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Pre-clinical drug-drug interactions (DDIs) of gefitinib with/without losartan and selective serotonin reuptake inhibitors (SSRIs): citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, and venlafaxine

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ABSTRACT

Objective: To evaluate drug-drug interactions (DDIs) between gefitinib with/without losartan and selective serotonin reuptake inhibitors (SSRIs).

Methods: In vitro supersomes were used to identify CYP isoenzymes (CYP1A2, 2C9, 2C19, 2D6, and 3A4) involved in drug metabolism, and *in vitro* pooled cryopreserved primary human hepatocytes were employed to investigate DDIs.

Results: The isoenzymes that showed drug degradation are listed in parentheses beside the respective drug: gefitinib (CYP2D6, 3A4, 1A2, 2C9, and 2C19), losartan (CYP2C9 and 3A4), citalopram (CYP2D6, 2C19, 3A4, and 2C9), fluoxetine (CYP2D6, 2C9, and 2C19), fluvoxamine (CYP2D6, 2C9, and 2C19), paroxetine (CYP2D6, 3A4, and 2C9), sertraline (CYP2D6, 2C9, 2C19, 1A2, and 3A4), and venlafaxine (CYP2D6 and 2C19).

DDIs from human hepatocytes assays revealed that gefitinib had significant metabolic changes in (1:1) combination with paroxetine or sertraline (p-value = 0.042 and 0.025 respectively) and (1:1:1) combination with losartan and fluoxetine, fluvoxamine, paroxetine, or sertraline (p-value = 0.009, 0.027, 0.048, and 0.037 respectively). Losartan showed significant changes in (1:1:1) combination with gefitinib and fluoxetine or sertraline (p-value = 0.026 and 0.008 respectively). Fluoxetine, fluvoxamine, and paroxetine underwent significant changes in (1:1:1) combination with gefitinib and losartan (p-value = 0.003, 0.022, and 0.046 respectively). Sertraline had significant changes within all combinations: DDIs with gefitinib alone and in combination with gefitinib and losartan (p-value = 0.009 and 0.008 respectively). Citalopram and venlafaxine appeared to be unaffected by any combination.

Conclusion: The study provides a clear proof-of concept for *in vitro* metabolic DDI testing. While identifying compounds by their inhibition potential can help better predict their metabolism, it cannot resolve problems that arise from DDIs since the overall degree of effectiveness is unknown. As shown in this study, gefitinib has been identified as a weak CYP2C19 and 2D6 inhibitor, however, gefitinib can have significant DDIs with sertraline. Furthermore, multiple drug combinations (1:1:1) can change the significance of previously determined DDIs in (1:1) combination. Thus, *in vitro* assays can potentially provide better guidance for multidrug regimens with minimal risk for DDIs.

1. Introduction

Gefitinib, erlotinib, and afatinib are three widely used epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) for treating advanced non-small cell lung cancer (NSCLC) with proven efficacy. Over the years, there have been many comparison studies on the three drugs (Siegel-Lakhai et al., 2005; Li et al., 2007; Takeda et al., 2015; Burotto et al., 2015; Yang et al., 2017; Kimura et al., 2018; Xu et al., 2109; Luong et al., 2021). From these drugs, gefitinib has been associated with a higher frequency of hepatotoxicity (Takeda et al., 2015; Burotto et al., 2015; Yang et al., 2017), however, gefitinib is more cost effective (Kimura et al., 2018). Pre-clinical drug-drug interactions (DDIs) of gefitinib or erlotinib with Cytochrome P450 (CYP) inhibiting drug fluoxetine and/or losartan showed that gefitinib is more susceptible than

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erlotinib to metabolic changes with fluoxetine and/or losartan (Luong et al., 2021). Additionally, gefitinib has a weak inhibition effect on CYP2C19 and 2D6, which should be considered during treatment (Swaisland et al., 2005; Xu and Li, 2019). As shown by a previous study, more personalized approaches to cancer therapeutics by understanding the molecular background of the cancer can lead to a more appropriate evaluation of the selected drugs used for treatment (Ziogas et al., 2017).

Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) have been used effectively for the treatment of major depressive disorders, anxiety disorders, hot flashes, and post-traumatic stress disorder (PTSD) (van Harten, 1993; Gibbons et al., 2012). They have also been extensively characterized in regards to CYP2D6 inhibition and drug-drug interactions (Brown, 2008; Lin and Lu, 1998; Nemeroff et al., 1996; Hamelin et al., 1996; Spina et al., 2008; Jin et al., 2016). Citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, and venlafaxine are all commonly used SSRIs, and their inhibitory effects on CYP-P450 isoenzymes are listed in Table 1. As previously reported, fluoxetine has an inhibitory effect on the metabolism of gefitinib via CYP2D6 and 3A4 (Luong et al., 2021). However, this is potentially an issue because gefitinib is metabolized mainly by CYP2D6 and CYP3A4 (Xu and Li, 2019; Luong et al., 2021). Additionally, in multiple combinations of gefitinib, fluoxetine, and losartan (in 1:1:1, multiple combination) with hepatocytes, the metabolism of gefitinib and losartan were significantly inhibited (Luong et al., 2021). Furthering complicating the issue is the fact that most of these drugs are metabolized by different enzymes, leading to a variety of metabolites. Fluoxetine is primarily metabolized via N-demethylation by CYP2D6, 2C9, and 3A (von Moltke et al., 1997; Margolis et al., 2000), citalopram by CYP3A4, 2D6, and 2C19 catalyzed N-demethylation, didemethylation, and N-oxide (Kobayashi et al., 1997; von Moltke et al., 1999, 2001; Olesen and Linnet, 1999; Sangkuhl et al., 2011), fluvoxamine by oxidative demethylation, oxidative deamination, and N-acetylation, and the specific CYP isoenzymes involved in the metabolism remain to be identified (van Harten, 1995), paroxetine by demethylenation of its methylenedioxy group by CYP2D6 (Bloomer et al., 1992), sertraline by N-demethylation by CYP2B6, 2C9, 2C19, 2D6, and 3A4 (Kobayashi et al., 1999; Greenblatt et al., 1999; Xu et al., 1999;

