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Original scientific paper

Preclinical assessment of ulixertinib, a novel ERK1/2 inhibitor

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Abstract

Ulixertinib (BVD-523) is a novel and selective reversible inhibitor of ERK1/ERK2. In xenograft studies it inhibited tumor growth in BRAF-mutant melanoma and colorectal xenografts as well as KRAS-mutant colorectal and pancreatic models. Ulixertinib is currently in Phase I clinical development for the treatment of advance solid tumors. The objective of the study is to assess the metabolic stability (in various pre-clinical and human liver microsomes/hepatocytes), permeability, protein binding, CYP inhibition, CYP induction and CYP phenotyping of ulixertinib. We have also studied the oral and intravenous pharmacokinetics of ulixertinib in mice, rats and dogs. Ulixertinib was found to be moderately to highly stable in various liver microsomes/hepatocytes tested. It is a medium permeable (2.67 x 10^{-6} cm /sec) drug and a substrate for efflux (efflux ratio: 3.02) in Caco-2 model. Ulixertinib was highly bound to plasma proteins. CYPs involved in its limited metabolism and it is CYP inhibition IC_{50} ranged between 10-20 µM. Post oral administration ulixertinib exhibited early T_{max} (0.50-0.75 h) in mice and rats indicating absorption was rapid, however in dogs T_{max} attained at 2 h. The half-life ($t_{½}$) of ulixertinib by intravenous and oral routes ranged between 1.0-2.5 h across the species. Clearance and volume of distribution by intravenous route for ulixertinib were found to be 6.24 mL/min/kg and 0.56 L/kg; 1.67 mL/min/kg and 0.36 L/kg and 15.5 mL/min/kg and 1.61 L/kg in mice, rats and dogs, respectively. Absolute oral bioavailability in mice and rats was > 92 %, however in dogs it was 34 %.

Keywords

Ulixertinib; ERK inhibitor; In vitro studies; Pharmacokinetics; Mice; Rats; Dogs

Introduction

The extracellular-signal-regulated kinases, ERK1 and ERK2 (ERK1/2), play a critical role in the RAS/RAF/MEK signaling pathway, that controls several fundamental cellular processes, driving proliferation, differentiation and cell survival [1]. RAS/RAF/MEK signaling pathway is frequently activated by mutations in upstream targets such as BRAF, RAS and receptor tyrosine kinases [2]. Most of the resistance mechanisms to BRAF and MEK inhibitors ultimately lead to increase in phosphorylation of ERK1/2 suggesting the importance of this node in the RAS/RAF/MEK pathway even in the resistance setting [3]. RAS activating mutations have been reported in about 90 % of pancreatic carcinomas, followed by colon carcinomas (50 %), lung cancers and myeloid leukemia cases (30 % each) [4]. Therefore, inhibition of

ERK1/2 offers a promising strategy to address both innate and acquired resistance to BRAF and MEK inhibitors in various solid tumors. Ulixertinib (BVD-523, VRT752271; Figure 1) is a first-in-class novel small molecule, which potently and selectively inhibits ERK1/2 kinases in a reversible, ATP-competitive fashion. Inhibition of ERK1/2 leads to proliferation inhibition (by decreasing the expression of VEGFA and VEGFR2 at mRNA and protein level), cell cycle arrest and apoptosis in lymphoma cell lines [5]. Ulixertinib inhibits tumor growth *in vivo* in BRAF-mutant melanoma and colorectal xenografts as well as in KRAS-mutant colorectal and pancreatic models [6]. In clinical studies, ulixertinib was well tolerated by patients with advanced solid tumors. In an oral Phase-I dose escalation study (having 9 doses) with an end point to determine the dose limited toxicities (DLT), maximum tolerated dose (MTD) along with pharmacokinetic profile and preliminary efficacy assessment it was administered in a dose range of 10-900 mg in a b.i.d regimen. Ulixertinib showed linear pharmacokinetics up to 600 mg (b.i.d), this was found to be MTD [7].

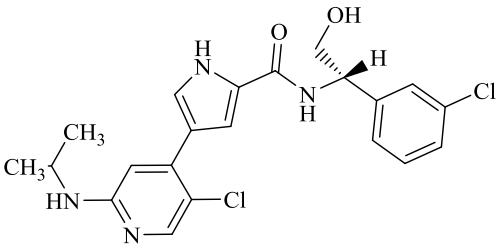

Figure 1. Structural representation of ulixertinib

To date there is no publication on preclinical pharmacokinetics of ERK1/2 inhibitors, hence we felt that preliminary data on various *in vitro* ADME studies and *in vivo* pharmacokinetics in mice, rats and dogs of ulixertinib will help researchers in this field to provide the differentiating features in this class. This paper describes various *in vitro* experiments done to (a) assess the metabolic stability of ulixertinib in various preclinical species and human liver microsomes and hepatocytes (b) determine the permeability in Caco-2 cells (c) determine the unbound fraction in preclinical species and human plasma (d) CYP inhibition (e) CYP induction and (f) CYP phenotyping of ulixertinib. Moreover, a series of *in vivo* experiments in Balb/C mice, Sprague Dawley rats and Beagle dogs were performed to characterize and understand pharmacokinetic parameters and absolute oral bioavailability of ulixertinib across the species.

