

Inhibition of Human Cytochromes P450 *in vitro* by Ritonavir and Cobicistat

A thesis

Submitted by

Md Amin Hossain

In partial fulfillment of the requirements
for the degree of

Master of Science

In

Pharmacology and Drug Development

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

May, 2016

Advisor

David J Greenblatt, MD

Abstract

Like ritonavir, cobicistat is also a potent inhibitor of cytochrome P450 3A enzymes, including subtype CYP3A4. Both of them have the ability to inhibit liver enzymes that metabolize other medications used to treat HIV, like atazanavir, darunavir and elvitegravir. Ritonavir and cobicistat have been using in highly active antiretroviral therapy (HAART) combination for boosting activity that suppresses HIV replication. The mechanism of action behind the antiretroviral boosting drug is to inhibit human cytochrome (mainly CYP450) isoforms which may lead to increase HIV antiviral drug's plasma concentration. Primary objective of this study is to see whether ritonavir and cobicistat inhibit any other cytochrome isoforms and to figure out what is the inhibition mechanism of these two drugs. This paper evaluates the inhibiting effect of ritonavir and cobicistat on different cytochrome isoforms, including CYP2B6 and CYP2C19 using an *in vitro* model based on human liver microsomes. Ritonavir inhibited CYP2C19 and CYP2B6 activity with mean IC_{50} 20.69 μ M and 15.41 μ M accordingly. Cobicistat also inhibited CYP2C19 and CYP2B6 activity with mean IC_{50} 6.45 μ M and 10.17 μ M accordingly. For CYP2B6 and CYP2C19, percent control values (ritonavir and cobicistat) for pre-incubation phase were always higher than without pre-incubation one. This indicates that, the inhibition mechanism is not time-dependent (mechanism based) rather time independent in this study. In summary, cytochrome isoforms (CYP2C19 and CYP2B6) were significantly inhibited in not time-dependent manner by ritonavir and cobicistat.

Acknowledgements

It would not be possible to finish my lab work if I was not helped by my lab co-workers Tim Tran and Tianmeng Chen, lab officer Yanli Zhao and last but not the least masters graduate Lei Cao. They were always so helpful all the time with any difficulties I faced. It is hard to come to a summary with a good outcome for a specific research in a very short time. Even though, I could have done many experiments with significant results. And for this success of my lab works, I would like to give a very special thanks to my PI Professor David Greenblatt whose dedication and guidance was always with me. Without his direction, dedication, all the new techniques, method of solving a critical situation, lab supply, funding and cooperation it would not be possible to finish my graduate thesis.

Table of Contents

Abstract	ii
Acknowledgements.....	iii
List of Tables.....	v
List of Figures.....	vi
Abbreviations.....	vii
Introduction.....	1
Method.....	4
Human Liver Microsome (HLM) preparation.....	4
Reagent Preparation.....	5
<i>In Vitro</i> study design.....	6
Incubation Procedures.....	7
<i>In Vitro</i> metabolism of Bupropion via CYP2B6	7
Pre-incubation.....	10
<i>In Vitro</i> metabolism of S-Mephenytoin via CYP2C19	11
Mobile Phase.....	11
Co-factor	12
HPLC Basics.....	12
Analysis of data.....	14
Results.....	15
CYP2B6 (Bupropion hydroxylation)	16
CYP2C19 (S-Mephenytoin hydroxylation)	19
Discussion and Conclusions.....	23
References	25

List of Tables

Table 1 (Analytical methods used in the study).....	14
---	----

List of Figures

Fig. 1. HPLC chromatogram of CYP2B6.....	09
Fig. 2. HPLC chromatogram of CYP2C19.....	11
Fig. 3. A simple HPLC model.....	12
Fig. 4. Different positive control inhibitor for different CYP showing inhibition.....	15
Fig. 5. OH-Bupropion formation by CYP2B6 HLM789.....	16
Fig. 6. OH-Bupropion formation by CYP2B6 HLM866.....	17
Fig. 7. OH-Bupropion formation by CYP2B6 HLM870.....	18
Fig. 8. OH-Bupropion formation by CYP2B6 HLM871.....	19
Fig. 9. OH-Mephenytoin formation by CYP2C19 HLM866.....	20
Fig. 10. OH-Mephenytoin formation by CYP2C19 HLM870.....	21
Fig. 11. OH-Mephenytoin formation by CYP2C19 HLM9511.....	22
Fig. 12. OH-Mephenytoin formation by CYP2C19 HLM Pooled.....	23

Abbreviations

HPLC: High pressure liquid chromatography

HLM: Human Liver Microsome

Rito: Ritonavir

Cobi: Cobicistat

HIV: Human immunodeficiency virus

AIDS: Acquired Immune Deficiency Syndrome

IC₅₀: Inhibitory Concentration at 50% concentration producing 50% reduction in reaction velocity

TDI: Time dependent inhibition

DDI: Drug-Drug Interaction

IS: Internal standard

ADE: Adverse Drug Events

FDA: Food and Drug Administration

CYP: Cytochrome P

PI: Protease inhibitor

P: Pre-incubation

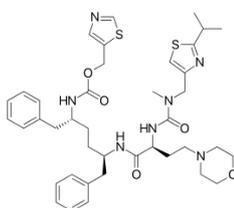
NP: No Pre-incubation

Introduction

Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) are caused by Human Immunodeficiency Virus (HIV). An infected person may experience influenza-like illness, and as the disease progresses, it reduces competence of the immune system and might cause susceptibility to tuberculosis. Approximately 37 million people around the world currently are infected with HIV. In 2008, approximately 1.2 million people were living with HIV which resulted in about 17,000 deaths in United States [1]. The primary treatment of HIV/AIDS usually includes antiretroviral drug therapy. As HIV/AIDS is increasing day by day in the world, it has become a great area of interest for doing further research work. There are several different classes of antiretroviral drugs which act on different stages of the HIV life cycle. These drugs can work alone in human. However they are quickly metabolized. A common remedy is, a combination of drugs for treating HIV/AIDS also known as Highly Active Antiretroviral Therapy (HAART) is used. They have been sometimes used with other protease inhibitors. The most commonly used antiretroviral boosting drugs are ritonavir and cobicistat. These drugs were tested against cytochrome 3A4, but this study will help to find out other isoforms with which they show a drug-drug interaction [2]. Furthermore, determination of the IC_{50} of these two drugs will give us an idea about their inhibition mechanism by comparing with and without pre-incubation methods. This comparison study between cobicistat and ritonavir may show their mechanism of inhibition.

Inhibition of cytochrome P450 enzymes is the most common mechanism in drug-drug interactions. The inhibitory effect can be reversible or irreversible (time dependent). Time-dependent inhibition of CYP450 is a particularly concern, since *de novo* synthesis of enzyme is required to restore activity. Most inhibitory drugs produce competitive, noncompetitive or mixed inhibition. On the other hand, time-dependent inhibition (TDI) has change in IC₅₀ due to pre-incubation with inhibitor. Ritonavir and cobicistat are known to inhibit CYP3A4 but we are looking for more CYP isoforms to determine whether they are inhibited by these drugs [2]. For that, some different drugs were taken which are metabolized by different cytochromes.

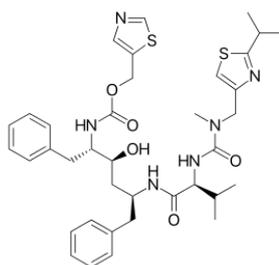
Drug interaction is a major consideration while using these antiretroviral boosting drugs for treating AIDS. As depression frequently accompanies HIV infection, bupropion which is a potent atypical antidepressant might be co-administered with antiretroviral drug [3]. At the same time, s-mephenytoin might also be used along with antiretroviral boosting drug which acts as an anticonvulsant.



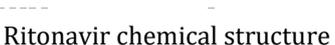
Cobicistat chemical structure

Though cobicistat has no anti HIV activity of its own, it plays a major role in reducing the clearance of other antiretroviral drugs when it is given in a combination in HAART therapy. Cobicistat was first approved in August 2012 in combination with elvitegravir, emtricitabine, tenofovir disoproxil. Later,

elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide was approved by the FDA in November 2015 in USA [4]. Additionally, there are also some fixed dose combinations of this drug. Protease inhibitors darunavir and atazanavir were approved by the FDA to be used in a combination with cobicistat in January 2015. This drug has a potent mechanism based inhibition activity against cytochrome P450 3A, including subtype 3A4. Its co-administration with other drugs that are CYP3A substrates may lead to expected boosting effect and unintentional drug-drug interaction. In comparison to ritonavir, cobicistat has



better solubility and also it has more selectivity towards CYP3A inhibition than other CYPs.