Hamelin et al., 1996; Wang et al., 2001; DeVane et al., 2002; Obach et al., 2005) venlafaxine by O-demethylation by CYP2D6 (Otton et al., 1996; Fogelman et al., 1999; Sangkuhl et al., 2014). Also, some of these agents have been shown to exhibit substantial differences in pharmacokinetics in subjects who lack CYP2D6 or CYP2C19, such as fluoxetine, paroxetine, and venlafaxine (Hamelin et al., 1996; Lessard et al., 1999; Liu et al., 2001; Charlier et al., 2003; Lindh et al., 2003; Yu et al., 2003).

Lack of knowledge on drug-drug interactions can be fatal. Acknowledging the importance of DDIs, this study was designed to further evaluate DDIs of gefitinib with/without losartan and SSRIs in order to determine whether gefitinib alters the pharmacokinetics of losartan or SSRIs, and if SSRIs with/without losartan alters the pharmacokinetics of gefitinib. The goals of the study were to identify which SSRIs least inhibit the metabolism of gefitinib, to study multiple drug combinations (gefitinib with/without losartan and SSRIs) and their effects on individual drug metabolism, and to perform a pre-clinical profile for SSRIs with CYP (CYP1A2, 1C9, 2C19, 2D6, and 3A4) screening assays for an in-depth understanding of their potential DDIs.

2. Materials and methods

Assays were performed according to guidance by the manufacturer, and are similar to those previously described (Jin et al., 2014, 2016; Luong et al., 2021). The combination of gefitinib with/without losartan and fluoxetine was repeated as before (Luong et al., 2021), and the same fluoxetine stock solution was used. Gefitinib, losartan, and other SSRIs were prepared with fresh powder. The drug concentrations in this study were based on previously reported IC_{50} values (Luong et al., 2021). GraphPad Prism 8.4.3 (La Jolla, CA, USA) software was used for data analysis, graph plotting, and nonlinear regression ([inhibitor] vs. response, in which a variable slope with four parameters was used to fit the data to a model to determine IC_{50} values).

2.1. Materials

The mixed-gender 10 donor pooled cryopreserved primary human hepatocytes with 5 million cells, hepatocyte thawing buffer, and incubation buffer were purchased from BIOIVT elevating science (Baltimore, MD). Gefitinib, losartan, SSRIs (citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, and venlafaxine), and CYP-specific substrates (phenacetin, mephenytoin, tolbutamide, dexamethorphan, nifedipine) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant pooled human supersomes for CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, and the NADPH regeneration system solutions A and B were obtained from Corning Life and Sciences (Tewksbury, MA).

2.2. In vitro human hepatocytes DDI of gefitinib with/without losartan and SSRIs

Pooled cryopreserved 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 $50{\times}g$ 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 seeded onto 24-well culture plates, with approximately 0.5×106 viable cells/mL per well, before adding the drug(s).

Gefitinib was premixed with/without losartan and citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, or venlafaxine 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. Samples were collected after 0 and 3 h by quenching 120 μL aliquots with equal volumes of acetonitrile containing the internal standard. The samples were vortexed

Table 1
List of drugs utilized in this study.

Inhibitory Effects of Study Drugs on Cytochrome P-450 Isoenzymes						Metabolism Pathway
Agent	CYP 1A2	CYP 2C9	CYP 2C19	CYP 2D6	CYP 3A4	CYP Pathway
Gefitinib	0	0	+	+	0	CYP 2D6 *, 3A4*, 1A2, 2C9, 2C19
Losartan	–	–	–	–	–	CYP 2C9*, 3A4
Citalopram	0	0	0	+	0	CYP 2D6*, 2C19*, 3A4, 2C9
Fluoxetine	+	++	+/++	+++	+/++	CYP2D6*, 2C9, 2C19
Fluvoxamine	+++	++	+++	+	++	CYP 2D6*, 2C9, 2C19
Paroxetine	+	+	+	+++	+	CYP 2D6*, 3A4, 2C9
Sertraline	+	+	+	+/++	+	CYP 2D6*, 2C9*, 2C19*, 1A2, 3A4
Venlafaxine	0	0	0	+	+	CYP 2D6*, 2C19*

0 = minimal/no inhibition + = mild/weak inhibition ++ = moderate inhibition.
+++ = potent/strong inhibition – = unknown * Major CYP Pathway.
Table was adapted from Brown (2008); Lin and Lu, 1998; Nemeroff et al. (1996); Hamelin et al. (1996); Swaisland et al. (2005); Spina et al. (2008); Jin et al. (2016); Xu and Li, 2019; Luong et al. (2021); and the results from the CYP1A2, 2C9, 2C19, 2D6, and 3A4 screening.

for 30 s, and then centrifuged at 1300×g at 4 °C for 15 min. Supernatants were stored at 4 °C until analysis. Samples were run after they were all collected within 4 h.

