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VT204 in Vitro Protein Binding and Tissue Distribution Study

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Abstract: *By employing equilibrium dialysis combined with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to determine the plasma protein binding rates of KRAS-G12C inhibitor VT204 across five species (human, cynomolgus monkey, beagle, rat and mouse) and compare their differences. Additionally, validated HPLC-MS/MS methods were used to investigate the tissue distribution of VT204 in tumor-bearing mice. The results demonstrated: At three test concentrations (1, 3, and 10 μmol/L), human, cynomolgus monkey, and beagle exhibited plasma protein binding rates exceeding 95%. In contrast, rats (at 3μmol/L and 10μmol/L) and mice (at 1μmol/L) showed binding rates below 95.0%. Following intragastric administration of VT204 at 20 mg/kg, the compound distributed widely across all tested tissues, with peak concentrations achieved 1 hour post-administration. The tissue distribution profile (in descending order of concentration) was: small intestine > liver > tumor > kidney > heart > lung > spleen > brain.*

Keywords: *KRAS-G12C; VT204; protein binding rate; HPLC-MS/MS*

1. Introduction

Lung cancer remains the leading cause of cancer-related deaths globally. It is primarily classified into two subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with NSCLC accounting for approximately 85% of all lung cancer cases[1][2]. Currently, the standard treatments for NSCLC still rely on conventional modalities such as surgery, radiotherapy, and chemotherapy[3]. However, these therapies are often limited by frequent postoperative complications, substantial side effects, and suboptimal efficacy. There is therefore an urgent clinical need for more precise treatment strategies—specifically targeted therapy and immunotherapy—to effectively combat NSCLC while minimizing damage to healthy tissues. Recent studies have identified ten key mutated genes associated with NSCLC: TP53, KRAS, STK11, EGFR, BRAF, RBM10, ROS1, MET and RET. Among these, EGFR and KRAS have emerged as the most well-validated therapeutic targets in clinical practice[3]. KRAS is the most prevalent mutated oncogene in lung adenocarcinoma, with G12C mutations accounting for 30–40% of all KRAS mutation cases. Owing to the structural characteristics of the KRAS protein and its high affinity for GTP, it is difficult to bind with conventional small-molecule drugs, earning it the designation of an "undrug% and research fields [4][5][6].

The plasma protein binding rate refers to the percentage of a drug bound to plasma proteins (relative to the total drug concentration in plasma) following administration. It is a crucial pharmacokinetic parameter that reflects the distribution, metabolism, and excretion of drugs in the body [7]. It also serves as a key indicator for evaluating therapeutic efficacy and clinical safety [8]. When a drug enters the body, it forms non-covalent bonds with plasma proteins to generate bound drug molecules. Only the free form of the drug can penetrate lipid-based biological barriers to act on target organs and tissues, thereby exerting pharmacological effects. However, the binding of drugs to plasma proteins is reversible: the bound and free forms maintain a dynamic equilibrium. As the free form is eliminated through distribution and metabolism, the bound form dissociates to replenish the free fraction and sustain therapeutic effects [9]. In this study, equilibrium dialysis was used to determine the in vitro plasma protein binding rate of VT204—a novel KRAS-G12C inhibitor independently developed by Suzhou Wentian Medical Technology Co., Ltd.—across multiple species (human, cynomolgus monkey, beagle, rat and mouse). The findings clarified the pharmacodynamic and

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pharmacokinetic properties of VT204 in vivo, providing a theoretical basis for predicting free drug concentrations in humans during clinical trials. Additionally, a mouse xenograft model using NCI-H358 cells was established to evaluate VT204's tissue penetration capacity and potential toxicity risks, while supporting investigations into its in vivo mechanism of action.