Materials and methods

Materials

Ulixertinib (purity >99 %) was purchased from MedKoo Biosciences, Inc., NC, USA. HPLC grade acetonitrile, formic acid and methanol were purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. Preclinical species and human liver microsomes and hepatocytes were purchased from Life Technologies (Gibco®), Inchinnan, UK. Substrates and inhibitors of CYP isoforms like midazolam, quinidine, diclofenac, bufuralol, omeprazole, verapamil, rifampicin, warfarin, tolbutamide, phenacetin, ketoconazole, sulfaphenazole, nootkatone, furafylline and digoxin were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. All other chemicals/reagents were of research grade and used without further purification.

In vitro studies

Metabolic stability in liver microsomes

Metabolic stability is defined as the percentage of parent compound lost over time in the presence of a metabolically active test system. The incubation mixtures consisted of liver microsomes (0.5 mg microsomal protein/mL), ulixertinib (1 µM) or positive control (verapamil, 1 µM). The reactions were initiated by adding 20 µL of 10 mM NADPH [8]. Reactions without NADPH (0 and 30 min) were also incubated to rule out non-NADPH metabolism or chemical instability in the incubation buffer [8]. All reactions were terminated using 200 µL of ice-cold acetonitrile containing internal standard (200 ng/mL of phenacetin) at 0, 5, 15 and 30 min. The vials were centrifuged at 3000 rpm (Eppendorf 5424R, Germany) for 15 min. The supernatants thus obtained were analyzed on LC-MS/MS to monitor the disappearance of ulixertinib.

Metabolic stability in hepatocytes

After checking the viability of cryopreserved hepatocytes, they were distributed 1 million viable cells per mL. An aliquot of 100 µL of thawed hepatocytes cell suspension was aliquoted into different vials labeled as 0, 30, 60, 120 and 180 min (mouse, rat, dog or human hepatocytes) and ulixertinib (3 µM). Imipramine or naloxone (3 µM) was used as a positive control [9]. The vials were pre-incubated in a 37 °C water bath for 5 min. All reactions were terminated using 200 µL of ice-cold acetonitrile containing internal standard (200 ng/mL of phenacetin) at specified time points. The vials (having reaction mixture terminated contents) were centrifuged at 3000 rpm (Eppendorf 5424R, Germany) for 15 min. The supernatants thus obtained were analyzed on LC-MS/MS to monitor the disappearance of ulixertinib. Clearance rate and half-life were calculated using the obtained data.

Caco-2 permeability

Caco-2 human intestinal epithelial cells were plated in 24-Transwell® dual chamber plates (Millipore, Billerica, MA, USA) (cell density of 80,000 cells/cm^2 on day-1). The permeability studies were conducted with the monolayers cultured for 21 to 22 days. The integrity of each Caco-2 cell monolayer was certified by trans epithelial electrical resistance (TEER) test (pre-experiment) and by determining the permeability of reference compound i.e., Lucifer yellow. Caco-2 cell monolayers with TEER values greater than 500 Ω cm^2 were considered for experimentation. Digoxin (5 µM) was used as a positive control for P-gp substrate [10]. The concentration of ulixertinib used in the assay was 10 µM. HBSS Buffer was used as the medium for the transport assay and the final concentration of DMSO in spiking solution was 0.05 %. The bi-directional permeability study was initiated by adding an appropriate volume of HBSS buffer containing ulixertinib to respective apical and basolateral chambers (n=2) [11]. An aliquot of sample (100 µL) was taken from both chambers at 0 and 60 min of the incubation period and to this equal volume of acetonitrile with internal standard (200 ng/mL of phenacetin) was added, mixed gently and centrifuged at 4000 rpm (Eppendorf 5424R, Germany) for 10 min. An aliquot of 100 µL was subsequently transferred to the auto-sampler and injected for analysis on LC-MS/MS.

Plasma protein binding

To evaluate the ability of ulixertinib to bind the plasma proteins, the most common approach of plasma protein binding using equilibrium dialysis was used [12]. Ulixertinib was tested at a final concentration of 3 µM in mouse, rat and dog plasma. An aliquot of 150 µL plasma containing ulixertinib was added in first half (plasma side) of the well of 96-well micro-equilibrium dialysis device. An aliquot of 150 µL of 100 mM

sodium phosphate buffer pH 7.4 was added in the second half (buffer side) of the well of 96-well HT equilibrium dialysis device. The plate containing plasma and buffer was equilibrated at 37 ± 1 °C for 4.5 h, with constant shaking at 120 rpm on an orbital shaker. Samples were collected from respective halves after the completion of incubation time. The proteins were precipitated using organic solvents. The samples were subjected to centrifugation and the supernatants were analyzed analysis on LC-MS/MS.

