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Pre-clinical drug-drug interaction (DDI) of gefitinib or erlotinib with Cytochrome P450 (CYP) inhibiting drugs, fluoxetine and/or losartan

Thu-Lan T. Luong [a,*], Michael J. McAnulty [a], David L. Evers [a], Brian J. Reinhardt [a], Peter J. Weina [a,b]

[a] Walter Reed National Military Medical Center, Biomedical Laboratory, Department of Research, 8901 Rockville Pike, Bethesda, MD 20889, United States
[b] Defense Health Headquarters, 7700 Arlington Blvd, Falls Church, VA 22042, United States

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ABSTRACT

Objective: To evaluate drug-drug interactions (DDIs) between gefitinib or erlotinib with fluoxetine, and/or losartan.

Methods: Human pooled microsomes, supersomes, and cryopreserved human hepatocytes were used to monitor DDIs *in vitro*. RED (Rapid Equilibrium Dialysis) protein binding was employed to investigate other pharmacokinetics.

Results: Gefitinib is significantly metabolized by Cytochrome P450 (CYP) 2D6 and CYP3A4, with less than 80% of the drug remaining. Erlotinib is significantly metabolized by CYP3A4, CYP2D6, and CYP1A2. Although gefitinib and erlotinib were metabolized by the same CYP isoenzymes, the metabolites formed from degradation of the two drugs were different.

Fluoxetine inhibited CYP2D6 and CYP3A4 metabolism of gefitinib with an IC_{50} of 65.12 ± 1.88 μM and 4.11 ± 2.26 μM, respectively. Fluoxetine also inhibited CYP2D6 and CYP3A4 metabolism of erlotinib with an IC_{50} of 7.06 ± 1.54 μM and 4.57 ± 1.22 μM, respectively.

For hepatocytes, fluoxetine affected the metabolism of gefitinib or erlotinib, while losartan had no effect. Gefitinib and erlotinib inhibited the metabolism of fluoxetine and losartan. Two-drug combinations involving gefitinib or erlotinib with fluoxetine or losartan yielded insignificant (p-value ≥ 0.05) differences in metabolism. However, combinations involving three drugs yielded significant degrees of inhibition (p-value ≤ 0.05). Three drug combinations involving fluoxetine and losartan with gefitinib or erlotinib yielded significant degrees of inhibition of the metabolism of gefitinib, but not for that of erlotinib.

Conclusion: As could be predicted by previous studies involving the inhibitory effect of fluoxetine on CYP3A4 and CYP2D6, and studies involving CYP metabolism of gefitinib and erlotinib, the tests performed here confirmed that fluoxetine has an inhibitory effect on metabolism of gefitinib or erlotinib by the main CYP isoenzymes involved. This study suggests a variable inhibitory effect of fluoxetine particularly on CYP2D6 activity towards gefitinib or erlotinib; erlotinib metabolism is less affected. Likewise, the combination of fluoxetine and losartan does not significantly affect hepatocyte metabolism of erlotinib, but does for that of gefitinib. The results presented in this study thus indicate a need for DDI assays to involve multiple drugs to properly study multidrug regimens.

1. Introduction

The treatment paradigm for metastatic non-squamous, non-small-cell lung cancer is continually changing. Algorithms published only 6 months ago are outdated today and differ dramatically from those published a few years ago. New driver mutations continue to appear in oncogene addiction strategies employed by cancer cells, so the development of therapies to target such oncogenes is ongoing. Patient survival is improving as treatments become more personalized and effective (Melosky 2018). Lung cancer chemotherapy may be done as monotherapy or in combination with other therapies. Combination therapies involve different types of treatment and concurrent use of multiple drugs.

In the United States, lung cancer occurs in all races, in males and females, and particularly in people over 50 years of age, reports the Center for Disease Control and Prevention (CDC, 2016). Older adults and people with severe underlying medical conditions like heart, lung disease, or diabetes seem to be at higher risk for developing more

* Corresponding author at: 8901 Rockville Pike, Bethesda, MD 20889, United States.
E-mail address: thu-lan.t.luong.civ@mail.mil (T.-L.T. Luong).

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serious complications from COVID-19 and other illnesses (CDC, 2019). As a result, chemotherapy requires patients to take more than one type of drug, pointing to the need to optimize drug combinations that offer the best chance of success.

Gefitinib, erlotinib and afatinib are three widely used epidermal, growth-factor receptor tyrosine kinase inhibitors (EGFR TKIs) for first-line treatment of patients with non-small cell lung cancer with proven efficacy (Melosky, 2018; Takeda et al., 2015; Burotto et al., 2015; Yang et al., 2017). Adverse events leading to treatment withdrawal occur significantly more often with afatinib or gefitinib than with erlotinib. The most common withdrawal adverse events were skin toxicity, interstitial lung disease, and hepatotoxicity (Takeda et al., 2015; Burotto et al., 2015; Yang et al., 2017). In the United States, gefitinib is approved for orphan use only.

