

# Direct and Sequential Bioactivation of Pemigatinib to Reactive Iminium Ion Intermediates Culminates in Mechanism-Based Inactivation of Cytochrome P450 3A<sup>S</sup>

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## ABSTRACT

We recently established the mechanism-based inactivation (MBI) of cytochrome P450 3A (CYP3A) by the fibroblast growth factor receptor (FGFR) inhibitors erdafitinib and infigratinib. Serendipitously, our preliminary data have also revealed that pemigatinib (PEM), another clinically approved FGFR1-3 inhibitor, similarly elicited time-dependent inhibition of CYP3A. This was rather unexpected, as it was previously purported that PEM did not pose any metabolism-dependent liabilities due to the absence of glutathione-related conjugates in metabolic profiling experiments conducted in human liver microsomes. Here, we confirmed that PEM inhibited both CYP3A isoforms in a time-, concentration-, and cofactor-dependent manner consistent with MBI, with inactivator concentration at half-maximum rate constant, maximum inactivation rate constant, and partition ratio of 8.69 and 11.95  $\mu\text{M}$ , 0.108 and 0.042  $\text{min}^{-1}$ , and approximately 44 and approximately 47 for CYP3A4 and CYP3A5, respectively. Although the rate of inactivation was diminished by coinubation with an alternative substrate or direct inhibitor of CYP3A, the inclusion of nucleophilic trapping agents afforded no such protection. Furthermore, the lack of catalytic activity recovery following dialysis and oxidation with potassium ferricyanide

coupled with the absence of a spectrally resolvable peak in the Soret region collectively implied that the underlying mechanism of inactivation was not elicited via the formation of pseudo-irreversible metabolite-intermediate complexes. Finally, utilizing cyanide trapping and high-resolution mass spectrometry, we illuminated the direct and sequential oxidative bioactivation of PEM and its major *O*-desmethylated metabolite at its distal morpholine moiety to reactive iminium ion hard electrophilic species that could covalently inactivate CYP3A via MBI.

## SIGNIFICANCE STATEMENT

This study reports for the first time the covalent MBI of CYP3A by PEM and deciphered its bioactivation pathway involving the metabolic activation of PEM and its major *O*-desmethylated metabolite to reactive iminium ion intermediates. Following which, a unique covalent docking methodology was harnessed to unravel the structural and molecular determinants underpinning its inactivation. Findings from this study lay the foundation for future investigation of clinically relevant drug–drug interactions between PEM and concomitant substrates of CYP3A.

## Introduction

Multiple lines of evidence have demonstrated that genomic aberrations in fibroblast growth factor receptors (FGFR) (i.e., gene amplification or chromosomal translocations) may result in its constitutive activation and drive oncogenesis or development of resistance to anticancer therapies in

several solid tumors (Turner and Grose, 2010; Babina and Turner, 2017). Consequently, pharmacological ablation of FGFR signaling pathways is gaining prominence as a promising treatment modality in oncology (Dienstmann et al., 2014; Ghedini et al., 2018). At present, three FGFR-selective inhibitors are clinically used in the armamentarium against FGFR-driven malignancies (Chae et al., 2017; Markham, 2019; Hoy, 2020). One such agent is pemigatinib (PEM) (Fig. 1A), a highly potent and selective inhibitor of FGFR1-3 that has garnered accelerated approval by the U.S Food and Drug Administration in 2020 for the treatment of advanced or metastatic cholangiocarcinoma harboring FGFR2 fusions or rearrangements (Liu et al., 2020).

Previous pharmacokinetic studies have revealed that PEM undergoes extensive hepatic metabolism by cytochrome P450 3A enzymes (CYP3A) – with majority of the dose recovered as the *O*-desmethylated metabolite (Fig. 1B) (Ji et al., 2021). Collectively, the cytochrome P450

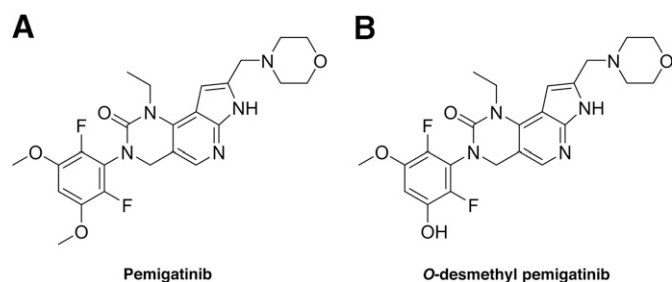
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**ABBREVIATIONS:** DDI, drug–drug interaction; EPI, enhanced product ion; ESI, electrospray ionization; FGFR, fibroblast growth factor receptor; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GSH, glutathione; KCN, potassium cyanide;  $K_i$ , inactivator concentration at half-maximum inactivation rate constant;  $k_{\text{inact}}$ , maximum inactivation rate constant;  $k_{\text{obs}}$ , observed first-order rate constant of inactivation; LC/MS/MS, liquid chromatography tandem mass spectrometry; MBI, mechanism-based inactivation; MIC, metabolite-intermediate complex; MS, mass spectrometry; NL, neutral loss; OBS, orthosteric binding site; P450, cytochrome P450; PEM, pemigatinib; rhCYP3A4, recombinant human cytochrome P450 3A4; ROS, reactive oxygen species;  $t_{1/2}$ , half-life; TDI, time-dependent inhibition.



**Fig. 1.** Chemical structure of (A) pemigatinib (PEM) and its (B) major *O*-desmethylated metabolite.

(P450) superfamily serves as one of the major driving forces of oxidative metabolism in the human body (Guengerich, 2001; Zanger and Schwab, 2013). It is estimated that the metabolism of more than 80% of all marketed drugs can be ascribed to just six P450 isoforms (Zanger et al., 2008), thereby entrenching their significance in drug disposition. However, due to their wide substrate and catalytic promiscuity, there is broad consensus that P450 could also inadvertently perpetrate metabolic activation of drugs, which may result in idiosyncratic adverse drug reactions (Guengerich, 2001). Also termed as bioactivation, the reaction results in the generation of electrophilic, reactive intermediates that, when liberated, may covalently alkylate to nucleophilic centers on biologic macromolecules or disrupt cellular redox homeostasis via the production of reactive oxygen species (ROS) (Stephens et al., 2014).

Apart from their potential overt toxicological consequences, the reactive intermediate generated could also be sequestered via covalent adduction to the P450 apoprotein and/or prosthetic heme or through coordination with the heme ferrous, which could engender time-dependent inhibition (TDI) of its catalytic activity via mechanism-based inactivation (MBI) (Ho et al., 2015). As the loss of enzymatic activity incited by MBI is irreversible and persists *in vivo* even after the perpetrator drug has been systemically cleared from the body and is only restored upon *de novo* synthesis of the implicated enzyme, the extent of drug–drug interactions (DDI) tends to be more profound than with a direct reversible inhibitor (Bjornsson et al., 2003). Furthermore, covalent modification of the P450 protein may constitute neoantigens and trigger autoimmune reactions leading to immune-mediated toxicities (Masubuchi and Horie, 2007). Due to the manifold ramifications of MBI in pharmacology and toxicology, it becomes clinically important to discern if a drug elicits MBI of P450, which is in turn characterized by the following salient features: time- and cofactor-dependency of inactivation, saturable kinetics of inactivation, protection against inactivation by a competing substrate, lack of protection by exogenous nucleophiles or scavengers of ROS, irreversibility of inactivation, and a 1:1 binding stoichiometry (Silverman, 1995). Notably, our group has recently characterized the MBI and mapped the bioactivation pathways of erdafitinib and infigratinib (two other FDA-approved FGFR inhibitors) by CYP3A (Tang et al., 2021a; Tang et al., 2021b). Intriguingly, our preliminary data have also hinted that PEM similarly elicited such metabolism-dependent inhibition of CYP3A. This was rather unexpected, as it was previously purported by the manufacturer that PEM did not pose any bioactivation-dependent liabilities due to the absence of glutathione (GSH)-related conjugates in reactive metabolite screens conducted in human microsomal fractions (Wu et al., 2021). Although the majority of reactive metabolites (i.e., epoxides, quinones, quinone-imines, quinone-methides, etc.) are soft electrophiles and can be effectively trapped by GSH, electrophilic species arising from the oxidative bioactivation of cyclic tertiary amines or primary alcohols (i.e., iminiums and aldehydes) are less polarizable and can only be trapped by a nucleophile of comparable “hardness” (Pearson, 1963). Consequently,

we posited that PEM is metabolically activated by CYP3A to a hard electrophile, thereby allowing it to elude GSH trapping and detection in the aforementioned metabolic profiling assay.

In this study, we revealed for the first time that PEM is an archetypal MBI of CYP3A4 and CYP3A5 and further confirmed our postulations through cyanide trapping and high-resolution mass spectrometry that it is liable to be directly and sequentially bioactivated at its distal morpholine moiety to reactive iminium ion intermediates that are likely implicated in the covalent MBI of CYP3A.

## Materials and Methods

**Chemicals and Reagents.** PEM and erdafitinib were procured from MedChem Express (Monmouth Junction, NJ). Potassium ferricyanide was obtained from VWR International (Leuven, Belgium). Dexamethasone, ketoconazole, potassium cyanide (KCN), rivaroxaban, GSH, and catalase were acquired from Sigma-Aldrich (St. Louis, MO). Human recombinant P450 3A4 and 3A5 superomes (rhCYP3A4 and rhCYP3A5) coexpressing cytochrome *b*<sub>5</sub> and NADPH P450 reductase and the NADPH-regenerating system comprising NADP<sup>+</sup> and glucose-6-phosphate (G6P) (NADPH A) and glucose-6-phosphate dehydrogenase (G6PDH) (NADPH B) were commercially purchased from Corning Gentest (Woburn, MA). High-performance liquid chromatography–grade acetonitrile was procured from Tedia Company Inc. (Fairfield, OH). Ultrapure water (type I) was prepared in-house using a Milli-Q water purification system (Millipore Corporation, Bedford, MA). All other commercially available chemicals were of analytical or high-performance liquid chromatography grade.