2. Material

2.1 Drugs and Reagents

VT204 (purity 98%, Suzhou Wentian Pharmaceutical Technology Co., Ltd.); Terfenadine (purity 98.6%, Guangzhou Jiatu Technology Co., Ltd.); Ethanol (analytical grade, Shanghai Titan Technology Co., Ltd.); Phosphate Buffered Salt Solution (PBS) (Beijing SolaBIO Technology Co., Ltd.); Tween 80 (Shanghai Aladdin Biochemical Technology Co., Ltd.); Fetal Bovine Serum (FBS) (Thermo Fisher); RPMI-1640 culture medium (Biosharp); Penicillin-Streptomycin (P.S.) (Thermo Fisher); Trypsin-EDTA (Thermo Fisher); Matrix Gel (Martigel) (Corning Cellgro); Formic acid (LC/MS grade, Thermo Fisher Scientific); Ammonium acetate (HPLC grade, Thermo Fisher Scientific); Acetonitrile (HPLC grade, Thermo Fisher Scientific); Methanol (HPLC grade, Thermo Fisher Scientific); Ultra-pure water (custom-made by Suzhou Xuhui Testing Co., Ltd.'s fully automatic ultrapure water system); Dimethyl sulfoxide (DMSO) (HPLC grade, Thermo Fisher Scientific).

2.2 Instruments

High-performance liquid chromatograph (LC-20ADXR), API 4000 triple quadrupole tandem mass spectrometer (AB SciTech); fully automatic ultrapure water system (Suzhou Jianfeng Instrument Equipment Co., Ltd.); analytical balance (Secura 225D-1CN, Sartorius); high-speed refrigerated centrifuge (Centrifuge 5417R, Eppendorf); biological sample homogenizer (Hangzhou Aosheng Instrument Co., Ltd.); vortex mixer (Vortex-Genie2, Scientific Industries); 96-well balanced dialysis unit (HTDialysis LLC); balanced dialysis membrane (HTDialysis LLC).

2.3 Blank matrix

Blank plasma samples were obtained from mice, rats and beagles (EDTA-K2 anticoagulated; Shanghai Shengchang Biotechnology Co., Ltd.), as well as human blank plasma (EDTA-K2 anticoagulated; Zibo Municipal Hospital) and cynomolgus monkey blank plasma (EDTA-K2 anticoagulated; Suzhou Liao Biological Technology Co., Ltd.).

2.4 Experimental mice and experimental animals

NCI-H358 (CL-0400) tumor-bearing SPF-grade BALB/c nude mice (Wuhan Punosai Life Science Co., Ltd.) and naive SPF-grade BALB/c nude mice (Shanghai Shengchang Biotechnology Co., Ltd.)—both male and female, aged 6–8 weeks and weighing 16–18 g—were used in this study. The animals were housed in individually ventilated cages (IVCs) with adequate ventilation, under controlled conditions of 20–26℃ temperature, 40–70% relative humidity, and a 12-hour light/dark cycle. The animal use license number is SCXK (Shanghai) 2021-0002.

3. Methods

3.1 HPLC-MS/MS Analysis Method

Chromatographic conditions: ZORBAX Eclipse Plus C18(2.1mm×50mm column (3.5μm, Agilent); column temperature 40℃, autosampler temperature 4℃;the mobile phase was 5mmol/ L ammonium acetate (containing 0.1% formic acid) aqueous solution-methanol. Gradient elution program: Gradient elution (0–0.5 min, 35% B; 1.0–2.3 min, 85% B; 3.0 min, 35% B); the flow rate was 0.5 mL/min and the injection volume was 2μL.

Mass spectrometry conditions: electrospray ion source (ESI), voltage (IS) 5500V, ion source temperature (TEM) 550℃; atomizing gas pressure (GS1) 55p.s.i; auxiliary heating gas pressure (GS2) 55p.s.i; collision gas pressure (CAD) 10p.s.i; curtain air pressure (CUR) 25p.s.i; the detection method was multiple reaction detection mode (MRM) with positive ion scanning. VT204 ion pair was m/z

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515.0→452.0, uncluster voltage was 100V, collision voltage was 37V. The ion pair of terfenadine was m/z 472.0→436.4, the uncluster voltage was 66V, and the collision voltage was 45V.

3.2 Balanced Dialysis Sample Processing

(1) Preparation of standard solution: weigh the VT204 and terfenadine reference substances precisely. After quality correction using the correction factor, dissolve them in dimethyl sulfoxide (DMSO) to prepare 1 mg/mL stock solutions, and store them at-20℃.

Using acetonitrile as the diluent, the 1 mg/mL terfenadine stock solution was diluted to 40 ng/mL to prepare the internal standard solution for this experiment, which was stored at-20℃.