Ritonavir was first approved by the FDA on March 1, 1996 [6]. The total death rate from HIV was decreased after ritonavir came to the market. It was developed as an inhibitor of  used in HAART combinations for antiretroviral therapy. The use of antiretroviral drugs can inhibit different stages of the HIV life cycle, which leads to durable and stable suppression of viral load. This leads to increased CD4 T cell counts, so ultimately reduces the mortality rate.

CYPs are major enzymes involved in drug metabolism, responsible for 75% of total metabolism [7]. Many drugs may decrease the activity of various

cytochromes by directly inhibiting the activity of the CYP. This is a major source of drug-drug interactions as changes in CYP enzyme activity may affect metabolism of various drugs. Antiretroviral drugs are principally metabolized by the cytochrome P450 3A subfamily (CYP3A) in liver and intestine, resulting in low systemic exposure after oral administration. Ritonavir and cobicistat are antiretroviral boosting agents which help to boost bioavailability. Studies have shown that ritonavir enhances the pharmacokinetics of important antiviral drugs which are CYP3A substrates like elvitegravir and narlaprevir [8]. Studies have shown that, elvitegravir is metabolized by CYP3A4 in liver and intestine. When it dosed alone, 400mg twice daily was required to achieve a reasonable plasma level. Whereas, when elvitegravir was co-administered with 100 mg of ritonavir, only once daily dosage was needed to achieve plasma concentration. However, there are some disadvantages of ritonavir, including unwanted drug-drug interactions and lipid disorders.

The purpose of this study is to introduce the reader to an updated evidence-based drug-drug interaction with a view to treat HIV/AIDS. Besides previous studies have shown that ritonavir potentially inhibit CYP2B6 with a 50% decrease of reaction velocity (IC_{50}) value of 2.2 μ M [9]. However this study may give an idea about interactions with other isoforms and this may potentially be helpful in the field of treating HIV infected patients.

Method

Human Liver Microsome (HLM) preparation

All liver samples from individual human donors with no known disease were provided by the International Institute for the Advancement of Medicine; the

Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN); or the National Disease Research Interchange (Philadelphia, PA). Based on in vitro phenotyping studies, all the liver samples were CYP2B6, CYP2C19 normal metabolizer phenotype. Microsome pellets were prepared through suspension in 0.1M Potassium Phosphate buffer which contained 20% glycerol and were kept at -80C until uses.

Reagent Preparation

Chemical reagents which were used for incubation procedure and HPLC analysis were purchased commercially. Most of the substrates, inhibitors, positive controls and internal standards were purchased from Sigma-Aldrich, USA. All stock solutions were made with methanol and kept at -18°C. Solutions were diluted further in methanol for experimental use.

The stock solutions of both ritonavir and cobicistat were kept at -18°C, working concentrations were made fresh weekly from stock. For both ritonavir and cobicistat, working concentration is 25 µM. Those working concentration were made by diluting stock using methanol.

Quinidine, clopidogrel, ticlopidine and omeprazole were used as positive controls for different CYPs. Bupropion and s-mephenytoin were used as substrates. OH-Bupropion and OH-mephenytoin were metabolite products. Trazodone and phenacetin were used as internal standard for the assays.

Potassium phosphate buffer, acetonitrile, and methanol were used in mobile phase in appropriate ratio depending upon analysis.

In all experiments methanol was used to make stock solution and also to make working concentrations.

***In Vitro* study design**

In vitro techniques were studied to evaluate the effect of ritonavir and cobicistat on human cytochromes P450. Comparative studies of mechanism of inhibition of these two drugs were carried out. Ritonavir and cobicistat are supposedly to have inhibitory effect on CYP3A but in this study both were also tried for different cytochromes as well. In order to find the mechanism of inhibition of these two drugs, the study had to go through three major steps for each of the CYPs. Major three steps are, (a) finding out the IC_{50} of positive control for each CYPs, (b) finding out the IC_{50} for both ritonavir and cobicistat, (c) finding the mechanism of inhibition of ritonavir and cobicistat for specific CYPs. In order to do the last step we need to do both pre-incubation and without pre-incubation steps. In order to find out whether they display time-dependent or independent inhibition, three major steps were studied for CYP2D6, CYP2B6 and CYP2C19. Moreover, to gather more accurate results, each of the CYPs was studied with four different human liver microsomes (HLM). The amount of substrate, inhibitor, internal standard, positive control and stop solution are different for each cytochrome. The first two steps are accomplished according to no pre-incubation method. However the last step was carried out in both pre and without pre-incubation method. A fixed concentration of index substrate and its specific inhibitor (Table 2) were used in studies of each specific CYP isoforms. Consecutive incubations included inhibitor-free controls, fixed concentrations of

the positive control inhibitor, and varying concentrations of ritonavir and cobicistat. Each individual incubation data point was carried out in duplicate using four or five different human liver samples.

Incubation Procedures

***In Vitro* metabolism of Bupropion via CYP2B6**

In order to determine the IC₅₀ of positive control clopidogrel, label seven sets of microcentrifuge reaction tubes (each set has two tubes) with different inhibitor's concentration (0, 0.01, 0.05, 0.1, 0.5, 1 and 0 μ M) then put 250 μ l of Bupropion (250 μ M) in all of them. All the chemicals and reagents were used when they achieved room temperature. 5 μ M Clopidogrel was added into all the vials according to the calculation except for the 0 μ M. All the vials were then taken into the freeze dryer for drying down. The main reason to dry down is to get rid of methanol as methanol will interrupt the reaction between HLM and substrate. They were kept inside the dryer until the solvent were evaporated. Human liver sample 866 was kept inside ice on a bowl. In the meanwhile, water bath was turned on with shaker. It was set at 37°C.

Bupropion final stock solution (250ml of 250 μ M) calculation was determined according to the table 1. According to the protocol final volume of liver microsomes should be 150 μ l. After HLM was made, it was kept in the ice bucket. At the same time, in another glass tube co factor was made. Every 4 mL cofactor contains: 4 mg NADP, 10 mg isocitric acid, 100 uL dehydrogenase, 1.9 mL 50mM incubation buffer (pH=7.5), 1 mL 50mM MgCl₂ and 1 mL 100mM phosphate buffer (pH=7.5). This glass tube was also kept inside the ice bucket. Then seven new different tubes were taken and marked with the previous concentration and

then 240µl cofactor, 150µl HLM, 210µl incubation buffer were added in all of them. They were spun vortex and then put in the incubator with shaker for 20 minutes at 37°C. After 20 minutes, all the vials were taken out from incubator and then 250µl was distributed in the dried (substrate and inhibitors are already in the dried vials) vials accordingly. In this step we will have to be very careful as the incubation timing for all the vials should be same so pipetting should be faster in this step so that we can get consistent results. After distributing, vials put back in the incubator for more 20 minutes. The basic reaction was between CYP2B6 and clopidogrel. Bupropion metabolizes through CYP2B6 at the same time clopidogrel tries to inhibit metabolizing bupropion by CYP2B6. So the outcome is to keep substrate in the system for long time.

We must be careful about the incubation time as prolong incubation might take the reaction reversible. After certain time the reaction was terminated using 100µl of stop solution for each reaction tubes (1.0N hydrochloric acid for stopping reaction and internal standard Trazodone 125µM). Trazodone and Hydrochloric acid should be in a mixture of 1:3. After putting the stop solution the tubes were vortex mixed and kept inside the ice bucket to further terminate the reactions. Ice helps to terminate the reaction as the HLM only takes part in reaction at 37°C. After keeping in the ice bucket for two minutes, all the tubes were placed in centrifuge for 10 minutes at 14,000g. At the same time, same count of HPLC tubes and capillary tubes were taken in the tube holder. Only supernatant was collected after centrifuge and put in the HPLC vial accordingly without disturbing the pellet at the bottom. Then they were kept in the freezer or directly in the HPLC for analysis. This is the first step for finding out the IC₅₀ for

positive control inhibitor. Bupropion is metabolized by CYP2B6 and clopidogrel is inhibitory positive control for CYP2B6. So the first step should be finding out the IC_{50} of positive control.