CYP inhibition

CYP inhibition potential of ulixertinib was assessed in human liver microsomes [Life Technologies (Gibco®), Inchinnan, UK] against CYP3A4, 2D6, 1A2, 2C9 and 2C19 in the following sequential steps. Standard reaction mixture (final volume 300 µL) contained 66.7 M potassium phosphate buffer (pH 7.4), protein [0.1 (for CYP3A4), 0.25 (for CYP2D6), 0.5 mg/mL (for CYP1A2, 2C9 and 2C19)] and ulixertinib (at 2.0, 10 and 20 µM, added as 0.9 µL DMSO solution with a final DMSO concentration of 0.1 %). The mixtures were pre-incubated at 37 ± 1 °C for 5 min. The reaction (in duplicate) was initiated by addition of 30 µL of NADPH (10 mM) [13]. Reaction was terminated at 10 min by adding 300 µL of ice cold acetonitrile with internal standard. The activity of liver microsomes was confirmed with positive controls i.e., monitoring the hydroxylation of midazolam, bufuralol, diclofenac and omeprazole for CYP3A4, 2D6, 2C9 and 2C19, respectively and deethylation of phenacetin for CYP1A2 (data not shown). The reaction mixtures (obtained from the above studies) were extracted, processed, analyzed on LC-MS/MS.

rhCYP metabolism

To evaluate the metabolism of ulixertinib, the most common approach of reaction phenotyping by using cDNA expressed enzyme system was used. Ulixertinib was incubated with a panel of individually-expressed recombinant human CYP enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4, purchased from Life Technologies (Gibco®), Inchinnan, UK expressed in baculovirus-infected insect cell membranes. The incubation mixture contained ulixertinib at a final concentration of 0.1 µM, expressed CYP enzyme (50 pmol/mL), phosphate buffer (66.7 mM, pH 7.4) in a total volume of 1 mL. The reaction was initiated by the addition of 20 µL of NADPH after pre-incubation of the aliquots (180 µL) marked 0, 5, 15, 30 and 60 min followed by incubation at 37 ± 1 °C. The reaction was terminated by the addition of equal volumes of ice cold acetonitrile with internal standard (200 ng/mL of phenacetin). The samples were centrifuged at 3000 rpm (Eppendorf 5424R, Germany) at 4 °C for 20 min to precipitate proteins. Supernatants were then transferred to clean vials and stored at -20 °C until analysis. Incubations without NADPH were used as negative controls. Control incubations using probe substrates for individual recombinant humans CYPs were included to check for appropriate incubation conditions and as positive control for activity [15]. The samples were analyzed on LC-MS/MS. Disappearance of ulixertinib was compared to that of control treatment (no CYP enzymes present) in order to assess the contribution of a given CYP in ulixertinib metabolism.

In vivo studies

Formulations

For oral administration a suspension formulation was prepared using Tween-80 and 0.5 % methyl cellulose. Weighed amount of ulixertinib was taken into a mortar and grinded into a fine powder with the help of a pestle. To this Tween-80 was added drop wise to wet the entire powder. Then slowly 0.5 % methyl cellulose was added with constant stirring to get a uniform suspension. The final strength of the suspension formulation was 10 mg/mL and it was administered to animal species at 10 mL/kg body weight. For *intravenous* (*i.v.*) administration a solution formulation was used (prepared using 10 % DMSO, 10 %

Solutol:absolute ethyl alcohol (1:1, v/v) and 80 % normal saline). The volume of administration for *intravenous* was 10 mL/kg for mice and 2 mL/kg for rats and dogs.

Animal experiments details

Institutional Animal Ethical Committee (IAEC) of Jubilant Biosys (IAEC/JDC/2017/121) nominated by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) approved the rodents pharmacokinetic experiments. Male Sprague Dawley rats (~7-8 weeks old) and BalbC mice (~6-10 weeks old) were purchased from Vivo Biotech, Hyderabad, India. Animals were quarantined in Jubilant Biosys Animal House for a period of 7 days with a 12:12 h light:dark cycles, had free access to rodent food (Altromin Spezialfutter GmbH & Co. KG., Im Seelenkamp 20, D-32791, Lage, Germany) and water *ad libitum*. For all the experimental work animals were kept for fasting (4 h for BalbC mice and 12 h for Sprague Dawley rats) and during this time they were allowed to take water *ad libitum*. Food was provided 2 h post-dose and water was allowed *ad libitum*.

IAEC of Palamur Biosciences, Telangana, India (1312/PO/RcBiBt/S/L/09/CPCSEA) approved studies conducted in dogs. Male Beagle dogs (~1.0-1.5 year old) were housed in Palamur Biosciences Private Limited animal house facility in a temperature (18-28 °C) and humidity (30-70 %) controlled room and fed with Pedigree standard pellet food and water *ad libitum* for one week before using for experimental purpose. For all the experimental work animals were kept for 12 h overnight fasting and during this time they were allowed to take water *ad libitum*. Food was provided 4 h post-dose and water was allowed *ad libitum*.

