Ritonavir and Efavirenz Significantly Alter the Metabolism of Erlotinib—an Observation in Primary Cultures of Human Hepatocytes That Is Relevant to HIV Patients with Cancer

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ABSTRACT

Erlotinib is approved for the treatment of non–small cell lung and pancreatic cancers, and is metabolized by CYP3A4. Inducers and inhibitors of CYP3A enzymes such as ritonavir and efavirenz, respectively, may be used as part of the highly active antiretroviral therapy drugs to treat patients with human immunodeficiency virus (HIV). When HIV patients with a malignancy need treatment with erlotinib, there is a potential of as-yet-undefined drug-drug interaction. We evaluated these interactions using human hepatocytes benchmarked against the interaction of erlotinib with ketoconazole and rifampin, the archetype cytochrome P450 inhibitor and inducer, respectively. Hepatocytes were treated with vehicle [0.1% dimethylsulfoxide, ritonavir (10 μ M)], ketoconazole (10 μ M), efavirenz (10 μ M), or rifampin (10 μ M) for 4 days. On day 5, erlotinib (5 μ M) was incubated with the above agents for another

24–48 hours. Concentrations of erlotinib and O-desmethyl erlotinib were quantitated in collected samples (combined lysate and medium) using liquid chromatography and tandem mass spectrometry. The half-life ($t_{1/2}$) of erlotinib increased from 10.6 ± 2.6 to 153 ± 80 and 23.9 ± 4.8 hours, respectively, upon treatment with ritonavir and ketoconazole. The apparent intrinsic clearance ($CL_{int, app}$) of erlotinib was lowered 16-fold by ritonavir and 1.9-fold by ketoconazole. Efavirenz and rifampin decreased $t_{1/2}$ of erlotinib from 10.3 ± 1.1 to 5.0 ± 1.5 and 3.4 ± 0.2 hours, respectively. Efavirenz and rifampin increased the $CL_{int, app}$ of erlotinib by 2.2- and 2-fold, respectively. Our results suggest that to achieve desired drug exposure, the clinically used dose (150 mg daily) of erlotinib may have to be significantly reduced (25 mg every other day) or increased (300 mg daily), respectively, when ritonavir or efavirenz is coadministered.

Introduction

Patients infected with human immunodeficiency virus (HIV) are highly prone to developing AIDS-defining malignancies (Kaposi's sarcoma, non-Hodgkin lymphoma, and cervical cancer) and non-AIDS-defining malignancies (anal, liver, and lung cancers and Hodgkin lymphoma) (Shiels et al., 2011). Highly active antiretroviral therapy (HAART) has significantly reduced the rates of AIDS-defining malignancies. As HIV patients live longer, the occurrence of non-AIDS-defining malignancies has increased. In these patients antineoplastic therapy has to be used to effectively treat malignancy along with HAART therapy to maintain antiretroviral activity (Rudek et al., 2011; Malfitano et al., 2012).

Many HAART drugs have complex drug interactions due to their ability to inhibit and/or induce cytochrome P450 (P450), uridine diphosphate glucuronyl transferases (UGTs), ATP-binding cassette efflux transporters, and solute carrier uptake transporters (Piscitelli and

Gallicano, 2001; Boffito et al., 2005). Among the various HAART drugs, the HIV protease inhibitor ritonavir is the most potent inhibitor of CYP3A (Sevrioukova and Poulos, 2010), whereas the non-nucleoside reverse transcriptase inhibitor efavirenz is a mixed type inducer and inhibitor of CYP3A (Smith et al., 2001). In clinical settings, inhibitory interaction is more readily hinted at by easily noticed toxicities, than by loss of efficacy due to induction. Indeed, well controlled clinical trials are lacking to identify treatment recommendations for the combination of anticancer agents and antiretrovirals.

Erlotinib is a small-molecule reversible inhibitor of epidermal growth factor receptor tyrosine kinase that is approved for the treatment of non–small cell lung cancer, and in combination with gemcitabine for the treatment of pancreatic cancer (Senderowicz et al., 2007; Kelley and Ko, 2008). Indeed, lung cancer is a common non-AIDS-defining cancer with an increase in standardized incidence ratio by 2.2–6.6 (e.g., 2.2–6.6 times the risk in the general population) (Deeken et al., 2012). In the US AIDS population, lung cancer is one of the top non-AIDS-defining cancers (Shiels et al., 2011). Erlotinib is orally bioavailable and primarily metabolized by CYP3A (Ling et al., 2006; Li et al., 2007) and to a lesser extent by CYP1A1/CYP1A2

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ABBREVIATIONS: AUC, area under the curve; DMSO, dimethylsulfoxide; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; LC-MS/MS, liquid chromatography–tandem mass spectrometry; M/P, metabolite-to-parent erlotinib; P450, cytochrome P450.

(Hughes et al., 2009). The side-effects profile of erlotinib includes burdensome skin (Ricciardi et al., 2009) and gastrointestinal tract, ocular, and rare but serious lung toxicities (Kobayashi et al., 2012; Borkar et al., 2013; Yoshida et al., 2013). Although tyrosine kinase inhibitors, such as erlotinib, do not commonly have the same life-threatening side effects as the cytotoxic anticancer drugs, they do suffer from wide interpatient variability in pharmacokinetics [% coefficient of variation for area under the curve (AUC) and trough level is 64 and 51%, respectively] (Gao et al., 2012). Underexposure often will go undetected with lack of response attributed to molecular resistance mechanisms, and overexposure will result in toxicities that significantly affect the quality of life of patients (Baker and Hu, 2009; Beumer, 2013).