2.3. CYP1A2, 2C9, 2C19, 2D6, and 3A4 screenings were conducted for gefitinib, losartan, and SSRIs

Gefitinib, losartan, or SSRIs were incubated with CYP1A2, 2C9, 2C19, 2D6, and 3A4 pooled human supersomes by adding 5 μL of 1 mM drugs (10 μM final concentration), 25 μL of solution B and 5 μL of solution A of NADPH regeneration system, and then 450 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 incubation, 15 μL of pooled human CYP supersomes were added. The solution was incubated at 37 °C for 1 min before sample collection. Samples (120 μL) were collected at 0, 60, and 120 min time points, and then quenched with an equal volume of acetonitrile containing the internal standard. The samples were vortexed for 30 s and centrifuged at 1300×g at 4 °C for 15 min. The supernatant was collected and stored at 4 °C until analysis. Samples were run after they were all collected within 3 h.

2.4. Liquid chromatography-mass spectrometry

Gefitinib, losartan, SSRIs, 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) methods were created to detect parent drugs. 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/min 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 9 min (for a total run time of 10 min). The ion source parameters were as follows: interface voltage 5 V (volts) for gefitinib and 4 V for erlotinib, nebulizing gas at 1.5 L/min, drying gas at 10.0 L/min, 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).

2.5. Statistics and data analysis

Parent drugs and metabolites were identified according to retention times and quantified by peak integration. Samples were prepared and run
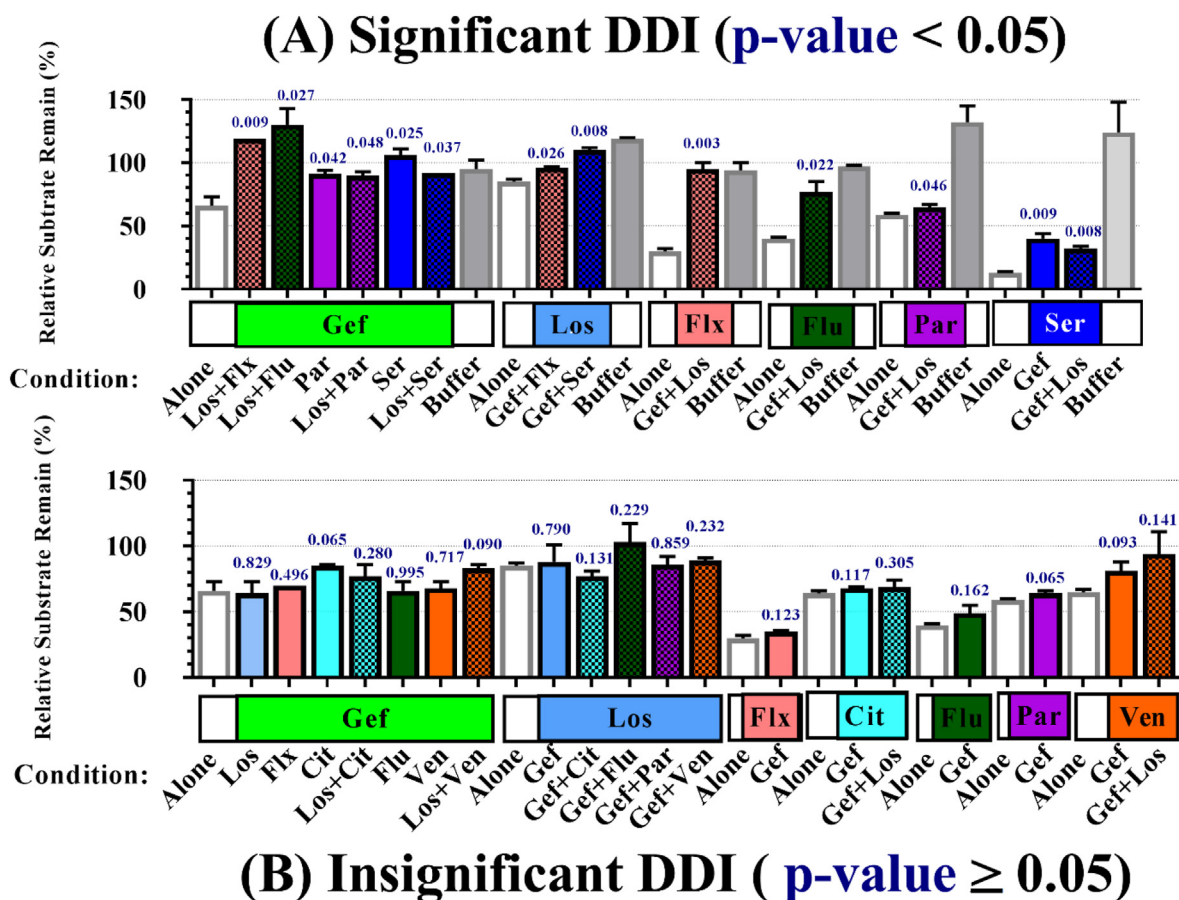