Pharmacokinetic studies

Oral bioavailability of ulixertinib was evaluated in male Balb/C mice, Sprague Dawley rats and Beagle dogs. Fasted mice (~4 h) and rats (overnight ~12 h) were divided into two groups (mice: 12/group; rats: 4/group), however in cases of dogs (n=2; fasted for ~12 h overnight) same dogs were dose after one week wash-out period (cross over design). In each species Group-1 animals received ulixertinib through *intravenous* route at a dose of 1 mg/kg; whereas Group 2 received ulixertinib through oral gavage at 10 mg/kg dose. Serial blood samples [100 µL in case of mice (sparse sampling; n=3 at each time point) and rats; 500 µL in dogs)] were collected from retro-orbital plexus at 0.12, 0.25, 0.5, 1, 2, 4, 8, and 24 h (intravenous administration) or at 0.25, 0.5, 1, 2, 4, 8, 10 and 24 h (oral administration) after drug administration. Blood samples were collected in tubes containing K_2.EDTA as the anticoagulant and centrifuged for 5 min at 14000 rpm in a refrigerated centrifuge (Biofuge, Heraeus, Germany) maintained at 4 °C for plasma separation and stored frozen at -80 ± 10 °C until analysis.

Sample processing

In vitro studies samples analysis

A Shimadzu (Shimadzu, Kyoto, Japan) SIL series LC system equipped with a degasser (DGU-20A3), isopump (LC-20 AD) and column oven (CTO-10AS) along with an auto-sampler (SIL-HTc) was used to inject 10 µL aliquots of the processed samples on a Atlantis C_{18} column (50 x 4.6 mm, 3 µm; Waters, Ireland, UK), which was maintained at 40 °C. The mobile phase system consisted of reservoir A (acetonitrile) and reservoir B (0.2 % formic acid in water) were run as per gradient program (0-0.1 min: 10 % A and 90 % B; 0.1-2.8 min: 100 % A; 2.9-4.0 min 10 % A and 90 % B). A flow rate of 0.8 mL/min with a 50 % splitter was used throughout the analytical run. Quantitation was achieved by MS/MS detection in positive ion mode for ulixertinib using an API-4000 Q Trap mass spectrometer (MDS Sciex, Toronto, Canada) equipped with a

Turboionspray™ interface at 450 °C temperature and 5500 V ion spray voltage. The source parameters viz., curtain gas, GS1, GS2 and CAD were set at 30, 35, 40 and 6 psi. The compound parameters viz., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were 81, 10, 49 and 15 V for ulixertinib and 80, 10, 29 and 14 V for the IS. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 433 precursor ion to the *m/z* 262 product ion for ulixertinib and *m/z* 180 precursor ion to the *m/z* 110 product ion for the IS. Quadrupole Q1 and Q3 were set on unit resolution. The dwell time was 150 msec.

Plasma samples processing and analysis

An aliquot of 50 µL plasma sample was precipitated with 200 µL of acetonitrile:methanol (1:1, v/v) enriched with internal standard (200 ng/mL of phenacetin) and centrifuged at 14,000 rpm for 5 min (Eppendorf 5424R, Germany) at 5 °C. Clear supernatant (125 µL) was transferred into vials and 5 µL of supernatant was injected onto LC-MS/MS system for analysis using a validated method [16]. The linearity range was 1.05-2096 ng/mL. In-study quality control (QC) samples, supplemented with concentrations of 3.14, 1048 and 1747 ng/mL of ulixertinib, were analysed with the unknowns. Plasma samples showing high concentration above the high calibration standard were diluted with appropriate animal species blank plasma to bring the concentration within linearity range.

For plasma samples analysis the criteria for acceptance of the analytical runs encompassed the following: (i) 67 % of the QC samples accuracy must be within 85-115 % of the nominal concentration (ii) not less than 50 % at each QC concentration level must meet the acceptance criteria. Following completion of the analysis both the linearity and quality control samples values were found to be within the accepted variable limits.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated by a non-compartmental method using Phoenix WinNonlin 7.0 software (Pharsight, Mountain View, CA, USA). Absolute oral bioavailability (F) was calculated using the relationship, F = [Dose (*i.v.*) x $AUC_{(0-\infty)oral}$ / Dose (oral) x $AUC_{(0-\infty)i.v.}$] x 100.

Results

In vitro studies

Metabolic stability in liver microsomes

Metabolic stability of ulixertinib in different species of liver microsomes is presented in Table 1. Ulixertinib was found to be moderately stable in mice and dog liver microsomes and highly stable rat and human liver microsomes.

Metabolic stability in hepatocytes

Hepatocyte stability of ulixertinib in different species is presented in Table 2. Ulixertinib was found to be highly stable in rat and human hepatocytes but moderately stable in mice and dog hepatocytes. The *in vivo* intrinsic clearance (Clint) was relatively low to moderate in rat and human hepatocytes but high in mice and dog hepatocytes.