Ketoconazole [the archetype inhibitor of CYP3A activity (Lu et al., 2007)] and rifampicin [the archetype inducer of CYP3A activity (Kozawa et al., 2009)] are commonly used in drug development to assess the potential vulnerability of drugs to P450 inhibition and induction, respectively. Both agents indeed have been reported to alter the exposure of erlotinib in patients (Abbas et al., 2003; Rakhit et al., 2008), and coadministration of CYP3A inhibitors/inducers are expected to result in serious adverse events or subtherapeutic concentrations of erlotinib, respectively. However, there are no reports on the extent of interaction between HAART drugs, ritonavir/efavirenz, and erlotinib. The objective of this study was to assess the potential effect of ritonavir and efavirenz on erlotinib using primary cultures of human hepatocytes, and benchmark these effects relative to the effects of ketoconazole and rifampin on the metabolism of erlotinib. The predicted change in oral clearance of erlotinib in the presence of ritonavir/efavirenz was also determined using *in vitro*–*in vivo* scale up, which will inform the starting dose of erlotinib in confirmatory phase I clinical trials of erlotinib in cancer patients with HIV on HAART therapy.

Materials and Methods

Erlotinib and dasatinib were purchased from LC Laboratories (Woburn, MA). Desmethyl erlotinib (OSI-420), [D₆]erlotinib, and ritonavir were procured from Toronto Research Chemicals (Toronto, ON, Canada). Ketoconazole was obtained from Janssen Research Foundation (Titusville, NJ). Hepatocytes maintenance medium was procured from Lonza, Inc. (Walkersville, MD). Organic solvents used for the extractions and the analytical assays were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals obtained from Sigma-Aldrich (St. Louis, MO).

Primary Human Hepatocyte Cultures. Human liver samples (*n* = 9) were obtained from Life Technologies (Grand Island, NY) or the Hepatocytes Transplantation Laboratory, University of Pittsburgh, or XenoTech, LLC (Lenexa, KS). These samples were collected immediately after the resection of the liver. Under sterile conditions a wedge of normal liver away from the tumor was resected for hepatocyte isolation. Hepatocytes were isolated by collagenase perfusion method and were cultured using hepatocyte maintenance medium supplemented with insulin (0.1 μ M), dexamethasone (0.1 μ M), 0.05% penicillin, 0.05% streptomycin, and 0.05% amphotericin B (Gramignoli et al., 2012). The viability of hepatocytes was determined using trypan blue cell exclusion assay and was observed to be >90% for all experiments. The hepatocytes were seeded at a density of 1.5×10^6 cells per well in six well plates that were previously coated with rat tail collagen. The cells were maintained at 37°C and in 5% carbon dioxide. Two hours after seeding, the medium was replaced with fresh medium to remove nonadherent cells. The culture medium was replaced daily with fresh culture medium. These serum-free cultures of human hepatocytes are suitable for drug-drug interaction studies as reported before (Komoroski et al., 2004). All the drug incubations were carried out 48 hours after seeding. The P450 induction potential of the hepatocytes used was also evaluated at the end of the study by measuring the activity of CYP3A (formation of testosterone 6 β -hydroxylation) in separate sets of untreated and rifampin (10 μ M) treated hepatocytes.

Time-Course of Erlotinib Metabolism. The time-course of erlotinib metabolism was assessed in the absence and presence of ritonavir or ketoconazole or efavirenz or rifampin. Hepatocytes were incubated daily with ritonavir (10 μ M), ketoconazole positive control (10 μ M), efavirenz (10 μ M), rifampin positive control (10 μ M), or vehicle control [0.1% dimethylsulfoxide (DMSO)] for 4 days. On day 5, erlotinib (5 μ M) was incubated along with the above agents for an additional 24 or 48 hours. Hepatocyte samples were collected at 0, 4, 8, 24, and 48 hours (for inhibition studies) or 0, 2, 4, 8, and 24 hours (for induction studies). To obtain the detectable amount of erlotinib in hepatocyte samples, the duration of incubation for induction studies was reduced to 24 hours and an additional sample was obtained at 2 hours. Hepatocyte samples were obtained by scraping the cells along with culture medium supernatant on ice and lysing the cells by sonication. Samples were stored at –80°C until analysis. Drug concentrations were selected based on the steady state maximum plasma concentration at clinically recommended doses in human (Yamamoto et al., 2008). Additionally, the cytotoxicity assay (MTT assay) was performed to ensure that the selected drug concentrations did not affect the viability of human hepatocytes (data not shown).

Recovery of Ritonavir/Efavirenz Mediated Inhibition/Induction of Erlotinib Metabolism. Hepatocytes were treated daily with ritonavir (10 μ M) efavirenz (10 μ M) or vehicle (0.1% DMSO) for 4 days. On day 5, hepatocytes were washed thrice with phosphate-buffered saline and fresh hepatocyte medium was added. Subsequently, erlotinib metabolism was assessed in the absence of ritonavir or efavirenz starting 0, 24, 48, 72, and 96 hours after the washout period. Erlotinib (5 μ M) was incubated with hepatocytes for 24 hours on each washout period. Samples were processed and stored as described before.

Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) Quantitation of Erlotinib and Desmethyl Erlotinib. Erlotinib and active desmethyl metabolite were quantitated with an Agilent 1100 Autosampler and Agilent 1100 Binary pump (Agilent Technologies, Santa Clara, CA) hyphenated to a Micromass Quatromicro triple-stage bench-top mass spectrometer (Waters, Milford, MA). Standard LC-MS/MS assays lack the ability to distinguish the isomers of *O*-demethylated erlotinib OSI-413 and OSI-420 (Messersmith et al., 2010). We used OSI-420 as reference standard to quantitate the composite signal of OSI-420 and OSI-413 in incubated samples, which is henceforth referred to as desmethyl erlotinib. Analytes were chromatographically separated on a Synergi polar reverse phase column (4 μ m, 2 mm $\times$ 100 mm) and kept at ambient temperature. The gradient mobile phase system was composed of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid). The initial mobile phase solvent was 35% solvent A and 65% solvent B pumped at a flow rate of 0.3 ml/min. Initial mobile phase conditions were held for 4 minutes. At 4.1 minutes, solvent A was increased to 90% along with a flow rate of 0.5 ml/min, where it was held until 6 minutes. At 6.1 minutes, mobile phase A was lowered back down to 35% with a flow rate of 0.3 ml/min and was maintained for 10 minutes, when the next sample was then injected. The settings of the mass spectrometer were as follows: capillary voltage 4 kV, cone voltage 40 V, and desolvation temperature 450°C. The multiple reaction monitoring *m/z* transitions monitored were 394.0 to 278.0, 400.0 to 278.0, 380.0 to 278.0, and 488.0 to 401.0 for erlotinib, [D₆]erlotinib, desmethyl erlotinib, and dasatinib, respectively. Aliquots of 100 μ l of hepatocyte medium were extracted with 100 μ l of acetonitrile, and 10 μ l of internal standard mix [500 ng/ml [D₆]erlotinib (internal standard for erlotinib) and 2 μ g/ml dasatinib (internal standard for desmethyl erlotinib)]. After vortexing and centrifugation, 10 μ l of supernatant was injected into the LC-MS/MS system. The ion chromatograms were integrated and quantified using Micromass MassLynx 4.0 software (Waters). The assay was linear between 10 and 5000 ng/ml with acceptable accuracy (92.8–112.0% and 96.0–109%) and precision (<9.0 CV% and <11.5 CV%) for erlotinib and desmethyl erlotinib, respectively, as determined from a triplicate calibration curve. The triplicate curve referred to represents independently prepared and analyzed samples from the same stock. That stock had been tested with an independently prepared stock earlier. The triplicate curve was not merely a reinjection of the same samples.

Data Analysis. Each study was performed in at least three different cultures of human hepatocytes in duplicate. The half-life (*t*_{1/2}) and area under the concentration-time curve from time 0–48 hours (AUC_{0–48 hours}) for erlotinib in

primary cultures of human hepatocytes were calculated noncompartmentally with WinNonlin Pro version 5.2 (Pharsight Corp., Cary, NC).

Calculation of Apparent Intrinsic Clearance. The apparent intrinsic clearance ($CL_{int, app}$) of erlotinib was calculated from the half-life of erlotinib in the absence and presence of ritonavir, ketoconazole, efavirenz, or rifampin in primary cultures of human hepatocytes using eq. 1 (Lu et al., 2006),

$$CL_{int, app} (\text{ml/min per kg}) = \frac{0.693}{t_{1/2}} \times \frac{\text{ml incubation}}{\text{number of cells}} \times \frac{130 \times 10^6 \text{ cells}}{\text{g liver}} \times \frac{20 \text{ g liver}}{\text{kg per body weight}} \quad (1)$$

where incubation volume is 1 ml and the number of cells is 1.5×10^6 .

Prediction of Hepatic Clearance. The hepatic clearance (CL_h) of erlotinib was predicted from $CL_{int, app}$ using the well-stirred liver model as follows (Witherow and Houston, 1999; Riley et al., 2005; Yamamoto et al., 2005),

$$CL_h = Q_h(1 - F_h) \quad (2)$$

Hepatic blood flow (Q_h) = 21 ml/min per kg, and hepatic availability (F_h) was calculated using eqs. 3–5:

$$F_h = \frac{Q_h}{\left(Q_h + f_B \times \frac{CL_{int, app}}{f_{u, inc}}\right)} \quad (3)$$

$$f_B = \text{fraction unbound in blood} = \frac{f_p}{R_B} \quad (4)$$

f_p = fraction unbound in plasma; R_B = blood-to-plasma concentration ratio

$$f_{u, inc} = \text{fraction unbound in incubation medium} = \frac{1}{1 + K_p(V_c/V_m)} \quad (5)$$

K_p = partition ratio = 3.2 (Minematsu and Giacomini, 2011); V_c = adherent cell volume = 0.0051 ml (Lodish et al., 2000); V_m = volume of culture medium = 1 ml.