Fig. 1. Hepatocytes DDIs of gefitinib (Gef) with/without losartan (Los) and SSRIs: citalopram (Cit), fluoxetine (Flx), fluvoxamine (Flu), paroxetine (Par), sertraline (Ser), and venlafaxine (Ven). (A) Significant (p-value < 0.05) DDIs and (B) insignificant (p-value ≥ 0.05) DDIs interacting in various combinations after 3 h of incubation at 10 μM concentrations. Assays performed 'alone' included only a single drug for reference, and negative controls "buffer" were performed with INVITROGRO KHB buffer. The assays were performed with duplicate injection, and the results reported as mean ± SD. Gefitinib metabolism was significantly affected in the (1:1:1) combination of losartan with fluoxetine or fluvoxamine, and in all (1:1) and (1:1:1) combinations of paroxetine or sertraline with/without losartan. However, gefitinib metabolism was not significantly affected in any (1:1) or (1:1:1) combination of citalopram or venlafaxine with/without losartan. Welch's T-test was performed to determine two-tailed unadjusted p-values for statistical significance.

in duplicate injection for CYP isoenzymes and hepatocytes. Metabolite identification data (peak area counts) was extracted by mass per charge (m/z) for parent and potential metabolites from the full scan. Hepatocytes and CYP metabolism data (relative substrate remaining %) were extracted by SIM for parent drug (gefitinib, losartan, and SSRIs). In addition, each assay was run with a known drug as a positive control for enzyme activity. Nifedipine was used as the positive control for CYP3A4 and hepatocyte assays, phenacetin was used for CYP1A2, tolbutamide was used for CYP2C9, S-mephenytoin was used for CYP2C19, and dextromethorphan was used for CYP2D6. 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. Welch's T-test was performed to determine two-tailed unadjusted p-values for statistical significance.

3. Results

3.1. In vitro human hepatocytes DDI of gefitinib with/without losartan and SSRIs: fluoxetine (Flx), citalopram (Cit), fluvoxamine (Flu), paroxetine (Par), sertraline (Ser), and venlafaxine (Ven)

Drug compounds were delivered to the cells at a 10 μM final concentration for all assays. Fig. 1A shows the significant DDIs ($p < 0.05$), and Fig. 1B shows the insignificant DDIs ($p \geq 0.05$). Gefitinib had significant metabolic changes in (1:1) combination with paroxetine or sertraline (p-value = 0.042 and 0.025 respectively) and in (1:1:1) combination with losartan and fluoxetine, fluvoxamine, paroxetine, or sertraline (p-value = 0.009, 0.027, 0.048, and 0.037 respectively). However, gefitinib showed insignificant (p-value $\geq$ 0.05) metabolic changes in (1:1) with losartan, fluoxetine, citalopram, fluvoxamine, or venlafaxine, and (1:1:1) with losartan and citalopram or venlafaxine (Fig. 1 and Table 2). Losartan was affected significantly in (1:1:1) combination with gefitinib and fluoxetine or sertraline (p-value = 0.026 and 0.008 respectively), but insignificantly with remaining combinations: (1:1) gefitinib and (1:1:1) gefitinib with citalopram, fluvoxamine, paroxetine, or venlafaxine. Fluoxetine, fluvoxamine, and paroxetine showed the same results: significant changes were observed in (1:1:1) combination with gefitinib and losartan (p-value = 0.003, 0.022, and 0.046 respectively), but not in (1:1) combination with gefitinib alone. Sertraline is the only drug whose metabolism significantly changed in all combinations (DDIs with gefitinib alone and combination of gefitinib and losartan (p-value = 0.009 and 0.008 respectively)). No significant effects were noted for citalopram and venlafaxine in any of the tested combinations.

The metabolic profiles of gefitinib, losartan, and SSRIs were generated using data gathered from the hepatocytes (alone) assays (Fig. 2 and Table 3). Potential metabolites were observed for gefitinib (m/z-433, 445, 472), losartan (m/z-439), citalopram (m/z-311, 338, 341, 352), fluoxetine (m/z-296, 337), fluvoxamine (m/z-305, 346), sertraline (m/z-292, 333), and venlafaxine (m/z-264, 305) (Fig. 2 and Table 3). There were no potential metabolites observed for paroxetine.

3.2. CYP1A2, 2C9, 2C19, 2D6, and 3A4 screenings were conducted for gefitinib, losartan, and SSRIs

Compounds were incubated with CYP1A2, 2C9, 2C19, 2D6, and 3A4 at a 10 μM final concentration to measure parent degradation and metabolite formulation. Fig. 3 shows the parent degradation from CYP screening with gefitinib and losartan (Fig. 3A), fluoxetine and fluvoxamine (Fig. 3B), paroxetine and sertraline (Fig. 3C), and citalopram and venlafaxine (Fig. 3D). From this screening, the metabolism of each compound was characterized: gefitinib (observed through CYP2D6, 3A4, 1A2, 2C9, and 2C19), losartan (CYP2C9 and 3A4), fluoxetine (CYP2D6, 2C9, and 2C19), fluvoxamine (CYP2D6, 2C9, and 2C19), paroxetine (CYP2D6, 3A4, and 2C9), sertraline (CYP2D6, 2C9, 2C19, 1A2, and 3A4), citalopram (CYP2D6, 2C19, 3A4, and 2C9), and venlafaxine (CYP2D6 and 2C19) (Fig. 3, Table 1-metabolism pathway).

Table 2

Drug-drug interactions of gefitinib (Gef) with/without losartan (Los) and SSRIs: fluoxetine (Flx), citalopram (Cit), fluvoxamine (Flu), paroxetine (Par), sertraline (Ser), and venlafaxine (Ven). (A) Gefitinib, (B) losartan, and (C) SSRIs results. Hepatocytes were incubated with the respective drugs, and then the amount remaining of the drugs (in relation to the amount measured at 0 h) were determined after 3 h. The assays were performed with duplicate injection, and the results reported as mean ± SD. Welch's T-test was performed to determine two-tailed unadjusted p-values for statistical significance. Results showing statistical significance (p-value < 0.05) when compared to the drug alone.