**Substrate Depletion of PEM by CYP3A4 and CYP3A5.** Unless otherwise stated, all incubations described in this work were performed in 96-well plates. Incubation mixtures comprising 20 pmol/ml rhCYP3A4/5, 1  $\mu$ M PEM, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared in triplicates. After prewarming at 37°C for 5 minutes, the reaction was initiated via the addition of NADP<sup>+</sup>/G6P. The final primary incubation mixture (100  $\mu$ l) contained less than 1% v/v organic solvent. Subsequently, at various time intervals (0, 5, 12.5, 30, 45, 60, 80, and 100 minutes), an 80- $\mu$ l aliquot of each incubation mixture was withdrawn and quenched with equal volumes of ice-cold acetonitrile spiked with internal standard (0.1  $\mu$ M erdafitinib). The quenched samples were then centrifuged at 4000g at 4°C for 30 minutes. Thereafter, aliquots of the supernatant were withdrawn to quantify the amount of PEM remaining using liquid chromatography–tandem mass spectrometry (LC/MS/MS).

**Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A.** Enzyme inactivation kinetic assays were previously optimized and performed as outlined in our recent works using rivaroxaban as a clinically relevant probe substrate of CYP3A (Tang et al., 2021a; Tang et al., 2021b). Briefly, primary incubation mixtures comprising 40 pmol/ml rhCYP3A4/5, PEM (0, 1, 2.5, 5, 15, and 25  $\mu$ M), G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared in triplicates. After preincubating at 37°C for 5 minutes, the reaction was initiated via the addition of NADP<sup>+</sup>/G6P. The final primary incubation mixture (100  $\mu$ l) contained less than 1% v/v organic solvent. Subsequently, at various preincubation intervals (0, 3, 8, 15, 22, and 30 minutes), a 5- $\mu$ l aliquot of each primary incubation mixture was withdrawn and diluted 20-fold into a prewarmed secondary incubation mixture comprising 50  $\mu$ M rivaroxaban ( $\sim 4\times$  Michaelis constant), an NADPH-regenerating system (1 mM), and 100 mM potassium phosphate buffer (pH 7.4). The secondary incubation mixtures were then further incubated at 37°C for an additional 2 hours, after which an 80- $\mu$ l aliquot was immediately withdrawn and quenched with equal volumes of ice-cold acetonitrile spiked with internal standard (4  $\mu$ M dexamethasone). The quenched samples were centrifuged at 4000g at 4°C for 30 minutes to obtain the supernatant for LC/MS/MS analysis. Negative control experiments were performed by substituting NADP<sup>+</sup>/G6P with 100 mM potassium phosphate buffer (pH 7.4).

**Calculation of MBI Kinetic Parameters ( $K_I$  and  $k_{inact}$ ).** To derive the inactivation kinetic parameters ( $K_I$  and  $k_{inact}$ ), the mean of triplicate peak area ratios was used to calculate the natural logarithm of percentage residual P450 enzyme activity normalized to vehicle, which was then plotted against preincubation time for each PEM concentration. The resulting data points were fitted to linear regression and the observed first-order inactivation rate constant ( $k_{obs}$ ) was derived from the slope of the initial linear decline in CYP3A activity for each PEM concentration. Following which, a plot of  $k_{obs}$  against PEM concentrations

[I] allowed the fitting of inactivation kinetic parameters ( $K_I$  and  $k_{\text{inact}}$ ) to non-linear least square regression based on eq. 1 in GraphPad 8.0.2 (San Diego, CA)

$$k_{\text{obs}} = \frac{k_{\text{inact}} \times [I]}{K_I + [I]} \quad (1)$$

where  $k_{\text{inact}}$  represents the maximal inactivation rate constant,  $K_I$  is the concentration of the inactivator at half-maximum inactivation rate constant, and  $[I]$  is the *in vitro* concentration of PEM. Equation 1 assumes that there is negligible change of  $[I]$  during the incubation period and that the loss of enzyme activity is purely commensurate with inactivation by PEM. The ratio of  $k_{\text{inact}}$  to  $K_I$  was determined by dividing the mean values of  $k_{\text{inact}}$  by  $K_I$ . Lastly, the time required for half of the enzyme molecules to be inactivated ( $t_{1/2}$ ) was determined by eq. 2.

$$t_{1/2} = \frac{\ln 2}{k_{\text{inact}}} \quad (2)$$

**Partition Ratio.** Primary incubation mixtures consisting of 100 pmol/ml rhCYP3A4/5, PEM (0, 1, 2.5, 5, 15, and 25  $\mu\text{M}$ ), G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared in triplicates. After prewarming the mixture at 37°C for 5 minutes, the reaction was initiated via the addition of NADP<sup>+</sup>/G6P and incubated for 45 minutes to allow inactivation to go into completion. The final primary incubation mixture (50  $\mu\text{l}$ ) contained less than 1% v/v organic solvent. Thereafter, aliquots of the primary incubation mixture were withdrawn and transferred to the secondary incubation mixture (similar to that prepared for the inactivation experiments) and incubated at 37°C for another 2 hours. Samples were then quenched, centrifuged, and assayed for residual enzyme activity as described above. The partition ratio was determined as described in our previous study (Tang et al., 2021c). Briefly, the percentage of residual CYP3A activity was plotted against the molar ratio of PEM to CYP3A4/5 concentration. The turnover number (partition ratio + 1) was obtained by extrapolating the intercept of the linear regression line plotted at lower ratios with the straight line plotted at higher ratios to the abscissa. Finally, the partition ratio was back calculated by subtracting the turnover number by 1.

**Substrate Protection.** To investigate if enzyme inactivation could be amenable to substrate protection, testosterone (an alternative CYP3A substrate) at a concentration of 100 and 200  $\mu\text{M}$  (equivalent to 1:4 and 1:8 molar ratio of PEM to testosterone) or ketoconazole (a potent reversible inhibitor of CYP3A) at a concentration of 0.1 and 1  $\mu\text{M}$  (corresponding to  $\sim 1\times$  and  $\sim 10\times$  its  $K_I$  value), was introduced separately in triplicates to the primary incubation mixture comprising 40 pmol/ml rhCYP3A4/5, 25  $\mu\text{M}$  PEM, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4). The enzymatic reaction was initiated by the addition of NADP<sup>+</sup>/G6P after prewarming at 37°C for 5 minutes. Aliquots were withdrawn at different preincubation time points (0, 3, 8, and 15 minutes), transferred to the secondary incubation mixture, and subsequently quenched, centrifuged, and assayed for residual CYP3A enzymatic activity as described above. Primary incubation mixtures that obviated the addition of either testosterone, ketoconazole, or both PEM and testosterone or ketoconazole served as the negative controls.

**Effect of Exogenous Nucleophile and Scavenger of ROS on Inactivation.** Primary incubation mixtures containing 40 pmol/ml rhCYP3A4/5, 25  $\mu\text{M}$  PEM, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were individually fortified with GSH (2 mM) or KCN (1 mM). After preincubating at 37°C for 5 minutes, the enzymatic reaction was initiated via the addition of NADP<sup>+</sup>/G6P. At specific preincubation time points (0, 3, 8, and 15 minutes), aliquots were transferred to the secondary incubation mixtures and subsequently quenched, centrifuged, and assayed for residual CYP3A enzymatic activity as described above. Negative controls were prepared without both PEM and GSH/KCN or only without GSH/KCN in the primary incubation mixture. Furthermore, parallel experiments involving the incorporation of catalase (800 U/ml) in place of the exogenous nucleophiles were also performed to investigate the specific effects of scavenger of ROS on the inactivation of CYP3A.

**Reversibility of Inactivation.** The reversibility of CYP3A inactivation was interrogated by two distinct yet complementary approaches, namely equilibrium dialysis and oxidation by potassium ferricyanide, as described in detail in our previous works (Tang et al., 2021a; Tang et al., 2021b). In the dialysis experiments, triplicate primary incubation mixtures comprising 40 pmol/ml rhCYP3A4/5, 25  $\mu\text{M}$  PEM, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were preincubated at 37°C for 5 minutes. Enzymatic reaction was initiated by the addition of NADP<sup>+</sup>/G6P and allowed to proceed for 30 minutes. After which, a 5- $\mu\text{l}$  aliquot was transferred to 95  $\mu\text{l}$  of the secondary incubation mixture yielding a

20-fold dilution. Concurrently, 90  $\mu\text{l}$  of the remaining primary incubation mixture was transferred to a Slide-A-Lyzer mini dialysis device (0.1 ml, molecular weight cutoff of 10,000; Pierce Chemical Co., Rockford, IL) and placed in a glass beaker filled with 500 ml of ice-cold 100-mM potassium phosphate buffer (pH 7.4). The buffer system was maintained on ice (4°C) with constant gentle stirring and accompanied by one fresh buffer change at the second hour. After 4 hours, 5  $\mu\text{l}$  of the dialyzed mixture was transferred to each prewarmed secondary incubation well. All secondary mixtures were further incubated at 37°C for 2 hours and subsequently assayed for residual CYP3A enzymatic activity as previously described.

In the experiments involving potassium ferricyanide, a series of three sequential incubations were performed. Briefly, the primary incubation comprised 40 pmol/ml rhCYP3A4/5, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) in the presence or absence of 25  $\mu\text{M}$  PEM. Following initiation of the reaction with the addition of NADP<sup>+</sup>/G6P and incubation at 37°C for either 0 or 30 minutes, 20  $\mu\text{l}$  of the primary incubation mixture was aliquoted into an equal volume of secondary incubation mixture containing 100 mM potassium phosphate buffer (pH 7.4) with or without 2 mM potassium ferricyanide. The secondary mixtures were then allowed to incubate at 37°C for another 10 minutes. Thereafter, 10  $\mu\text{l}$  of the mixture was withdrawn and diluted 10-fold into a tertiary incubation mixture containing 50  $\mu\text{M}$  rivaroxaban (probe substrate), an NADPH-regenerating system (1 mM), and 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture was further incubated at 37°C for another 2 hours and subsequently assayed for residual CYP3A activity as previously described. The percentage of CYP3A metabolic activity remaining after 0 or 30 minutes' incubation with PEM compared with the corresponding controls in the absence of PEM was calculated using eqs. 3 and 4, respectively.

$$\% \text{ control}_{0\text{min}} = \frac{v_{(0\text{min}, (+) \text{PEM})}}{v_{(0\text{min}, (-) \text{PEM})}} \times 100 \quad (3)$$

$$\% \text{ control}_{30\text{min}} = \frac{v_{(30\text{min}, (+) \text{PEM})}}{v_{(30\text{min}, (-) \text{PEM})}} \times 100 \quad (4)$$

where  $v$  represents the residual CYP3A activity. Thereafter, percentage restoration of metabolic activity of CYP3A was derived by subtracting  $\% \text{ control}_{30\text{min}}$  in the presence of potassium ferricyanide with the corresponding values obtained in the absence of potassium ferricyanide.

