde group of houttuynin was much more reactive than its ketone group under the conditions used in the present study (pH 7.4, 37°C) due to steric and electronic reasons.

LC/MSⁿ analysis of P3 displayed a molecular ion M⁺ at m/z 489, 342 Da higher than that of the lysine standard, indicating the addition of two molecules of houttuynin to lysine residues with the loss of three water molecules. The MS/MS spectrum of P3 gave only one fragment ion at m/z 360 (Figure 5C). P3 was confirmed to be a houttuynin-lysine pyridinium adduct by comparing its chromatographic behavior, MSⁿ and NMR data with the prepared standard.

P4 showed the protonated molecule [M+H]⁺ at m/z 314, 180 Da higher than that of the aspartate standard. This indicated the addition of one houttuynin molecule to an N-terminal aspartate residue accompanied by the loss of one water molecule. In the MS/MS spectrum (Figure 5D), a similar mass fragment pattern to that of P2 was displayed. The major fragment ions at m/z 160 and 142 were derived from C_α-C_β bond cleavage in houttuynin. Other fragment ions formed by the neutral loss of water (-18 Da), formic acid (-46 Da), and acetic acid (-60 Da) were also observed. As a result, P4 was tentatively identified as the Schiff's base adduct of houttuynin and N-terminal aspartate formed by aldehyde carbonyl addition reaction.

P5 exhibited similar MS behavior with P3. LC/MSⁿ analysis of P5 gave a molecular ion at m/z 476, which was 342 Da higher than that of aspartate, and a major fragment ion at m/z 360 (Figure 5E). This allowed us to assign P5 as the pyridinium derivative of houttuynin with N-terminal aspartate formed by the addition of two molecules of houttuynin to

