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Article

Pharmacokinetic Studies of a Novel Multikinase Inhibitor for Treating Cancer by HPLC–UV

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Small-molecule inhibitors are promising antitumor drugs. We have designed and synthesized a novel multi-targeted inhibitor, 2-methylcarbamoyl-4-{4-[3-(trifluoro-methyl)benzamido]phenoxy}pyridinium (SKLB610), that potently inhibits human tumor growth. In the study, the pharmacokinetic profile of SKLB610 was investigated. A simple, rapid and sensitive high-performance liquid chromatography–ultraviolet detection method was developed for the determination of SKLB610 in rat plasma. Samples were extracted with methanol and SKLB610 was separated on a C18 column using a mobile phase system consisting of 55% acetonitrile and 45% water, with ultraviolet detection at 270 nm. Sorafenib was used as the internal standard. The retention times of SKLB610 and the internal standard were 5.6 and 8.1 min, respectively. The quantification limit was 67 ng/mL. The calibration curves were linear over a concentration range of 0.1–50 μg/mL. The inter-day and intra-day accuracy and precision were within ± 10%. The recovery and stability of the assay were evaluated from spiked rat plasma. The method was successfully applied to a pharmacokinetic study of SKLB610 in rats. The pharmacokinetic profile of SKLB610 indicated that the oral formulations should be further optimized to improve bioavailability and intravenous formulation of SKLB610 should be developed.

Introduction

Targeted cancer therapies of small-molecule inhibitors against one or more of tumor-associated targets have become one of the major research areas for drug development of cancer therapies (1). We have designed and fully synthesized a novel multi-targeted inhibitor 2-methylcarbamoyl-4-{4-[3-(trifluoro-methyl) benzamido]phenoxy}pyridinium (SKLB610) that could potently and selectively inhibit activities of tumor-associated multikinases, primarily including Polo-like kinase 1 (PLK1) (2–5), vascular endothelial growth factor receptor 2 (VEGFR2) (6, 7) and platelet-derived growth factor receptor (PDGFR) (8, 9). Our previous research (10) proved that SKLB610 could inhibit the proliferation of a panel of human cancer cells and the growth of several human tumor xenografts in mice, including human non-small cell lung cancer cell line A549 and human colorectal cancer cell line HCT116. SKLB610 can also inhibit angiogenesis both *in vitro* and *in vivo*. Moreover, the acute toxicity test found that no adverse effect level value of SKLB610 was greater than 3 g/kg in rats after intraperitoneal administration of suspension. All of these data suggested that SKLB610 is a potential candidate as an anticancer agent and is worth further investigation.

It is necessary and urgent to investigate the pharmacokinetic profile of SKLB610, which will benefit in developing a suitable formulation for its further clinic application. No method has been developed to determine SKLB610 in plasma. Therefore, the aim of the present work is to develop a rapid and effective isocratic chromatographic procedure for the determination of SKLB610 in plasma, and then to study the *in vivo* pharmacokinetics of SKLB610 in rats using this method.

Experimental

Chemicals and reagents

SKLB610 (MW = 405.37) was synthesized in the State Key Laboratory of Biotherapy, Sichuan University (China) (purity > 98%). Sorafenib (MW = 637.03), used as internal standard (IS), was purchased from Shanghai Targsense Scientific Co. (China). Acetonitrile [high-performance liquid chromatography (HPLC) grade] was obtained from Tedia (Fairfield, OH) and used without further purification. All other reagents and solvents used in this experiment were of the highest purity commercially available. The pure water was prepared using the Millipore purification system (Molsheim, France).

Preparation of calibration standard and quality control samples

Stock standard solutions of SKLB610 and sorafenib were prepared by dissolving each substance in methanol to yield final concentrations of 50 and 10 μg/mL. The working standard SKLB610 solutions were prepared by diluting its stock standard solution with methanol to a range from 0.1 to 50 μg/mL. The calibration curve solutions were prepared as follows: 100 μL of the working solution was added to 1.5 mL Eppendorf centrifuge tubes and evaporated to dryness by a gentle stream of nitrogen (CM-24 nitrogen evaporators, Thomson Instrument Company; Oceanside, CA) in a 37°C water bath, and then 100 μL of blank rat plasma was added into the previously described tubes and vortexed for 15 s in a Liquid Fast Vortex-5 Mixer 104 (Qilinbeier Apparatus Co.; China) to obtain the concentrations of 0.1, 0.25, 0.5, 1, 2, 5, 10, 25 and 50 μg/mL.

The quality control (QC) samples were separately prepared in the same way at concentrations of 0.25, 2 and 25 μg/mL.