Prediction of Oral Clearance. Erlotinib has been reported to be well-absorbed from the gastrointestinal tract (Ranson et al., 2010). Consequently, the intestinal clearance is assumed to be negligible for erlotinib, particularly in the presence of ritonavir or ketoconazole. Therefore, the fraction absorbed (f_a) and fraction of dose escaping gut wall metabolism (F_g) are assumed to be 1 for the prediction of oral clearance using eq. 6 (Chiba et al., 2009):

$$CL_{oral} = \frac{CL_h}{f_a \times F_g \times F_h} \quad (6)$$

The bias of CL_{oral} prediction was estimated from the geometric mean ratio of the predicted and observed values, and calculated as average fold-error (*afe*) from eq. 7, whereas the precision of the prediction was assessed from root mean squared prediction error (*rmse*) using eqs. 8 and 9, respectively (Kilford et al., 2009).

$$afe = 10^{\left| \frac{1}{N} \sum \log \frac{\text{Predicted}}{\text{Observed}} \right|} \quad (7)$$

$$mse = \frac{1}{N} \sum (\text{Predicted} - \text{Observed})^2 \quad (8)$$

$$rmse = \sqrt{mse} \quad (9)$$

Statistical Analysis. All the results are expressed as mean $\pm$ S.D. The statistical difference among the different treatment groups were analyzed by nonparametric Friedman analysis of variance followed by Dunn's multiple comparison post-hoc test. A $P < 0.05$ was considered as statistically significant. Data with two groups were analyzed by Wilcoxon signed rank

TABLE 1
Demographic profiles of human liver donors

Liver ID	Age	Gender	Race	Diagnosis
HH 2011	69	Female	Caucasian	Metastatic colon cancer
HH 2014	52	Female	Caucasian	Colorectal cancer
Hu 1522	67	Female	Caucasian	Colorectal cancer
HH 2018	68	Male	Caucasian	Cholangiocarcinoma
HH 2019	68	Male	Caucasian	Metastatic colon cancer
Hu 1488	31	Male	Caucasian	Colorectal cancer
Hu 1527	31	Female	Caucasian	Adenoma
Hu 1524	58	Male	Caucasian	Colorectal cancer
HH 12-010	31	Female	Caucasian	Adenoma
Hu 1594	64	Male	Caucasian	Cholangiocarcinoma
H 1161	60	Female	Caucasian	Anoxia
Hu 1595	31	Female	Caucasian	Focal nodular hyperplasia

nonparametric test. While considering the large variability in cytochrome P450 activities in human (Lin and Lu, 2001), obtaining a statistical significance from just three different batches of human liver hepatocytes is practically very difficult. If the fold change in responses is ≥ 1.5 -fold, we considered the data to be clinically significant rather making a decision from statistical significance of the results for this type of studies.

Results

The time-course of erlotinib metabolism in the absence and presence of HAART drugs ritonavir or efavirenz was assessed in

Fig. 1. Time-course of erlotinib depletion (A) and formation of desmethyl erlotinib (B) in primary cultures of human hepatocytes treated with DMSO (○), ritonavir (●) or ketoconazole (□). Data are representative of three to six individual experiments each performed in duplicate. Error bars represent standard deviation.

TABLE 2

Area under the concentration-time curve for erlotinib depletion and desmethyl erlotinib formation in primary cultures of human hepatocytes treated with vehicle (DMSO), ritonavir, or ketoconazole and incubated with erlotinib (5 μ M)

Treatment	Liver ID	AUC _{0-48h}	
		Erlotinib $\mu\text{g}\cdot\text{h}/\text{ml}$	Desmethyl Erlotinib $\mu\text{g}\cdot\text{h}/\text{ml}$
DMSO	HH 2011	22.7	10.2
	Hu 1522	42.7	9.7
	HH 2014	11.9	6.0
	Hu 1594	25.3	4.1
	H 1161	31.3	3.4
	Hu 1595	23.7	3.2
	Mean $\pm$ S.D.	26.3 $\pm$ 10.2	6.1 $\pm$ 3.2
Ritonavir	HH 2011	102.2	3.3
	Hu 1522	190.8	4.4
	HH 2014	85.7	1.7
	Hu 1594	84.9	1.4
	H 1161	80.3	1.1
	Hu 1595	76.7	1.2
	Mean $\pm$ S.D.	103.4 $\pm$ 43.7*	2.2 $\pm$ 1.4*
Ketoconazole	Ratio to DMSO	4.2 $\pm$ 1.6	0.4 $\pm$ 0.1
	Hu 1594	58.9	3.9
	H 1161	61.0	4.1
	Hu 1595	44.9	3.2
	Mean $\pm$ S.D.	55.0 $\pm$ 8.7	3.7 $\pm$ 0.5
	Ratio to DMSO	2.1 $\pm$ 0.2	1.1 $\pm$ 0.1

* $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

primary cultures of human hepatocytes and compared with that of the corresponding positive controls, ketoconazole and rifampin. The demographics of human liver donors are shown in Table 1. All the batches of human hepatocytes used in this study retained P450-induction potential as confirmed by the greater than 2.5-fold increase in CYP3A activity upon treatment with rifampin, and the CYP3A4/5 activities in control hepatocytes ranged from 51 to 357 pmol/min per million cells (based on formation of 6- β -hydroxy testosterone from testosterone) at the end of the study period.

The average concentration-time profile for erlotinib and the formation of desmethyl erlotinib in primary cultures of human hepatocytes treated with vehicle, ritonavir, and positive control (ketoconazole) are shown in Fig. 1. The individual and mean values of AUC_{0-48h} for the time-course of metabolism of erlotinib and the formation of desmethyl erlotinib in the absence and presence of ritonavir or ketoconazole in human hepatocytes are shown in Table 2. Treatment with ritonavir showed a 4.2-fold increase in the exposure (AUC_{0-48h}) of erlotinib with a corresponding 2.9-fold reduction in the formation of desmethyl erlotinib in human hepatocytes as compared with vehicle treatment. Although ketoconazole treatment moderately increased the exposure of erlotinib by 2.1-fold, the formation of desmethyl erlotinib was not significantly affected (Table 2).