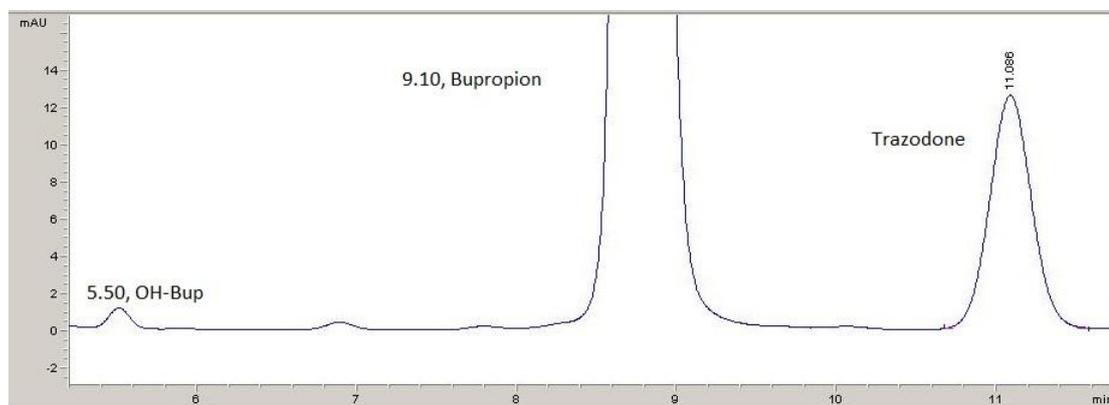


Figure. 1. HPLC chromatogram of in vitro biotransformation of Bupropion by CYP2B6

The second step is a little bit different than the first step. Now we will have to figure out the IC_{50} of ritonavir and cobicistat using IC_{50} as positive control. In this step 8 sets of vials for ritonavir and 8 sets of vials for cobicistat were taken. In general, for both ritonavir and cobicistat the usual concentrations are 0, 1, 5, 10, 20, 50, IC_{50} and 0 μM . Substrate and inhibitors were placed in the tubes and set in the dryer. The rest of the experiment is same as step one. The main difference between these two steps is inhibitor. For first step, clopidogrel was used as established positive control and for second step, both rito and cobi were used as inhibitor. The above procedure is without pre-incubation method for this assay.

Basic method for doing third step is similar to first two steps. However, third step includes both pre-incubation and without pre-incubation method. No pre-incubation method already described earlier.

Pre-incubation

In pre-incubation method, basically inhibitor and HLM get more time to react together. So as a result inhibitor gets more time to inhibit specific cytochrome (CYP2B6 in this case). When the mixture of HLM, incubation buffer, co-factor and inhibitor was distributed in the substrate tube after first incubation, the substrate was expected to form less metabolite. Because the inhibitor gets more time to react with HLM but on the other hand without pre-incubation step, inhibitor does not get more time to react with HLM. This is called time-dependent inhibition. That means the percent control from pre-incubation step should have more inhibition if it follows time-dependant inhibition. To perform third step of the assay, we need to take positive control IC_{50} from first step, IC_{50} of Ritonavir and Cobicistat from second step. Two sets of vial were taken to perform no pre-incubation and pre-incubation method. Each set should contain five vials to perform the experiment. For no pre-incubation step, the procedure was the same exactly described earlier. But for pre-incubation set of vials, inhibitor and substrate were taken separately then put in the dryer. HLM, incubation buffer and co-factor were added in the dried inhibitor vials. Then they were set in the incubator for 20 minutes and after that 250 μ l from each of the vials were added to the substrate only vials for further 20 minutes incubation. Rest of the procedure was done in same way as first step.

There were some basic differences in different HLMs and CYPs. For example sometimes some HLM does not respond well to metabolism for specific cytochrome isoforms. CYP2C19 takes longer time than regular incubation time which is almost 60 minutes in compare to CYP2B6. If we incubate for

conventional 20 minutes, no metabolite peaks will come in the HPLC analysis. So we will have to consider which HLM and CYP we are working with.

***In Vitro* metabolism of S-Mephenytoin via CYP2C19**

This *in vitro* study of CYP2C19 was conducted exactly same as CYP2B6 except for some few parameters and chemicals. At first, S-Mephenytoin (45 μ l of 229 μ M stock) as substrate, Omeprazole 62.5 μ M as inhibitor, 500 μ M Phenacetin as internal standard and 500 μ M Phenacetin & Acetonitrile (1:30) were used as stop solution mixture. All other parameters are given in the table 1.

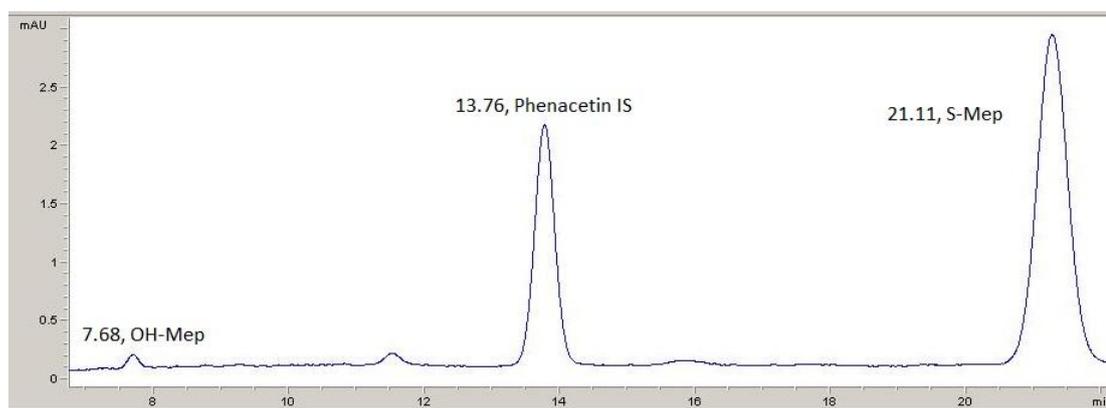


Figure 2. HPLC chromatogram of *in vitro* biotransformation of S-Mephenytoin by CYP2C19

Mobile Phase

1L (85%) of distilled water and $KPHO_4$ were taken in a vial and then placed on a electrical stirrer. When all the particles was dissolved, it was filtered using vacuum to another fresh vial and then pH was maintained to 3.0 using HCl (sometimes KOH or NaOH depending on pH). 15% acetonitrile (176.4ml) was added into the vial as an organic phase. Ultrasonic bath can be used to get rid of bubbles.

Ratio of mobile phase for both the cytochromes are described in the table 1.

Co-factor

For every 4ml of co-factor, 4 mg of β -NADPH β -Nicotinamide adenine dinucleotide phosphate sodium salt, 10 mg of DL-Isocitric acid, 1.9 ml of incubation buffer (50mM), 1 ml of 50mM MgCl₂, 1ml of 100mM phosphate buffer (ph=7.5) and finally 100 μ M phosphate buffer were added together.

HPLC Basics

Agilent 1200 series isocratic HPLC with Waters μ Bondpack C18 10 μ m 125A 3.9X300mm column was used to perform all the analyses in this study. Detection method, mobile phase, injection volume, run time and flow rate are described in table 1.

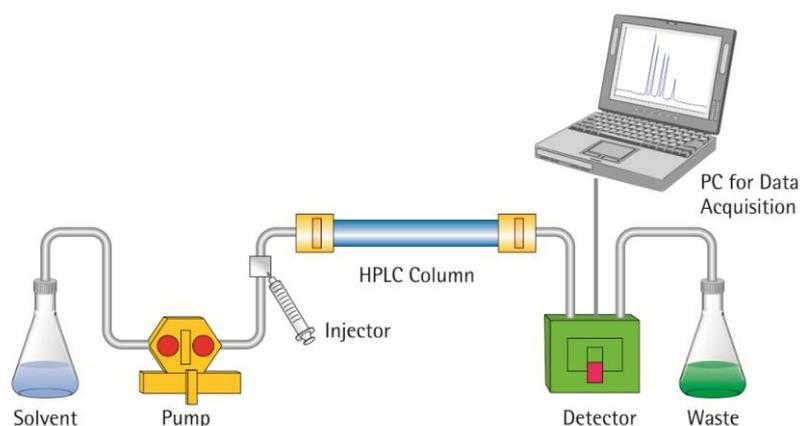


Figure. 3. A simple HPLC showing its main parts for performing assay Basically isocratic HPLC deals with one mobile phase and usually it is used for analysis of one or two compounds in a single run. In a reverse phase system, mobile phase is polar. So when any polar compound passes through column it goes faster as it likes the mobile phase (as both of them are polar). At the same time, the compound which is non polar it will try to stay with stationary phase as

it is non polar. The polar compound which will leave the column first, will be appeared on the most left of chromatogram but non polar compound which will try to stay in the stationary phase will appear in the chromatogram on right. Two mobile phases could also be considered as isocratic as long as the composition is the same. However, 90% polar and 10% non polar mixture makes less polar than before. As a result the more non polar compound will go faster than before. That means the total retention time of the whole chromatogram will be shorter than before. So this is the reason, we add organic phase (acetonitrile) when we see peaks at delayed retention time (more right portion of the chromatogram). More organic phase makes the mobile phase more non polar, so non polar compound does not want stay longer in the column as a result we get our desired peak within a shorter time [10].

