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Characterization of Preclinical in Vitro and in Vivo Pharmacokinetic Properties of KPLA-012, a Benzopyranyl 1,2,3-Triazole Compound, with Anti-Angiogenetic and Anti-Tumor Progressive Effects

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Abstract : KPLA-012, a benzopyranyl 1,2,3-triazole compound, is considered a potent HIF-1α inhibitor based on the chemical library screening, and is known to exhibit anti-angiogenetic and anti-tumor progressive effects. The aim of this study was to investigate the pharmacokinetic properties of KPLA-012 in ICR mice and to investigate in vitro characteristics including the intestinal absorption, distribution, metabolism, and excretion of KPLA-012. The oral bioavailability of KPLA-012 was 33.3% in mice. The pharmacokinetics of KPLA-012 changed in a metabolism-dependent manner, which was evident by the low recovery of parent KPLA-012 from urine and feces and metabolic instability in the liver microsomes. However, KPLA-012 exhibited moderate permeability in Caco-2 cells (3.1×10^{-6} cm/s) and the metabolic stability increased in humans compared to that in mice (% remaining after 1 h; 47.4% in humans vs 14.8% in mice). Overall, the results suggest that KPLA-012 might have more effective pharmacokinetic properties in humans than in mice although further studies on its metabolism are necessary.

Keywords : KPLA-012, benzopyranyl 1,2,3-triazole compound, pharmacokinetics, metabolic instability

Introduction

Hypoxia-inducible factors (HIFs), master regulators for oxygen (O2) homeostasis, mediate several transcriptional changes in response to low O2 tension.[1,2] HIF-1 consists of an O2-sensitive α subunit and a constitutively expressed β subunit.[3] HIF-1α is constantly synthesized, but is rapidly degraded under normoxic conditions. This process is initiated by the hydroxylation of two proline residues (402 and/or 564) in the HIF-1α subunit. Subsequently, HIF-1α binds to the von Hippel- Lindau tumor suppressor (pVHL) protein, resulting in HIF-1α ubiquitination and proteasomal degradation.[3,4] However, under hypoxic conditions, the stabilized HIF-1α dimerizes with HIF-1β and translocated into the nucleus. The HIF-1α/β heterodimer binds to the hypoxia response elements (HREs) of the target gene promoters, consequently, activating the transcription of downstream genes involved in angiogenesis, metastasis, apoptosis, and glycolysis.[5-7] Upon adapting to hypoxic condition, the tumor cells promote the transcription of genes associated with angiogenesis, metabolism, cell proliferation, survival, pH regulation, and cell migration. Therefore, as master regulators of O2 homeostasis, HIF-1α has emerged as an attractive target to develop novel cancer therapeutics.[5,8]

An earlier study has reported benzopyranyl 1,2,3-triazole compound as a potent HIF-1α inhibitor, by the reporter gene assay.[1] This benzopyranyl 1,2,3-triazole compound, named KPLA-012, hydroxylated HIF-1α and increased HIF-1α ubiquitination, and also decreased HIF-1α target gene expression and vascular endothelial growth factor (VEGF) secretion in a dose-dependent manner. Furthermore, this compound inhibited VEGF-induced in vitro angiogenesis in human umbilical vein endothelial cells (HUVECs). It also inhibited chick chorioallantoic membrane angiogenesis in vivo. In the allograft assay, cotreatment with KPLA-012 and gefitinib significantly inhibited tumor growth and angiogenesis.[1]

Therefore, the aim of this study was to elucidate the druggability of KPLA-012. Therefore, we performed in vivo pharmacokinetic (PK) study in mice and investigated the in vitro absorption, distribution, and metabolism of KPLA-012. Furthermore, we correlated the in vivo animal PK properties with those of human.

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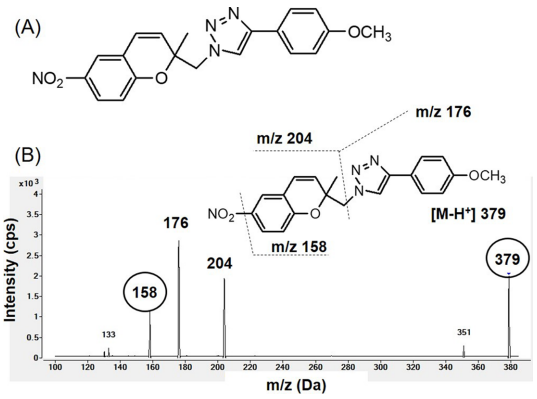

Figure 1. (A) Structure of KPLA-012 and (B) product ion spectra of KPLA-012

Experimental

Chemicals and reagents

KPLA-012 (Figure 1A) was synthesized with a purity of > 99.0% and confirmed by nuclear magnetic resonance spectroscopy and mass spectroscopy (MS). Propranolol (purity > 99.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of reagent or analytical grade.