Components	(A) Gefitinib (Gef)		(B) Losartan (Los)		(C) SSRIs	
	(%)	P-Value	(%)	P-Value	(%)	P-Value
Alone (Gef, Los, Flx)	66 ± 7	–	85 ± 2	–	30 ± 2	–
Gef + Los	64 ± 9	0.829	88 ± 13	0.790		
Gef + Flx	70 ± 0	0.496			35 ± 1	0.123
Gef + Los + Flx	119 ± 0	0.009	96 ± 1	0.026	95 ± 5	0.003
Alone (Cit)	–	–	–	–	64 ± 2	–
Gef + Cit	85 ± 1	0.065	–	–	68 ± 1	0.117
Gef + Los + Cit	77 ± 9	0.280	77 ± 4	0.131	69 ± 5	0.305
Alone (Flu)	–	–	–	–	40 ± 1	–
Gef + Flu	66 ± 7	0.995	–	–	49 ± 6	0.162
Gef + Los + Flu	130 ± 13	0.027	103 ± 14	0.229	77 ± 8	0.022
Alone (Par)	–	–	–	–	59 ± 1	–
Gef + Par	91 ± 3	0.042	–	–	64 ± 2	0.065
Gef + Los + Par	90 ± 3	0.048	86 ± 6	0.859	65 ± 2	0.046
Alone (Ser)	–	–	–	–	13 ± 1	–
Gef + Ser	106 ± 5	0.025	–	–	40 ± 4	0.009
Gef + Los + Ser	92 ± 0	0.037	110 ± 2	0.008	32 ± 2	0.008
Alone (Ven)	–	–	–	–	65 ± 2	–
Gef + Ven	68 ± 5	0.717	–	–	81 ± 7	0.093
Gef + Los + Ven	83 ± 3	0.090	89 ± 2	0.232	94 ± 17	0.141

The potential metabolites that were observed from CYP1A2, 2C9, 2C19, 2D6, and 3A4 screening assays with gefitinib, losartan, and SSRIs were recorded as "+" in Table 3. Notable metabolites from drug exposure within the CYP assays was as follows: gefitinib m/z-433 (CYP2D6, 2C9, and 3A4), gefitinib m/z-445 (CYP3A4), gefitinib m/z-472 (CYP3A4, 2C9, 2C19, and 1A2) (Table 3, Luong et al., 2021), losartan m/z-439 (CYP2C9 and 3A4), citalopram m/z-311, 341, and 352 (CYP2D6, 2C19, 3A4, and 2C9), citalopram m/z-338 (CYP2D6 and 2C19), fluoxetine m/z-337 (CYP2D6, 2C9, and 2C19), fluoxetine m/z-296 (CYP2D6), fluvoxamine m/z-305 and 346 (CYP2D6), sertraline m/z-292 and 333 (CYP2D6, 2C9, 2C19, 1A2, and 3A4), venlafaxine m/z-264 (CYP2D6 and 2C19), venlafaxine m/z-305 (CYP2C19) (Table 3).

4. Discussion

Identifying the strength of an inhibitor can help to better predict the metabolism of a compound. An example of this can be constructed from the common lung cancer drug gefitinib. This drug acts as a weak inhibitor of CYP2C19 and 2D6 (Swaisland et al., 2005; Xu and Li, 2019), therefore, drugs that are metabolized via CYP2C19 or 2D6 could potentially be affected when taken in combination with gefitinib. Fig. 3 shows that all SSRIs are metabolized via CYP2D6, with citalopram, fluvoxamine, sertraline, and venlafaxine also being metabolized via CYP2C19. If all interactions are considered the same, by this theory, the metabolism of all SSRIs will be affected when used in combination with gefitinib even if it is a weak inhibitor. However, as shown from the described assays (Fig. 1 and Table 2), the results are not the same for all drugs. While indeed (1:1) DDIs of gefitinib and SSRIs yielded insignificant results for most of the drugs, sertraline was an exception. Considering gefitinib is metabolized via CYP2D6, 3A4, 1A2, 2C9, and 2C19 (Fig. 3), in which CYP2D6 and
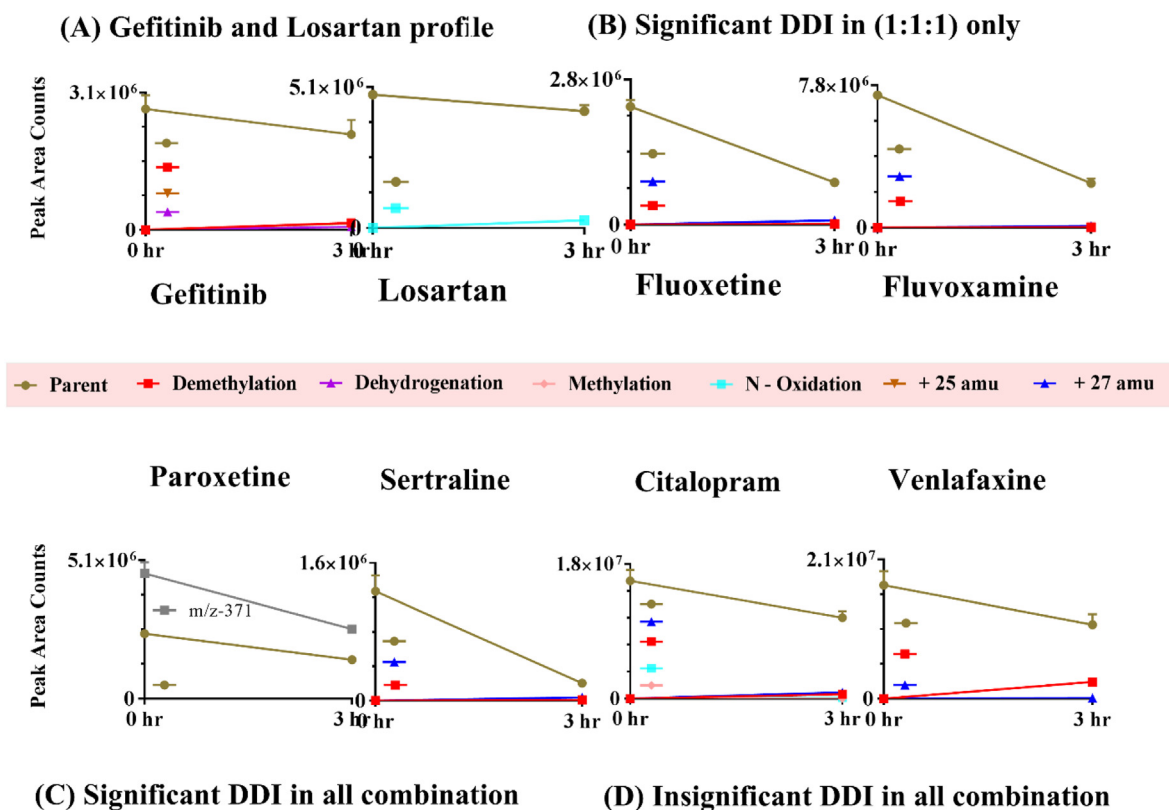
Hepatocytes Metabolites Identification