Agilent ChemStation software was used to analyze the data. Flow rate, injection volume and run time were selected using the software according to the table 1. Integration and calibration were done for analyzing the data. Integration helps to integrate all the desired peaks in the chromatogram. Calibration helps to specify the range of retention time where a specific peak is suppose to have. In other way we can say calibration is to set the standard for the specific peak in specific area. Percent controls were calculated using Microsoft excel. Each of the different concentration of inhibitors has shown different percentage of metabolite formation.

Analysis of data

After getting metabolite formation rates, these data were analyzed using SigmaPlot. Reaction velocities with addition of inhibitor were expressed as a percentage ratio (R_v) of the control velocity with no inhibitor present. The relationship of inhibitor concentration (C) and R_v was analyzed by nonlinear regression (SigmaPlot 13.0) to determine the concentration producing a 50% decrement in reaction velocity (IC_{50}), based on the following equation [9]:

$$R_v = 100 \left(1 - \frac{E_{\max} C^b}{C^b + IC^b} \right)$$

Iterated variables were: E_{\max} , the maximum degree of inhibition; IC , the inhibitor concentration producing an R_v value of 50% of $(100 - E_{\max})$; and b , an exponent.

The actual IC_{50} was calculated as:

$$IC_{50} = IC / (2E_{\max} - 1)^{1/b}$$

IC_{50} values were determined based on mean data points across four or five liver samples at corresponding inhibitor concentrations. The IC_{50} value determined by this method is independent of the mode of inhibition. SigmaPlot 13.0 was used to find all the IC_{50} of the assay.

Table 1: Analytical methods used in the study

CYP Isoform	Substrate	Product	Mobile Phase	Detection mode	Positive Control Inhibitor
2B6	Bupropion (250 μ M)	OH-bupropion	15% CH ₃ CN, 85% buffer pH 3.0	U.V. 214 nm Inj vol 10 μ l Flow 1.2ml/m Stop 23mins	Clopidogrel (0.1 μ M) Ticlopidine
2C19	S-mephenytoin (25 μ M)	4'-OH-mephenytoin	20 % CH ₃ CN, 80% buffer	U.V. 230 nm Inj vol 20 μ l Flow 1.3ml/m Stop 23mins	Omeprazole (10.0 μ M)

Aqueous buffer is 50mM Potassium Phosphate

Results

All positive control inhibitors (Table 1, Fig 4) produced the anticipated degree of inhibition of their respective CYP isoforms.

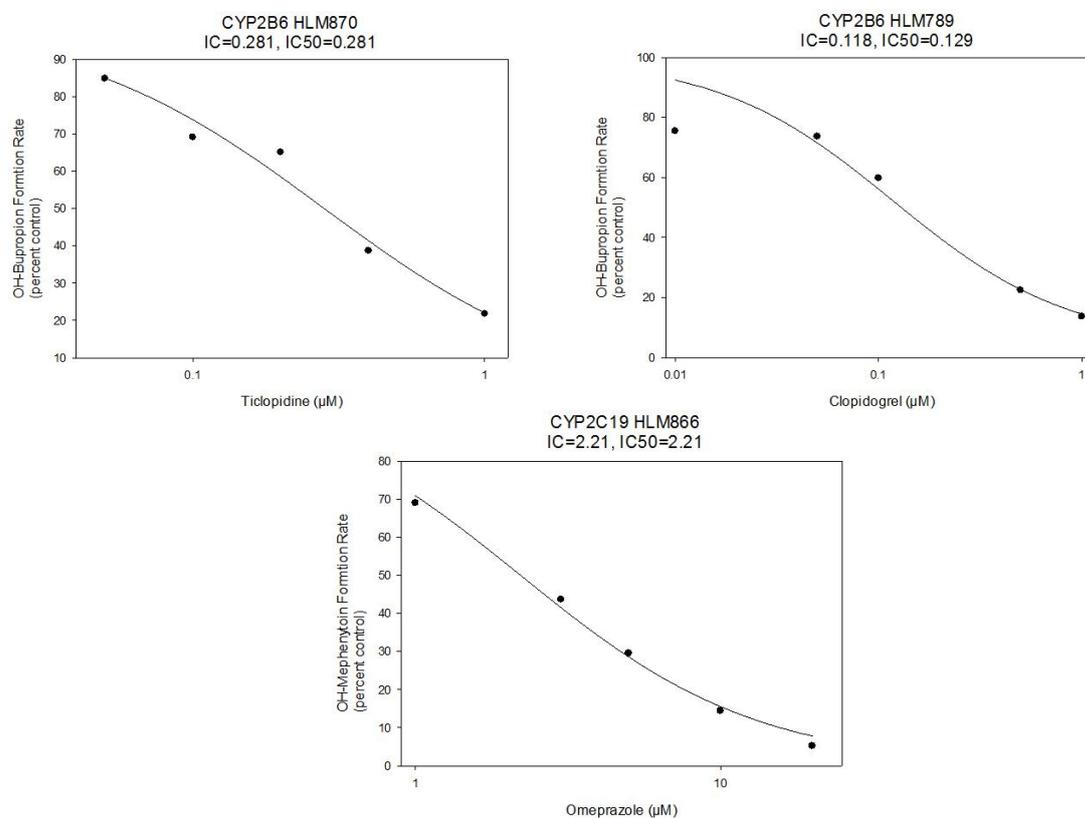


Figure 4. Different positive control inhibitor for different CYP showing inhibition

The calculated mean IC_{50} value for omeprazole was $2.21 \mu\text{M}$ which is similar to previous studies (relative contributions of CYP2C19 and CYP2C9). Fig 4 shows both ticlopidine (IC_{50} 0.281) and clopidogrel (IC_{50} 0.129) were potent inhibitors for CYP2B6.

CYP2B6 (Bupropion hydroxylation)

In both cases, ritonavir and cobicistat inhibited CYP2B6 activity. Cobicistat inhibited OH-bupropion formation rate with IC₅₀ value of 3.57 μM (without pre-incubation) in HLM 789. Ritonavir also inhibited CYP2B6 activity with IC₅₀ value of 3.50 μM (without pre-incubation). It makes clear from the graph that these drugs were not indicating time-dependent (mechanism based) inhibition. Same results goes for HLM866, ritonavir showed inhibition with IC₅₀ 6.61 and cobicistat showed IC₅₀ 5.74. There is no significant change in IC₅₀ so they are not time-dependent inhibition.