(2) Sample processing at the administration end: prepare the VT204 stock solution through stepwise dilution with 40% methanol to final concentrations of 100, 300, and 1000 μmol/L. Prepare the analyte working solutions (1, 3, and 10 μmol/L) by mixing the stock solution with blank plasma from five species at a volume ratio of 1:99. Transfer 150μL of each working solution to the dosing port of a pre-treated dialysis unit, and incubate at 37±1℃ in a CO_2incubator for 6 hours. After incubation, take 10μL of the sample from the dosing port and mix it with 90μL of phosphate buffer and 600μL of internal standard solution. Vortex thoroughly, then centrifuge at 15,400×g for 10 minutes at 4℃. Collect the supernatant and inject it into the HPLC-MS/MS system for analysis.

(3) Sample processing at the receiving end: add 150μL of blank phosphate buffer to the receiving chamber of the dialysis device, and incubate according to the conditions of the administration end. After incubation, collect 90μL of the sample from the receiving chamber, then add 10μL of blank plasma from the corresponding species and 600μL of internal standard solution. Vortex thoroughly, centrifuge at 15,400×g for 10 minutes at 4℃, and collect the supernatant for HPLC-MS/MS injection analysis.

(4) Preparation of reference substance: a phosphate buffer was prepared by dissolving one PBS tablet in 100 mL of ultrapure water containing 2 μL of Tween 80. Subsequently, add the blank plasma of five species according to a volume ratio of 1:99 respectively into the phosphate buffer to prepare the blank bio-matrix, which was used as the blank matrix for both the standard curve and quality control samples.

Using 40% methanol-water as the dilution, the VT204 stock solution was gradually diluted to 0.1, 0.2, 1, 2, 4, 10,20, and 40μmol/L standard curve working solution and 0.3, 16, and 32μmol/L quality control working solutions ;a series of calibration and quality control working solutions were added to the above blank biological matrix at a volume ratio of 1:19 to dilute them into 5, 10,50,100,200,500, 1000, and 2 000 nmol/L standard curve samples and 15,800 and 1 600 nmol/L quality control samples ; 10μL of each standard curve and quality control sample were mixed with 90μL of blank PBS buffer and 600μL of internal standard solution. After vortex mixing, the mixture was centrifuged at 15,400×g for 10 minutes at 4℃., then take the supernatant for HPLC-MS/MS sample analysis.

3.3 Data Statistics

The formula for calculating the free drug (fu) concentration of the compound in plasma and its binding rate (fb) in 100% plasma is:

$$\mathrm{fu} = \frac{C_R}{C_D} \times 100\% \quad (1),$$

$$\mathrm{fb} = 100\% - \mathrm{fu} \quad (2)$$

C_R: Compound concentration at the receiving end. C_D: Compound concentration on the administration side;

$$\mathrm{Recovery}(\%) = \frac{C_R + C_D}{C_1} \times 100\% \quad (3)$$

C_1: Concentration of reference sample compound

Evaluation criteria for data results are presented in Table 1[10].

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Table 1: Classification and acceptance criteria of compounds

Parameters	Classification	Standard
fb(%)	Low	fb<50%
	Middle	50% ≤fb< 95%
	High	95% ≤fb< 99%
Recovery(%)	Extremely high	fb≥99%
	Sufficient	70%-130%

3.4 Sample processing methods

(1) Animal tumor model experiments: NCI-H358 cells were cultured in vitro using RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37℃with 5% CO_2. Cells were passaged based on growth status. When reaching the exponential growth phase, cells were harvested and counted for animal inoculation. Log-phase NCI-H358 cells were digested with EDTA-trypsin. After digestion, the cells were resuspended in 200μL of 50% serum-free RPMI-1640 medium matrix gel mixture, with the entire process performed on ice to maintain cell viability. Subsequently,200μLof the aforementioned cell suspension containing5×10^6cells was subcutaneously injected into the right neck and dorsal regions of nude mice. This procedure was repeated for 9 male mice to establish tumor models.

(2) Tumor animal experiments: when tumors reach an average volume of approximately 200 mm^3, mice were selected based on tumor size and body weight. A single intragastric administration of 20 mg/kg was given, followed by whole blood collection at 1h, 4h, and 24h post-administration (3 mice per group). EDTA-K2-prepared plasma was stored at-80°C. After blood collection, each group underwent CO_2euthanasia and cervical dissection. Organs (heart, liver, lung, spleen, kidney, small intestine, brain, and tumor) were rapidly frozen in liquid nitrogen. Tissue samples were homogenized using a homogenizer with a 1:4 (g: ml) methanol-water mixture, and the homogenate was stored at-80°C for subsequent analysis.