Caco-2 permeability

Table 3 shows the rate of transport (P_{app}) of ulixertinib and digoxin from A → B and B → A along with efflux ratio values. The findings suggested that ulixertinib is a medium permeable compound and may be a

substrate of an active efflux transporter.

Table 1. Metabolic stability data of ulixertinib at 1 µM in liver microsomes of various preclinical species

Species	Percent metabolized	$t_{½}$ (min)	mCl_{int} (µL/min/mg)	Cl_{int} (mL/min/kg)
Mice	44.5	36	19.3	27.7
Rat	16.4	122	5.7	7.6
Dog	35.5	52	13.3	9.1
Human	12.8	142	9.8	5.4

Table 2. Hepatocyte stability data of ulixertinib at 3 µM in liver hepatocytes of various preclinical species

Species	Percent metabolized	$t_{½}$ (min)	hCL_{int} (mL/min/106 cells)	CL_{int} scaled (mL/min/kg)
Mice	79	78	8.9	41.3
Rat	27	410	1.7	7.1
Dog	76	73	9.1	16.4
Human	37	306	2.3	5.0

Table 3. Permeability data of ulixertinib in Caco-2 cell monolayers

		% Recovery		Permeability (x 10^{-6} cm/sec)		Efflux ratio
Compound	Concentration (µM)	B → A	A → B	B → A	A → B	B → A/ A → B
Ulixertinib	10	79	65	8.09	2.67	3.02
Digoxin	5.0	98	75	10.5	1.00	10.5

Plasma protein binding

Ulixertinib had a very high binding in rat plasma followed by mouse and dog plasma with fraction unbound of 0.001, 0.003 and 0.050, respectively. The stability and recovery of ulixertinib in plasma was good across the species tested.

CYP inhibition

The predicted IC_{50} values of ulixertinib were determined for CYP-specific hydroxylation of midazolam, bufuralol, diclofenac and omeprazole for CYP 3A4, 2D6, 2C9 and 2C19, respectively and deethylation of CYP1A2. Ulixertinib did not show notable inhibition against these enzymes and the predicted IC_{50} values of ulixertinib were found to be ~10 and 20 µM for CYP 3A4 and 2D6, respectively indicating it is a weak inhibitor against these two CYPs. Furthermore, its inhibitory effect was much weaker against CYP1A2, 2C9 and 2C19 as its IC_{50} was > 20 µM. Thus, when compared with known inhibitors, ulixertinib shows less significant inhibition of CYP enzyme activity *in vitro* (Table 4).

CYP induction

Ulixertinib was tested at two different concentrations (1 and 10 µM). The readings suggested no or very less percent activation compared against rifampicin, a known CYP3A4 inducer.

Table 4. IC_{50} values for CYP450 inhibition by ulixertinib and other known CYP inhibitors

	IC_{50} (µM)				
Inhibitor	**CYP3A4**	**CYP2D6**	**CYP2C9**	**CYP2C19**	**CYP1A2**
Ulixertinib	~10	~20	>20	>20	>20
Ketoconazole	0.08	NT	NT	NT	NT
Quinidine	NT	0.12	NT	NT	NT
Sulphaphenazole	NT	NT	0.72	NT	NT
Nootkatone	NT	NT	NT	1.02	NT
Furafylline	NT	NT	NT	NT	0.58

rhCYP metabolism

Data following incubations with different CYP isozymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4) considered in the panel suggested very minimal contribution of CYP1A2, 2C9, 2C19, 2D6 and 3A4 towards the metabolism of ulixertinib involving oxidation and dealkylation. This data is corroborating with the work reported by Germann et al [6].

Pharmacokinetic studies

The profiles of plasma concentration for ulixertinib following single oral and *intravenous* administration to mice, rats and dogs are shown in Figure 2A and 2B, respectively. The estimates of pharmacokinetic parameters in these species are summarized in Table 5.