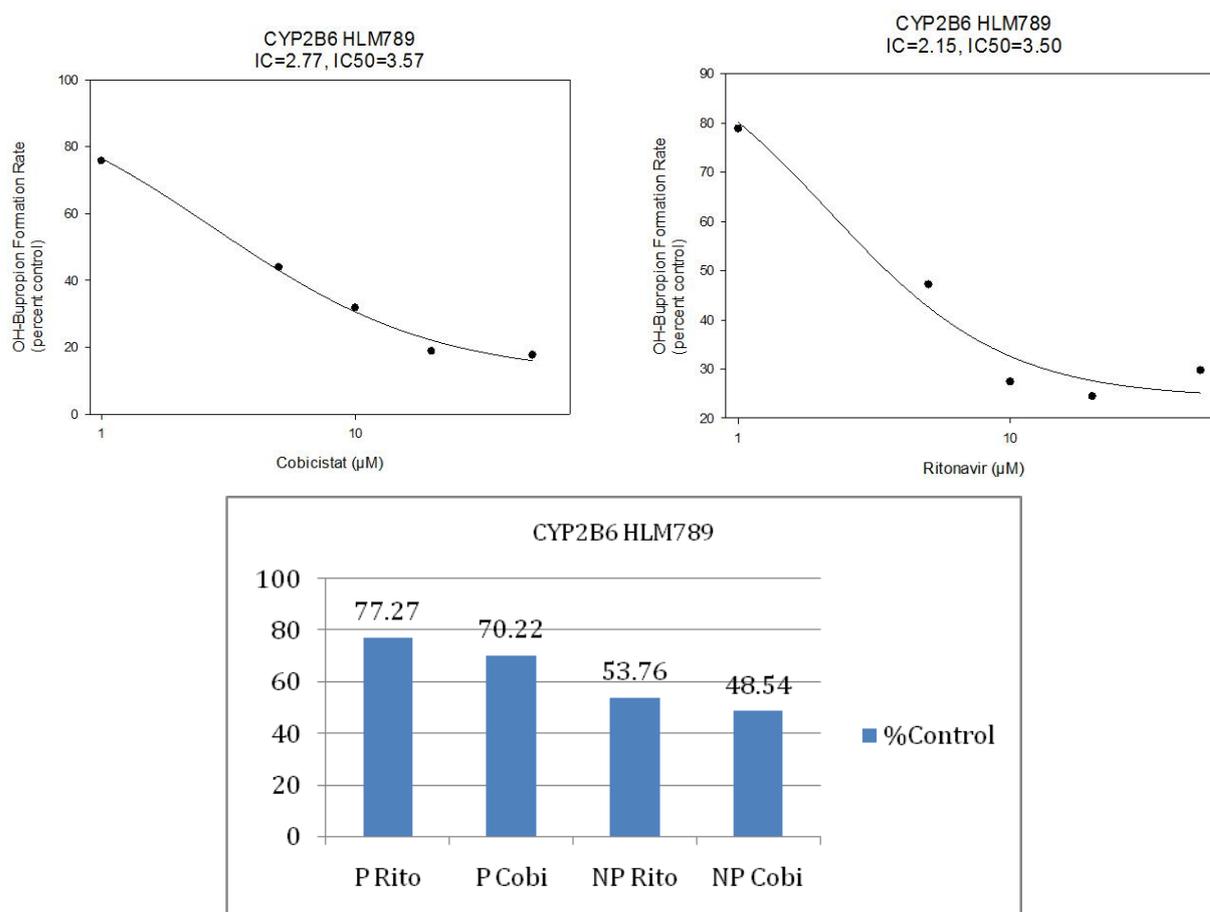


Figure 5. Rates of OH-Bupropion formation from Bupropion with either Ritonavir or Cobicistat in HLM789 and comparison between pre-incubation and without pre-incubation

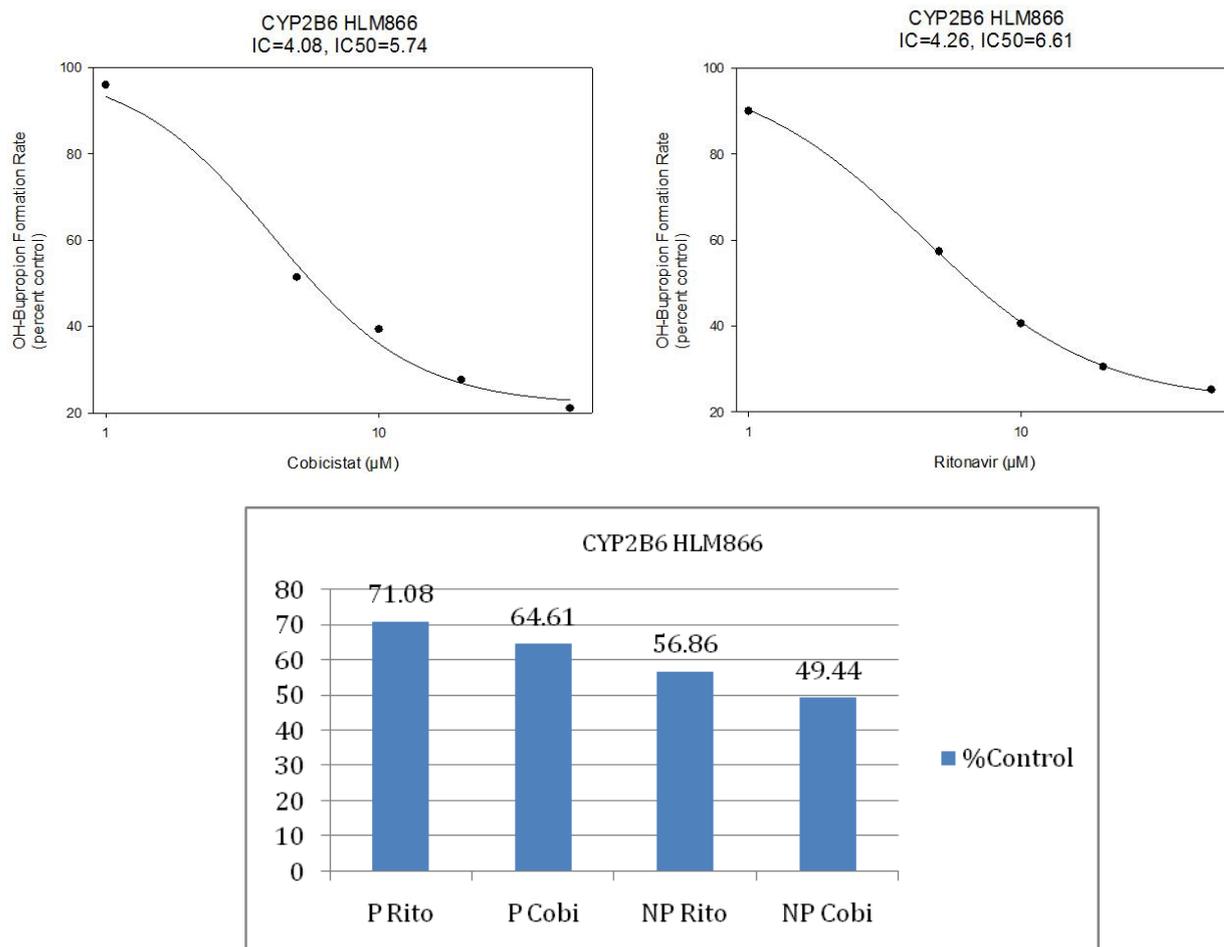


Figure 6. Rates of OH-Bupropion formation from Bupropion with either Ritonavir or Cobicistat in HLM866 and comparison between pre-incubation and without pre-incubation

Besides, the comparison study between pre-incubation and no pre-incubation shows the percent control for no pre-incubation is much lower than the pre-incubation one. This demonstrates these inhibition mechanism is not time-dependent.

For HLM 870, they have the similar IC_{50} , when they were not pre-incubated. Cobicistat showed a great inhibition with IC_{50} value of 17.26 μM . On the other hand, ritonavir also inhibited CYP2B6 and IC_{50} was 22.25 μM (Figure 7). For HLM

871, ritonavir gave inhibition with IC_{50} 29.29 and cobicistat gave IC_{50} 14.12. In both case, inhibition was not enhanced. And also we can look at the P and NP graphs, which show there is a significant difference between pre and no pre-incubation. As a result, ritonavir and cobicistat do not consider time-dependent inhibition in CYP2B6 study.

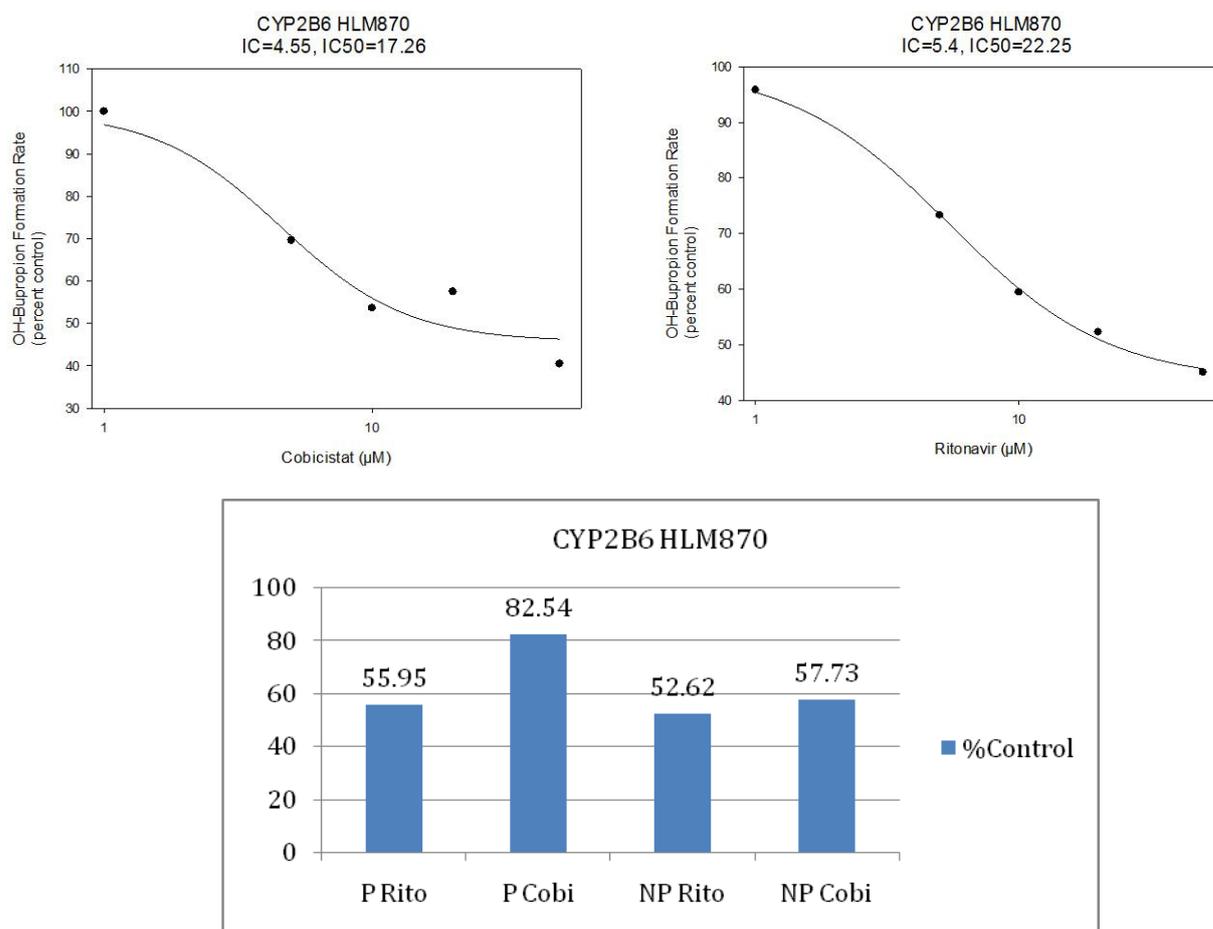


Figure 7. Rates of OH-Bupropion formation from Bupropion with either Ritonavir or Cobicistat in HLM870 and comparison between pre-incubation and without pre-incubation