Gefitinib and erlotinib share a common chemical backbone structure and exhibit similar disposition characteristics in humans after oral administration (Li et al., 2007; Siegel-Lakhai et al., 2005). Both drugs have similar oral bioavailability and undergo extensive metabolism primarily by Cytochrome (CYP) P450 isoenzymes (Li et al., 2007; Frohna et al., 2006; Swaisland et al., 2005; Ling et al., 2006; McKillop et al., 2005; McKillop et al., 2004). Both drugs are metabolized primarily by CYP3A4, CYP3A5, and CYP1A1. CYP2D6 is involved in gefitinib metabolism, whereas CYP 1A2 is considerably involved in erlotinib metabolism. Gefitinib is more susceptible to CYP3A mediated metabolism than erlotinib, which may contribute to the higher apparent oral clearance observed for gefitinib. Metabolism by hepatic and extrahepatic CYP1A may represent a determinant of pharmacokinetic variability and response for both drugs. The differences in metabolizing enzyme profiles suggest there may be differences in drug-drug interaction potential. Furthermore, stimulation of CYP3A4 may likely play a role in drug interactions for erlotinib and gefitinib (Li et al., 2007).

Since the efficacy of gefitinib or erlotinib has been linked with CYP3A4, CYP3A5, CYP2D6, and CYP1A2 metabolism, drugs that inhibit these cytochromes could result in pharmacokinetic alterations and reduced gefitinib and erlotinib efficacy. The purpose of this study is to evaluate DDIs between gefitinib or erlotinib with the CYP inhibitor drugs fluoxetine and/or losartan (the most commonly used hypertension drug). Fluoxetine is an anti-depressant with mild CYP1A2, moderate CYP2C9, mild/moderate CYP2C19, potent CYP2D6, and mild/moderate CYP3A4 inhibition according to Brown, 2008. Fluoxetine is metabolized by CYP2C19 and CYP2D6. Losartan is the first orally available angiotensin-receptor antagonist without agonist properties that has a favorable DDI profile, as evidenced by the lack of clinically relevant interactions between this drug and a range of inhibitors and stimulators of the CYP system according to Sica et al., 2005. Losartan is metabolized by CYP2C9 and CYP3A4. The aims of the study are (1) to generate a complete preclinical profile for gefitinib and erlotinib, (2) to study DDIs between gefitinib or erlotinib with fluoxetine and/or losartan to determine (i) whether gefitinib or erlotinib alters the pharmacokinetics of fluoxetine and/or losartan, and (ii) whether fluoxetine and/or losartan alters the pharmacokinetics of gefitinib or erlotinib, and (3) to determine whether *in vitro* assays can adequately describe DDIs.

2. Material and methods

Most assays were performed according to guidance by the manufacturer and are similar to those described by Jin et al., 2013 and Jin et al., 2016.

2.1. Materials

The mixed-gender 200 donor pooled cryopreserved primary human hepatocytes with 5 million cells, hepatocyte thawing buffer, and incubation buffer were purchased from In Vitro Technologies (Baltimore, MD). Gefitinib, erlotinib, CYP-specific substrates (phenacetin, S-mephenytoin, tolbutamide, dextromethorphan, nifedipine), and CYP inhibitors (fluoxetine and losartan) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human pooled supersomes for CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, pooled human microsomes, and the NADPH regeneration system solutions A and B were obtained from Corning Life and Sciences (Tewksbury, MA). Pooled human plasma was purchased from Innovative Research (Novi, MI). RED base plate, RED device inserts, and RED dialysis buffer were purchased from Thermo Scientific.

2.2. In vitro metabolism of gefitinib and erlotinib with human pooled microsomes

Gefitinib or erlotinib were incubated with human pooled microsomes by combining 10 μL of 1 mM drugs (10 μM final concentration), 50 μL of solution B and 10 μL of solution A of NADPH regeneration system, and 900 μL of 0.1 M phosphate buffer (pH 7.4). The solution was gently mixed by pipetting and was incubated at 37 °C for 5 min. After 5 min incubation, 30 μL of human pooled microsomes were added. The solution was incubated at 37 °C for 1 min before sample collection. Samples (150 μL) were collected at 0, 30, 60, and 120 min time points, and quenched with an equal volume of acetonitrile. The samples were vortexed for 30 s, then centrifuged at 1,300 × g at 4 °C for 15 min. Supernatants were collected and stored at 4 °C until analysis. Samples were run after all samples were collected within 3 h.

2.3. CYP1A2, 2C9, 2C19, 2D6, and 3A4 screening for gefitinib and erlotinib with human pooled supersomes

Gefitinib or erlotinib was incubated with CYP1A2, 2C9, 2C19, 2D6, and 3A4 human pooled supersomes by adding 10 μL of 1 mM drugs (10 μM final concentration), 50 μL of solution B and 10 μL of solution A of NADPH regeneration system, and then adding 900 μL of 0.1 M phosphate buffer (pH 7.4). The solution was gently mixed by pipetting and incubated at 37 °C for 5 min. After 5 min incubation, 30 μL of human pooled CYP supersomes were added. The solution was incubated at 37 °C for 1 min before sample collection. Samples (150 μL) were collected at 0, 30, 60, and 120 min time points, and quenched with an equal volume of acetonitrile. The samples were vortexed for 30 s, and then centrifuged at 1,300 × g at 4 °C for 15 min. Supernatant was collected and stored at 4 °C until analysis. Samples were run after all samples were collected within 3 h.

2.4. CYP DDIs with CYP inhibitor and supersomes

Gefitinib or erlotinib at 10 μM final concentration (5 μL of 1 mM drugs) was mixed with 5 μL of CYP inhibitor drug at the concentration to be tested, 25 μL of solution B and 5 μL of solution A of the NADPH regeneration system, and 445 μL of 0.1 M phosphate buffer (pH 7.4). The solution was gently mixed by pipetting and incubating at 37 °C for 5 min. After 5 min incubation, 15 μL of CYP supersomes were added. The solution was incubated at 37 °C for 1 min before sample collection. Samples were collected at time point 0 and 60 min by mixing 150 μL aliquots with an equal volume of acetonitrile to quench. The quenched samples were vortexed for 30 s, then centrifuged at 1,300 × g at 4 °C for 15 min Supernatants were collected and stored at 4 °C until analysis. Samples were run after all samples were collected within 2 h.