(3) Homogenate Sample Pre-treatment: a ccurately pipet 50μL of homogenate and add 300μL of internal standard solution. Vortex mix and incubate for 3 minutes, then centrifuge at15,400×g for 10 minutes at 4℃. Dilute the supernatant with 85% methanol-water mixture (1:1 v/v) and vortex thoroughly. Inject into HPLC-MS/MS for analysis.

4. Results

4.1 Plasma protein binding rate study

The plasma protein binding rate of VT204 was detected by equilibrium dialysis. Five blank substrates of mouse, rat, beagle dog, cynomolgus monkey and human plasma were used to prepare three samples at three concentrations (1, 3, 10μmol/L), and each concentration was prepared in three aliquots in parallel. At the end of the incubation, the concentration of CD at the drug administration end and the sample concentration of CR at the receiving end were measured. The results of plasma protein binding rate of VT204 in five species were calculated according to the formula, as shown in Table 2. The results showed that the recovery rates of VT204 in the plasma substrates of the five species were between 71.2 and 98.0%, which met the requirements of the test. In the study of plasma protein binding rate, the plasma protein binding rate of VT204 at all concentrations of human, monkey and dog was higher than 95.0 %, which had a high plasma protein binding rate. The binding rates of VT204 in the plasma of rats and mice were different at different concentrations. The binding rates of VT204 in the plasma of rats at low concentration (1μmol/L) were 86.6%, and those in mice at medium concentration (3μmol/L) and high concentration (10μmol/L) were 92.4% and 93.9%, respectively. The remaining concentrations of the two species showed a high degree of protein binding.

Table 2: Plasma protein binding rate results of VT204 in different species

Species	Spiked Con. (μmol/L)	fu %	fb %	Recovery %
Human	1	0.7	99.3	71.2
	3	1.3	98.7	73.9
	10	3.9	96.1	75.9

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Monkey	1	4.9	95.1	74.0
	3	2.9	97.1	81.2
	10	6.7	93.3	84.4
Dog	1	3.7	96.3	80.6
	3	2.8	97.2	90.0
	10	1.8	98.2	83.5
Rat	1	13.4	86.6	80.7
	3	2.4	97.6	80.5
	10	2.3	97.7	79.9
Mouse	1	4.8	95.2	94.1
	3	7.6	92.4	95.3
	10	6.1	93.9	98.0

4.2 Tissue Distribution Study of VT204 in Tumor-Mediated Mice

The tumor mouse model was used as the research object. A single dose of 20mg/kg VT204 was given by gavage, and then the tissue samples of the mice were processed to determine the drug content. Subsequently, tissue samples from the mice were processed and analyzed for drug concentration, with results presented in Table 3.The distribution of VT204 exposure in each tissue is shown in Figure 1. The results of the bar chart showed that VT204 had strong penetration and was evenly distributed in all tissues and organs. It was found that the concentration of VT204 in all tissues reached the peak at 1h after administration, and then showed a downward trend at 4h and 24h after administration. At the same time, we found that VT204 could not be detected in all tissues except tumors 24h after administration. The results showed that VT204 was absorbed synchronously into the tissues, distributed widely and had targeting property, and had no accumulation except in the tumor tissues. The distribution degree of VT204 was in the order of small intestine > liver > tumor > kidney > heart > lung > spleen > brain.

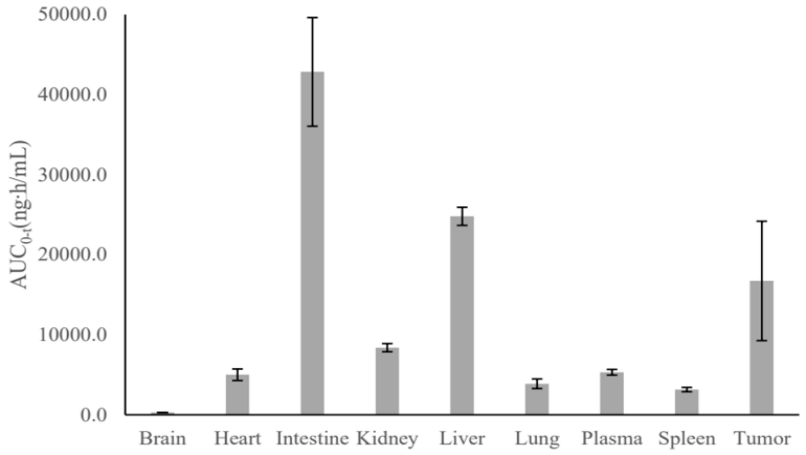

Figure 1: illustrates the tissue distribution of VT204 following a single intragastric administration of 20 mg/kg.