Fig. 2. Hepatocytes metabolites identification profiles of (A) gefitinib and losartan, (B) fluoxetine and fluvoxamine, (C) paroxetine and sertraline, (D) citalopram and venlafaxine from "alone" hepatocytes data. Potential metabolites were identified with single drug incubation after 3 h of incubation at 10 μM concentrations. The legend shows the potential metabolites from highest to lowest ionization detection. Table 3 provides the mass per charge for parent and potential metabolites. The assays were performed with duplicate injection, and the results reported as mean ± SD. Gefitinib metabolism was significantly affected in the (1:1:1) combination of losartan with fluoxetine or fluvoxamine, and in all (1:1) and (1:1:1) combinations of paroxetine or sertraline with/without losartan. However, gefitinib metabolism was not significantly affected in any (1:1) or (1:1:1) combination of citalopram or venlafaxine with/without losartan (Fig. 1).

Table 3
Potential metabolites of gefitinib, losartan, and SSRIs: citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, and venlafaxine from hepatocytes and CYP1A2, 2C9, 2C19, 2D6, and 3A4 screening. Observed potential metabolite is recorded as "+". Potential metabolites were previously described for gefitinib (McKillop et al., 2004, 2005), losartan (Sica et al., 2005), citalopram (Sangkuhl et al., 2011), fluoxetine (Deodhar et al., 2021), fluvoxamine (van Harten, 1995), sertraline (Obach et al., 2005; DeVane et al., 2002), and venlafaxine (Sangkuhl et al., 2014).

Parent Drugs	Metabolites Information	Potential Metabolite	Hepatocytes	CYP 1A2	CYP 2C9	CYP 2C19	CYP 2D6	CYP 3A4
Gefitinib (m/z-447)	Demethylation	m/z-433	+		+		+	+
	Dehydrogenation	m/z-445	+					+
	(+25 amu)	m/z-472	+	+	+	+		+
Losartan (m/z-423)	N-Oxidation	m/z-439	+		+			+
Citalopram (m/z-325)	Demethylation	m/z-311	+		+	+	+	+
	N- Methylation	m/z-338	+			+	+	
	N-Oxidation	m/z-341	+		+	+	+	+
	(+27 amu)	m/z-352	+		+	+	+	+
Fluoxetine (m/z-310)	Demethylation	m/z-296	+				+	
	(+27 amu)	m/z-337	+		+	+	+	
Fluvoxamine (m/z-319)	Demethylation	m/z-305	+				+	
	(+27 amu)	m/z-346	+				+	
Sertraline (m/z-306)	Demethylation	m/z-292	+	+	+	+	+	+
	(+27 amu)	m/z-333	+	+	+	+	+	+
Venlafaxine (m/z-278)	Demethylation	m/z-264	+			+	+	
	(+27 amu)	m/z-305	+			+		