The half-life, % erlotinib metabolized over 48 hours, metabolite-to-parent erlotinib (M/P) to AUC_{0-48h} ratio and time to reach maximum (T_{max}) concentration of desmethyl erlotinib in the absence and presence of either ritonavir or ketoconazole in human hepatocytes are shown in Table 3. Treatment with ritonavir significantly increased the half-life of erlotinib by 16-fold as compared with treatment with vehicle. Additionally, the % erlotinib metabolized and M/P to AUC_{0-48h} ratio were substantially inhibited, 80 and 91% respectively, by ritonavir as compared with treatment with vehicle. T_{max} of desmethyl erlotinib was increased from 12 hours to 48 hours upon ritonavir treatment. Ketoconazole treatment resulted in only a moderate increase (1.9-fold) in half-life, and a 22% reduction in erlotinib metabolized and a 49% reduction in M/P to AUC_{0-48h} ratio as compared with treatment with vehicle. Treatment with ketoconazole increased the T_{max} of desmethyl erlotinib from 12 hours to 48 hours.

The average concentration-time profile for erlotinib and the formation of desmethyl erlotinib in primary cultures of human hepatocytes treated with vehicle control (DMSO), efavirenz, and rifampin (inducer positive control) are shown in Fig. 3. The individual and mean values of AUC_{0-24h} for the time-course of metabolism of erlotinib and the formation of desmethyl erlotinib in the presence and absence of efavirenz or rifampin are presented in Table 4. Efavirenz treatment decreased the exposure (AUC_{0-24h}) of erlotinib by 3-fold and moderately

TABLE 3

Half-life ($t_{1/2}$), % erlotinib metabolized after 48 hours, M/P to AUC ratio, T_{max} of desmethyl erlotinib, and apparent intrinsic clearance ($\text{CL}_{\text{int, app}}$) of erlotinib in primary cultures of human hepatocytes treated with vehicle (DMSO), ritonavir, or ketoconazole and incubated with erlotinib (5 μ M)

Treatment	Liver ID	$t_{1/2}$	% Erlotinib Metabolized after 48 h	M/P to AUC _{0-48h} ratio	T_{max} of Desmethyl Erlotinib	$\text{CL}_{\text{int, app}}$
		<i>h</i>			<i>h</i>	<i>ml/min per kg</i>
DMSO	HH 2011	8.4	98	0.45	8	2.38
	Hu 1522	10.3	97	0.23	4	1.94
	HH 2014	7.2	100	0.50	4	2.78
	Hu 1594	11.3	95	0.16	24	1.77
	H 1161	14.1	91	0.11	24	1.42
	Hu 1595	12.4	93	0.14	8	1.61
	Mean $\pm$ S.D.	10.6 $\pm$ 2.6	96 $\pm$ 3	0.26 $\pm$ 0.17	12.0 $\pm$ 9.5	1.98 $\pm$ 0.51
Ritonavir	HH 2011	111.8	20	0.03	48	0.18
	Hu 1522	117.5	22	0.02	48	0.17
	HH 2014	301.3	11	0.02	48	0.07
	Hu 1594	115.5	16	0.02	48	0.17
	H 1161	188.1	15	0.01	48	0.11
	Hu 1595	86.0	28	0.02	48	0.23
	Mean $\pm$ S.D.	153.4 $\pm$ 80.1*	19 $\pm$ 6*	0.02 $\pm$ 0.01*	48.0 $\pm$ 0.0*	0.15 $\pm$ 0.06*
Ketoconazole	Ratio to DMSO	16.2 $\pm$ 12.8	0.2 $\pm$ 0.1	0.09 $\pm$ 0.03	6.7 $\pm$ 4.5	0.08 $\pm$ 0.04
	Hu 1594	28.9	64	0.07	48	0.69
	H 1161	23.3	74	0.07	48	0.86
	Hu 1595	19.4	81	0.07	48	1.03
	Mean $\pm$ S.D.	23.9 $\pm$ 4.8	73 $\pm$ 8	0.07 $\pm$ 0.003	48.0 $\pm$ 0.0†	0.86 $\pm$ 0.17
	Ratio to DMSO	1.9 $\pm$ 0.6	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1	3.3 $\pm$ 2.3	0.6 $\pm$ 0.1

* $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

TABLE 4

Area under the concentration-time curve for erlotinib depletion and desmethyl erlotinib formation in primary cultures of human hepatocytes treated with vehicle (DMSO), efavirenz, or rifampin and incubated with erlotinib (5 μ M)

Treatment	Liver ID	AUC 0–24h	
		Erlotinib	Desmethyl Erlotinib
		μ g·h/ml	μ g·h/ml
DMSO	HH 2018	21.2	2.3
	HH 2019	27.3	2.3
	HH 1488	22.9	2.2
	Mean $\pm$ S.D.	23.8 $\pm$ 3.2	2.2 $\pm$ 0.04
Efavirenz	HH 2018	8.7	1.8
	HH 2019	5.9	1.1
	HH 1488	12.4	2.1
	Mean $\pm$ S.D.	9.01 $\pm$ 3.2	1.7 $\pm$ 0.6
	Ratio to DMSO	0.39 $\pm$ 0.16	0.74 $\pm$ 0.24
Rifampin	HH 2018	3.8	1.5
	HH 2019	4.3	1.9
	HH 1488	3.7	1.7
	Mean $\pm$ S.D.	3.9 $\pm$ 0.4*	1.7 $\pm$ 0.2
	Ratio to DMSO	0.17 $\pm$ 0.07	0.75 $\pm$ 0.09

* $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

increased the formation of desmethyl erlotinib by 1.5-fold as compared with treatment with vehicle. Treatment with rifampin resulted in a substantial 6-fold decrease in the exposure of erlotinib and moderate 1.3-fold increase in the formation of desmethyl erlotinib.