**Spectral Difference Scanning.** Incubation mixtures (200  $\mu\text{l}$ ) containing 200 pmol/ml rhCYP3A4/5, 25  $\mu\text{M}$  PEM, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared and prewarmed at 37°C for 5 minutes. Thereafter, the enzymatic reaction was initiated via the addition of NADP<sup>+</sup>/G6P and immediately scanned from 400 to 500 nm at 5-minute intervals over a 1-hour duration using a Hidex Sense microplate reader (Hidex, Turku, Finland) thermostated at a constant 37°C. The spectral differences were obtained by comparing the UV absorbances between the sample and reference wells, which consisted of vehicle in place of PEM. Additionally, the degree of metabolite-intermediate complex (MIC) formation was also quantitatively evaluated by measuring the absorbance difference between 454 and 490 nm with time.

**Cyanide Trapping Assay.** Incubation mixtures (250  $\mu\text{l}$ ) containing 50 pmol/ml rhCYP3A4, 25  $\mu\text{M}$  PEM, G6PDH, 1 mM KCN, and 100 mM potassium phosphate buffer (pH 7.4) were prepared and prewarmed at 37°C for 5 minutes. The reaction was then initiated via the addition of NADP<sup>+</sup>/G6P and incubated at 37°C for 1 hour, after which an equal volume of ice-cold acetonitrile was added to quench the reaction. The resulting mixture was centrifuged at 14,000g at 4°C for 15 minutes, after which the supernatant was transferred to a new microcentrifuge tube and dried under a gentle stream of nitrogen gas (TurboVap LV; Caliper Life Science, Hopkinton, MA). The residue was subsequently reconstituted with 60  $\mu\text{l}$  of acetonitrile–water mixture (3:7), vortexed and centrifuged at 14,000g at 4°C for 15 minutes. The resulting supernatant was then carefully removed and transferred to a fresh vial for LC/MS/MS analysis. Samples that omitted the inclusion of PEM in the incubation mixture served as the negative controls.

**In Silico Protein Structure and Ligand Preparation.** To interrogate the plausible structural and molecular determinants underscoring the observed MBI of CYP3A elicited by PEM and the apparent discrepancies in inactivation potencies between CYP3A4 and CYP3A5, we employed 61 high-resolution crystal structures ( $<3 \text{ \AA}$ ) of human CYP3A4 retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank database and 30 structures for human CYP3A5, which consisted of the two reported high-resolution crystal

structures and an ensemble of 28 molecular dynamics simulation frames generated from our previous trajectory clustering analyses (Tang et al., 2021d). For each protein structure obtained, only chain A was considered. All cocrystallized ligands, except for the heme cofactor, were removed from the structure, followed by reverting any mutated amino acid residues back to their wild-type counterparts, after which, the protein structures were processed with the protein preparation wizard in Maestro (Schrödinger, NY), and the missing residues and loop segments were added to reconstruct the protein. Lastly, hydrogen atoms were added to the structure, and ionization states of titratable groups were determined. Concurrently, three-dimensional coordinates of PEM were obtained from the PubChem database (Kim et al., 2019), and multiple conformers of PEM were generated using LigPrep (Schrödinger, NY). The particular conformer with the lowest energy was then selected for covalent docking simulations.

**In Silico Binding Site Prediction and Covalent Docking.** First, potential ligand-binding pockets for each of the included CYP3A4 and CYP3A5 structures were identified using the site recognition software Sitemap (Schrödinger, New York, NY). As the flexibility of F-F' loop, a critical region of the CYP3A protein oriented directly above the active site – was previously alluded by our studies to be intricately involved in its inactivation, we identified a list of lysine and serine residues in the vicinity of the orthosteric binding site (OBS) encapsulated by the F-F' and C-terminal loops. The docking of PEM was performed in two sequential stages; PEM was first noncovalently docked in the binding pockets containing the accessible lysine and serine residues using GLIDE (Friesner et al., 2004). If noncovalent docking was successful, covalent docking was then performed for the accessible residues within the pocket using CovDock (Zhu et al., 2014) in the Schrödinger suite. The covalent docking scores obtained were then combined, sorted, and ranked for each accessible residue.

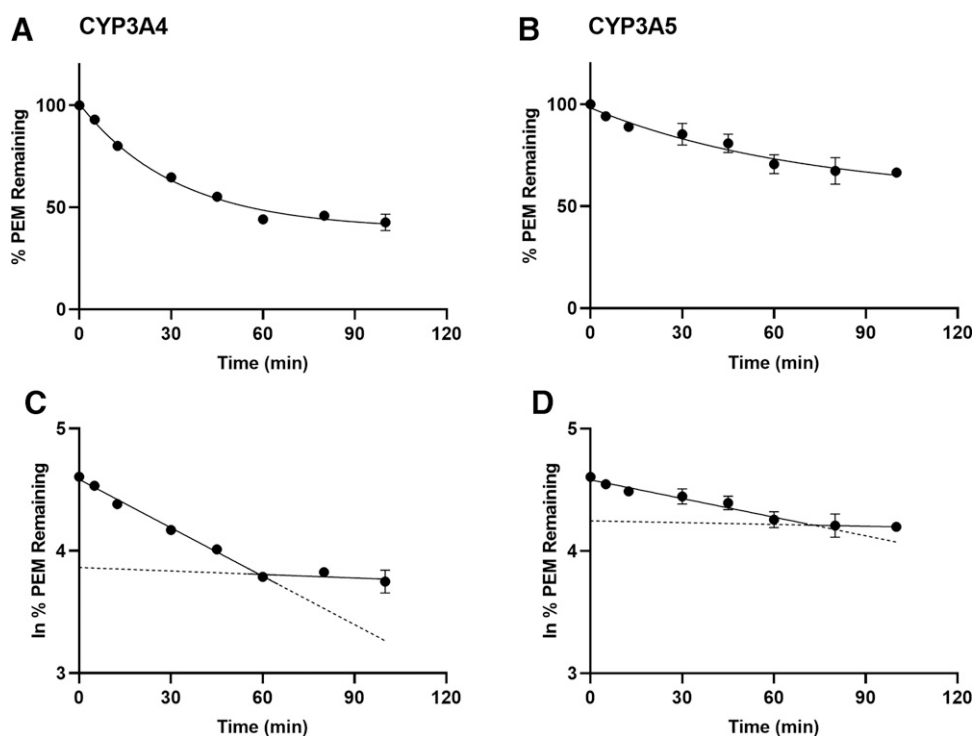
**Measurement of PEM and Residual P450 Activity by LC/MS/MS.** All samples were analyzed using the liquid chromatography tandem mass spectrometry (LC/MS/MS) system consisting of an Agilent 1290 Infinity ultra-high-pressure liquid chromatography (Agilent Technologies Inc., Santa Clara, CA) bridged with an QTRAP 3500 tandem mass spectrometry (MS/MS) (AB SCIEX, Framingham, MA). Chromatographic separation of PEM and erdafitinib (internal standard) in the substrate depletion assay was achieved with an ACQUITY ultra-performance liquid ethylene bridged hybrid C<sub>18</sub>, 1.7  $\mu$ m, 2.2  $\times$  100-mm column (Waters, Milford, MA), whereas a shorter but similar 50-mm C<sub>18</sub> column was used for the chromatographic separation of the rest of the analytes and internal standards described in this work. The aqueous mobile phase (A) was 0.1% formic acid in water, whereas the organic mobile phase (B) was 0.1% formic acid