2.5. In vitro metabolism of gefitinib and erlotinib with pooled hepatocytes

Cryopreserved pooled human primary hepatocytes were flash thawed in a water bath for 1 min. One vial of the thawed hepatocytes was added to 48 mL of pre-warmed thawing INVITROGRO HT medium

buffer, mixed thoroughly by gentle pipetting, and centrifuged at 50 × g at room temperature for 5 min. The supernatant was discarded by pouring in one motion. The cell pellet was loosened by gently swirling the centrifuge tube, then the hepatocytes were resuspended in INVITROGEN KHB buffer. The hepatocytes were then seeded onto 24-well culture plates, with approximately 0.5 × 10^6 viable cells/mL per well, and incubated in a humidified incubator for 30 min prior to the addition of drug(s), to allow the hepatocytes to adjust to the incubator conditions.

Gefitinib or erlotinib were premixed with fluoxetine and/or losartan in DMSO to establish the same condition before incubating with hepatocytes. Following the addition of 5 μL drugs at 1 mM (10 μM final concentration), the solution was gently mixed by pipetting, then incubated at 37 °C for 1 min. Samples were collected after 0 and 4 h by quenching 120 μL aliquots with equal volumes of acetonitrile with internal standard. The samples were vortexed for 30 s, and then centrifuged at 1,300 × g at 4 °C for 15 min. Supernatants were stored at 4 °C until analysis. Samples were run after all samples were collected within 5 h.

2.6. In vitro human plasma rapid equilibrium dialysis (RED) protein binding assay

Assays were performed according to guidance by the manufacturer. Gefitinib or erlotinib was mixed with 1 mL pooled human plasma at a 10 μM concentration. This mixture (300 μL) was dispensed into the sample chamber (red ring) and 500 μL dialysis buffer was put in the buffer chamber. The plate was sealed, then incubated at 37 °C on an up and-down shaker shaking at 20 rpm for 4.0 h. Samples were collected by removing 100 μL from each buffer and plasma chamber. The collected buffer samples were mixed with 100 μL plasma, and the collected plasma samples were mixed with 100 μL dialysis buffer. Then, 200 μL of acetonitrile containing internal standard was added to all samples. The samples were vortexed for 30 s, then centrifuged at 1,300 × g at 4 °C for 15 min. Supernatants were stored at 4 °C until analysis. Samples were run after all samples were collected within 5 h.

2.7. Liquid chromatography-mass spectrometry

Gefitinib, erlotinib, fluoxetine, losartan, and metabolites were detected and quantified using a Shimadzu LCMS-2020 liquid chromatograph mass spectrometer equipped with a Shimadzu SIL-20A auto sampler, two LC-20AD pumps and an SPD-M20A Photodiode Array detector (PDA). The mass spectrometer was a single quadrupole equipped with electrospray ionization and corona discharge needle sources for a dual ionization source interface.

A positive full scan (mass range from 250 to 650 amu) and single ion monitor (SIM) method were created to detect parent drugs and metabolites formed. A 10 μL aliquot of the supernatant was injected onto a 3.9 × l50 mm C-l8 reverse-phase column (Waters, catalog WAT046980) equipped with an analytical guard column (Phenomenex, catalog KJ0-4282) at a flow rate of 0.4 mL/minute at a temperature of 37 °C. The mobile phase (A) was 0.1% formic acid in water and the mobile phase (B) was 0.1% formic acid in acetonitrile. The gradient ran at 5% B for 1 min, ramped to 95% B for 2 min and held at 95% B for 4 min, then returned to the initial start condition 5% B at 6 min for a total run time of 7 min. The ion source parameters were: interface voltage 5 V (volts) for gefitinib and 4 V for erlotinib, nebulizing gas at 1.5 L/minute, drying gas at 10.0 L/minute, desolvation line temperature of 200 °C, heat block temperature at 350 °C, and detector voltage at 1.0 kV. These parameters were set after tuning and calibrating according to a tuning solution (Shimadzu, catalog 225–14985-01), parent method development by injection, and metabolite identification from a 30-minute full scan (mass range from 200 to 650 amu) detected from preliminary *in vitro* human microsome studies.

2.8. Statistics and data analysis

Parent drugs and metabolites were identified according to retention times and quantified by peak integration. Samples were prepared and run in duplicate for microsomes, supersomes, and hepatocytes. Sample were prepared and run in triplicate for RED protein binding according to manufacture. For microsomal studies, drugs of interest were run with enzyme, without enzyme, denatured microsomes, and buffer as negative controls to confirm retention of parent compound stability in the tested buffer, and to refute the possibility of degradation through other metabolic pathways (no degradation was detected in denatured microsomes or in microsomes without NAPH enzyme solution A and B). In addition, each assay was run with a control drug as a positive control for enzyme activity. Nifedipine was used as this control for microsomal, CYP3A4, and hepatocyte assays to determine the enzyme activity. Phenacetin was used as the control drug for CYP1A2, tolbutamide was used for CYP2C9, S-mephenytoin was used for CYP2C19, and dextromethorphan was used for CYP2D6 enzyme activities. The percentage of parent drug remaining was determined using the ratio of integrated peak area at a time point over integrated peak area of the zero time point. The half maximal inhibitory concentration (IC_{50}) values were determined from plots of percent parent drug remaining against fluoxetine concentrations. GraphPad Prism 8.4.3 (La Jolla, CA, USA) software was used for data analysis and graph plotting, and nonlinear regression in the form of [Inhibitor] vs. response – Variable slope (four parameters) was used to fit the data to a model to determine IC_{50} values. Welch's T-test was performed to determine two-tailed unadjusted p-values for statistical significance. Protein binding was calculated using the equation % Bound = 100% - % Free, where % Free = (Concentration buffer chamber/Concentration plasma chamber) × 100%.