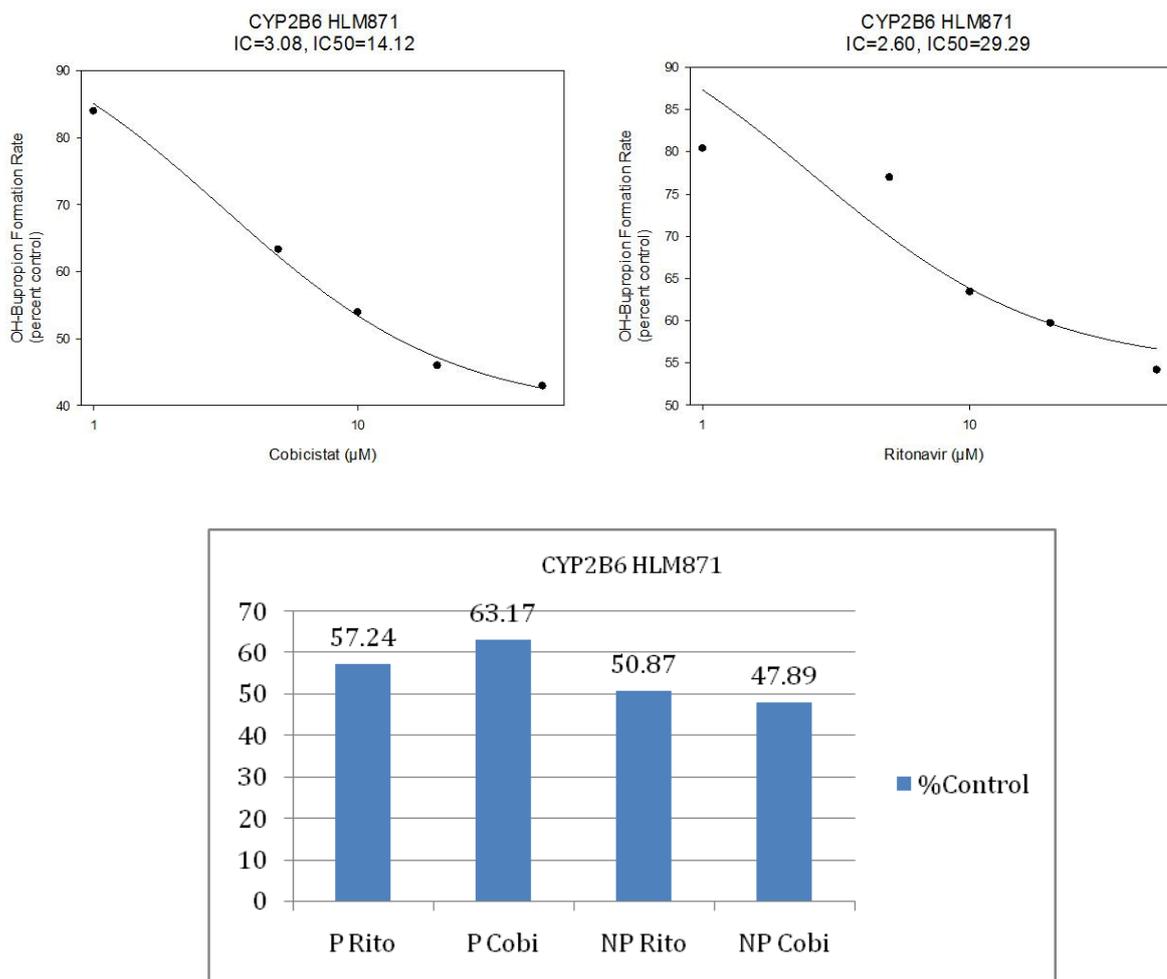


Figure 8. Rates of OH-Bupropion formation from Bupropion with either Ritonavir or Cobicistat in HLM871 and comparison between pre-incubation and without pre-incubation

CYP2C19 (S-Mephenytoin hydroxylation)

For CYP2C19, ritonavir and cobicistat inhibited CYP2C19 activity. Cobicistat inhibited OH-Mephenytoin formation rate with IC₅₀ value of 7.67 μM (without pre-incubation) in HLM 866. Ritonavir also inhibited CYP2C19 activity with IC₅₀ value of 7.15 μM (without pre-incubation). Both of their inhibitory concentrations are similar here. According to the graphs these drugs were not indicating time-dependent (mechanism based) inhibition as percent control of no pre-incubation was way too lower than the pre-incubation one.

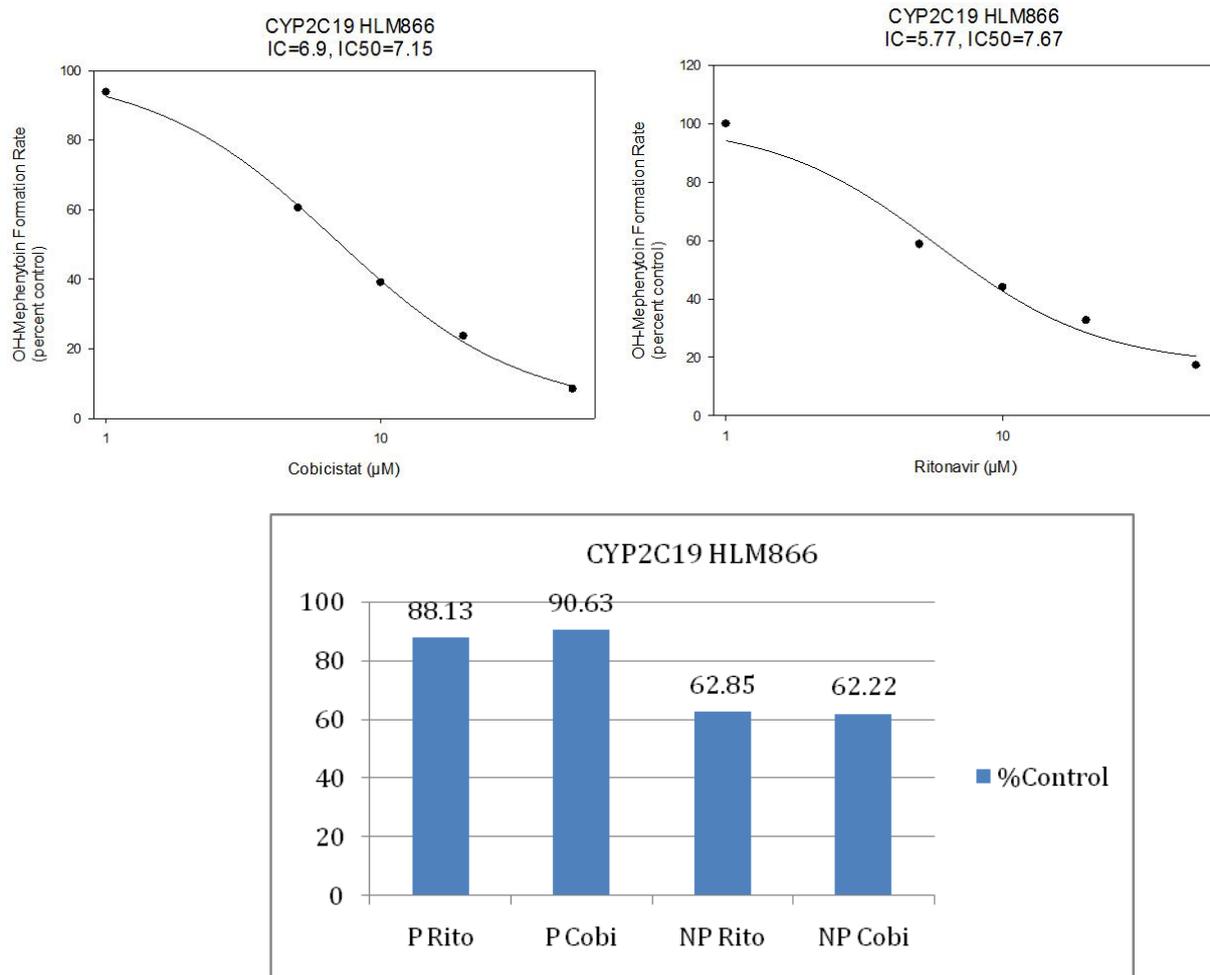


Figure 9. Rates of OH-Mephenytoin formation from S-Mep with either Ritonavir or Cobicistat in HLM866 and comparison between pre-incubation and without pre-incubation