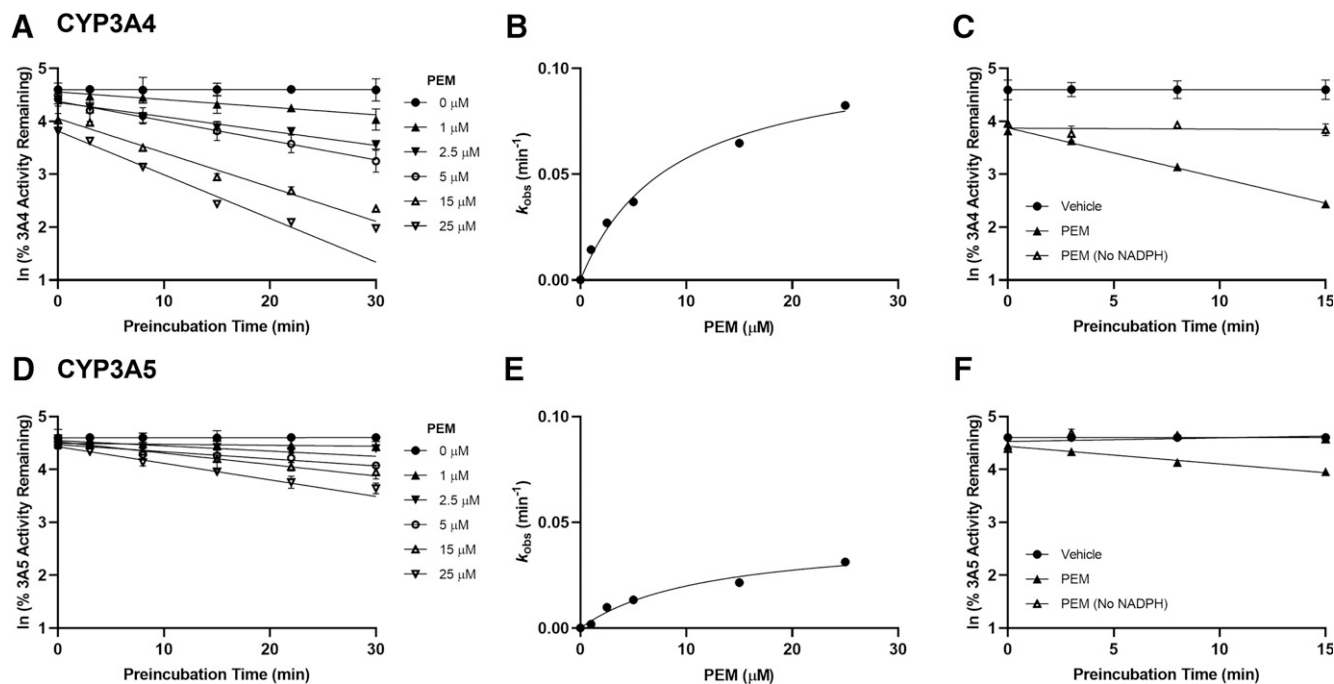
in acetonitrile. Mobile phases were delivered at a flow rate of 0.5 ml/min. The column and sample temperature were thermostated at 45°C and 4°C, respectively. The gradient elution conditions were as follows: linear gradient from 20% to 80% B (0–1.20 min), isocratic at 100% B (1.21–2.0 min), and isocratic at 20% B (2.01–2.50 min). All analytes were detected in positive electrospray ionization (ESI) mode. The source-dependent mass spectrometry (MS) parameters were as follows: ion spray voltage = 5500 V; source temperature = 500°C; curtain gas = 25 psi; ion source gas 1 (sheath gas) = 30 psi; ion source gas 2 (drying gas) = 30 psi. The multiple reaction monitoring transitions and compound-dependent MS parameters of the analytes are summarized in Supplemental Table 1. Chromatographic peak integration was performed using MultiQuant software version 3.0 (Applied Biosystems, Foster City, CA). For all LC/MS/MS analyses, the peak area of the analyte was expressed as a ratio to the peak area of the internal standard.

**Detection of Cyano Adducts by LC/MS/MS.** Cyano adducts of putative reactive electrophilic intermediate of PEM were analyzed using the LC/MS/MS system comprising an Agilent 1290 Infinity ultra-high-pressure liquid chromatography (Agilent Technologies Inc., Santa Clara, CA) interfaced with a QTRAP 5500 MS/MS (AB SCIEX, Framingham, MA). Chromatographic separation was achieved on an ACQUITY ultra-performance liquid ethylene bridged hybrid C<sub>18</sub>, 1.7  $\mu$ m, 2.2  $\times$  100-mm column (Waters, Milford, MA). The aqueous mobile phase (A) was 0.1% formic acid in water, whereas the organic mobile phase (B) was 0.1% formic acid in acetonitrile. Mobile phases were delivered at a flow rate of 0.35 ml/min. The column and sample temperature were set at 45°C and 4°C, respectively. The gradient elution conditions were as follows: isocratic at 10% B (0–2 min), linear gradient, 10–70% B (2.01–12.0 min), linear gradient, 70–90% B (12.01–14.0 min), isocratic at 90% B (14.01–18.0 min), isocratic at 10% B (18.01–20.0 min). An information-dependent acquisition experiment was conducted to detect PEM-derived cyano conjugates via constant neutral loss (NL) scan of 27 Da in positive ESI mode. Enhanced product ion (EPI) scan was prospectively performed for all potential cyano adducts identified. The source-dependent MS parameters used were as follows: ion spray voltage = 4500 V; source temperature = 500°C; curtain gas = 30 psi; ion source gas 1 (sheath gas) = 50 psi; ion source gas 2 (drying gas) = 50 psi.

Accurate mass measurement of the PEM-derived cyano adducts identified in the cyanide-trapping experiments was performed using an ACQUITY ultra-high-pressure liquid chromatography (Waters, Milford, MA) coupled to an Orbitrap Exploris 480 MS (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation was achieved on an ACQUITY ultra-performance liquid ethylene

**Fig. 2.** Substrate depletion of PEM by CYP3A. Percentage of PEM remaining against time in the presence of (A) CYP3A4 and (B) CYP3A5 plotted on a linear scale and the corresponding substrate depletion graphs of PEM in the presence of (C) CYP3A4 and (D) CYP3A5 as plotted on a semilogarithmic scale. Each point in (A–D) represents the mean and S.D. of triplicate experiments.





**Fig. 3.** Time- and concentration-dependent inactivation of (A) CYP3A4 and (D) CYP3A5 by PEM using rivaroxaban as probe substrate. Nonlinear regression of observed first-order inactivation rate constants ( $k_{\text{obs}}$ ) versus PEM concentration yielded  $K_I$  and  $k_{\text{inact}}$  values of (B)  $8.69 \pm 1.62 \mu\text{M}$  and  $0.108 \pm 0.008 \text{ min}^{-1}$  for CYP3A4 and (E)  $11.95 \pm 4.41 \mu\text{M}$  and  $0.042 \pm 0.007 \text{ min}^{-1}$  for CYP3A5. Cofactor NADPH-dependent inactivation of (C) CYP3A4 and (F) CYP3A5 by PEM. Each point in (A, C, D, and F) represents the mean and S.D. of triplicate experiments.

bridged hybrid C<sub>18</sub>, 1.7  $\mu\text{M}$ ,  $2.2 \times 100\text{-mm}$  column (Waters, Milford, MA). The mobile phases, flow rate, temperature, and gradient elution conditions were identical to those described earlier in this section. The injection volume was 3  $\mu\text{L}$ , and the analytes were ionized by ESI in positive ion mode under the following conditions: sheath gas = 50 arbitrary units; auxiliary gas = 10 arbitrary units; sweep gas = 1 arbitrary unit; S-lens = 50; ion transfer tube temperature =  $325^\circ\text{C}$ ; and vaporizer temperature =  $350^\circ\text{C}$ , whereas a normalized collision energy of 20% was applied to fragment all ions. The Orbitrap-MS data were acquired and further processed using Xcalibur 4.4 and Freestyle 1.7 software (Thermo Fisher Scientific, San Jose, CA).

## Results

**Substrate Depletion of PEM in CYP3A4 and CYP3A5.** To elucidate any potential TDI of CYP3A by PEM, we first monitored the depletion of PEM over time by CYP3A4 and CYP3A5. Our results demonstrated that although both CYP3A isoforms were capable of metabolizing PEM, CYP3A4 yielded slightly more efficient metabolism with  $42.64\% \pm 4.05\%$  of PEM remaining after 100 minutes (Fig. 2A) compared with  $66.55\% \pm 2.18\%$  in CYP3A5 incubations (Fig. 2B). Notably, two distinct phases were observed in the log-transformed substrate depletion profile of PEM for both CYP3A isoforms (Figs. 2C and 2D). Consequently, two different elimination rate constants (termed  $k_{\text{fast}}$  and  $k_{\text{slow}}$ , respectively) were calculated for the substrate depletion of PEM by CYP3A4 and CYP3A5. A comparison of the elimination rate

constants in the initial portion of incubation revealed that PEM was metabolized approximately 2.6 times faster by CYP3A4 as compared with CYP3A5 (i.e.,  $k_{\text{fast, CYP3A4}} = 0.0132 \pm 0.0005 \text{ min}^{-1}$  compared with  $k_{\text{fast, CYP3A5}} = 0.0051 \pm 0.0006 \text{ min}^{-1}$ ). However, after approximately 60 minutes of incubation, there was a dramatic reduction in its rate of metabolism (i.e.,  $k_{\text{slow, CYP3A4}} = 0.0009 \pm 0.0013 \text{ min}^{-1}$  and  $k_{\text{slow, CYP3A5}} = 0.0005 \pm 0.0029 \text{ min}^{-1}$ ), which alludes to plausible TDI of CYP3A by PEM.

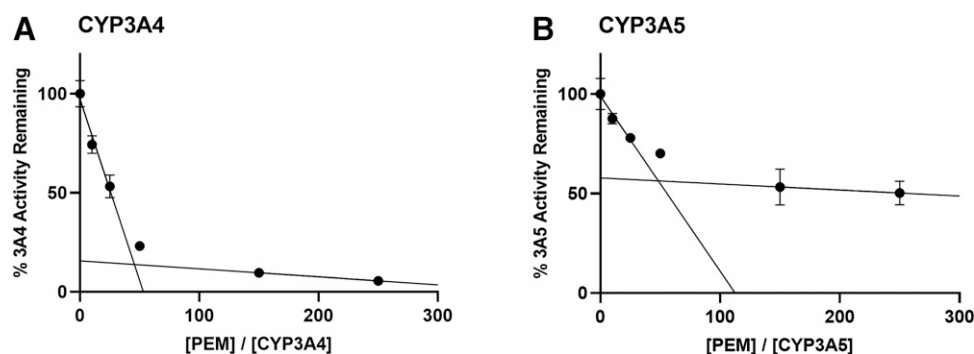
**Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A.** We then followed-up with a deeper quantitative characterization of its inactivation kinetics using rivaroxaban, a clinically relevant and moderately sensitive probe substrate of CYP3A. Our findings revealed that PEM inactivated CYP3A4- and CYP3A5-mediated rivaroxaban hydroxylation in a time- and concentration-dependent manner (Figs. 3A and 3D), with the most profound loss of enzyme activity obtained when 25  $\mu\text{M}$  PEM was preincubated with CYP3A4 for 30 minutes. As the  $k_{\text{obs}}$  determined from the slopes of each of the included concentrations of PEM approached a maximum inactivation rate constant ( $k_{\text{inact}}$ ) (Figs. 3B and 3E), it denoted that the loss of CYP3A activity elicited by PEM is saturable and exhibited pseudo-first-order kinetics. The inactivation kinetic parameters ( $K_I$  and  $k_{\text{inact}}$ ) of PEM derived from the Kitz-Wilson plot (Kitz and Wilson, 1962) were  $8.69 \pm 1.62 \mu\text{M}$  and  $0.108 \pm 0.008 \text{ min}^{-1}$  for CYP3A4 and  $11.95 \pm 4.41 \mu\text{M}$  and  $0.042 \pm 0.007 \text{ min}^{-1}$  for CYP3A5. This in turn yielded respective

TABLE 1

CYP3A inactivation kinetic parameters for pemigatinib derived using morpholinone hydroxylation of rivaroxaban as a surrogate marker of residual CYP3A activity. Data are presented as means  $\pm$  S.D.