Pharmacokinetic study

All animal procedures were approved by the Animal Care and Use Committee of the Kyungpook National University (Permission no. 2015-47-0). The ICR mice (7--8-weeks old, 25-30 g) were purchased from the SAMTAKO bio Korea Inc. (Osan, Korea).

Blood samples (0.1 mL) were collected via retro-orbital blood collection using heparinized capillary tube at 0.08, 0.25, 1, 1.5, 2, 4, 8, and 24 h following the intravenous administration of KPLA-012 (2 mg/kg) via the tail vein. Blood samples (0.1 mL) were collected via retro-orbital blood collection using heparinized capillary tube at 0.25, 0.5, 1, 1.5, 2, 4, 8, and 24 h following oral administration (10 mg/kg) using oral gavage.

The blood was centrifuged at 13000 rpm to separate the plasma for 10 min, and the plasma sample was stored at -80°C in aliquots of 20 μL.

In the experiment using metabolic cages, the urine and feces samples were collected for 24 h following the administration of KPLA-012. The total volume of urine sample was measured and stored at -80°C in aliquots of 50 μL. The feces samples were weighed, 20% homogenate was prepared, and 100 mg of 20% feces homogenate was stored at -80°C until further analysis.

Plasma, urine and feces samples were added to 250 μL of acetonitrile containing 10 ng/mL of propranolol as an internal standard (IS). After vortexing for 15 min and centrifuging at 13200 rpm for 5 min, an aliquot (5 μL) of the supernatant was injected directly into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

LC-MS/MS analysis of KPLA-012

The concentration of KPLA-012 in the plasma and transport medium was analyzed using an Agilent 6430 Triple Quad LC-MS/MS system (Agilent, Wilmington, DE, USA) coupled with an Agilent Infinity 1260 series high performance liquid chromatography (HPLC) system. The separation was performed using a Luna C18 column (2 mm i.d. × 150 mm, 4 μm, Phenomenex, Torrance, CA, USA) using a mobile phase consisting of acetonitrile and double distilled water (90:10, v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min. Retention time was 2.52 min for KPLA-012, and 1.85 min for propranolol (IS) and total run time was 4.0 min. The mass spectra were recorded by electrospray ionization in the positive mode. Quantification was performed by multiple reaction monitoring (MRM) at m/z 379 → 158 for KPLA-012 (Figure 1B) and m/z 260 → 116 for propranolol.

Protein binding

The plasma protein binding of KPLA-012 (1000 ng/mL) in mice and humans was determined using a rapid equilibrium dialysis kit (ThermoFisher Scientific Korea, Seoul, Korea) according to the protocol of the manufacturer. Briefly, 100 μL of mouse and human plasma samples containing 1000 ng/mL KPLA-012 were added to the sample chamber of a semipermeable membrane (molecular weight cut-off 8000 Da) and 300 μL of PBS was added to the outer buffer chamber. Four hours after incubation at 37°C on a shaking incubator at 300 rpm, aliquots (50 μL) were collected from both the sample and buffer chambers and treated with equal volumes of fresh PBS and plasma, respectively, to match the sample matrices. The matrix-matched sample (100 μL) was added to 300 μL of acetonitrile containing 10 ng/mL propranolol. After vortexing for 15 min and centrifuging at 13200 rpm for 5 min, the supernatant was injected directly into the LC-MS/MS system.

We also determined the human plasma protein binding of 1 μM atenolol, 1 μM ranitidine, and 1 μM propranolol as positive and negative controls, respectively, following the same procedure.[9] The protein binding (%) of atenolol, ranitidine, and propranolol was 5.2%, 20.4%, and 89.0%, respectively, indicating the feasibility of this system.