Apparatus and HPLC procedure

The HPLC system consisted of a LC-20AD pump, a DGU-20A5 degasser, a SIL-20AC auto sampler, a CTO-20A column oven and a SPD-M20A diode array detector. Chromatographic

separations were carried out on a Thermo C18 reverse phase column (150×4.6 mm, 5 μm), equipped with a Sunfire TM C18 guard column (20×4.6 mm, 5 μm) (Waters Technologies, Ireland). The mobile phase consisted of a premixed isocratic mixture of acetonitrile and water (55:45, v/v). It was newly prepared using double distilled deionized water, filtered through a 0.22-μm membrane filter and degassed via an online degasser. The injection volumes for samples and standards were 20 μL and eluted at a flow rate of 1.0 mL/min at 35°C. The eluent was monitored at 270 nm.

Sample preparation procedure

To 100 μL of rat plasma (blank, calibration standard, QC samples or unknowns), 10 μL of sorafenib (IS) stock standard solution was added and vortexed for 15 s, and then 200 μL of methanol was added and vortexed for 20 s. The mixture was centrifuged at 13,000 rpm for 10 min in a 5414 D centrifuge (Eppendorf, Germany) to precipitate the proteins. Twenty microliters of supernatant was injected into the HPLC for analysis.

Validation of the method

For method validation, selectivity, linearity, quantification limit, precision, accuracy, stability and recovery were determined according to Food and Drug Administration (FDA) guidelines (11).

The method selectivity was verified by comparing the chromatograms of the blank plasma sample and plasma sample spiked with SKLB610 and IS.

Calibration curves were constructed by linear least-squares regression analysis plotting of peak-area ratio (SKLB610/IS) versus the drug concentrations.

The lower limit of quantification (LLOQ) was defined as the lowest plasma concentration in the calibration curve that can be quantitatively measured with acceptable precision and accuracy (within 20%).

The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.25, 2 and 25 μg/mL. The intra-day accuracy and precision was evaluated with five aliquots of each sample, accompanied by a single calibration curve. The inter-day accuracy and precision was evaluated with five aliquots of each sample in three consecutive assays. The accuracy of the method was expressed as the relative error (RE) by comparing calculated to nominal concentrations. The precision was expressed as relative standard deviation (RSD).

The stability of SKLB610 was evaluated by analyzing rat plasma containing SKLB610 at 0.25, 2 and 25 μg/mL after storage at 4°C (auto-sampler) for 24 h and after three freeze-thaw cycles.

The efficiency of the extraction procedure was observed at low (0.25 μg/mL), medium (2 μg/mL) and high (25 μg/mL) concentrations. Six extraction samples were prepared by the same method as the calibration curve solutions, and the relative control samples were prepared in the same way, except that blank plasma was substituted by water. The recovery was calculated by comparing the respective peak areas of the extracted samples to the control samples. The efficiency of the extraction procedure over the IS was determined at a single concentration of 1 μg/mL.

Animal experiment and sample collection

Male Wistar rats (body weight 200 ± 20 g) were supplied by the Experimental Animal Center (Sichuan University, China) and kept in environmentally controlled quarters (approximately 25°C) for at least one week before use. All experimental procedures and animal care complied with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, and were approved by the Institutional Animal Care and Use Committee of Sichuan University.

The pharmacokinetic study of SKLB610 was carried out via two routes of administration (oral administration and intravenous administration). SKLB610 suspension for oral administration (IG) was prepared by dispersing SKLB610 into aqueous solution containing 0.5% sodium carboxymethylcellulose (w/v). SKLB610 solution for intravenous administration (IV) was obtained by dissolving SKLB610 in mixed solvents (dimethylsufoxide–saline = 9:1, v/v).

Rats were fasted for 12 h with free access to water before administration and divided randomly into two groups. SKLB610 suspension and SKLB610 solution at a dose of 50 mg/kg SKLB610 were administered. A 250-μL blood sample was collected into heparinized tubes by retro-orbital puncture at 0.083, 0.25, 0.5, 1, 2, 3, 4, 8, 10, 12 and 24 h after a single dose by administration of 50 mg/kg SKLB610 ($n = 6$). The blood samples were centrifuged at 7,000 rpm for 10 min to obtain the plasma. The plasma samples were stored at −20°C until analysis.

The plasma concentrations of SKLB 610 were determined by the developed HPLC method. The pharmacokinetic parameters were calculated by the pharmacokinetics–intelligent analysis module of DAS 2.1.1 software. Total areas under the curve (AUC) after intravenous administration and oral administration were determined by extrapolation from time 0 to infinity, respectively. The oral bioavailability of SKLB610 was estimated using the ratio of dose-normalized AUC values following oral and IV administrations.