3. Results

3.1. Gefitinib metabolism profile

The human microsome assays provided confirmation for gefitinib being extensively metabolized by cytochrome P450. Three potential metabolites (*m/z*-433 or o-desmethyl gefitinib, *m/z*-445, and *m/z*-472) were identified from pooled human microsomes, and then in assays involving CYP and hepatocytes. To evaluate the contribution of individual CYPs to the overall gefitinib metabolic profile, gefitinib was screened with the five most significant isoenzymes. CYP2D6, 3A4, 2C9, and 1A2 contributed to the metabolism of gefitinib (Fig. 1). A wide range of metabolites were formed, in order of greatest to least abundance: *m/z*-433 was observed from CYP2D6, 2C9, 2C19 and 3A4; *m/z*-472 was observed from CYP3A4, 2C9, 2C19, and 1A2; and *m/z*-445 was observed only from CYP3A4 (Table 1A).

3.2. Erlotinib metabolism profile

The human microsome assays provided confirmation for erlotinib also being extensively metabolized by cytochrome P450. Three potential metabolites (*m/z*-380-O-demethylation, *m/z*-410-oxidation, and *m/z*-428-hydroxylation) were identified from pooled human microsomes, and then in CYP and hepatocyte assays. To evaluate the contribution of individual CYPs to the overall erlotinib metabolic profile, erlotinib was screened with the five most significant isoenzymes. CYP3A4, 2D6, 1A2, 2C9, and 2C19 contributed to the metabolism of erlotinib (Fig. 1). A wide range of metabolites were formed, in order of greatest to least abundance: *m/z*-410 was observed from CYP3A4, 1A2, 2D6, and 2C9; *m/z*-380 was observed from CYP2D6, 3A4, 1A2, 2C9, then 2C19; *m/z*-428 was observed from 2D6, 1A2, and 3A4 (Table 1B).

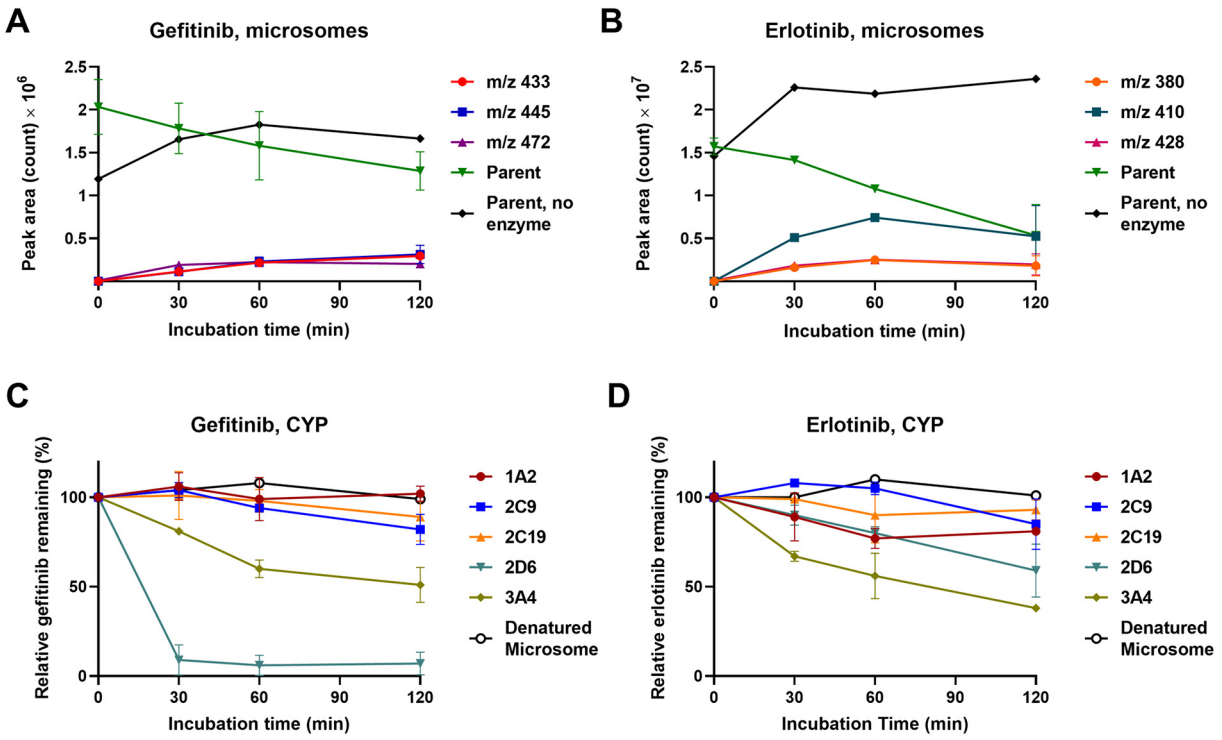

Fig. 1. Pre-clinical *in vitro* metabolism profiles for gefitinib and erlotinib. (A) Gefitinib, parent-*m/z*-447 degradation, and formation of three potential metabolites: *m/z*-433 or o-desmethyl gefitinib, *m/z*-445, and *m/z*-472 in human pooled microsomes. (B) Erlotinib, parent-*m/z*-394 degradation, and formation of three potential metabolites: *m/z*-380-O-demethylation, *m/z*-410-oxidation, and *m/z*-428-hydroxylation in human pooled microsomes. (C) Contribution of individual CYP isoenzymes to the metabolism of gefitinib. (D) Contribution of individual CYP isoenzymes to the metabolism of erlotinib. The assays were performed in duplicate, and the results reported as mean ± SD.

