

Hong K. Kim, Virginia Borges, Dongmei Zhou, Li-Tain Yeh  
 Ardea Biosciences, Inc., San Diego, CA

## Overview

### Purpose

❖ Biosynthesis and isolation of two oxidative and two conjugated (glucuronide and glutathione) metabolites of VRX-480773

### Methods

- ❖ *In vitro* synthesis of two oxidative metabolites of VRX-480773 using microsomes
- ❖ *In vitro* synthesis of glucuronide and glutathione conjugate metabolites of VRX-480773 using liver S9 incubation and cofactors (1 mM of UDPGA and 5 mM of Glutathione)
- ❖ Isolation of metabolites by solid-phase extraction and semi-prep HPLC
- ❖ Structural identification by LC-MS/MS and NMR

### Results

- ❖ The structures of two oxidative metabolites, and glucuronide and glutathione conjugates of VRX-480773 were identified by LC-MS/MS and NMR
- ❖ Glutathione conjugate was produced by rat S9 incubation with cofactors, while glucuronide conjugate was produced by human S9 incubation with cofactors
- ❖ 10 – 20 µg of isolated metabolites were sufficient for 1D proton NMR using 2.5 mm probe.

## Introduction

VRX-480773, 2-[5-bromo-4-(4-cyclopropyl-naphthalen-1-yl)-4H-[1, 2, 4]triazol-3-ylsulfanyl]-N-(2-chloro-4-sulfamoyl-phenyl)-acetamide, is a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) and active *in vitro* against most of the clinically relevant NNRTI-resistant viruses. Since metabolites often differ in pharmacological activity, toxicity, and pharmacokinetic characteristics, the structural determination and the assessment of biological activity of the major metabolites are becoming major components of modern drug discovery programs. However, it is often difficult to chemically synthesize the major metabolites especially in the discovery stage. Therefore, this study describes the utilization of *in vitro* systems for the biosyntheses of the major oxidative and conjugated metabolites of VRX 480773 followed by their isolation and structural determination by LC-MS/MS and NMR.

## Methods

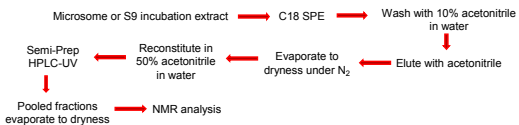
### LIVER MICROSOMAL INCUBATION

VRX-480773 (50 µg) + microsomes (1 mg/mL)  
 + PBS (100 mM, pH 7.4)  
 ↓ 37 °C, 3 min  
 + NADPH (1 mM)  
 ↓ 37 °C, 60 min  
 + 5 mL acetonitrile  
 ↓ Centrifuge at 10,000 rpm,  
 10 min  
 Metabolite isolation

### S9 INCUBATION

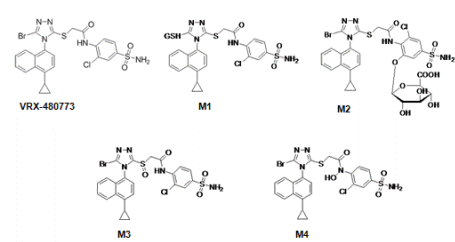
VRX-480773 (50 µg) + S9 (1 mg/mL)  
 + PBS (100 mM, pH 7.4) + 5 mM GSH  
 + 1mM UDPGA + 1 µg/mL alamethicin  
 ↓ 37 °C, 3 min  
 + NADPH (1 mM)  
 ↓ 37 °C, 60 min  
 + 5 mL acetonitrile  
 ↓ Centrifuge at 10,000 rpm,  
 10 min  
 Metabolite isolation