3A4 are the major (with less than 80% of the drug remaining) and CYP1A2, 2C9, and 2C19 are minor (Luong et al., 2021; Xu and Li, 2019), drugs that inhibit CYP2D6 and 3A4 performance could potentially affect the overall metabolism of gefitinib. As for the remaining SSRIs, metabolic inhibition effects (weak, moderate, strong) have also been characterized:

fluoxetine (strong CYP2D6, weak/moderate CYP3A4), paroxetine (strong CYP2D6, weak CYP 3A4), sertraline (weak/moderate CYP2D6, weak 3A4), and fluvoxamine (weak CYP2D6, moderate CYP3A4). Continuing with the theory mentioned above, the metabolism of gefitinib should also be significantly affected when used in combination with fluoxetine,
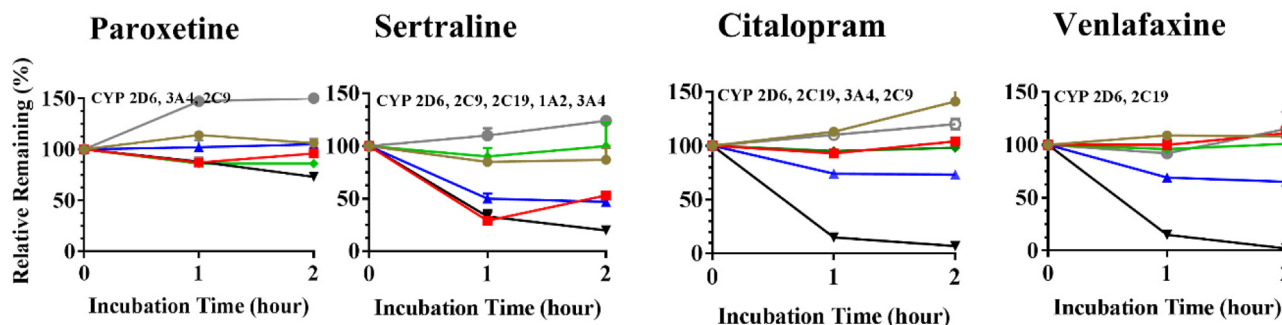
CYP 1A2, 2C9, 2C19, 2D6, and 3A4 Screen

(A) Gefitinib and Losartan profile
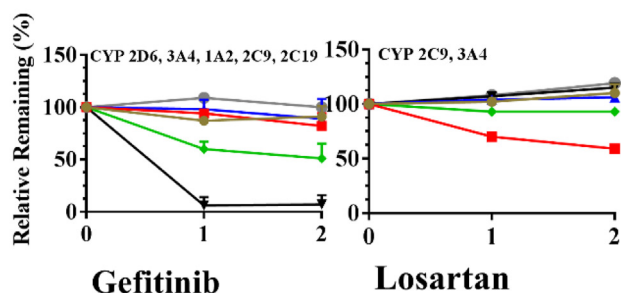
(B) Significant DDI in (1:1:1) only
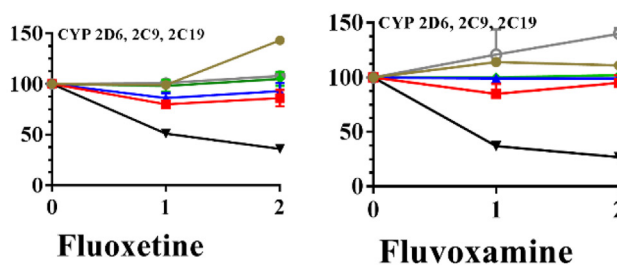
(C) Significant DDI in all combination

(D) Insignificant DDI in all combination

Fig. 3. Pre-clinical *in vitro* CYP1A2, 2C9, 2C19, 2D6, and 3A4 screening profiles of (A) gefitinib and losartan, (B) fluoxetine and fluvoxamine, (C) paroxetine and sertraline, (D) citalopram and venlafaxine. The CYP metabolism pathway was recorded from major to minor. The assays were performed with duplicate injection, and the results reported as mean ± SD. Gefitinib metabolism was significantly affected in the (1:1:1) combination of losartan with fluoxetine or fluvoxamine, and in all (1:1) and (1:1:1) combinations of paroxetine or sertraline with/without losartan. However, gefitinib metabolism was not significantly affected in any (1:1) or (1:1:1) combination of citalopram or venlafaxine with/without losartan (Fig. 1).

fluvoxamine, paroxetine, or sertraline. But from this study, the results show that the theory is true for paroxetine and sertraline, but not for fluoxetine and fluvoxamine (Fig. 1 and Table 2). Moreover, it can be seen that citalopram and venlafaxine do not effect gefitinib metabolism, which is in agreement with the theory drawn from Table 1.

The results concerning sertraline, fluoxetine, and fluvoxamine in combination with gefitinib did not agree with the predictions. Like paroxetine, sertraline affects the metabolism of gefitinib in (1:1) and in (1:1:1) combinations with losartan. Like citalopram, fluvoxamine, and venlafaxine, sertraline is metabolized by CYP2C19 and 2D6. However, unlike other SSRIs, only sertraline is affected in (1:1) with gefitinib. The CYP screening profile (Fig. 3, Table 1-metabolism pathway) showed that sertraline was significantly degraded with CYP2D6, 2C9, and 2C19, while other SSRI degradations were observed with mostly CYP2D6. One explanation for this noted behavior is that the combination of both drugs being weak inhibitors of CYP2D6 with CYP2C19 caused an increase of DDIs. Moreover, fluvoxamine is a strong CYP1A2 and CYP2C19 inhibitor, a moderate CYP3A4 and CYP2C9 inhibitor, and a weak CYP2D6 inhibitor, while gefitinib is a weak CYP2D6 and CYP2C19 inhibitor. Looking at gefitinib's CYP profile (Fig. 3A), gefitinib was more strongly metabolized by CYP2D6 as compared to CYP3A4. Therefore, moderate CYP3A4 and weak CYP2D6 inhibition is not significant enough to observe a notable