Plasma and Microsmal Stability

The stability of KPLA-012 (1000 ng/mL) in the plasma (mouse and human) and microsomal fraction (mouse and human) was measured. An aliquot (50 μL) of plasma and liver microsomal solution of mouse and human origin containing KPLA-012 (1000 ng/mL) and incubated for 0, 0.5, 1, 1.5, and 2 h. After incubation, 50 μL of plasma and liver microsomal sample was treated with equal volumes of fresh buffer and plasma, respectively. The matrix-matched sample (100 μL) was added to 300 μL of

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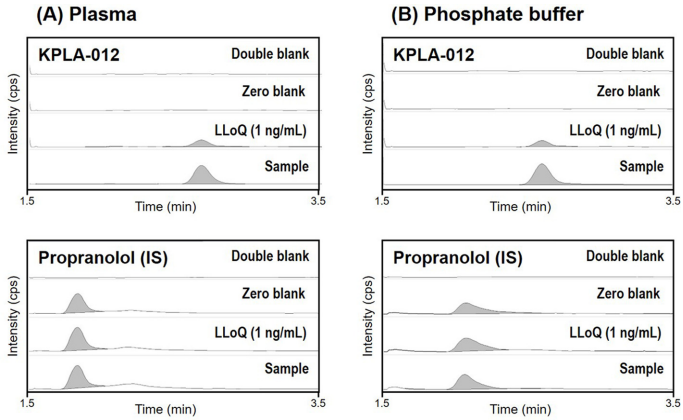

Figure 2. Representative MS/MS chromatograms in (A) mouse plasma and in (B) phosphate buffer.

acetonitrile containing 10 ng/mL propranolol. After vortexing for 15 min and centrifuging at 13200 rpm for 5 min, an aliquot (5 μL) of the supernatant was injected directly into the LC-MS/MS system.

We also determined the metabolic stability of 1 μM propranolol and 1 μM metformin as positive and negative controls, respectively, by the same procedure.[10] The percent of remaining propranolol and metformin after a 60 min incubation period was 12.2% and 63.4% of the initial concentration, indicating the feasibility of this system.

Caco-2 permeability

Caco-2 cells (passage no 42-48; purchased from ATCC, Rockville, MD, USA) were seeded on collagen-coated 12-transwell membranes at a density of 5×10^5 cells/mL and maintained at 37°C in a humidified atmosphere with 5% CO2/ 95% air for 21 d. The culture medium was replaced every 2 d and the permeability assay was conducted as previously described.[11,12] Briefly, HBSS medium containing marker compounds (caffeine, propranolol, ofloxacin, and atenolol) (2, 5, 10, and 50 μM, respectively) or KPLA-012 (20 μM) was added to the donor side and aliquots (0.4 mL) from the receiver side were collected at 15, 30, 45, and 60 min and stored at -80°C until further analysis.

The concentration of caffeine, propranolol, ofloxacin, and atenolol in the samples was measured simultaneously following the same method described previously.[12] To analyze KPLA-012, 200 μL of thawed samples was added to 600 μL of acetonitrile containing 10 ng/mL propranolol. After vortexing for 15 min and centrifuging at 13200 rpm for 5 min, the supernatant was injected directly into the LC-MS/MS system.

Data Analysis

The PK parameters were determined by the non-compartmental analysis (WinNonlin® 2.0; Pharsight, Mountain View, CA, USA).[13] The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) values were obtained from the plasma concentration-time curves. The area under the plasma concentration-time curve from zero to infinity (AUC) was calculated by the trapezoidal-extrapolation method. Oral bioavailability (BA) was calculated by dividing AUC_{PO}, which was normalized with KPLA-012 dose (10 mg/ kg) by AUC_{IV}, which was also normalized by the IV dose of KPLA-012 (2 mg/kg). The apparent permeability (P_{app}) was calculated by dividing the initial transport rate of KPLA-012 by initial drug concentration in the donor side and the surface area of the insert. All data are expressed as mean ± SD.

Statistical analysis was evaluated by student t-test. In all cases, a difference was considered be significant when $P<0.05$.

Results and Discussion

LC-MS/MS analysis of KPLA-012 in plasma samples and in phosphate buffer

For the analysis of KPLA-012 in the small amount of preclinical in vitro ADME and in vivo mouse plasma samples, we developed analytical method for KPLA-012 in plasma samples and in phosphate buffer. Product ion scan results were shown in Figure 1B and the representative MS/MS chromatograms for KPLA-012 in plasma and in phosphate buffer samples were shown in Figure 2. The concentrations of KPLA-012 in plasma and in phosphate buffer were analyzed with the standard curves ranged from 1 ng/mL to 1000 ng/mL and intra- and inter-day precision and accuracy had coefficients of variance of less than 15%.