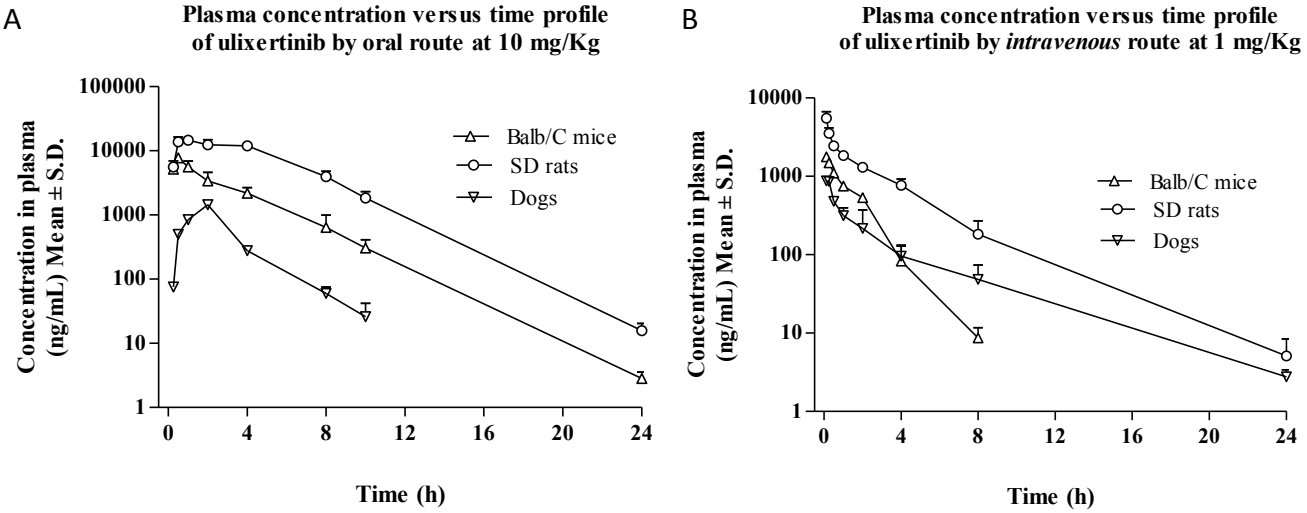

Figure 2. Plasma concentration-time profiles of ulixertinib after (A) oral (10 mg/kg) and (B) *intravenous* (1 mg/kg) administration to male Balb/C mice (mean ± S.D, n = 12), Sprague Dawley rats (mean ± S.D, n = 4) and Beagle dogs (mean, n = 2).

In the mice, plasma concentrations of ulixertinib decreased mono-exponentially manner after 1 mg/kg *intravenous* administration. The clearance was 6.24 mL/min/kg [7 % hepatic blood flow (HBF)]. The *in vivo* clearance was bit over predicted by the *in vitro* microsomes (~4-fold) and hepatocytes (~6-fold) scaled clearance. Ulixertinib had a high volume of distribution of 0.56 L/kg in mice, which is 38 times higher than that of total body water (TBW) of 0.015 L/kg [17]. The terminal $t_{1/2}$ was found to be 1.04 h. Post oral administration maximum plasma concentrations (C_{max}: 7768 ng/mL) attained at 0.5 h indicating rapid absorption from gastrointestinal tract. The terminal $t_{1/2}$ (2.06 h) determined after oral administration was longer than that after *intravenous* administration (1.04 h), which may indicate multiple sites absorption. The $AUC_{0-\infty}$ attained post oral dose was 24460 ng*h/mL. The oral bioavailability in mice at 10 mg/kg was 92 % (Table 5).

Table 5. Pharmacokinetic parameters of ulixertinib after *intravenous* and oral administration to mice, rats and dogs at 1 and 10 mg/kg, respectively

Strain	Route of adm.	Dose (mg/kg)	$AUC_{0-\infty}$ (ng h/mL)	C_{max}/C_o (ng/mL)	T_{max} (h)	$t_{½}$ (h)	Cl (mL/min/kg)	V_d (L/kg)	*F* (%)
BalbC mice	*Intravenous*	1.0	2672	1918	---	1.04	6.24	0.56	---
	Oral	10	24460	7768	0.50	2.06	---	---	91.6
Sprague Dawley rats	*Intravenous*	1.0	10179 ± 1528	6644 ± 1812	---	2.52 ± 0.07	1.67 ± 0.25	0.36 ± 0.06	---
	Oral	10	98421 ± 14005	15026 ± 2098	0.75 ± 0.29	2.02 ± 0.17	---	---	97.7
Beagle dogs	*Intravenous*	1.0	1091	1033	---	1.21	15.5	1.61	---
	Oral	10	3687	1442	2.00	1.29	---	---	33.8

In the rats, plasma concentrations of ulixertinib decreased mono-exponentially manner after 1 mg/kg *intravenous* administration. The clearance was found to be 1.67 mL/min/kg (3 % of HBF), which is in good agreement with *in vitro* (microsomes and hepatocytes) scaled clearance. The volume of distribution (0.36 L/kg) of ulixertinib in rat is approximated to TBW. The terminal $t_{1/2}$ was found to be 2.52 h. In rats also post oral administration early T_{max} of 0.75 h suggesting that ulixertinib has a rapid absorption from gastrointestinal tract. The $AUC_{0-\infty}$ and C_{max} attained post oral dose was 98421 ng*h/mL and 15026 ng/mL, respectively. The terminal $t_{1/2}$ determined after oral administration is 2.02 h, which is comparable to *intravenous* route half-life. The oral bioavailability in rat at 10 mg/kg was 98 % (Table 5).