P450 Isoform	$K_I$ ( $\mu\text{M}$ )	$k_{\text{inact}}$ ( $\text{min}^{-1}$ )	$k_{\text{inact}}/K_I$ ( $\text{min}^{-1}\text{mM}^{-1}$ )	$t_{1/2}$ (min)	Partition Ratio
CYP3A4	$8.69 \pm 1.62$	$0.108 \pm 0.008$	12.4	6.4	~44
CYP3A5	$11.95 \pm 4.41$	$0.042 \pm 0.007$	3.5	16.5	~47

**Fig. 4.** Partition ratio for the inactivation of (A) CYP3A4 and (B) CYP3A5 by PEM, determined by extrapolating the intercept of the linear regression line at lower ratios and the straight line for the high ratios to the  $x$ -axis, was estimated to be 44 and 47, respectively. Each point in (A and B) represents the mean and S.D. of triplicate experiments.



$k_{\text{inact}}/K_I$  ratios of 12.4 and 3.5  $\text{min}^{-1}\text{mM}^{-1}$  and inactivation  $t_{1/2}$  of 6.4 and 16.5 minutes. Taken together, our results demonstrated that PEM was a more potent inactivator of CYP3A4 than CYP3A5. All reported values of  $K_I$ ,  $k_{\text{inact}}$ ,  $k_{\text{inact}}/K_I$  ratio, and  $t_{1/2}$  are summarized in Table 1. Additionally, as the intentional omission of cofactor NADPH nullified the loss of CYP3A-mediated rivaroxaban hydroxylase activity (Figs. 3C and 3E), it implied that metabolic activation of PEM was a key molecular-initiating event that preceded the inactivation of CYP3A.

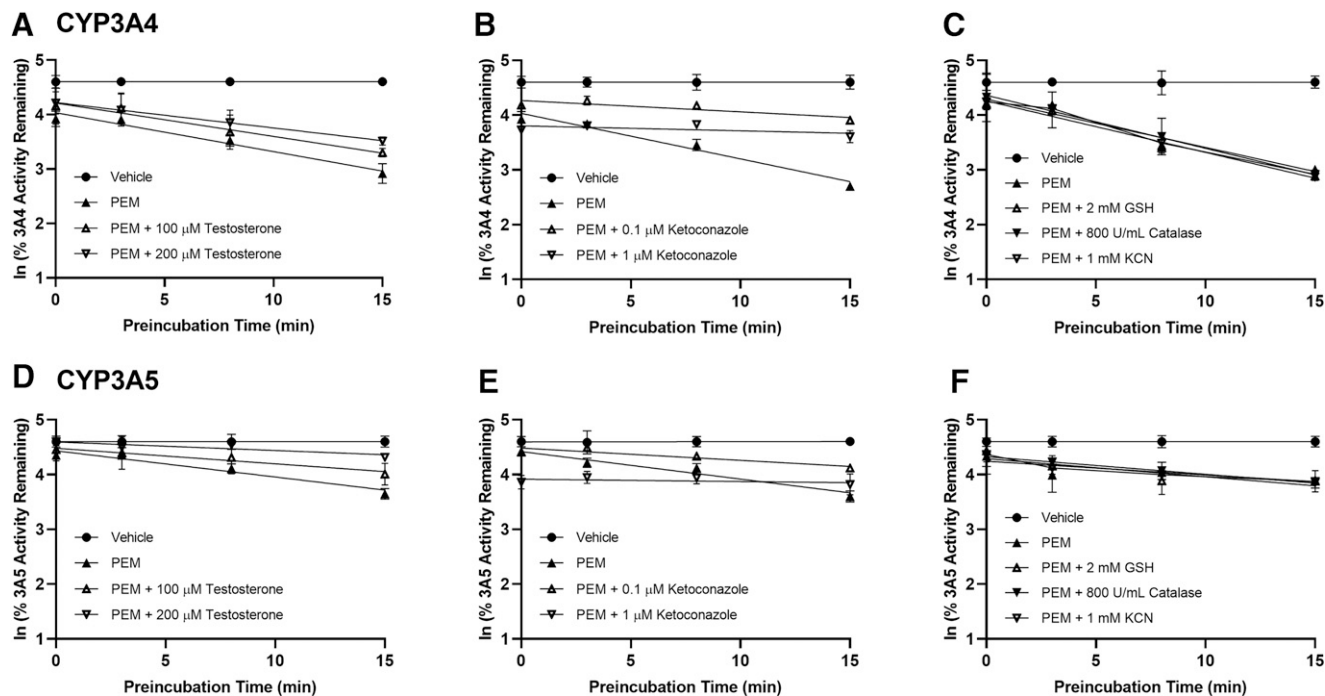
**Partition Ratio.** A previously described titration method (Silverman, 1995) was adopted, which determined the turnover number for the inactivation of CYP3A4 and CYP3A5 to be approximately 45 and 48, respectively (Figs. 4A and 4B). This in turn corresponded to a partition ratio of approximately 44 and 47 (Table 1).

**Substrate Protection.** The inactivation of CYP3A by PEM was protected in the presence of both an alternative substrate and direct inhibitor. Wherein incubation with either testosterone (Figs. 5A and 5D) or ketoconazole (Figs. 5B and 5E) diminished the rate of inactivation by PEM as evident by the attenuated rate of enzyme inactivation with time. Additionally, the degree of substrate protection appeared to be dose dependent, with the complete abolishment of inactivation when 1  $\mu\text{M}$

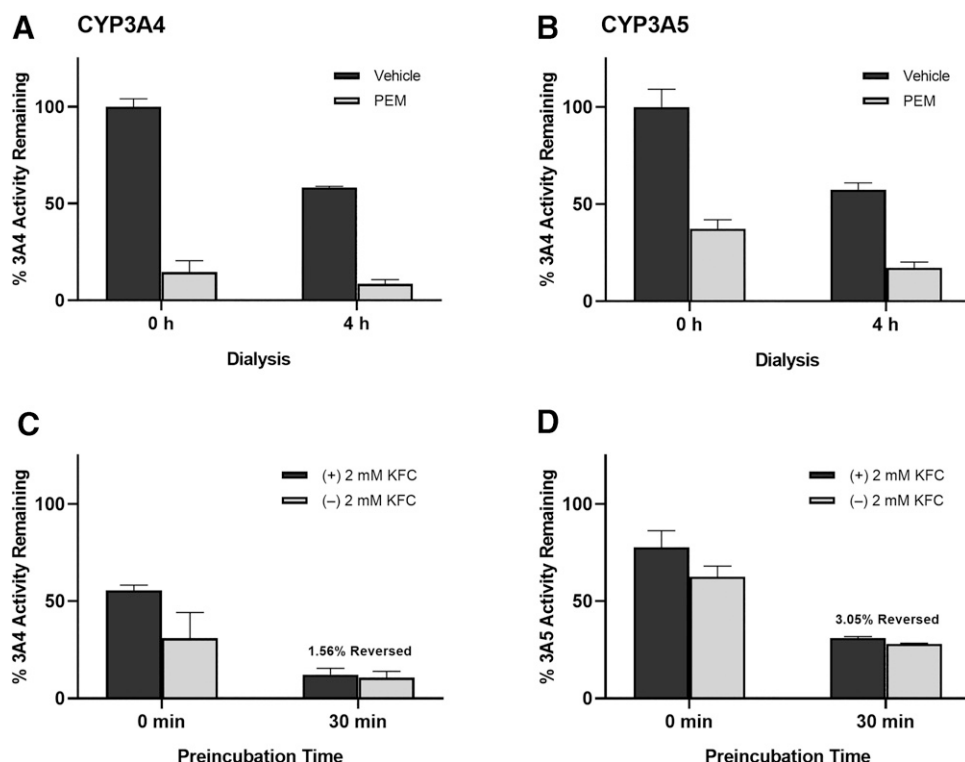
ketoconazole was coincubated with PEM and CYP3A in the primary incubation mixture.

**Effect of Exogenous Nucleophile and Scavenger of ROS on Inactivation.** Conversely, the inclusion of nucleophilic trapping agents (i.e., GSH and KCN) and catalase afforded no protection from inactivation. As evident in Figs. 5C and 5F, CYP3A was inactivated to a similar extent in incubation mixtures comprising PEM alone.

**Reversibility of Inactivation.** To decipher whether the inactivation of CYP3A by PEM is pseudo-irreversible or irreversible, the specific nature of inactivation was interrogated via equilibrium dialysis and oxidation with potassium ferricyanide. Our findings revealed that the magnitude of CYP3A activity was not restored after dialysis at 4°C for 4 hours (Figs. 6A and 6B). Rather, the marginal decrease in residual enzyme activity observed after dialysis could be rationalized by enzymatic degradation that occurred during the course of dialysis, as was further confirmed in vehicle control experiments. Furthermore, potassium ferricyanide only marginally restored the metabolic activity of CYP3A4 and CYP3A5 by 1.56%  $\pm$  0.01% and 3.05%  $\pm$  0.39% respectively after a 30-minute preincubation with 25  $\mu\text{M}$  PEM (Figs. 6C and 6D). Taken together, our



**Fig. 5.** Inactivation of CYP3A4 and CYP3A5 was attenuated in the presence of (A and D) an alternative CYP3A substrate testosterone and (B and E) a direct CYP3A inhibitor ketoconazole (KTC). (C and F) Conversely, the presence of GSH, KCN, or catalase did not protect against enzymatic inactivation. Each point in (A–F) represents the mean and S.D. of triplicate experiments.