Table 1A
Potential metabolites of gefitinib. Chronological metabolite peak area average values for metabolites generated from digestion of gefitinib by CYP1A2, 2C9, 2C19, 2D6, and 3A4. Potential metabolites *m/z*-433 and *m/z*-445 were described in McKillop et al., 2004.

	Potential Metabolite *m/z*-433				
Time (min)	1A2	2C9	2C19	2D6	3A4
0	———	———	———	1,981,682	———
30	———	20,384	———	3,516,826	———
60	———	61,302	———	3,468,314	14,933
120	———	119,717	———	3,831,381	21,485
	Potential Metabolite *m/z*-445				
Time (min)	**1A2**	**2C9**	**2C19**	**2D6**	**3A4**
0	———	———	———	———	17,339
30	———	———	———	———	94,770
60	———	———	———	———	165,864
120	———	———	———	———	213,786
	Potential Metabolite *m/z*-472				
Time (min)	**1A2**	**2C9**	**2C19**	**2D6**	**3A4**
0	———	———	———	———	58,573
30	———	20,407	———	———	67,578
60	16,832	27,478	16,916	———	70,696
120	32,718	69,861	42,916	———	104,194

3.3. Gefitinib with fluoxetine CYP 2D6 and 3A4 DDI

CYP screening revealed that CYP2D6 and 3A4 were the two most significant isoenzymes contributing to gefitinib metabolism. Fluoxetine is known to have potent (strong) CYP2D6, and mild/moderate CYP3A4 inhibition. Therefore, assays were designed to investigate DDIs between gefitinib and fluoxetine in terms of CYP2D6 and 3A4 enzyme activity. A fixed concentration of gefitinib at 10 μM was incubated in the presence or absence of varying concentrations of fluoxetine from 0 to 100 μM for 60 min with CYP2D6 or 3A4. As the fluoxetine concentration increased, the relative percent of remaining gefitinib also increased, suggesting a reduction in gefitinib metabolism as a result of CYP2D6 and 3A4 inhibition (see Fig. 2). As the fluoxetine concentration was increased in DDI assays involving CYP2D6, the relative percent of gefitinib remaining increased, with a concomitant decrease in formation of the *m/z* 433 metabolite and increase in the *m/z* 472 metabolite (which was not found in the CYP2D6 screening without fluoxetine). As the fluoxetine concentration was increased in DDI assays with CYP3A4, the relative percent of gefitinib remaining also increased, with concomitant decreases in all three metabolites *m/z* 433, 445, and 472. The IC_{50} of fluoxetine interacting with gefitinib was calculated to be 65.12 ± 1.88 μM for CYP2D6 and 4.11 ±

Table 1B
Potential metabolites of erlotinib. Chronological metabolite peak area average values for metabolites generated from digestion of erlotinib by CYP1A2, 2C9, 2C19, 2D6, and 3A4. Potential metabolites *m/z* – 380, 410 and 428 were described in Li et al., 2006.

	Potential Metabolite *m/z*-380				
Time (min)	**1A2**	**2C9**	**2C19**	**2D6**	**3A4**
0	108,348	0	0	65,295	93,393
30	415,278	11,536	8087	612,799	774,338
60	619,740	50,952	26,111	1,271,090	1,170,381
120	1,263,945	115,739	70,971	2,351,243	1,612,580
	Potential Metabolite *m/z*-410				
Time (min)	**1A2**	**2C9**	**2C19**	**2D6**	**3A4**
0	266,372	0	0	4379	209,429
30	916,728	0	0	22,415	2,539,687
60	994,106	15,930	0	50,026	4,184,976
120	1,278,061	18,521	13,046	3,629,281	5,354,720
	Potential Metabolite *m/z*-428				
Time (min)	**1A2**	**2C9**	**2C19**	**2D6**	**3A4**
0	52,792	0	0	29,906	71,720
30	160,290	0	0	294,934	368,855
60	177,138	0	0	601,321	596,530
120	257,224	10,804	5327	1,831,510	979,895