In the dogs also plasma concentrations of ulixertinib decreased mono-exponentially manner after 1 mg/kg *intravenous* administration. The clearance was 15.5 mL/min/kg (~50 % HBF). The *in vivo* clearance was in good agreement with *in vitro* (microsomes and hepatocytes) predicted scaled clearance. The apparent volume of distribution 1.61 L/kg of ulixertinib in dogs is approximated to TBW. The terminal $t_{1/2}$ was found to be 1.21 h. In dogs post oral administration, maximum plasma concentrations (1442 ng/mL) of ulixertinib observed at 2.00 h, indicating it is a delayed T_{max} (when compared with T_{max} value of rodents). The $AUC_{0-\infty}$ was 3687 ng h/mL. The terminal $t_{1/2}$ determined after oral administration was 1.29 h. The oral bioavailability in dogs at 10 mg/kg was 34 % (Table 5).

Discussion

RAS/RAF/MEK signaling pathway is frequently activated in many cancers [2] and clinical efficacy of BRAF and MEK inhibitors confirms targeting RAS/RAF/MEK pathway has therapeutic potential and great promise [18]. Most of the resistance mechanisms to BRAF and MEK inhibitors ultimately lead to increase in phosphorylation of ERK1/2 [3]. Therefore, inhibition of ERK1/2 offers a promising strategy to address both innate and acquired resistance to BRAF and MEK inhibitors in various solid tumors. Ulixertinib is a novel compound, which selectively and potently inhibits ERK1/2 kinases in a reversible, ATP-competitive fashion. As a single-agent it inhibits tumor growth *in vivo* in BRAF-mutant melanoma and colorectal xenografts as well as in KRAS-mutant colorectal and pancreatic models [6]. In Phase-1 clinical studies, ulixertinib was well tolerated by patients with advanced solid tumors. Ulixertinib showed linear pharmacokinetics up to 600 mg (b.i.d), this was found to be MTD [7]. To the best of our knowledge there is no preclinical pharmacokinetic data reported for ERK1/2 inhibitors in the literature. Preclinical pharmacokinetics of a novel target class drug has great influence on the development and investigation of potential candidates for the further drug design, which necessitates a through exploration and understanding of pharmacokinetic disposition. This is important since *in vivo* pharmacokinetic behavior and pharmacokinetic-pharmacodynamic correlation act as surrogates for clinical effectiveness of potential drug candidates. In this paper we report the metabolic stability, permeability, protein binding, CYP inhibition, CYP induction, CYP phenotyping and pharmacokinetics in mice, rats and dogs for ulixertinib.

The metabolic stability data suggested that ulixertinib is moderately to highly stable under *in vitro* conditions using liver microsomal and hepatocytes preparations of various pre-clinical species and humans. The Caco-2 cell monolayer transport assay is well established test system for estimating drug absorption [19]. This Caco-2 cell monolayer system generated P_{app} value in absorptive direction for ulixertinib was 2.67 x 10^{-6} cm/sec, indicating it is a moderately permeable compound, however the P_{app} value in secretive direction appeared to much higher (8.09 x 10^{-6} cm/sec), suggesting involvement of efflux transporters in the Caco-2 monolayer. Our results indicate that ulixertinib weakly inhibits the major CYPs tested and IC_{50} values are greater than 20 µM (for most of CYPs tested). Ulixertinib did not show propensity to induce CYP34. So, CYP inhibition or induction is likely to represent a clinically significant risk of drug-drug interactions. Ulixertinib oral absorption in mice and rats was not limited by efflux as bioavailability in these two species was > 92 %. However in dogs the oral bioavailability was 34 % and this may be due to high unbound concentrations (fu 0.05 in dog plasma *vs* 0.001 and 0.003 in mice and rat plasma, respectively) in plasma and extensive metabolism in dogs thus ulixertinib had relatively high plasma clearance. In addition, higher volume of distribution of mice indicates that ulixertinib is highly distributed in mice compared to rats and dogs. Ulixertinib demonstrated high plasma clearance in dogs compared to rats and mice, which is 50, 3.0 and 7.0 % of HBF, respectively and was very much predicted by the *in vitro* experiment, indicating the difference in hepatic metabolism.

Conclusions

In conclusion, the preclinical data gathered in this work provided evidence that ulixertinib has excellent oral bioavailability in rodents and acceptable bioavailability in dogs, adequate metabolic stability and devoid of drug-drug interactions liability and different mechanism of action may provide differentiating features from other oncology compounds in this class. Overall, looking at the ulixertinib preclinical data presented by us and its current clinical data provide a good platform for researches to develop novel ERK1/2 inhibitors with potential benefit as a single or concomitant solid tumor therapy.

Conflict of interest: The authors have no conflict of interest

References

[1] A. Akinleye, M. Furqan, N. Mukhi, P. Ravella, D. Liu. MEK and the inhibitors: from bench to bedside. *Journal of Hematology & Oncology* **6** (2013) 27.

[2] D. Mattallanas, P. Crespo. New druggable targets in the Ras pathway? *Current Opinion in Molecular Therapeutics* **12** (2010) 674-683.

[3] M. Warthaka, C.H. Adelmann, T.S. Kaoud, R. Edupuganti, C, Yan, W.H. Johnson Jr, S. Ferguson, C.D. Tavares, L.J. Pence, E.V. Anslyn, P. Ren, K.Y. Tsai, K.N. Dalby. Quantification of a Pharmacodynamic ERK End Point in Melanoma Cell Lysates: Toward Personalized Precision Medicine. *ACS Medicinal Chemistry Letters* 5 (2015) 47-52.