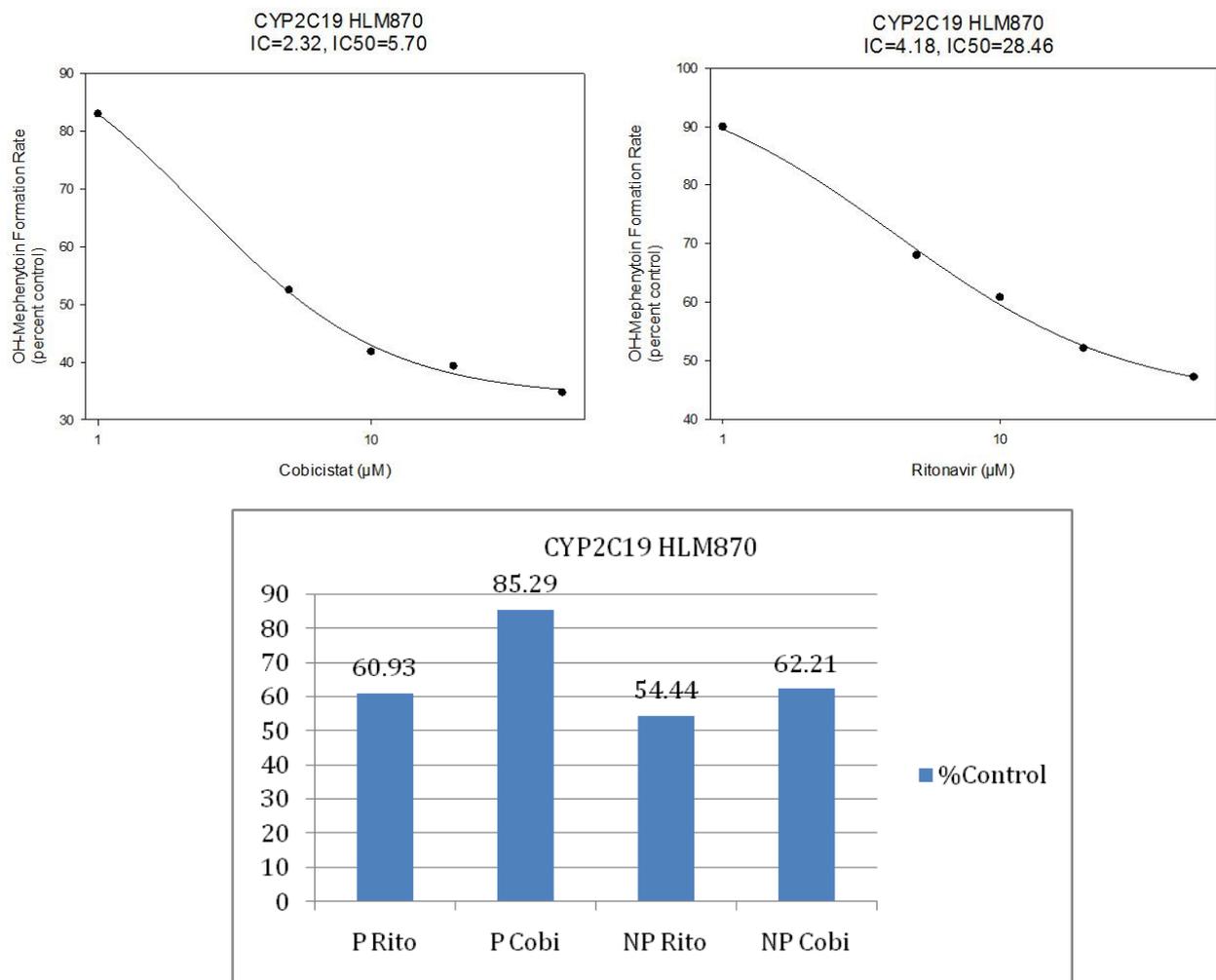


Figure 10. Rates of OH-Mephenytoin formation from S-Mep with either Ritonavir or Cobicistat in HLM870 and comparison between pre-incubation and without pre-incubation

For HLM870 and HLM 9511, we can conclude that they are not showing time-dependent inhibition as the percent control values for pre-incubation are not lower than the without pre-incubation one. Pooled HLM (Figure 11) showed IC₅₀ 4.95 (cobicistat), 30.24 (ritonavir) and pre-incubation data showed that CYP2B6 does not follow time-dependent inhibition.

For CYP2B6 and CYP2C19, percent control values (ritonavir and cobicistat) for pre-incubation phase were always higher than without pre-incubation one. This

indicates that, the inhibition mechanism is not time-dependent (mechanism based) rather time independent in this study.

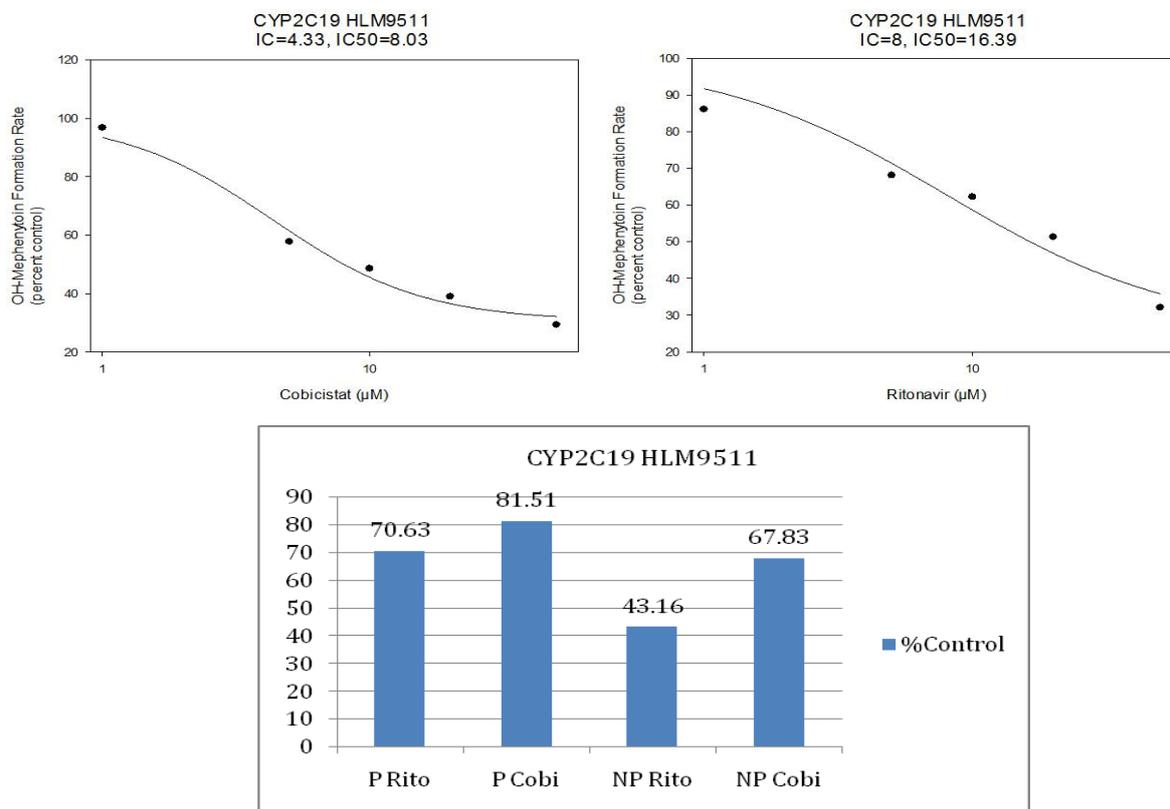


Figure 11. Rates of OH-Mephenytoin formation from S-Mep with either Ritonavir or Cobicistat in HLM9511 and comparison between pre-incubation and without pre-incubation