Pharmacokinetic profiles of KPLA-012

The PK profile of KPLA-012 after a single intravenous and oral administration is shown in Figure 3. The PK parameters of KPLA-012 are listed in Table 1. Intravenous and oral dose of KPLA-012 (10 mg/kg) was decided based on the therapeutic intraperitoneal dose (5 mg/kg)[1] and detection limit of KPLA-012 (1 ng/mL) in this study. The half-life of intravenously and orally administered KPLA-012 was 4.6 ± 0.73 and 2.8 ± 0.32 h, respectively. The T_{max} was 2.0 h with oral administration. The AUC of intravenous and oral administration was 205.5 ± 23.0 and 343.4 ± 129.9 ng·h/ mL, respectively, yielding a 33% absolute BA of KPLA-012.

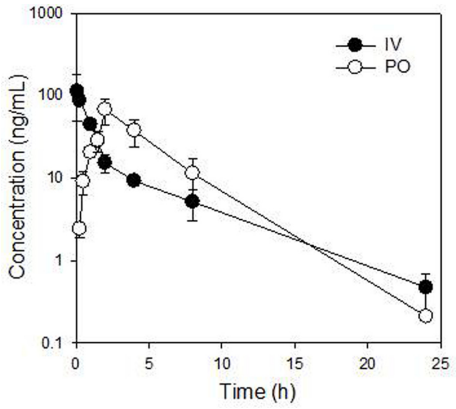

Figure 3. Plasma concentration-time profile of KPLA-012 after intravenous (IV, ●) and oral administration (PO, ○) of KPLA-012 at a single dose of 2 mg/kg and 10 mg/kg, respectively. Each data represents the mean ± SD from four different mice per group.

Table 1. Pharmacokinetic parameters of KPLA-012 following IV and PO administration of KPLA-012 in mice

Parameters	IV (2 mg/kg)	PO (10 mg/kg)
Half-life (T1/2)	4.6 ± 0.7	2.8 ± 0.3
C_{max} (ng/mL)	119.4 ± 60.2	68.2 ± 24.2
T_{max} (ng/mL)	0.14 ± 0.1	2
AUC (ng·h/mL)	205.5 ± 23.0	343.3 ± 129.9
MRT (h)	3.9 ± 1.0	4.7 ± 0.2
CL(mL/min/kg)	163.7 ± 19.3	
$V_{d,ss}$ (L/kg)	38.1 ± 9.2	
BA (%)		33
Excreted in urine (%)	0.002 ± 0.0007	0.0008 ± 0.0007
Excreted in feces (%)	0.01 ± 0.003	3.3 ± 3.0

Each data represents the mean ± SD from four different mice per group.

Although the BA value is not significant, it showed that the compound can be developed in an orally administered dosage form. The systemic clearance (CL) and volume of distribution at steady-state ($V_{d,ss}$) after the intravenous administration was 163.7 ± 19.3 mL/min/kg and 38.1 ± 9.2 L/kg, respectively, suggesting the elimination of drugs from the systemic circulation at high rates.

The cumulative excreted amounts of KPLA-012 are listed in Table 1. Less than 1% of KPLA-012 dose was recovered in the parent form from the urine and feces samples for 24 h following the intravenous administration of KPLA-012. Similarly, KPLA-012 was excreted in urine and feces at less than 1% and 3.3%, respectively, for 24 h following its oral administration. The excretion results were consistent with the high clearance of KPLA-012 (163.7 ± 19.3 mL/min/kg), suggesting the metabolic instability of this compound in the liver and intestine. Therefore, we investigated the plasma level and metabolic stability of KPLA-012.

Table 2. Plasma protein binding of KPLA-012

Species	Protein bound (%)
Mouse	97.7 ± 0.1
Human	99.4 ± 0.1*

Each data represents the mean ± SD of three independent experiments.
* $p < 0.05$, significant compared with mouse by student's t-test.

Table 3. Stability of KPLA-012 in the plasma and liver microsomes

	Species	% remaining after 1h	Half-life (h)
Plasma	mouse	82.0 ± 5.2	9.9 ± 5.5
	human	91.3 ± 6.5*	20.2 ± 5.0*
Liver micro-somes	mouse	14.8 ± 1.5	0.5 ± 0
	human	47.4 ± 7.5*	1.1 ± 0.1*

Each data represents the mean ± SD of three independent experiments.
* $p < 0.05$, significant compared with mouse by student's t-test.

Protein binding

As shown in Table 2, the protein binding percent of KPLA-012 in the plasma of mice and humans was 97.7 ± 0.1% and 99.4 ± 0.1%, respectively. KPLA-012 was highly bound to the plasma protein of mice and humans, with slightly higher protein binding in human pasma.

