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LC-ESI-MS/MS identification and characterization of ponatinib *in vivo* phase I and phase II metabolites

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ABSTRACT

Ponatinib (Iclusig®) is a multi-targeted tyrosine kinase inhibitor (TKIs). It is active against T315I and other BCR-ABL mutants. Investigation of *in vivo* metabolism of ponatinib was done using Sprague Dawley rats by giving one oral dose of PNT (4.7 mg/kg) to each rat and urine samples were gathered at several time intervals from dosing. Filteration of urine samples was done through 0.45 μm syringe filters. Phase separation using ACN was applied for extraction of ponatinib related metabolites. Characterization and identification of one *in vivo* phase II metabolite and thirteen *in vivo* phase I of PNT were done using LC-MS/MS. Phase I metabolic reactions were reduction, *N*-demethylation, hydroxylation, *N*-oxidation, oxidation and amide hydrolysis. Phase II metabolic reaction was glucuronidation of hydroxyl benzyl metabolites of ponatinib. The major *in vivo* metabolic reactions were α hydroxylation and α oxidation at piperazine ring. Literature review revealed no articles that have been published on *in vivo* metabolism of ponatinib in Sprague Dawley rats or ponatinib *in vivo* phase I and phase II metabolites structural characterization and identification.

1. Introduction

Tyrosine kinases act as “on” or “off” switch in many cellular functions. They are enzymes that catalyze the transfer of ATP γ phosphate to hydroxyl groups of tyrosine on intented proteins [1]. Tyrosine kinase enzymes control the activity inside the cell by regulating important processes. Cancer abnormal proliferation characteristics usually are driven by growth factor receptor-mediated signaling [2, 3].

Ponatinib (PNT) was approved in the U.S. and EU. PNT (Fig. 1), as a multi-targeted TKIs, is active against T315I and other BCR-ABL mutants [4]. PNT acts against imatinib-resistant mutants and against FGFR1-derived fusion kinases [5, 6]. The FDA approved PNT in 2012, but stopped sales on 31 October 2013 because of “the emergant risk of life-threatening severe narrowing of blood vessels and blood clots [7–10]. Iclusig returned market by FDA approval on Dec. 20, 2013 with a clear Black Box Warning [11–13].

Studying of drug metabolism is a crucial step in drug discovery, and is considered the factor which usually determines the ability of the developed drug to reach market and clinical use [14, 15]. Elimination of xenobiotics and endogenous compounds were initially done by increasing their hydrophilicity by metabolism. Metabolites are usually less toxic than their parent molecules, but sometimes bioactivation occurred forming reactive intermediates that are very reactive and toxic [16, 17].. *In vitro* and *in vivo* methedologies were used in studying drug metabolism. *In vivo* ponatinib metabolism was done by single oral dose administration to Sprague Dawley rats kept inside metabolic cages, and the consequent collection of urine samples that contain ponatinib and its *in vivo* metabolites.

In vivo metabolism of cyclic tertiary amines containing drugs usually generates many oxidative metabolites including N-oxygenation, *N*-dealkylation, α-carbonyl formation, ring hydroxylation and ring opening metabolites which formed through an iminium ion intermediate [18, 19]. The chemical structure of PNT contains *N*-methyl piperazine ring which is considered cyclic tertiary amine [20, 21]. *In vivo* metabolism of PNT generates more oxidized metabolites comparing to *in vitro* metabolism of PNT. The results from *in vivo* metabolism of PNT showed eight new metabolites. The major metabolic pathway *in vivo* metabolism was hydroxylation at the *N*-methyl piperazine ring while in case of *in vitro* metabolism was *N*-demethylation [6]. Oxidized cyclic tertiary amines are very easily dehydrated forming iminium species which are considered very reactive electrophiles that may explain the observed clinical side effects of PNT [21–23].

In vivo metabolism of PNT in human after single oral dose was discussed in human [24].but in our work, we gave more details about structure identification and characterization of the formed metabolites and we efficiently used low resolution LC-MS/MS technique in the identification of ponatinib *in vivo* metabolites using Sprague Dawely rats as an animal model. *In vivo* metabolism of ponatinib in Sprague Dawely rat gave similar results to what happened inside human body that approved the

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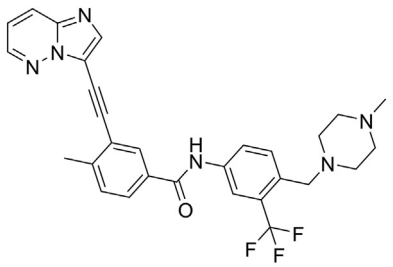

Fig. 1. Chemical structure of PNT.

applicability of studying *in vivo* metabolism using Sprague Dawely as an animal model [25–27].