The half-life, % erlotinib metabolized in 8 and 24 hours, M/P to AUC_{0–24h} ratio, and time to reach maximum ($T_{\max}$) concentration of desmethyl erlotinib in the presence and absence of either efavirenz or rifampin are presented in Table 5. Efavirenz treatment resulted in a 2.2-fold decrease in half-life of erlotinib as compared with vehicle control. Both the % erlotinib metabolized after 8 hours and M/P to AUC_{0–24h} ratio were increased approximately 2-fold by efavirenz. Because the % erlotinib metabolized after 24 hours was already greater than 80% in hepatocytes treated with vehicle, the magnitude of difference between efavirenz or rifampin treatment and vehicle control was minimal. $T_{\max}$ of desmethyl erlotinib was decreased from 24 hours to ~6 hours upon efavirenz treatment. Rifampin treatment resulted in a 3-fold reduction in the half-life, and a 2.3-fold increase in % erlotinib metabolized, and a 4.5-fold increase in M/P to AUC_{0–24h}

Fig. 2. Predicted human hepatic or oral clearance (CL_{hep} or CL_{oral}) of erlotinib from primary cultures of human hepatocytes treated with DMSO, ritonavir, or ketoconazole. * $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

ratio compared with vehicle control. Treatment with rifampin decreased the $T_{\max}$ of desmethyl erlotinib from 24 hours to 2 hours.

The individual and mean apparent intrinsic clearance ($CL_{\text{int, app}}$) values of erlotinib in primary cultures of human hepatocytes treated with either vehicle (DMSO), ritonavir, or ketoconazole are shown in Table 3 and these values upon treatment with vehicle, efavirenz, or rifampin are presented in Table 5. The $CL_{\text{int, app}}$ of erlotinib was substantially decreased 16-fold by ritonavir and moderately reduced 1.9-fold by ketoconazole. In case of efavirenz or rifampin treatment, the $CL_{\text{int, app}}$ of erlotinib was increased 2.2- and 3-fold, respectively. The erlotinib human CL_{hep} values were predicted from $CL_{\text{int, app}}$ using the well-stirred liver model and are represented in Figs. 2 and 4. Additionally, human CL_{oral} values were also predicted from CL_{hep} and

TABLE 5

Half-life ($t_{1/2}$), % erlotinib metabolized after 8 and 24 hours, M/P to AUC ratio, $T_{\max}$ of desmethyl erlotinib, and apparent intrinsic clearance ($CL_{\text{int, app}}$) of erlotinib in primary cultures of human hepatocytes treated with vehicle (DMSO), efavirenz, or rifampin and incubated with erlotinib (5 μ M)

Treatment	Liver ID	$t_{1/2}$	% Erlotinib Metabolized after 8 h	% Erlotinib Metabolized after 24 h	M/P to AUC 0–24h Ratio	$T_{\max}$ of Desmethyl Erlotinib	$CL_{\text{int, app}}$
		h				h	ml/min per kg
DMSO	HH 2018	9.1	49	86	0.11	24	2.2
	HH 2019	11.0	41	79	0.08	24	1.8
	Hu 1488	10.7	39	79	0.10	24	1.9
	Mean $\pm$ S.D.	10.3 $\pm$ 1.1	43 $\pm$ 5	81 $\pm$ 4	0.097 $\pm$ 0.015	24.0 $\pm$ 0.0	2.0 $\pm$ 0.2
Efavirenz	HH 2018	5.6	88	97	0.21	8	3.6
	HH 2019	3.3	93	100	0.18	2	6.1
	Hu 1488	6.2	70	94	0.17	8	3.2
	Mean $\pm$ S.D.	5.0 $\pm$ 1.5	84 $\pm$ 12	97 $\pm$ 3	0.18 $\pm$ 0.02	6.0 $\pm$ 3.5	4.3 $\pm$ 1.6
	Ratio to DMSO	0.5 $\pm$ 0.2	2 $\pm$ 0.3	1.2 $\pm$ 0.1	2 $\pm$ 0.3	0.3 $\pm$ 0.1	2.2 $\pm$ 1.0
Rifampin	HH 2018	3.5	98	100	0.39	2	5.7
	HH 2019	3.2	96	100	0.44	2	6.2
	Hu 1488	3.6	96	100	0.45	2	5.7
	Mean $\pm$ S.D.	3.4 $\pm$ 0.2*	97 $\pm$ 1*	100 $\pm$ 0.1	0.43 $\pm$ 0.03*	2.0 $\pm$ 0.0	5.8 $\pm$ 0.3*
	Ratio to DMSO	0.3 $\pm$ 0.1	2.3 $\pm$ 0.2	1.2 $\pm$ 0.1	4.5 $\pm$ 1	0.1 $\pm$ 0.0	3.0 $\pm$ 0.4

* $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

Fig. 3. Time-course of erlotinib depletion (A) and formation of desmethyl erlotinib (B) in primary cultures of human hepatocytes treated with DMSO (○), efavirenz (●), or rifampin (□). Data are representative of three individual experiments each performed in duplicate. Error bars represent standard deviation.

are shown in Figs. 2 and 4. Under the given experimental conditions and assumptions for the predictions, the values of both CL_{hep} and CL_{oral} are similar and therefore they are presented in the same figure (Figs. 2 and 4). The parameters used for the prediction of hepatic clearance and the comparisons of observed and predicted CL_{oral} values for erlotinib with prediction errors are shown in Table 6.