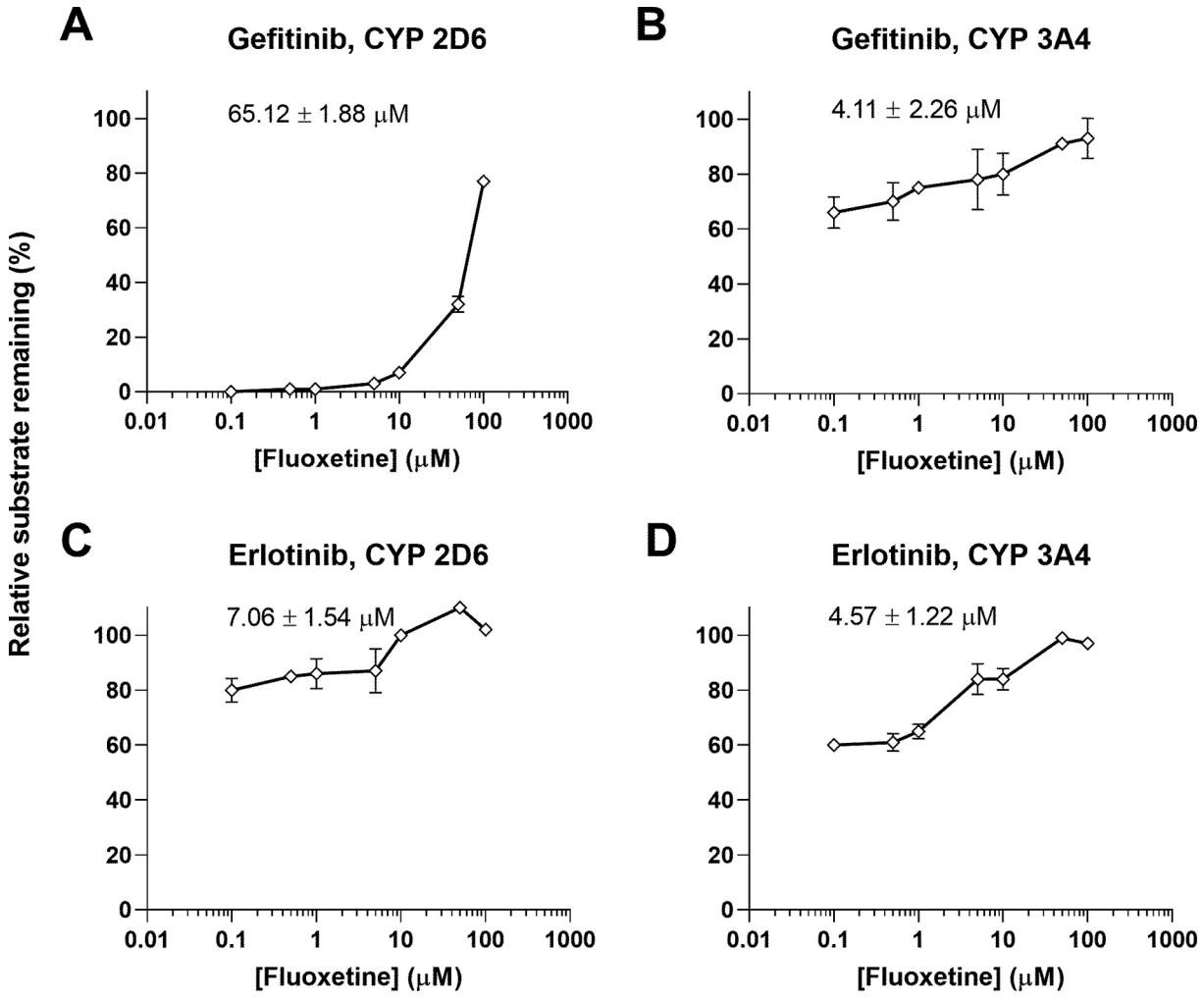

Fig. 2. Gefitinib or Erlotinib DDI with fluoxetine. CYP2D6 and CYP3A4 DDIs in 60 min incubations between gefitinib or erlotinib at 10 μM concentration with fluoxetine (potent CYP2D6, mild/moderate CYP3A4 inhibitor) at various (0.1 to 100 μM) concentrations. For metabolism of gefitinib, fluoxetine inhibits activity of (A) CYP2D6 and (B) CYP3A4. For metabolism of erlotinib, fluoxetine inhibits activity of (C) CYP2D6 and (D) CYP3A4. Calculated IC_{50} values for the inhibitory action by fluoxetine are shown. The assays were performed in duplicate, and the results reported as mean ± SD. GraphPad Prism 8.4.3 (La Jolla, CA, USA) software was used for data analysis and graph plotting, and nonlinear regression in the form of [Inhibitor] vs. response. Variable slope (four parameters) was used to fit the data to a model to determine IC50 values.

2.26 μM for CYP3A4. Further monitoring of fluoxetine showed that CYP2D6 contributes to fluoxetine metabolism too.

3.4. Erlotinib with fluoxetine drug-drug interaction

CYP screening showed that CYP3A4, 1A2, and 2D6 were the three most significant isoenzymes contributing to erlotinib metabolism. With fluoxetine's known potent (strong) CYP2D6 and mild/moderate CYP3A4 inhibition, assays were designed to investigate DDIs between erlotinib and fluoxetine in terms of CYP2D6 and 3A4. A fixed concentration of erlotinib at 10 μM was incubated in the presence or absence of varying concentrations of fluoxetine from 0 to 100 μM for 60 min with CYP2D6 or 3A4. The results (Fig. 2) overall illustrate that as the fluoxetine increased, the relative percent of erlotinib remaining also increased, suggesting a reduction of erlotinib metabolism as a result of inhibition of CYP2D6 and 3A4. More specifically, the formation of all three metabolites *m/z* 380, 410, and 428 decreased with decreases in metabolism of erlotinib, as the concentration of fluoxetine was increased. The IC_{50} of fluoxetine interacting with erlotinib was calculated to be 7.06 ± 1.54 μM for CYP2D6 and 4.57 ± 1.22 μM for CYP3A4. The additional monitoring of fluoxetine showed that CYP2D6 also contributes to fluoxetine metabolism.