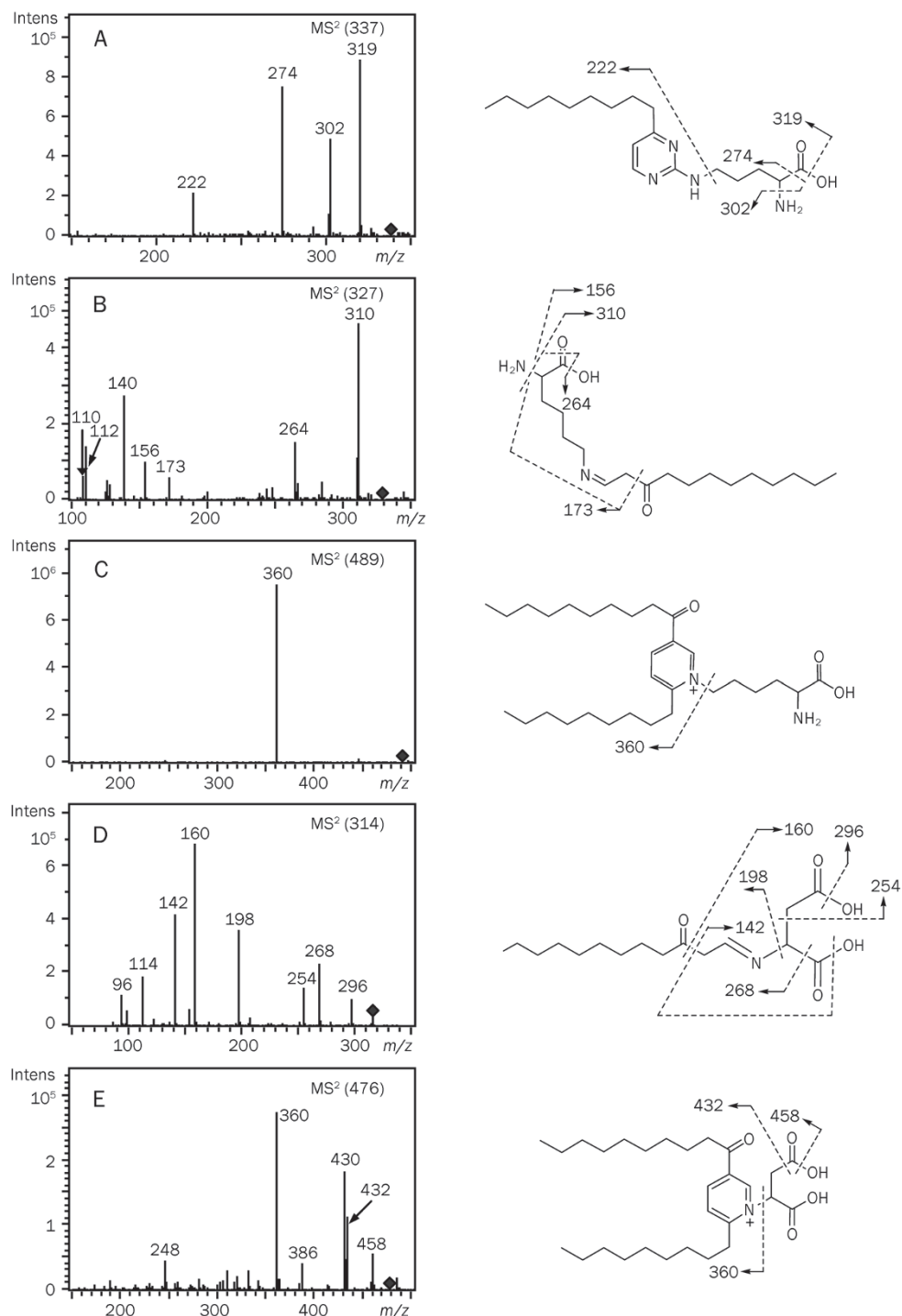


Figure 5. MS/MS spectra of the pronase E digest of HSA with houttuynin. (A) [M+H]⁺ at m/z 337 (P1, arginine adduct); (B) [M+H]⁺ at m/z 327 (P2, lysine adduct); (C) M⁺ at m/z 489 (P3, lysine adduct); (D) [M+H]⁺ at m/z 314 (P4, aspartate adduct); (E) M⁺ at m/z 476 (P5, aspartate adduct).

aspartate residues with the loss of three water molecules.

Identification of adducts of houttuynin with *in vivo* proteins

The blood, plasma and tissues collected at 10 min after an iv administration of [1-¹⁴C]sodium houttuynifonate were digested by pronase E and analyzed by LC/MSⁿ method. Compared with the blank samples, only one houttuynin-related adduct was detected in lung tissue (Figure 7). The adduct was assigned as an arginine-derived pyrimidine adduct by com-

paring its chromatographic behavior and MSⁿ spectra with the product P1 from the pronase E-digested houttuynin-HSA adducts.

Discussion

Accumulating evidence suggests that the covalent binding and formation of drug-protein adducts are related to drug organ toxicities of the liver, kidneys and lungs as well as to hypersensitivity reactions and carcinogenesis^[2,31]. Several pharma-

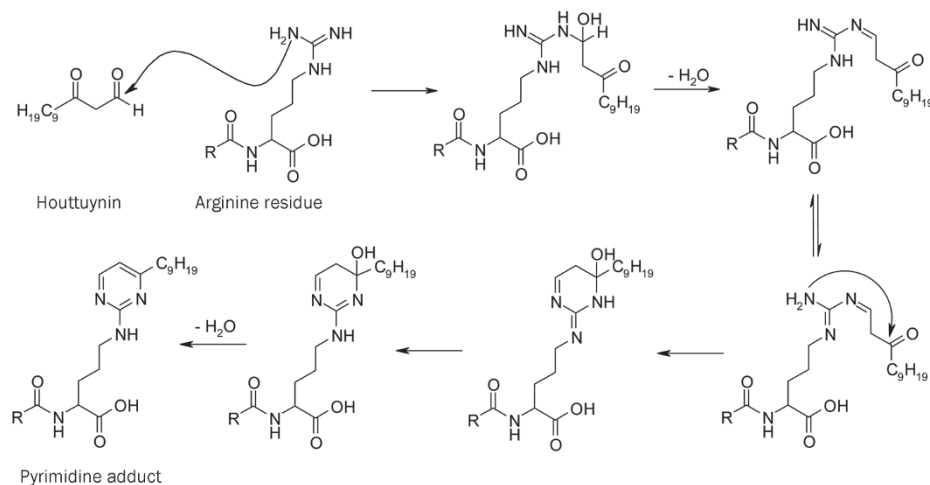


Figure 6. Proposed mechanism for the formation of the houttuynin-arginine adduct.