The recovery of ritonavir-mediated inhibition and efavirenz-mediated induction of erlotinib metabolism is shown in Fig. 5. A minimum of approximately 48 hours was required for the recovery of both ritonavir- and efavirenz-mediated induction of erlotinib metabolism in primary cultures of human hepatocytes.

Discussion

Erlotinib is primarily metabolized by CYP3A and based on its interaction with ketoconazole and rifampin, erlotinib is expected to undergo significant interactions with agents that modulate the activity of CYP3A. Several HAART drugs have been reported to alter the activity of CYP3A (Makinson et al., 2010). However, the magnitude of an interaction between erlotinib and drugs used as part of HAART are not completely understood at this time. Given that primary cultures of human hepatocytes are a suitable in vitro model to assess the potential drug-drug interactions (Li and Jurima-Romet, 1997), the present study evaluated the extent of interaction of HAART drugs ritonavir/efavirenz on erlotinib in primary cultures of human hepatocytes.

Fig. 4. Predicted human hepatic or oral clearance (CL_{hep} or CL_{oral}) of erlotinib from primary cultures of human hepatocytes treated with DMSO, efavirenz, or rifampin. * $P < 0.05$ versus DMSO; nonparametric Friedman analysis of variance followed by Dunn's multiple comparison test.

The results from this study revealed that ritonavir substantially inhibited the metabolism of erlotinib (4.2-fold) and reduced the exposure of desmethyl erlotinib (2.9-fold). This can be explained by the potent CYP3A inhibitory effects of ritonavir (Katzenmaier et al., 2011). The inhibition of erlotinib depletion was more pronounced than the inhibition of desmethyl erlotinib formation/clearance. Erlotinib metabolism is predominantly mediated by CYP3A4 and, to a lesser extent, by CYP1A1/1A2. Desmethyl erlotinib represents only 5% of circulating metabolites in plasma of humans dosed with erlotinib (Ling et al., 2006) and can be formed by both CYP3A4/5 and CYP1A1/1A2 (Li et al., 2007). Ritonavir is a potent CYP3A4 inhibitor but was reported to have only insignificant inhibitory effects on CYP1A1/1A2 (Eagling et al., 1997), and this may explain the differential magnitude of the effect of ritonavir on erlotinib depletion and desmethyl erlotinib

TABLE 6

The parameters used for the prediction of hepatic clearance and the comparisons of observed and predicted CL_{oral} values for erlotinib with prediction errors

Observed CL_{oral} values (1.9, 3.3, 1.1, 5.3, 5.75, 4.08) were obtained from literature ($N = 6$) (Messersmith et al., 2004; Miller et al., 2007; Rudin et al., 2008; van Erp et al., 2009; Padda et al., 2012). Predicted CL_{oral} values (0.853, 0.696, 0.997, 0.793, 0.651, 0.668) were obtained from six different vehicle-treated human hepatocytes.

Parameters	Values
K_p	3.12
f_p	0.08
R_B	1
f_b	0.08
Mean observed CL_{oral} (l/hr)	3.57
Mean predicted CL_{oral} (l/hr)	0.78
Bias of prediction (<i>afe</i>)	0.25
Precision of prediction (<i>rmse</i>)	2.92

K_p , partition ratio; f_p , fraction unbound in plasma; R_B , blood to plasma concentration ratio; f_b , fraction unbound in blood; CL_{oral} , oral clearance; *afe*, average fold error; *rmse*, root mean square error.

Fig. 5. Recovery of ritonavir-mediated inhibition (top) and efavirenz-mediated induction (bottom) of erlotinib metabolism in primary cultures of human hepatocytes. Data are representative of three individual experiments each performed in duplicate. Error bars represent standard deviation.

formation/elimination. Despite its relevance as a pharmacologically active metabolite, given that it represents only a small percentage of circulating metabolites, any observed changes in the exposure of desmethyl erlotinib may not be interpreted as representative of the metabolism of erlotinib. All the tested inhibitors or inducers have been reported as having either no or weak effects on CYP1A1/CYP1A2 activity, except ketoconazole, which has a moderate inhibitory effect on CYP1A2 (Eagling et al., 1997; Runge et al., 2000; Backman et al., 2006; Lu et al., 2007; Xu and Desta, 2013). Our data demonstrate that ketoconazole increased the exposure of erlotinib by 2.1-fold. These results are in agreement with clinical observations, where ketoconazole increased the exposure of erlotinib almost 2-fold (Rakhit et al., 2008). Despite the increase in exposure of erlotinib, the exposure of desmethyl erlotinib was not affected by ketoconazole. This may be explained by the inhibition of both the formation and elimination of desmethyl erlotinib by ketoconazole, resulting in net effect of no change in the exposure of desmethyl erlotinib.

Efavirenz decreased the exposure of erlotinib (61%) and modestly increased (~26%) the exposure of desmethyl erlotinib. The effect on the depletion of erlotinib was greater than the effect on the elimination/formation of desmethyl erlotinib. This may be due to the mixed type effects (induction and inhibition) of efavirenz on CYP3A4/1A2 activity. Our results suggested that rifampin decreased the exposure of erlotinib in primary cultures of human hepatocytes by 83%. Similar effects were observed in clinical studies (erlotinib product characteristics; http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_Product_Information/hum-an/000618/WC500033994.pdf). Furthermore, the rifampin-mediated increase in erlotinib depletion was greater

than the increase in formation or elimination of desmethyl erlotinib. This may be due to rifampin-mediated induction of enzymes responsible for the metabolism of both erlotinib and its metabolite desmethyl erlotinib in human hepatocytes.