### ISOLATION OF METABOLITES



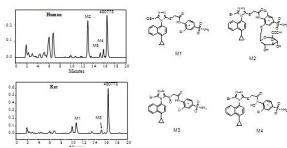
	HPLC PROFILING	SEMI-PREP	LC-MS/MS
HPLC	Shimadzu 10A	Shimadzu 10A	Agilent 1100
Detector	Shimadzu UV 254 nm, INUS β-ram Radio	Shimadzu UV 254 nm, INUS β-ram Radio	ABMDS Sciex API4000 mass spectrometer
Column	Kromasil C4 (4.6 × 250 mm, 5 µm)	Agilent ZORBAX XDB C18 (9.4 × 250 mm, 5 µm)	Phenomenex C8, (150 × 4.6 mm, 5 µm)
Injection volume (µL)	50-100	500	2.0
Run time (min)	30	25	30
Flow rate (mL/min)	0.8 mL	2.0	1.0
Mobile phase	A: 10 mM ammonium acetate: methanol (95.5, v/v) B: 10 mM ammonium acetate: methanol (5.95, v/v)	A: 10 mM ammonium acetate: methanol (95.5, v/v) B: 10 mM ammonium acetate: methanol (5.95, v/v)	A: 0.1% acetic acid in water B: 0.1% acetic acid in acetonitrile
Elution	Gradient	Gradient	Gradient
MS/MS			Turbo Ion Spray Positive ion mode IS temperature: 550°C IS voltage: 6000 V Declustering potential: 96 V Collision energy: 30 eV

## Structures

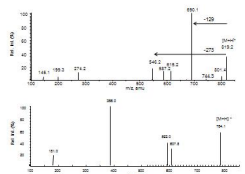


## Results

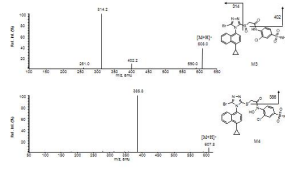
### HPLC Profile of [3H]-480773 (Phase I and II Metabolites, S9/Cofactors)



### MS/MS Spectra of M1 and M2

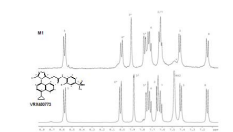


### MS/MS Spectra of M3 and M4



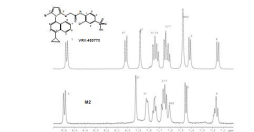
M1: The LC-MS showed an isotope ratio of 2:1 at m/z 819: 821 indicating the loss of bromine ion.

### NMR Spectral Expansion of the Aromatic Proton Region of VRX-480773 and M1



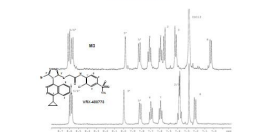
Except for the glutathione signals, there was no apparent difference between M1 (VRX-480773) in the one-dimensional 1D proton NMR spectra. No addition of an original peak found in the proton NMR spectrum of M1, indicating that the C-6' position of the glutathione conjugation.

### NMR Spectral Expansion of the Aromatic Proton Region of VRX-480773 and M2



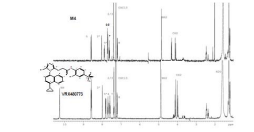
The 1D proton NMR spectrum of M2 showed similar proton pattern to that of VRX-480773 except for the loss of an-NH signal. The NMR data indicated that the site of glucuronide conjugation is the C-6' position of the amino-phenylbenzene end-moiety.

### NMR Spectral Expansion of the Aromatic Proton Region of VRX-480773 and M3



The 1D proton NMR spectrum of M3 showed similar proton pattern to that of VRX-480773. Major differences included significant downfield chemical shift changes for those signals assigned to H-2 (oxidation), significant up-field chemical shift changes for those signals assigned to H-4 (oxidation). These chemical shift changes can be attributed to the de-shielded or shielded effects of the resulted SO<sub>2</sub> group due to oxidation.

### NMR Spectra of VRX-480773 and M4



The 1D proton NMR spectrum of M4 showed similar proton pattern to that of VRX-480773 except for the absence of the signal of major chemical shift of proton H' signal to upfield, and small downfield shift of CH<sub>2</sub> signals.

## Conclusions

- ❖ Two oxidative metabolites (M3 and M4), and glutathione (M1) and glucuronide (M2) conjugates of VRX-480773 were isolated following incubation with rat and human liver microsomes or S9 with cofactors.
- ❖ The isolated metabolites were pure based on UV trace (254 nm).
- ❖ The structures of four metabolites (M1, M2, M3, M4) were identified by LC-MS/MS and NMR.
- ❖ Authentic standard (M3) was prepared based on MS/MS and NMR data.
- ❖ 10-20 µg of isolated metabolites were sufficient for 1D proton NMR analysis using 2.5 mm probe.