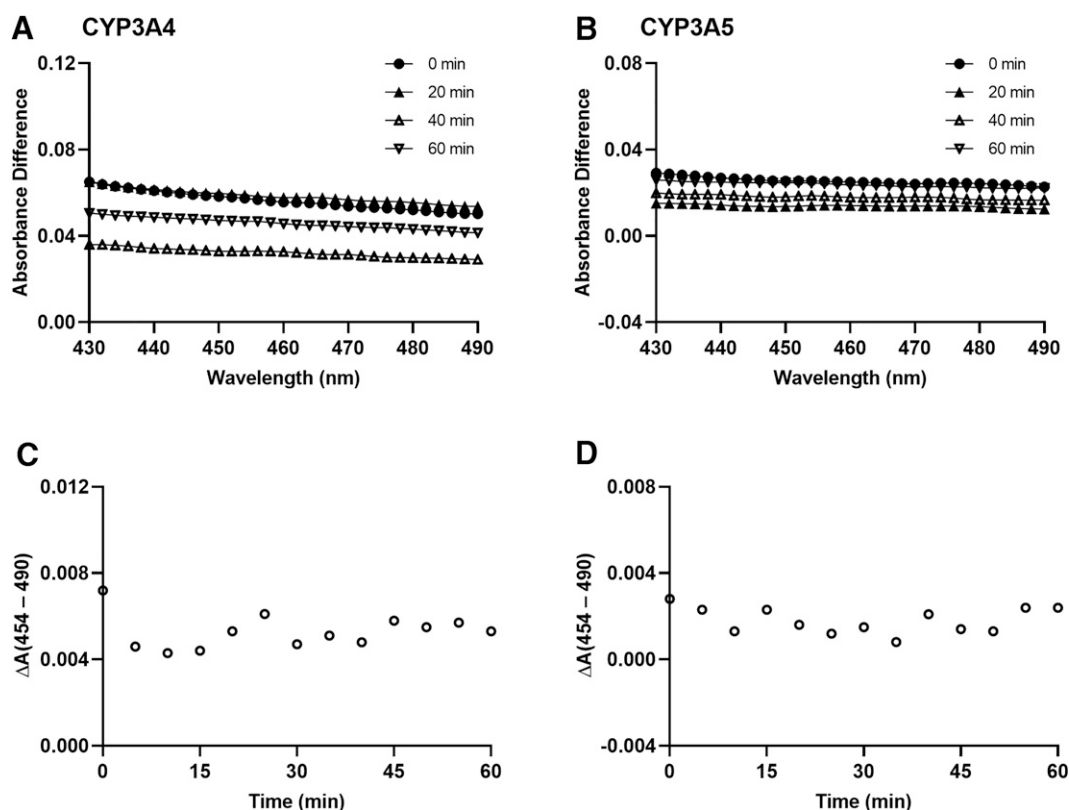


**Fig. 6.** Percentage activity of (A) CYP3A4 and (B) CYP3A5 remaining did not increase after extensive dialysis at 4°C for 4 hours. Similarly, potassium ferricyanide (KFC) only restored the metabolic activity of (C) CYP3A4 and (D) CYP3A5 by a modest  $1.56\% \pm 0.01\%$  and  $3.05\% \pm 0.39\%$  after a 30-minute incubation with 25  $\mu\text{M}$  PEM. Results from all four graphs depict the mean and S.D. of three independent experiments conducted in triplicates.

results collectively substantiated the irreversible inactivation of CYP3A by PEM.

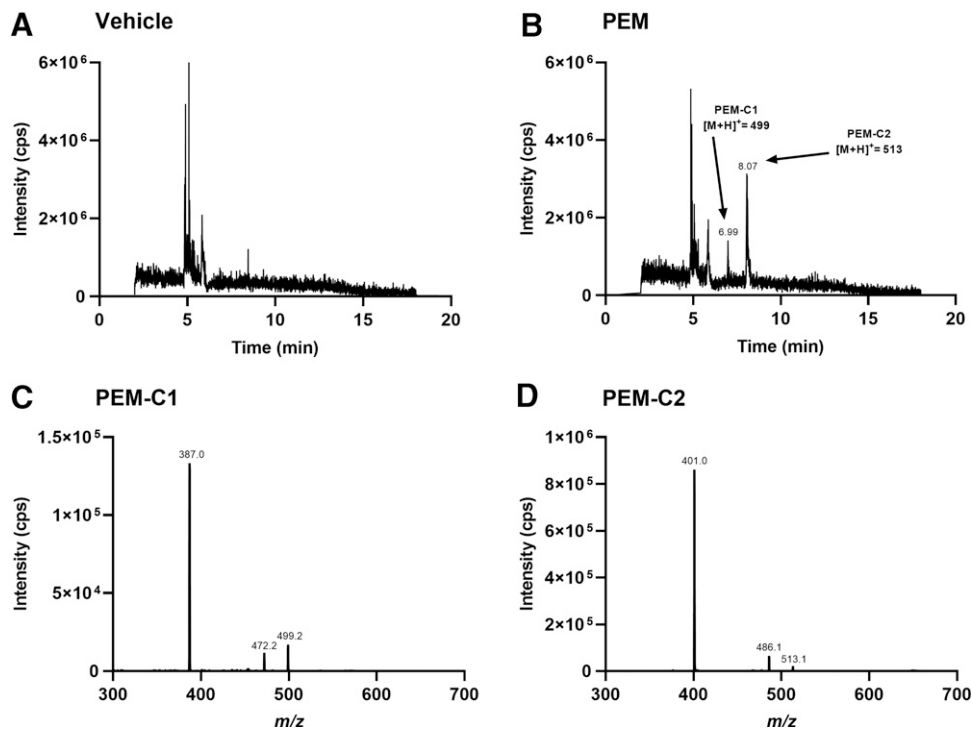
**Spectral Difference Scanning.** Consistent with our previous findings, there was an absence of a spectrally detectable peak in the Soret region (448–458 nm), associated with the formation of pseudo-irreversible

MIC, when incubation mixtures containing PEM and CYP3A were scanned from 400 to 500 nm at 5-minute intervals for 1 hour (Figs. 7A and 7B) (Polasek and Miners, 2008). Moreover, tracking the increase in absorbance between 454 nm and the isosbestic point at 490 nm further substantiated the lack of MIC formation with PEM (Figs. 7C and 7D). On



**Fig. 7.** Spectral difference measured over 60 minutes failed to elicit a Soret peak in the absorbance ranges of 448–458 nm for (A) CYP3A4 and (B) CYP3A5 incubated with 25  $\mu\text{M}$  PEM. Similarly, a comparison of the absorbance at the reference of 454 nm against the isosbestic point at 490 nm failed to demonstrate an increase in the extent of MIC formation over time in (C) CYP3A4 and (D) CYP3A5 incubation mixtures.

**Fig. 8.** Total ion chromatogram for constant NL scan of 27 Da in positive ESI mode of (A) vehicle and (B) pemigatinib incubations in the cyanide trapping assay. Notably, two peaks corresponding to PEM-derived cyano adducts PEM-C1 (retention time: 6.99 minute) and PEM-C2 (retention time 8.07 minute) were apparent in incubation mixtures containing PEM. Representative EPI MS/MS spectra of (C) PEM-C1 (*m/z* 499) and (D) PEM-C2 (*m/z* 513) formed in situ in the cyanide trapping assay.



the contrary, previous control experiments involving verapamil (a known pseudoirreversible MIC of CYP3A) produced a discernible peak and a time-dependent increase in absorbance at 454 nm (Tang et al., 2021b).

**Cyanide Trapping.** A previously described cyanide-trapping assay (Argoti et al., 2005) was conducted by fortifying reaction mixtures with the hard nucleophile KCN to uncover iminium ion intermediates of PEM trapped as stable cyano adducts. Remarkably, two peaks consistent with putative PEM-derived cyano conjugates, PEM-C1 (retention time, 6.99 min) and PEM-C2 (retention time, 8.07 min) with  $[M+H]^+$  ion at *m/z* of 499 and 513, respectively, were detected via a constant NL scan of 27 Da in positive mode but absent in vehicle-containing controls (Figs. 8A and 8B). The resultant EPI MS/MS spectra acquired further reinforced our postulations that these peaks corresponded to cyano adducts due to their characteristic collision-induced dissociation fragmentation pattern yielding neutral mass loss of 27 Da (corresponding to the loss of hydrogen cyanide) (Fig. 8C and D). To further elucidate the structures of PEM-C1 and PEM-C2, accurate mass measurements were performed. As expected, we were able to recapitulate the nominal mass patterns generated using the QTRAP-MS when the cyano adducts were subjected to accurate mass measurements using the Orbitrap-MS. The proposed elemental composition, theoretical and experimental exact *m/z*, and mass accuracy (in both  $\Delta Da$  and  $\Delta ppm$ ) of the PEM-C1 and PEM-C2 are summarized in Tables 2 and 3. Additionally, the representative product ion chromatogram, accurate mass MS/MS spectrum and proposed fragmentation pattern of the PEM-derived

cyano adducts are depicted in Figs. 9A–9D, whereas the corresponding Orbitrap-MS data for PEM are illustrated in Supplemental Fig. 1 and outlined in Supplemental Table 2.