In addition to the use of plasma steady state maximum concentration ($C_{ss, max}$) of erlotinib (5 μM), the projected $C_{ss, max}$ in liver ($C_{ss, max}$ in plasma × liver/plasma partition ratio; 5 μM × ~3 = 15 μM) was also evaluated in additional batches of human hepatocytes. We observed a concentration-independent interaction of erlotinib with ketoconazole and rifampin. Ritonavir treatment reduced the $CL_{int, app}$ of erlotinib from 1.98 ± 0.51 to 0.15 ± 0.06 ml/min per kilogram (using 5 μM erlotinib) and 2.37 to 0.17 ml/min per kilogram (using 15 μM erlotinib). At both the concentrations (5 and 15 μM), erlotinib showed a similar extent of interaction with ritonavir. Whereas efavirenz treatment increased the $CL_{int, app}$ of erlotinib from 2.2 ± 0.2 to 4.3 ± 1.6 ml/min per kilogram (using 5 μM erlotinib) and 2.3 to 2.9 ml/min per kilogram (using 15 μM erlotinib). The magnitude of efavirenz-mediated interaction with erlotinib was reduced at 15 μM concentration of erlotinib as compared with 5 μM erlotinib. This may be due to the concentration-dependent inhibition of CYP3A by erlotinib itself (Dong et al., 2011). At higher concentrations of erlotinib (15 μM), the extent of inhibition of CYP3A may be higher, thereby minimizing the efavirenz-mediated induction. Additional studies are warranted to confirm this concentration-dependent effect of erlotinib on efavirenz-mediated interactions.

We also evaluated the time required for the recovery of ritonavir-mediated inhibition, and efavirenz-mediated induction of the metabolism of erlotinib. Results from the recovery study revealed that both ritonavir-mediated inhibition (mechanism based, requiring de novo protein synthesis for recovery) (Zhou et al., 2005), and efavirenz-mediated induction of erlotinib metabolism were almost fully reversed after a 48-hour drug-free period. This suggests if there is a change in HAART therapy from those containing ritonavir or efavirenz, regular doses of erlotinib can be initiated in patients after 2 days. This should however be confirmed in further studies.

Human pharmacokinetic parameters can be reasonably predicted from in vitro human hepatocyte data using in vitro-in vivo extrapolation methods (Ring et al., 2011). The mean predicted human CL_{oral} of erlotinib from vehicle-treated primary cultures of human hepatocytes was approximately 5-fold lower than the clinically observed mean CL_{oral} of erlotinib. In addition to the hepatic CYP3A, intestinal CYP3A was also reported to be involved in the metabolism of erlotinib in human (Ling et al., 2006). The intestinal metabolism of erlotinib may be equivalent to or greater than liver metabolism. Since the value of the fraction of erlotinib that escaped from the gut metabolism (F_g) was not known, we have presumed F_g is equal to 1 in our prediction calculation. These may be responsible for the relatively higher observed human CL_{oral} of erlotinib than the erlotinib CL_{oral} predicted from primary cultures of human hepatocytes. Notably, the extent of underprediction in this study was comparable to predictions for several drugs using primary cultures of human hepatocytes in a similar manner (Riley et al., 2005; Chiba et al., 2009).

The present in vitro study suggests that the exposure of erlotinib in primary cultures of human hepatocytes was increased 2.1- and 4.2-fold by ketoconazole and ritonavir treatment, respectively. Predicted human CL_{oral} of erlotinib was reduced by approximately 1.9- and 16-fold with ketoconazole and ritonavir treatment, respectively. Previous clinical data demonstrated that the erlotinib exposure was increased nearly 1.7-fold and CL/F was reduced correspondingly with ketoconazole treatment in healthy volunteers (Rakhit et al., 2008). In the in vitro induction study, the erlotinib exposure was decreased 6- and 3-fold by rifampin and efavirenz treatment, respectively. Predicted human CL_{oral} of erlotinib was increased 3- and 2.2-fold upon rifampin and efavirenz

treatment, respectively. Clinical reports suggest that rifampin treatment reduces the erlotinib exposure approximately 3-fold in healthy volunteers. Further, the CL/F of erlotinib was increased 3-fold with rifampin treatment in humans (Abbas et al., 2003). Importantly, the extent of interactions between ketoconazole or rifampin and erlotinib predicted from primary cultures of human hepatocytes were comparable to the extent observed in clinical interaction studies. This increases the confidence of our in vivo prediction of the extent of the interaction of the HAART drugs ritonavir and efavirenz on erlotinib from human hepatocytes data.

In conclusion, the current investigations predict the extent of interaction of the HAART drugs ritonavir and efavirenz on erlotinib using human hepatocytes and compared these interactions with ketoconazole and rifampin, respectively. Finally, these studies suggest that the clinically used dose of erlotinib (150 mg daily) may have to be either substantially reduced (25 mg every other day) or moderately increased (300 mg daily) to achieve desired drug exposure when coadministered with ritonavir or efavirenz, respectively. The magnitude of the interactions predicted with efavirenz, and especially with ritonavir, provides the impetus and rationale for dose selection in confirmatory phase I dose-finding trials of erlotinib in combination with HAART through the AIDS Malignancy Consortium (AMC).

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