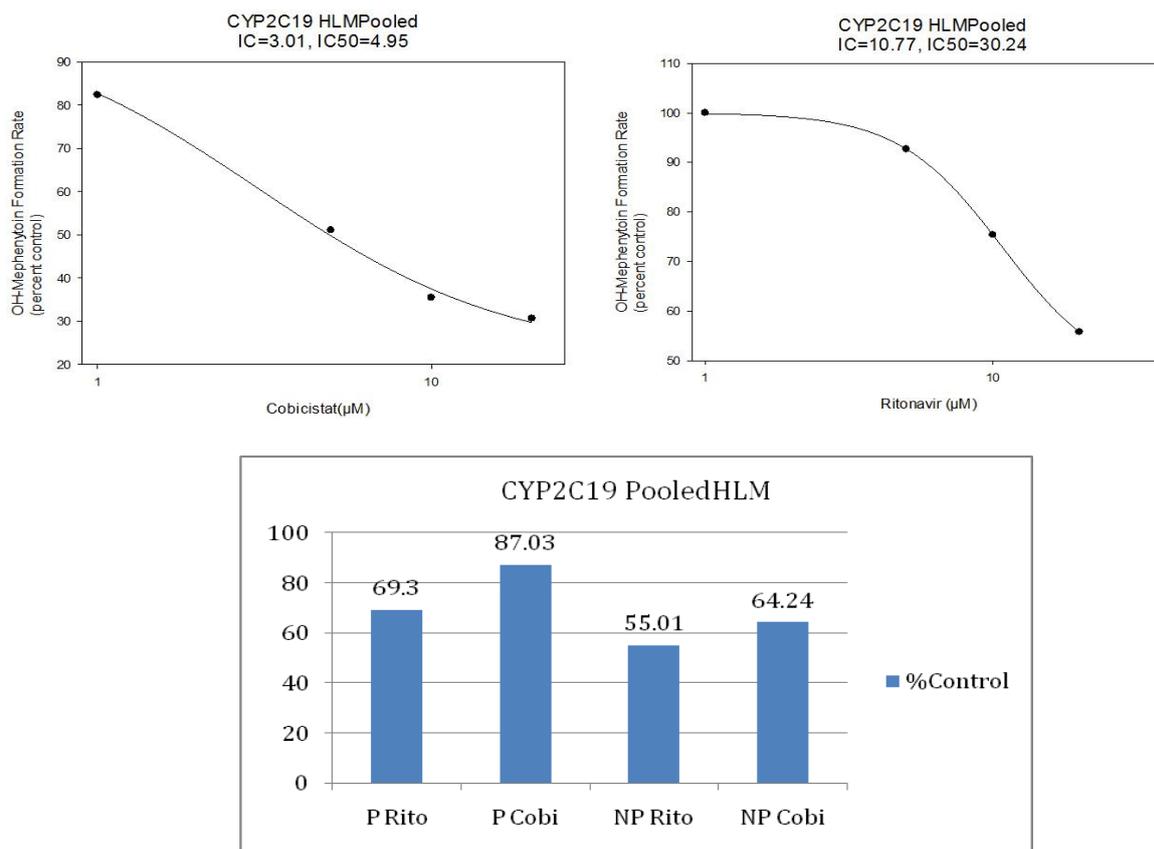


Figure 12. Rates of OH-Mephenytoin formation from S-Mep with either Ritonavir or Cobicistat in PooledHLM and comparison between pre-incubation and without pre-incubation

Discussion and Conclusions

Both ritonavir and cobicistat have the capability to inhibit human cytochrome isoforms *in vitro*. Our previous studies showed that these two antiretroviral boosting drugs were able to significantly inhibit CYP3A. In this study, both of them were able to inhibit CYP2B6 and 2C19 significantly with different IC₅₀ values, we can say they are nonspecific inhibitor of multiple cytochrome isoforms. Comparison study between pre-incubation and no pre-incubation shows the percent control for no pre-incubation is much lower than the pre-incubation one. As a result, this study also concludes that none of the

experiments showed time-dependent inhibition which means they are reversible and also they are likely to be noncompetitive. For making this study more accurate, each cytochrome was tested with different HLMs.

We also can conclude that these two drugs show potential drug-drug interaction where they can inhibit cytochromes. As a result delay the clearance of antiretroviral drugs. The first step of this study was to look for perfect positive control. Some positive controls do not work for all HLMs. So this study had to look for some other positive control which might work for all of our HLMs. The second step involved finding out IC_{50} of ritonavir and cobicistat for performing the later steps. The most important step was third one. In this step, we can figure out whether they show reversible or irreversible inhibition.

In some cases, we have seen that the inhibition potency of cobicistat is more than ritonavir in this study. If we carefully consider all of our P and NP data we can see there is some significantly increased inhibition of cobicistat at IC_{50} .

Some previous studies have shown that the reduction of bupropion (CYP2B6 substrate) exposure by ritonavir is dose related. Studies have also shown s-mephenytoin was inhibited via ritonavir (IC_{50} 23.5) which is very close to this study (mean IC_{50} 20.69). Besides, ritonavir inhibited CYP2C19 and CYP2B6 activity with mean IC_{50} 20.69 μ M and 15.41 μ M accordingly. Cobicistat also inhibited CYP2C19 and CYP2B6 activity with mean IC_{50} 6.45 μ M and 10.17 μ M accordingly which are very similar to previous studies [11].

The calculated mean IC₅₀ value found in this study (CYP2B6) is much larger than the IC₅₀ for ritonavir on CYP3A4 and also larger than ritonavir maximum plasma concentration C_{max} (0.51 µg/ml).

However, no potential study was done to see the DDI (IC₅₀) of bupropion and s-mephenytoin with cobicistat. Further studies can be done to see DDI of cobicistat and plasma concentration of cobicistat comparing with *in vitro* IC₅₀ in different isoforms.

References

1. Kallings LO. "The First Postmodern Pandemic: 25 years of HIV/ AIDS." J Intern Med Journal of Internal Medicine 263.3 (2008): 218-43.
2. Gao, Na, Bing Qi, Fang-jun Liu, Yan Fang, Jun Zhou, Lin-jing Jia, and Hai-ling Qiao. "Inhibition of Baicalin on Metabolism of Phenacetin, a Probe of CYP1A2, in Human Liver Microsomes and in Rats." PLoS ONE. Public Library of Science, n.d. Web. 06 Apr. 2016.
3. Judd FK, Mijch AM. "Depressive Symptoms in Patients with HIV Infection." Aust N Z J Psychiatry. 1996;30:104-109.
4. Lepist, E. -I.; Phan, T. K.; Roy, A.; Tong, L.; MacLennan, K.; Murray, B.; Ray, A. S. (2012). "Cobicistat Boosts the Intestinal Absorption of Transport Substrates, Including HIV Protease Inhibitors and GS-7340, in Vitro"

5. Zeldin, R. K. "Pharmacological and Therapeutic Properties of Ritonavir-boosted Protease Inhibitor Therapy in HIV-infected Patients." *Journal of Antimicrobial Chemotherapy* 53.1 (2003): 4-9.
6. "WHO Model List of Essential Medicines" (PDF). World Health Organization. October 2013. Retrieved 22 April 2014.
7. Guengerich FP (January 2008). "Cytochrome p450 and chemical toxicology". *Chem. Res. Toxicol.* **21** (1): 70–83.
8. German P, Warren D, West S, Hui J, Kearney BP. 2010. Pharmacokinetics and bioavailability of an integrase and novel pharmacoenhancer-containing single-tablet fixed-dose combination regimen for the treatment of HIV. *J. Acquir. Immune Defic. Syndr.* 55:323–329.
9. von Moltke LL, Greenblatt DJ, Court MH, Duan SX, Harmatz JS and Shader RI. "Inhibition of Alprazolam and Desipramine Hydroxylation In Vitro by Paroxetine and Fluvoxamine." *Journal of Clinical Psychopharmacology* 15.2 (1995): 125-31.
10. Waters. "How does HPLC works." http://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US
11. von Moltke LL, Greenblatt DJ, Grassi JM, Granda BW, Duan SX, Fogelman SM, Daily JP, Harmatz JS, and Shader RI. "Protease Inhibitors as Inhibitors of Human Cytochromes P450: High Risk Associated with Ritonavir." *The Journal of Clinical Pharmacology* 38.2 (1998): 106-11.
12. Lianhong X, Liu H, Murray BP, Callebaut C, Lee MS, Hong A, Strickley RG, Tsai LK, Stray KM, Wang Y, Rhodes GR, and Desai MC. "Cobicistat (GS-9350): A Potent and Selective Inhibitor of Human CYP3A as a Novel Pharmacoenhancer." *ACS Med. Chem. Lett. ACS Medicinal Chemistry Letters* 1.5 (2010): 209-13.