**Covalent Docking of PEM to CYP3A4 and CYP3A5.** As it was previously reported that iminium ion intermediates elicit MBI of P450 via apoprotein alkylation (Orr et al., 2012), we focused our efforts toward identifying susceptible residues in the periphery of the OBS. A total of five lysine (Lys173, Lys208, Lys209, Lys476, and Lys487) and one serine (Ser312) residues were identified in the vicinity of the OBS in CYP3A4 as accessible residues that were potentially amenable to covalent adduction by the PEM-derived iminium ion intermediate. Interestingly, our analyses also determined that these aforementioned residues were structurally conserved in CYP3A5. This prompted us to perform covalent docking of PEM on these identified residues in CYP3A4 and CYP3A5. Our findings revealed that for both CYP3A isoforms, the top covalent docking scores were obtained by docking PEM to Ser312. As illustrated in the respective molecular plots (Figs. 10A and 10B), PEM adducted to Ser312 in both CYP3A isoforms and adopted a binding pose that snugly occupied the OBS beneath the F-F' loop. On one hand, in CYP3A4, the adduct laid almost parallel to the F-F' loop and completely blocked the opening between the C-terminal and F-F' loop (Fig. 10A). On the other hand, in CYP3A5, the adduct pointed toward the F-F' loop and only partially occluded the opening to the OBS (Fig. 10B). Taken together, these results hinted that PEM covalent adducts possessed a greater propensity to impede substrate

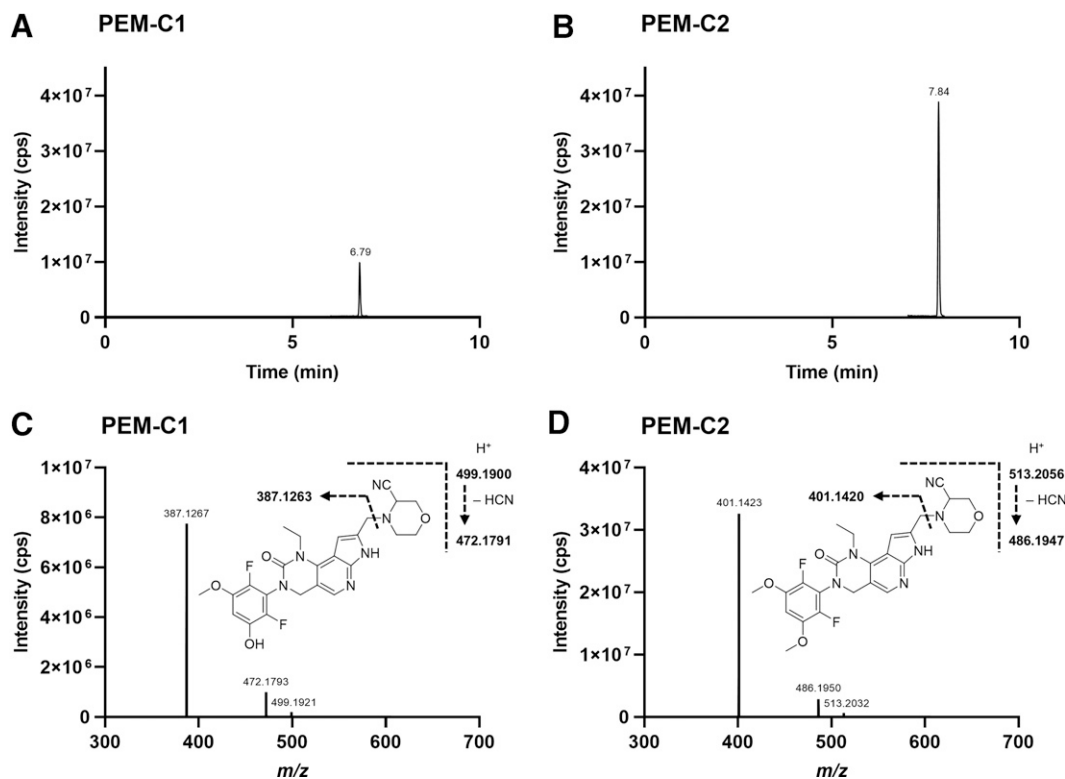
TABLE 2

Accurate mass measurement of the parent and product ions of pemigatinib-derived cyano adduct PEM-C1 using a mass tolerance of 5 ppm				
Proposed Elemental Composition	Theoretical <i>m/z</i>	Experimental <i>m/z</i>	Mass Accuracy $\Delta Da$	Mass Accuracy $\Delta ppm$
C <sub>24</sub> H <sub>25</sub> O <sub>4</sub> N <sub>6</sub> F <sub>2</sub>	499.1900	499.1921	0.0021	4.2
C <sub>23</sub> H <sub>24</sub> O <sub>4</sub> N <sub>5</sub> F <sub>2</sub>	472.1791	472.1793	0.0002	0.4
C <sub>19</sub> H <sub>17</sub> O <sub>3</sub> N <sub>4</sub> F <sub>2</sub>	387.1263	387.1267	0.0004	1.0

TABLE 3

Accurate mass measurement of the parent and product ions of pemigatinib-derived cyano adduct PEM-C2 using a mass tolerance of 5 ppm				
Proposed Elemental Composition	Theoretical <i>m/z</i>	Experimental <i>m/z</i>	Mass Accuracy $\Delta Da$	Mass Accuracy $\Delta ppm$
C <sub>25</sub> H <sub>27</sub> O <sub>4</sub> N <sub>6</sub> F <sub>2</sub>	513.2056	513.2032	−0.0024	−4.7
C <sub>24</sub> H <sub>26</sub> O <sub>4</sub> N <sub>5</sub> F <sub>2</sub>	486.1947	486.1950	0.0003	0.6
C <sub>20</sub> H <sub>19</sub> O <sub>3</sub> N <sub>4</sub> F <sub>2</sub>	401.1420	401.1423	0.0003	0.7





**Fig. 9.** (A) Representative product ion chromatogram of (A) PEM-C1 (retention time: 6.79 minute) and (B) PEM-C2 (retention time: 7.84 minute). Proposed accurate mass fragmentation pattern of (C) PEM-C1 and (D) PEM-C2. The exact mass MS/MS spectra depict the experimental  $m/z$  values whereas the chemical structures (inset) illustrate the theoretical accurate  $m/z$  values of the parent and product ions of the cyano adducts as outlined in Tables 2 and 3 using a mass tolerance of 5 ppm.

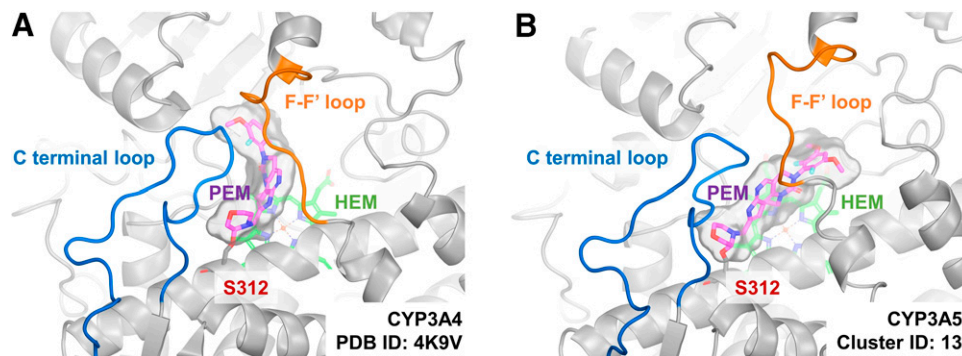
access to the catalytic heme in CYP3A4 than in CYP3A5. Consistent with these binding poses, our *in silico* findings also demonstrated that PEM interacted more favorably with Ser312 in CYP3A4 as compared with CYP3A5, as substantiated by their respective docking scores and calculated distances. In particular, the adduction of PEM to Ser312 generated a more favorable docking score in CYP3A4 (−8.5) than that in CYP3A5 (−7.2). Additionally, the minimum distance between the heavy atoms of the PEM adduct in Ser312 and the F-F' loop was calculated to be considerably shorter in CYP3A4 (2.9 Å) as compared with CYP3A5 (4.2 Å). Conversely, although our covalent docking simulations also revealed that PEM could form adducts with the five conserved lysine residues in both CYP3A isoforms, their binding poses and docking scores were found to be substantially less favorable as compared with that yielded at Ser312 in both isoforms (see Supplemental Fig. 2 and Supplemental Table 3 for more details). Hence, we are inclined to believe that the covalent adduction at Ser312 is likely to be more pertinent to mechanistically explain both the MBI of CYP3A by

PEM as well as to rationalize the observed differences in inactivation potencies between CYP3A4 and CYP3A5.

### Discussion

Although it was initially purported that PEM did not pose any metabolism-dependent liabilities (i.e., TDI) due to the absence of GSH-related conjugates in reactive metabolite screens, our findings presented here challenged the veracity of these previous inferences by establishing that PEM exhibited all the classic hallmarks of an archetypal MBI of CYP3A4 and CYP3A5. Following which, we also provided evidence that PEM could be directly and sequentially bioactivated to a reactive iminium ion intermediate that could covalently modify CYP3A and culminate in its MBI.

The  $K_I$  and  $k_{\text{inact}}$  are two important kinetic constants that are intrinsic to an MBI and are reflective of the enzyme-inactivator binding affinities at the molecular level. Consequently, the ratio of  $k_{\text{inact}}$  to  $K_I$  is



**Fig. 10.** Proposed covalent adduction of PEM to CYP3A4 and CYP3A5. Molecular plots illustrating the top scored binding poses for PEM adducts at (A) Ser312 of CYP3A4 (PDB ID: 4K9V) with a docking score of −8.5 and a minimum distance between the heavy atoms of PEM and the F-F' loop of 2.9 Å and (B) Ser312 of CYP3A5 (cluster 13) with a docking score of −7.2 and a minimum distance between the heavy atoms of PEM and the F-F' loop of 4.2 Å. Color code: F-F' loop, orange; C terminal loop, blue; heme moiety, green; PEM, pink stick with white surface.