Results

Method specificity

The chromatographic run time for the extracted plasma samples was 10 min. The retention times for SKLB610 and IS were 5.6 and 8.1 min, respectively. Figure 1 shows the representative HPLC chromatograms of blank plasma (Figure 1A), the standard solution containing at concentration of 2 μg/mL and IS at concentration of 1 μg/mL (Figure 1B), blank plasma spiked with SKLB610 at concentration of 2 μg/mL and IS at a concentration of 1 μg/mL (Figure 1C), the plasma at 1 h of a rat following a single oral administration of SKLB610 at 50 mg/kg (Figure 1D), and the plasma at 1 h of a rat following an single intravenous administration of SKLB610 at 50 mg/kg (Figure 1E). No endogenous interference was found to interfere with the SKLB610 and IS peaks.

Linearity

The standard curves of SKLB610 in rat plasma were linear in the concentration range of 0.1–50 μg/mL. A typical equation of the calibration curve was obtained as follows: $y = 0.297x +$

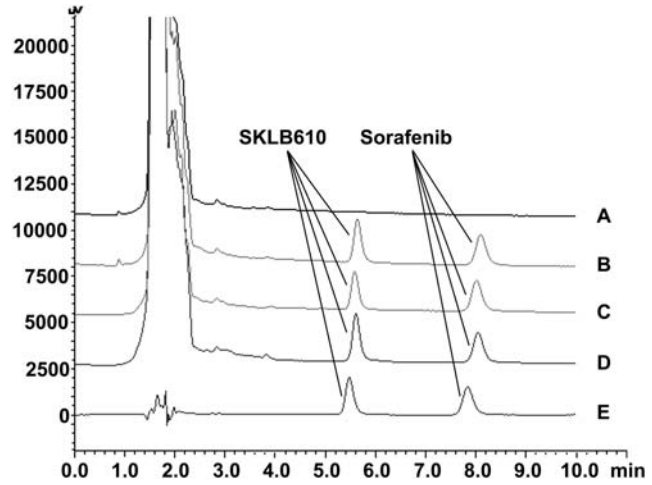

Figure 1. Representative HPLC chromatograms of blank plasma (A); standard solution containing at concentration of 2 μg/mL and IS at concentration of 1 μg/mL (B); blank plasma spiked with SKLB610 at concentration of 2 μg/mL and IS at concentration of 1 μg/mL (C); plasma of a rat at 1 h following an single oral administration of SKLB610 at 50 mg/kg (D); plasma of a rat at 1 h following an single intravenous administration of SKLB610 at 50 mg/kg (E).

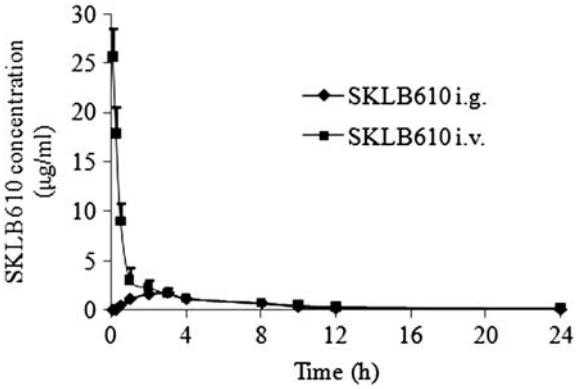

Figure 2. Pharmacokinetics of SKLB610 after intravenous administration (IV) and oral administration (IG) to rats. Animals received a dose of 50 mg/kg. Data were expressed as mean ± SD, $n = 6$ per time point.

0.231 ($r = 0.9999$), where y was the peak–area ratio of SKLB610 to IS and x was the plasma concentration of SKLB610, respectively.

LLOQ

The LLOQ for SKLB610 was 67 ng/mL, which was sufficient for rat pharmacokinetic studies following oral and intravenous administration of SKLB610.

Precision and accuracy

The intra-day and inter-day accuracy and precision results in rat plasma are summarized in Table I.

Stability

The stability of plasma samples was determined in five replicates. The results are displayed in Table II.