3.5. DDIs in human hepatocytes involving combinations of gefitinib or erlotinib with fluoxetine and losartan

Having established the inhibitory trends of fluoxetine over recombinant CYP2D6 and 3A4, gefitinib and erlotinib each were further assessed in primary human hepatocytes individually and in combinations with fluoxetine and/or losartan. Cell cultures were pre-incubated for 30 min, then the drug compounds were delivered to the cells at a 10 μM final concentration for both the drugs of interest and combination drugs. Gefitinib, erlotinib, fluoxetine, and losartan underwent insignificant (p-value ≥ 0.05) metabolic changes in two-drug combinations (Fig. 3 and Table 2). In three-drug combinations, only erlotinib underwent insignificant changes in all tested combinations. For example, the combining of gefitinib, fluoxetine, and losartan caused significant decreases in metabolism of gefitinib (p-value = 0.02) and losartan (p-value = 0.02), but insignificant decreases in metabolism of fluoxetine (p-value = 0.07). The combining of erlotinib, fluoxetine, and losartan caused insignificant decreases in metabolism of erlotinib (p-value = 0.17), fluoxetine (p-value = 0.02), and losartan (p-value = 0.05).

3.6. Protein binding

RED assays for protein binding to plasma proteins were in concordance with values in the literature for individual drugs (Table 2B). Gefitinib was found to be 97% ± 1% bound (compared with ~ 97% according to Li et al., 2006), erlotinib was 96% ± 0% bound (compared with 95% according to Christiansen et al., 2009), fluoxetine was 95% ± 1% bound (compared with 95% according to Van Harten 1993), and losartan was 99% ± 0% bound (compared with greater than 98%, according to Sica et al., 2005). The addition of the other drugs in combinations tested here had no significant effect on protein binding.

4. Discussion

The *in vitro* metabolic profiles of gefitinib and erlotinib (Fig. 1) found here agree with the findings by McKillop et al. (2004), Ling et al. (2006), and Li et al. (2007). Gefitinib is significantly metabolized by CYP2D6 and CYP3A4, with less than 80% of the drug remaining. Erlotinib is significantly metabolized by CYP3A4, CYP2D6, and CYP1A2 (at 60 min). Although gefitinib and erlotinib are metabolized by the same CYP isoenzymes, their metabolites differ (Table 1). The products of gefitinib metabolism are much more CYP specific compared with those of erlotinib. Therefore, it can be expected that CYP inducers or inhibitors will have a more profound effect on the metabolism and overall efficacy of gefitinib, and are more likely to incur DDIs with gefitinib compared with erlotinib.

DDIs are difficult to predict using data that involves only a single drug. According to Brown (2008), fluoxetine has mild CYP1A2, moderate CYP2C9, mild/moderate CYP2C19, potent CYP2D6, and mild/-moderate CYP3A4 inhibition. The CYP DDI assays here showed that, indeed, fluoxetine also inhibits the CYP3A4 degradation of gefitinib and erlotinib (IC_{50} values of 4.11 ± 2.26 μM and 4.57 ± 1.22 μM, respectively). However, fluoxetine has a variable inhibitory effect on CYP2D6 degradation dependent on the other drug in the DDI. Such is the case for inhibition of degradation of gefitinib and erlotinib

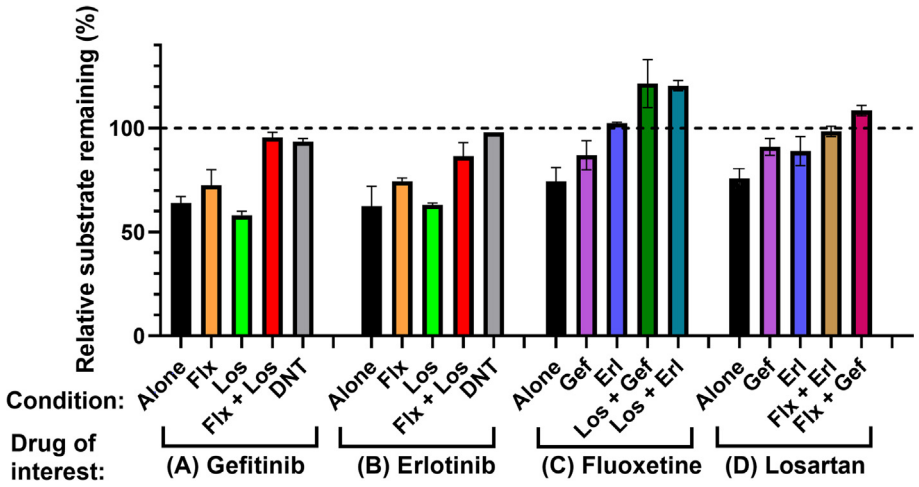

Fig. 3. Gefitinib, erlotinib, fluoxetine, and losartan hepatocytes data. Hepatocyte assays for metabolism of (A) gefitinib (Gef), (B) erlotinib (Erl), (C) fluoxetine (Flx), and (D) losartan (Los), interacting in various combinations after 4 h of incubation at 10 μM concentrations. Assays performed 'Alone' included only a single drug for reference, and negative controls 'DNT' were performed with denatured microsomes in INVITROGRO KHB buffer. The assays were performed in duplicate, and the results reported as mean ± SD. Significant differences from the 'Alone' controls (p-value ≤ 0.05) included: Fluoxetine and losartan inhibiting metabolism of Gefitinib, erlotinib inhibiting metabolism of fluoxetine, erlotinib and losartan inhibiting metabolism of fluoxetine, gefitinib and fluoxetine inhibiting metabolism of losartan, and erlotinib and fluoxetine inhibiting metabolism of losartan. The p values can be found in Table 2.