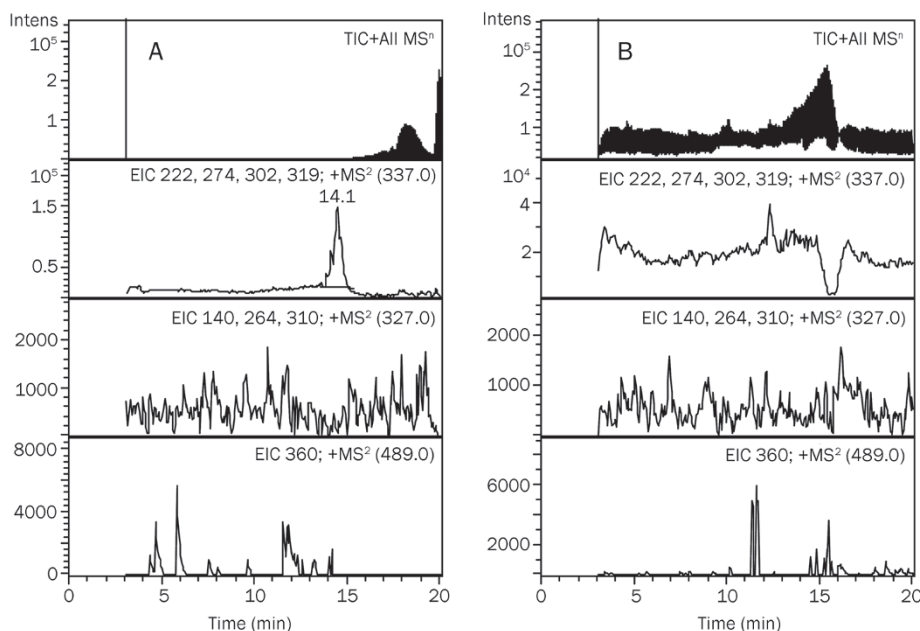


Figure 7. LC/MSⁿ analysis of the pronase E digest of lung tissue collected at 10 min after a 10 mg/kg iv dose of [1-¹⁴C]sodium houttuynifonate (A) and blank lung tissue (B).

ceutical companies, such as Merck and Pfizer, recommend that levels of covalent protein binding *in vivo* for a drug candidate should be <50 nmol drug equivalents/g protein, although they emphasized that other factors should also be considered on a case-by-case basis.

In the present work, the tissue distribution of houttuynin in rats was investigated. After an injection, houttuynin was distributed rapidly into different tissues and was eliminated slowly from the body. The drug concentrations in the lung were higher than those in any other tissues. The lung was identified as the target organ of houttuynin, which was consistent with its therapeutic efficacy for pneumonia. The total plasma radioactivity was compared with the houttuynin concentration measured by the LC/MS/MS method. It was found that the major drug-related component in plasma was unchanged houttuynin, not its metabolites. The low distribution level in the liver indicated that hepatic metabolism may

not be a significant pathway of houttuynin elimination. The covalent binding levels of houttuynin to the blood, plasma and tissue proteins were high, ranging from 254 to 1137 nmol equivalents/g protein, and were approximately 5 to 22 times higher than the acceptance criteria value of 50 nmol equivalents/g protein^[2]. The covalent binding level was closely correlated with houttuynin concentrations in rats.

HSA contains numerous nucleophilic residues that react with electrophiles. In addition, HSA is a common target of reactive species of drugs. In this experiment, HSA was used as a model protein to identify the structures of houttuynin-related adducts. The covalent binding of houttuynin with HSA was high and rapid, and the adducts formed mainly through a Schiff's base mechanism. Houttuynin selectively adducted with arginine guanidinium, lysine amines and the N-terminal amine of HSA under physiological conditions, and the resulting adducts contained a Schiff's base and pyridinium

formed by condensation reactions of houttuynin with lysine or N-terminal residues (1:1 or 2:1), as well as a pyrimidine adduct formed by a 1:1 houttuynin-arginine reaction. *In vivo*, only the houttuynin-arginine pyrimidine adduct was detected; the other adducts were not observed at a measurable concentration.

This study showed that β -keto aldehyde compounds are capable of covalently binding to cellular proteins. The major adduct in the study was houttuynin-arginine pyrimidine, mainly due to the alkalinity and nucleophilicity of arginine guanidinium. Similar pyrimidine adducts derived from methylglyoxal and arginine-containing proteins have been reported previously^[30]; however, little is known about their behaviors.

In conclusion, this study provided clear evidence that houttuynin is widely distributed into tissues after iv administration of sodium houttuynin, especially in lungs, and the β -keto aldehyde in houttuynin readily conferred covalent binding to tissue proteins. These findings may partially explain the idiosyncratic reactions to houttuynin injection. We should draw great attention to the safety of preparations containing houttuynin.

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Author contribution

Zhi-peng DENG and Xiao-yan CHEN were responsible for the study design, data analysis and paper writing. Zhi-peng DENG and Jian MENG conducted the experiments. Da-fang ZHONG was a senior advisor and provided valuable advice for this study and for writing the manuscript.

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