[4] J.L. Bos. Ras oncogenes in human cancer: a review. *Cancer Research* **49** (1989) 4682-4689.

[5] W.J. Ding, W.P. Tao, T. Zeng, J.J. Deng, H.B. Lei, W. Ge. Selective ERK inhibitor inhibits proliferation and induces apoptosis in lymphoma cell lines. *International Journal of Clinical Experimental Medicine* **9** (2016) 10955-10962.

[6] U. Germann, B. Furey, J. Roix, W. Markland, R. Hoover, A. Aronov, M. Hale, G. Chen, G. Martinez-Botella, R. Alargova, B. Fan, D. Sorrell, K. Meshaw, P. Shapiro, M. J. Wick, C. Benes, M. Garnett, G. DeCrescenzo, M. Namchuk, S. Saha, D.J. Welsch. The selective ERK inhibitor BVD-523 is active in models of MAPK pathway-dependent cancers, including those with intrinsic and acquired drug resistance. *Cancer Research* **75** (2015) abstract no. 4693.

[7] J.R. Infante, F. Janku, A.W. Tolcher, M.R. Patel, R.J. Sullivan, K. Flaherty, R.D. Carvajal, A.M. Varghese, D.J. Lee Wong, M. Sznol, J.A. Sosman, A. Wang-Gillam, H.A. Burris, A. Ribas, S.P. Patel, D.J. Welsch, S. Saha. Dose escalation stage of a first-in-class phase I study of the novel oral ERK 1/2 kinase inhibitor BVD-523 (ulixertinib) in patients with advanced solid tumors. *Journal of Clinical Oncology* **33** (2015) abstract no. 2506.

[8] R.S. Obach. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metabolism and Disposition* **27** (1999) 1350-1359.

[9] D.F. McGinnity, M.G. Soars, R.A. Urbanowicz, R.J. Riley. Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. *Drug Metabolism and Disposition* **32** (2004) 1247-1253.

[10] Z. Wang, C.E. Hop, K.H. Leung, J. Pang. Determination of in vitro permeability of drug candidates through a Caco-2 cell monolayer by liquid chromatography/tandem mass spectrometry. *Journal of Mass Spectrometry* **35** (2000) 71-76.

[11] P.V. Balimane, Y.H. Han, S. Chong. Current industrial practices of assessing permeability and P-glycoprotein interaction. *Journal of AAPS* **8** (2006) E1-13.

[12] I. Kariv, H. Cao, K.R. Oldenburg. Development of a high throughput equilibrium dialysis method. *Journal of Pharmaceutical Sciences* **90** (2001) 580-587.

[13] R.S Obach, R.L. Walsky, K. Venkatakrishnan, E.A. Gaman, J.B. Houston, L.M. Tremaine. The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *Journal of Pharmacology and Experimental Therapeutics* **316** (2006) 336-348.

[14] V. Chu, H.J. Einolf, R. Evers, G. Kumar, D. Moore, S. Ripp, J. Silva, V. Sinha, M. Sinz, A. Skerjanec . In vitro and in vivo induction of cytochrome p450: a survey of the current practices and recommendations: a pharmaceutical research and manufacturers of america perspective. *Drug Metabolism and Disposition* **37** (2009) 1350-1359.

[15] T.W. Harper, P.J. Brassil. Reaction phenotyping: current industry efforts to identify enzymes responsible for metabolizing drug candidates. *Journal of AAPS* **10** (2008) 200-207.

[16] R. Kumar, P.S. Suresh, G. Rudresh, M. Zainuddin, P. Dewang, R.R. Kethiri, S. Rajagopal, R. Mullangi. Determination of ulixertinib in mice plasma by LC-MS/MS and its application to a pharmacokinetic study in mice. *Journal of Pharmaceutical Biomedical Analysis* **125** (2016) 140-144.

[17] B. Davies, T. Morris. Physiological parameters in laboratory animals and humans. P*harmaceutical Research* **10** (1993) 1093-1094.

[18] K.T. Flaherty, J.R. Infante, A. Daud, R. Gonzalez, R.F. Kefford, J. Sosman, O. Hamid, L. Schuchter, J. Cebon, N. Ibrahim, R. Kudchadkar, H.A. Burris 3rd, G. Falchook, A. Algazi, K. Lewis, G.V. Long, I. Puzanov, P. Lebowitz, A. Singh, S. Little, P. Sun, A. Allred, D. Ouellet, K.B. Kim, K. Patel, J. Weber. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *New England Journal Medicine* **367** (2012) 1694-1703.

[19] L.M. Chan, S. Lowes, B.H. Hirst. The ABCs of drug transport in intestine and liver:efflux proteins limiting drug absorption and bioavailability. *European Journal of Pharmaceutical Sciences* **21** (2004) 25-51.