2. Chemicals and methods

2.1. Chemicals

All chemicals and animals details are previously mentioned [6] except tween 80 which was procured from Eurostar Scientific Ltd. (UK) and poly ethylene glycol 300 (PEG 300) which was procured from Sigma-Aldrich (USA).

2.2. In vivo metabolism of PNT in Sprague Dawley rats

2.2.1. Protocol for Sprague Dawley rat dosing

Male Sprague-Dawley rats (number = 6, average weight: 340 g, 4 weeks of age) were gifted from college of pharmacy animal house at King Saud University (KSU). Animals' were maintained according to the Animal Care Center instructions at KSU that were accepted by Local Animal Care and Use Committee of KSU. Each rat was individually housed in metabolism cage for 72 h before study begining in a 12 h light and dark cycles. PNT dose for each rat was individually calculated and formulated in (30% PEG 300, 4% DMSO, 5% Tween 80 and HPLC H_2O) as suspension for oral dosing of rats. Average dose of PNT (Iclusig®) in human was 45 mg/qDay. By using the upcoming equation [28–30]:

$$\text{Rat}\left(\frac{\text{mg}}{\text{kg}}\right) = \text{Human}\left(\frac{\text{mg}}{\text{kg}}\right) * \text{km ratio}$$

$$\text{Rat}\left(\frac{\text{mg}}{\text{kg}}\right) = 0.75 * {}^{37}/_{6}$$

$$\text{Rat}\left(\frac{\text{mg}}{\text{kg}}\right) = 4.7\left(\frac{\text{mg}}{\text{kg}}\right)$$

Sprague Dawley rat dose was 4.7 mg/Kg. Oral gavage syringes were used for administration of PNT doses. Urine samples were collected from special compartments in the metabolism cages at 6, 12, 18, 24, 48, 72, 96 and 120 h following PNT dosing and stored at (−20 °C).

2.2.2. Sample preparation

Thawing of collected urine samples was done at room temperature. All urine samples were filtered using syringe filters (pore size: 0.45 μm) to remove any solid particles. Extraction of PNT and its *in vivo* metabolites was done using liquid liquid extraction (LLE). Acetonitrile (ice cold) was added to each urine sample (1:1 v/v). Shaking was done by vortexing for one minute. Phase separation between an aqueous (Aq.) urine sample and acetonitrile (water-miscible solvent) into two layers was achieved by using ice cold ACN and storing the mixture at 4 °C overnight [31–33]. The lower temperature (4 °C) resulted in ACN/urine mixture separated into two layers. Also, the slightly acidic *p*H of urine (5–7) and high concentration of salt content of urine matrix helped in phase separation [34, 35]. Samples were transferred to ice bath for 20 min. to maintain phase separation [33].Both upper organic (org.) layer and Aq. layer were transferred in separate tubes and evaporated to dryness under nitrogen stream. Reconstitution of the dried extracts of both layers was done in 1 mL of mobile phase and then transferred to HPLC vials for LC-MS/MS analysis. Control urine samples were processed following the same method of extraction.

2.3. Chromatographic conditions

Fragmentation patterns of PNT, its' *in vivo* metabolites were studied using LC (Agilent RRLC 1200 connected to triple quadrupole mass detector (Agilent 6410 QqQ). The most optimized LC-MS/MS analytical parameters for identification and separation of the PNT and its *in vivo* metabolites in Sprague Dawley rat urine samples are mentioned in Table 1.

2.4. Identification of in vivo PNT metabolites

Extracted ion chromatograms (EIC) for the supposed *in vivo* PNT metabolites were utilized to locate metabolites peaks in the total ion chromatogram (TIC) of both extracted layers. Fragmentation studies

Table 1
Optimized chromatographic conditions of the supposed LC-MS/MS methodology.

LC conditions			MS/MS conditions	
HPLC model	Agilent 1200		MS detector	Agilent 6410 QQQ
Elution mobile phase	A: Aqueous (H_2O contains 10 mM Ammonium formate at pH: 4.2 adjusted with HCOOH acid)		Electrospray (ESI) source	ESI under positive mode
	B: Organic (ACN)			Flow rate (12 L/min)
	Elution rate: 0.2 mL/min.			Pressure (55 psi)
	Run time: 110 min.			Drying gas: N_2 gas
	Post Time: 15 min.			
Column	Type	Eclipse plus C_{18}		T: 350 °C
	Particle size	1.8 μm		
	Internal diameter	2.1 mm		
	Length	50 mm	Analyte	PNT, its' *in vivo* phase I and phase II metabolites
	Temperature:	24 °C		
Gradient elution	Time	%B	Collision cell	High purity N_2 at collision energy of 15 eV
	0	5		
	20	25	Capillary	Fragmentor voltage: 135 V
	60	50		
	80	90		
	90	90		Capillary voltage: 4000 V
	110	5		