change in (1:1) DDIs. Oddly, fluoxetine is a strong CYP2D6 inhibitor, a weak/moderate CYP3A4 and CYP2C19 inhibitor, a moderate CYP2C9 inhibitor, and a weak CYP1A2 inhibitor. Based on the information in Table 1, fluoxetine should be the strongest inhibitor of gefitinib metabolism compared to the other SSRIs. However, the results show that paroxetine and sertraline significantly inhibit gefitinib metabolism in all combinations: (1:1) gefitinib alone and (1:1:1) with gefitinib and losartan. Herein, the CYP data profile of fluoxetine shows similar to fluvoxamine. Indeed, fluoxetine and fluvoxamine showed significant DDIs only in (1:1:1) with gefitinib and losartan. It is most likely that other pathways are affected as well since the activation and metabolism of many drugs primarily involve three metabolic pathways: (1) cytochrome P450, (2) monoamine oxidase (MAO), and (3) flavin-containing monooxygenase (FMO) (Jacobson et al., 1987; Jin et al., 2014).

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 (Sica et al., 2005). Herein, Fig. 1 and Table 2 shows that (1:1:1) combination of losartan with gefitinib and fluoxetine or sertraline has significant DDI results. As seen from the assays performed for this study, losartan is metabolized by CYP2C9 and 3A4 (Fig. 3A), which is in agreement with previously

reported literature showing that losartan is primarily metabolized by CYP3A4, 2C9 and 2C10 isoenzymes (Sica et al., 2005). Because gefitinib may have a weak inhibitory effect on CYP2C19 and 2D6 (Swaisland et al., 2005; Xu and Li, 2019), the combination of gefitinib and losartan should not affect metabolism of either drug (confirmed in Fig. 1B). Fluoxetine has moderate CYP2C9 and weak/moderate CYP3A inhibition, while fluvoxamine has moderate CYP2C9 and CYP3A4 inhibition. With this information alone, fluoxetine and fluvoxamine should inhibit the metabolism of losartan, however, the results show that is true only with fluoxetine, and not fluvoxamine. Accordingly, sertraline has weak CYP2C9 and CYP3A4 inhibition that led to significant DDIs in (1:1:1) combination. While more studies will need to be performed to corroborate the information provided here, there is a solid foundation suggesting that DDIs should be more seriously considered to improve patient care.

Table 3 shows the potential metabolites for all the drugs. The metabolic pathway for the SSRIs are demethylation and (+27 amu). Only citalopram was observed to have two additional pathways: methylation and oxidation. There was no metabolite identification observed for paroxetine that met the metabolite definition (showed formulation over-time, not detected in zero time point nor in buffer). There was spontaneous degradation detected for paroxetine, but no breakdown products met the metabolite definition. In addition, m/z-371 (+41 amu adduct) was strongly detected in the paroxetine data (Fig. 2C). Moreover, paroxetine is the only drug that showed more degradation according to the hepatocyte data (59 ± 1% of remaining after 3 h incubation) as compared to CYP2D6, which is the major pathway (73 ± 2% of remaining after 2 h incubation). Herein, sertraline was observed to be strongly metabolized (with less than 80% of the drug remaining) by CYP2D6, 2C9, and 2C19, while other SSRIs were observed to be strongly metabolized by CYP2D6 only (fluoxetine, fluvoxamine, paroxetine), and CYP2D6 and 2C19 (citalopram and venlafaxine) (Table 1- metabolism pathway). Additionally, venlafaxine (insignificant DDIs with gefitinib) showed that the metabolic pathway involving demethylation had a higher ionization than the unknown (+27 amu) metabolic pathway as compared to other SSRIs.

The results from hepatocyte experiments indicated that gefitinib is likely prone to CYP 2D6-mediated drug-drug interactions when incubated with CYP inhibitors (Fig. 1 Table 2). While insightful, the translation into a pharmacological effect for gefitininb with CYP inhibitor co-administration is currently unknown. The IC$_{50}$ values for gefitinib with fluoxetine were calculated with CYP2D6 and 3A4, 65.12 ± 1.88 µM and 4.11 ± 2.26 µM respectively (Luong et al., 2021). From the hepatocytes results, paroxetine and sertraline were the most potent inhibitors with gefinitib. Further studies should be performed with paroxetine and sertraline with gefinitib to better determine if co-administration with gefitinib will have any pharmacological effects.

5. Conclusion

This study provides a clear insight into DDIs between gefitinib with/without losartan and SSRIs. The results show that identified compounds can be classified as weak, moderate, or strong inhibitors, which can help in predicting the overall metabolism of a compound (Fig. 1), but it cannot resolve problems that arise with DDIs since the overall degree of effectiveness is unknown, especially when used within multiple drug combinations. However, further testing with MAO and FMO assays, animal models, and clinical trials would be needed to confirm the results reported here. Nevertheless, *in vitro* hepatocytes assays can adequately describe DDIs, and can help determine which multidrug regimens could have minimized potential for DDIs.

Over the course of treatment, therapies to treat lung cancer can become resistant or ineffective. Thus, there is a need to discover drug combinations that may prolong the effectiveness of treatment versus a single therapeutic intervention. Additionally, many cancer patients often receive one or more targeted drug that must be taken in combination with other drugs in order to combat side effects. While these drugs are

tested individually, there is no current requirement to test for DDIs nor effectiveness. Commonly used drug combinations should be evaluated for DDIs so that doctors and patients alike can be aware of the potential side effects or treatment failures.

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CRediT authorship contribution statement

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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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