Table I
Intra-Day ($n = 5$) and Inter-Day ($n = 15$) Precision and Accuracy Analysis of SKLB610 in Rat Plasma

Concentration (μg/mL)		0.25	2	25
Accuracy	Mean bias (RE%)	3.8	2.5	1.3
Precision	Intra-day (RSD%)	3.0	2.2	1.7
	Inter-day (RSD%)	4.2	3.9	2.8

Table II
Stability of Plasma Samples Under Indicated Conditions ($n = 5$)

Concentration (μg/mL)		0.25	2	25
RE%	4°C, 24 h	1.4	1.1	0.5
	Three freeze-thaw cycles	4.1	3.5	2.1

Table III
Recoveries of SKLB610 and IS ($n = 5$)

	SKLB610			IS
Concentration (μg/mL)	0.25	2	25	1
Recovery (%)	84.9 ± 7.5	85.2 ± 6.4	87.7 ± 5.8	98.1 ± 2.6

Extraction recovery

All results of the extraction recovery tests are shown in Table III. Mean values of recoveries were 85.9% for SKLB610 and 98.1% for IS, respectively.

Method application

The present HPLC method was successfully applied to study the pharmacokinetics of SKLB610 in rats. The mean plasma concentration versus time curves are presented in Figure 2 ($n = 6$). The major pharmacokinetic parameters of SKLB610 were calculated by non-compartment model and demonstrated in Table IV. The oral bioavailability of SKLB610 delivered in suspensions was calculated to be approximately 40%.

Discussion

The chemical structures of SKLB610 and sorafenib are shown in Figure 3. The skeletal structures of two compounds are clearly similar. Therefore, sorafenib was selected as the IS of SKLB610. The extraction recoveries of SKLB610 and sorafenib were similar, which demonstrated that sorafenib was suitable to be used as the IS of SKLB610.

The HPLC method of SKLB610 determination was developed according to the quantification method of sorafenib. HPLC–tandem mass spectrometry (MS-MS) methods (12–14) and HPLC–ultraviolet (UV) assays (15–17) have been reported for sorafenib determination in human plasma, mouse plasma or mouse serum. HPLC–MS-MS equipments are expensive and not generally available (18). Thus, HPLC–UV assay was used in the current study. The RSD values of intra-day and inter-day accuracy and precision were less than 15% and the RE% values were all within ± 15%, which showed that the present method had a satisfactory accuracy and precision. The RE% values of stability of plasma samples under indicated conditions were all

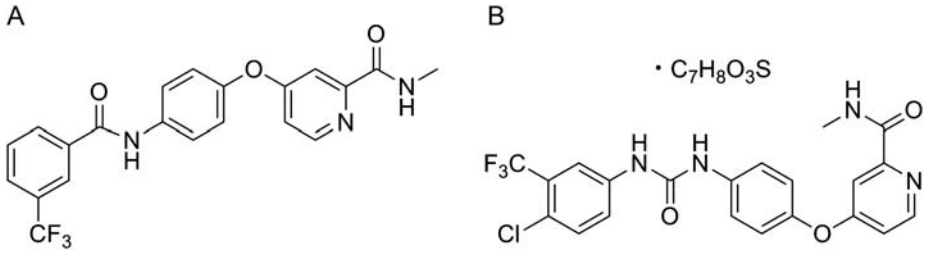

Figure 3. Chemical structures of SKLB610 (A); sorafenib (IS; B).

Table IV
Pharmacokinetic Parameters of SKLB610 in Rats for the Different Administration Routes

Parameters		C_{max} (μg)	T_{max} (h)	$T_{1/2}$ (h)	AUC (μg h/mL)	F (%)
Route	IV	25.60 ± 2.82	0.083	7.68 ± 4.10	29.15 ± 5.60	—
	IG	1.78 ± 0.14	2.75 ± 0.50	2.68 ± 0.78	11.63 ± 0.84	39.90

within ± 15%, which confirmed that plasma samples were stable at 4°C for 24 h and after three freeze–thaw cycles.

According to the oral bioavailability of SKLB610 delivered in suspensions, the novel formulations need be developed to further improve the bioavailability of SKLB610. The intravenous formulation of SKLB610 displayed higher concentration in blood, thereby suggesting that IV administration might be one of potential strategies for its clinic application. Our previous research has proved that SKLB610 was poorly water-soluble (only 0.34 μM saturation solubility in water). Thus, to develop an intravenous formulation containing enough SKLB610 to satisfy the dose acquirement of long-term toxicity test represents a real challenge.

Conclusion

A sensitive, selective and rapid HPLC method was developed for the quantification of SKLB610 in rat plasma. This method was proved to be linear over the range of 0.1–50 μg/mL. The method was successfully applied to the pharmacokinetic study of SKLB610 in rats. The pharmacokinetic profile of SKLB610 implied that the oral formulations should be further optimized to improve bioavailability and an intravenous formulation of SKLB610 must be developed.

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