Plasma and Metabolic Stability

When KPLA-012 was incubated for 2 h in human and mouse plasma, the percent of residual KPLA-012 compared with the initial concentration was 82.0 ± 5.2% in mouse plasma and 91.3 ± 6.5% in human plasma (Table 3), suggesting that KPLA-012 was stable in the plasma of both mice and humans. However, the percent of residual KPLA-012 compared with the initial concentration was 14.8 ± 1.5% in the mouse liver microsomes and 47.4 ± 7.5% in the human liver microsomes, and the half-life of KPLA-012 for metabolic degradation was 0.5 and 1 h in mice and humans, respectively (Table 3). This suggests that the metabolic instability of KPLA-012 in mouse and human liver microsomes. This metabolic instability of KPLA-012 might be attributed to the low recovery of KPLA in urine and feces (< 1%), which necessitates an understanding of possible metabolites of KPLA-012 and their corresponding metabolic enzymes. Although its stability has to be improved in terms of druggability of new chemical entities, the human microsomal stability of KPLA-012 was better than that of mouse microsomal stability. The hepatic intrinsic clearance of KPLA-012 from mice and humans was estimated to be 90.0 and 11.7 mL/min, respectively, using the microsomal elimination half-life and scaling factors reported previously.[14] Moreover, this estimated hepatic intrinsic clearance in mice suggested that the hepatic metabolism might play a critical role in eliminating KPLA-012. However, in humans, the contribution of hepatic metabolism to the overall clearance of KPLA-012 might be lower than that in mice.

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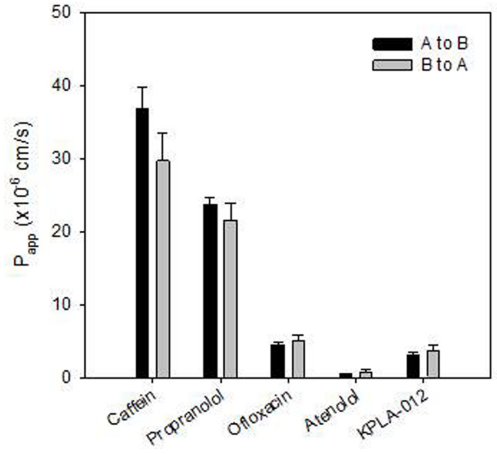

Figure 4. The apparent permeability (P_{app}) of caffeine, propranolol, ofloxacin, atenolol, and KPLA-012 from the direction of A to B and B to A was measured in Caco-2 cell monolayers. Bar represents the mean ± SD of three independent experiments.

Caco-2 cell Permeability

Considering the metabolic instability of KPLA-012 deduced from the microsomal degradation and low recovery of parent KPLA-012 from the urine and feces samples, the substantial BA of KPLA-012 indicated that this compound might have high permeability. To further investigate the permeability of KPLA-012, we determined the P_{app} values following either apical or basolateral loading of 20 μM KPLA-012 and the results are shown in Figure 4. Initially, caffeine, propranolol, ofloxacin, and atenolol were used as marker compounds for high, moderate, and low permeable drugs. As a positive control of high permeability, the absorptive P_{app} value of caffeine and propranolol was 36.8×10^{-6} and 23.7×10^{-6} cm/s, respectively, which is consistent with the findings of other studies.[15] As a positive control of moderate and poor permeability, the absorptive P^{app} value of ofloxacin and atenolol was 4.5×10^{-6} and 0.04×10^{-6} cm/s, respectively, which corroborates with the findings of other studies.[16]

The A to B and B to A permeability of KPLA-012 was 3.1×10^{-6} and 3.7×10^{-6} cm/s, respectively, indicating moderate permeability. The efflux ratio ($P_{app,BA}$ / $P_{app,AB}$) of KPLA-012 was 1.18, suggesting the least involvement of efflux pumps in the intestinal absorption of KPLA-012.

Conclusions

KPLA-012, a novel HIF-1α inhibitor, exhibited moderate oral BA (33%), large systemic clearance (163.7 ± 19.3 mL/min/kg), and low recovery in its parent form (< 1%) in mice. These PK results were consistent with the metabolic instability of KPLA-012 in the liver microsomes of mice, suggesting that metabolism plays a critical role in the PKs of KPLA-012. Moreover, the improved metabolic stability of this compound might contribute to a better PK property. Based on the in vitro Caco-2 permeability (moderate and efflux ratio of 1.18), protein binding (99.4%), and metabolic stability (47.4% remaining after 1 h incubation), KPLA-012 might have more effective PK properties in humans than in mice. However, further studies to improve the metabolic stability and to understand the metabolism of this compound are necessary.

Acknowledgments

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