Table 2
Drug-drug interaction of gefitinib or erlotinib with fluoxetine and/or losartan. (A) Hepatocyte assay results (mean ± SD). Hepatocytes were pre-incubated for 30 min. Then, monitored drug was then added in duplicate, and amount remaining (in relation to the amount measured at 0 h) determined after 4 h. Results showing statistical significance (p-value ≤ 0.05) compared with monitored drug alone. (B) RED assays for protein binding in triplicate (mean ± SD). No significant change in DDI.

Types of Assays	**Drug-Drug Interactions**	(A) Hepatocytes Data 4 h incubation		(B) RED Protein Binding Data 4 h of shaking	
Monitor Drugs	Components	Parent remain (%)	p-Value	Binding (%)	p-Value
Gefitinib (Gef)	Gef	65 ± 2	–	97 ± 1	–
	Gef + Flx	72 ± 10	0.41	99 ± 1	0.22
	Gef + Los	58 ± 3	0.28	97 ± 0	1.00
	Gef + Flx + Los	96 ± 4	0.02	100 ± 0	0.07
Erlotinib (Erl)	Erl	62 ± 13	–	96 ± 0	–
	Erl + Flx	75 ± 3	0.32	97 ± 1	0.73
	Erl + Los	63 ± 2	0.96	97 ± 1	0.45
	Erl + Flx + Los	86 ± 9	0.17	97 ± 1	0.35
Fluoxetine (Flx)	Flx	75 ± 9	–	95 ± 1	–
	Flx + Gef	87 ± 10	0.32	96 ± 1	0.70
	Flx + Gef + Los	122 ± 16	0.07	92 ± 2	0.20
	Flx + Erl	102 ± 1	0.05	96 ± 1	0.54
	Flx + Erl + Los	121 ± 3	0.02	96 ± 5	0.87
Losartan (Los)	Los	77 ± 6	–	99 ± 0	–
	Los + Gef	89 ± 4	0.15	99 ± 0	0.27
	Los + Gef + Flx	112 ± 13	0.02	99 ± 0	0.57
	Los + Erl	89 ± 10	0.31	99 ± 0	0.41
	Los + Erl + Flx	99 ± 4	0.05	99 ± 0	0.99

(IC_{50} values of 65.12 ± 1.88 μM and 7.06 ± 1.54 μM, respectively). Whether this result can be explained by erlotinib inhibiting fluoxetine degradation by CYP2D6, preventing the concentration and inhibitory effect of fluoxetine from decreasing, it remains to be tested. Nonetheless, the importance of determining the actual IC_{50} value is demonstrated here, as it gives more insight into potential DDIs when a patient must use a combination of drugs.

Compared with *in vitro* CYP assays, *in vitro* hepatocyte assays offer broader information concerning DDI. The hepatocyte assays performed here also shed light on any potential inhibitory effects of gefitinib and erlotinib on the metabolism of fluoxetine and losartan, as this remained unknown. Although the combination of gefitinib or erlotinib with fluoxetine or losartan does not significantly change metabolism (p-value ≥ 0.05), the addition of even a drug with a favorable DDI profile to these combinations may cause significant inhibition. For instance, metabolism of gefitinib is not significantly impaired in the presence of fluoxetine (p-value = 0.41) or losartan (p-value = 0.28), but is significantly diminished (p-value = 0.02) when all drugs are combined (gefitinib, fluoxetine, and losartan). Similarly, metabolism of losartan is not significantly impaired in the presence of gefitinib (p-value = 0.15) or erlotinib (p-value = 0.31), while the addition of fluoxetine to these combinations causes significant decreases in metabolism (gefitinib, fluoxetine, and losartan; p-value = 0.02) (erlotinib, fluoxetine, and losartan; p-value = 0.05). Metabolism of fluoxetine is insignificantly affected by gefitinib (p-value = 0.32) or gefitinib combined with losartan (p-value = 0.07), but is significantly decreased in the presence of erlotinib (p-value = 0.05) or erlotinib combined with losartan (p-value = 0.02). Only metabolism of erlotinib is not significantly affected by any of the drugs and combinations tested here: with fluoxetine (p-value = 0.32); with losartan (p-value = 0.96); and with fluoxetine and losartan (p-value = 0.17). However, erlotinib has an inhibitory effect on the metabolism of fluoxetine (p-value = 0.05), so caution should be advised with this combination.

For the protein binding, the addition of the other drugs in combinations tested here had no significant effect on protein binding. Gefitinib, erlotinib, fluoxetine, and losartan are high protein binding (>95%), therefore any changes to protein binding are unlikely.

5. Conclusion

The data presented in this study provides insight into potential DDIs during treatment of metastatic non-squamous non-small-cell lung cancer. The pre-clinical profiles for gefitinib and erlotinib found here are close to those described in the literature. The inhibitory effects (in terms of IC_{50} concentrations) of gefitinib or erlotinib on the metabolism of fluoxetine and/or losartan were determined. Overall, the study provides a clear insight of the pharmacokinetics of DDIs between gefitinib or erlotinib and fluoxetine and/or losartan. Further studies in animal models and clinical trials would be needed to confirm the results here. Nevertheless, it is safe to conclude *in vitro* assays can adequately describe DDIs, and can help determine multidrug regimens with minimized potential for DDIs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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