Table 3: Average tissue distribution of VT204 after a single intragastric administration of 20 mg/kg (Mean ± SD, n=3)

Tissue	Concentration (ng/g)			AUC_{0-t} (ng·h/mL)
	1h	4h	24h	
Heart	2102±436.8	539±344.0	-	5011±719.9
Liver	9900±1625.6	3333±2779.9	-	24800±1129.6
Spleen	1315±149.1	350±230.7	-	3154±268.9
Lung	1545±169.0	526±367.7	-	3879±603.0
Kidney	3520±416.4	902±519.1	-	8392±512.6
Intestine	17950±3627.3	4623±2128.5	-	42835±6785.5
Tumor	3663±558.0	1015±374.0	42.0±9.8	16728±7453.4
Brain	31.3±3.2	-	-	15.6±1.6
Plasma	1247±225.0	1883±514.2	-	5318±356.5
(ng/mL)				

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5. Discussion

This study employed a combination of equilibrium dialysis and HPLC-MS/MS to determine the plasma protein binding rates of VT204 (a KRAS-G12C inhibitor) at three concentrations (1, 3, and 10 μmol/L) across five species: humans, cynomolgus monkeys, beagles, rats and mice. Results showed that VT204 recovery rates in the matrices of all five species ranged between 71.2% and 98.0%, meeting experimental requirements and ensuring reliable data for plasma protein binding rate analysis. In the binding rate evaluation, humans, cynomolgus monkeys, and beagles exhibited high binding rates (over 95%) at all three concentrations (1, 3, and 10μmol/L), while rats (3μmol/L and 10μmol/L) and mice (1μmol/L) showed moderate binding rates (below 95%). The differences in binding rates across species and concentrations may be attributed to variations in plasma protein concentrations (e.g., albumin or α-1 acid glycoprotein), structural differences, or binding site specificity. VT204 demonstrates high plasma protein binding across species, indicating its potential as a drug reservoir. When the free drug is eliminated through distribution and metabolism, the bound drug can continuously release free drug molecules, maintaining relative stability and sustained efficacy. However, high plasma protein binding may increase drug side effects or toxicity. Therefore, monitoring free drug concentrations is essential during drug combination therapy. Furthermore, the small variation in protein binding rates across species suggests that VT204 may exhibit high consistency in its preclinical species and human pharmacokinetics, supporting its further development.

This study also investigated the tissue distribution of VT204 in tumor-bearing mice. Results showed that VT204 rapidly distributed to various tissues within 1 hour after administration, reaching peak levels before declining, which coincided with the peak time of blood concentration. Within 24 hours of administration, VT204 concentration was undetectable in all tissues except tumor tissue, demonstrating rapid clearance in non-tumor tissues and reduced organ toxicity risk, indicating good drug safety. The detectable concentration in tumor tissue likely stems from its unique physiological characteristics, such as high vascular permeability and abundant neovascularization, which help maintain drug concentration in tumors and enhance anti-tumor efficacy, providing crucial evidence for VT204's development as an anticancer agent. Notably, VT204 shows the highest concentration in the small intestine, suggesting absorption in this region, followed by liver and kidney tissues, indicating primary excretion through these organs. This requires further attention to potential toxicity risks. The lowest concentration was observed in brain tissue, with undetectable levels within 4 hours, indicating poor blood-brain barrier penetration and minimal central nervous system toxicity. Future clinical trials should consider including brain metastasis patients in the indication, and if so, explore combination therapies or optimized strategies.

6. Conclusion

VT204 exhibited plasma protein binding rates exceeding 86.6% across five species (rat, mouse, human, crab-eating macaque and beagle), with over 95% binding in most species and at various concentrations, demonstrating high protein binding. Following a single intragastric administration of 20 mg/kg to tumor-bearing mice, VT204 rapidly distributed through tissues in the following order: small intestine> liver> tumor> kidney> heart> lung> spleen> brain.

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