TABLE 4

Comparison of the CYP3A4 enzyme inactivation kinetic parameters between pemigatinib and other FGFR inhibitors using rivaroxaban hydroxylation as a marker reaction of CYP3A activity

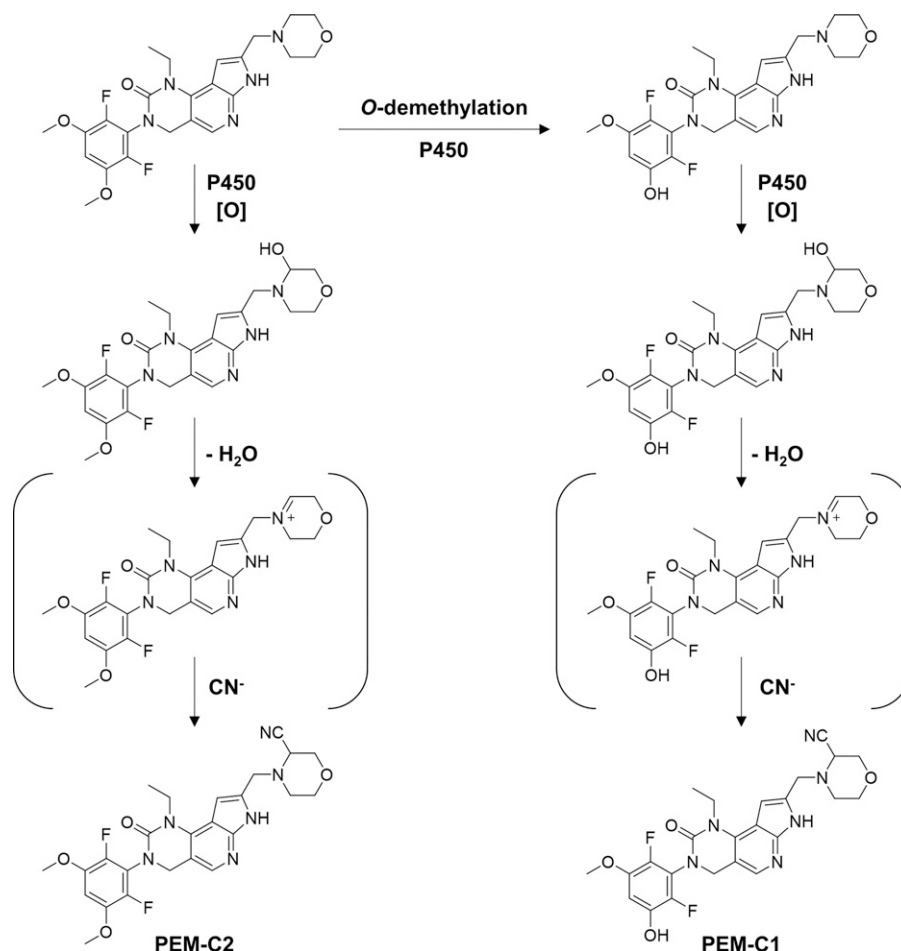
Compound	$K_I$ ( $\mu\text{M}$ )	$k_{\text{inact}}$ ( $\text{min}^{-1}$ )	$k_{\text{inact}}/K_I$ ( $\text{min}^{-1} \text{mM}^{-1}$ )	Reference
PEM	8.69	0.108	12.4	
Erdafitinib	4.01	0.120	29.9	(Tang et al., 2021a)
Infigratinib	4.17	0.068	16.4	(Tang et al., 2021b)

frequently harnessed in drug discovery to quantitatively assess the in vitro inactivation potency of a preclinical drug candidate (Orr et al., 2012). A comparison of the  $k_{\text{inact}}/K_I$  ratios derived for CYP3A4 revealed that the inactivation potency of PEM was largely comparable with the other FGFR inhibitors (Table 4). Conversely, juxtaposing the  $k_{\text{inact}}/K_I$  ratios between both CYP3A isoforms for PEM demonstrated that the inactivation of CYP3A4 was approximately 3.5-fold more potent than that in CYP3A5. It was previously established in a high-throughput TDI screen comprising 400 marketed drugs that CYP3A inactivators which possess a  $k_{\text{obs}}$  value of  $>0.020 \text{ min}^{-1}$  at a concentration of  $10 \mu\text{M}$  may constitute a potential in vivo DDI risk (Zimmerlin et al., 2011). Consistent with the trends observed in the aforementioned  $k_{\text{inact}}/K_I$  ratios, we estimated that the  $k_{\text{obs}}$  of CYP3A4 and CYP3A5 at  $10 \mu\text{M}$  were  $0.058 \text{ min}^{-1}$  and  $0.019 \text{ min}^{-1}$ , respectively. Taken together, it hinted that the inactivation of CYP3A4 by PEM is likely to be more pertinent from a clinical standpoint although it should be noted that at present there are no reported DDI data pertaining to PEM. At the

same time, due to the functional redundancy and overlapping substrate specificities of both CYP3A isoforms, it is plausible that the metabolism of PEM and other coadministered substrates of CYP3A may be preserved due to the less potent inactivation of CYP3A5. However, as the expression levels of CYP3A5 are highly polymorphic across different ethnicities, further studies are vital to mechanistically comprehend the full pharmacogenomic implications of our findings and how they may contribute to interindividual variability in PEM interactions. Additionally, the presence of MBI in PEM may potentially serve as a portentous predictor of reactive metabolite-induced toxicity and therefore warrants closer scrutiny.

Apart from its potency, the efficiency of an MBI can be also quantitatively evaluated by means of its partition ratio, which is defined as the number of inactivator molecules metabolized for every molecule of the enzyme inactivated. Partition ratios ranging from near zero to several thousand have been previously reported and hinge on a number of factors such as the reactivity of the intermediate generated through metabolic activation as well as its relative proximity to the targeted adduct/coordination site, among many others. As highly efficient inactivators are known to possess ratios of less than 50 (Lim et al., 2005), our data suggest that PEM are relatively efficient MBI of both CYP3A isoforms. Moreover, we also demonstrated that the inactivation of CYP3A by PEM could be attenuated in the presence of testosterone or ketoconazole, thereby implying that inactivation occurred within the enzymatic active site and could be diminished or even abolished by an alternative substrate or direct inhibitor competing for the same binding pocket. On the contrary, such similar protection was not observed in incubation

**Fig. 11.** Proposed bioactivation pathways of PEM and its *O*-desmethylated metabolite by CYP3A.



mixtures that were fortified with nucleophilic trapping agents, and this implied that reactive intermediate inactivated the enzyme before it is liberated from the active site and precludes the involvement of ROS in enzyme inactivation.

Although our experimental findings collectively supported the MBI of both CYP3A isoforms by PEM, the specific nature of inactivation still remained obfuscated. MIC, which stems from the formation of a coordinate bond between the reactive intermediate and heme ferrous iron are pseudo-irreversible and can be dissociated *in vitro* by means of dialysis or with a strong oxidizing agent which reverts the heme iron back to its ferric state and in doing so displaces the inactivator from the tight-binding complex and restores enzyme catalytic activity. Conversely, enzyme inactivation that arises from covalent alkylation is irreversible and cannot be mitigated by both aforementioned experimental approaches. Moreover, despite sharing approximately 85% sequence homology between CYP3A4 and CYP3A5, disparate modalities of inactivation have been reported for the same drug (Takakusa et al., 2011; Chan et al., 2012). As PEM possesses a tertiary amine substructure that is known to be capable of undergoing *N*-dealkylation to a reactive nitroso intermediate that can form MIC (Kamel and Harriman, 2013), it is imperative to individually delineate the nature of inactivation of both CYP3A isoforms by PEM. Here, our findings demonstrated that the loss of CYP3A activity is irrevocable and could not be recovered after dialysis. Similarly, the addition of potassium ferricyanide also did not restore enzyme activity by more than 20%, which is the predetermined threshold for MIC-forming inactivators (Watanabe et al., 2007). Finally, the lack of the diagnostic Soret peak in our spectral analyses further cemented our postulations that the inactivation of CYP3A by PEM was not mechanistically tied to the formation of pseudo-irreversible MIC but rather via irreversible covalent alkylation.

Consistent with a previous report by the drug's manufacturer (Wu et al., 2021), we also did not manage to evince any GSH conjugates in reactive metabolite screens performed in human liver microsomes or rhCYP3A4/5 (data not shown). This was rather peculiar as a core tenet underscoring MBI of P450 is the generation of chemically reactive metabolite(s) as the molecular-initiating event. Consequently, we surmised that the reactive intermediate of PEM implicated in the covalent modification of CYP3A may be a hard electrophile that cannot be efficiently trapped by GSH. Notably, one susceptible chemical motif in PEM that may be metabolically activated to such species is its distal morpholine ring, which is amenable to undergo oxidative bioactivation to an iminium ion intermediate (Mašič, 2011; Bolleddula et al., 2014). These reactive species generated *in situ* can then be readily trapped as stable cyano adducts by KCN and detected via NL of 27 Da in positive ESI mode (Argoti et al., 2005). Our postulations were indeed proven to be correct when we detected two putative adducts of PEM in our cyano-trapping assays. Moreover, the successful recapitulation of the nominal mass EPI spectral peaks for both adducts in our high-resolution Orbitrap-MS along with mass accuracies well below the mass tolerance threshold of 5 ppm allowed us to confidently ascertain that the direct and sequential bioactivation of PEM and its major *O*-desmethylated metabolite arose from P450-catalyzed hydroxylation at the  $\alpha$ -carbon of the morpholine nitrogen to an unstable carbinolamine that can undergo further dehydration to form the reactive iminium ion intermediate. Thereafter, the adduction of these hard electrophiles to cyanide yields the conjugates PEM-C1 and PEM-C2, respectively (Fig. 11).

Although the formation of reactive iminium ions from cyclic amines-containing compounds may occasionally be desirable, as evident in methoxy-4-morpholinyl doxorubicin, in which the iminium species potentiates its antineoplastic activity by facilitating DNA alkylation (Quintieri et al., 2005), more often than not, they are deleterious to its pharmacology (Kalgutkar, 2017). A well-established paradigm of this

phenomenon can be gleaned from the withdrawn cannabinoid receptor 1 antagonist rimonabant. Studies have shown that rimonabant was bio-activated by CYP3A4 to an iminium intermediate which resulted in extensive covalent binding to microsomal protein and cytotoxicity in human hepatocytes and SV40-THLE cells overexpressing CYP3A4 (Foster et al., 2013). More importantly, rimonabant was also discovered to incite TDI of CYP3A4 (Bergström et al., 2011). Consequently, we believe that the iminium species generated from PEM could likewise covalently modify CYP3A and culminate in its MBI. As such, covalent docking was conducted to elucidate plausible molecular determinants underpinning the MBI of CYP3A by PEM. Our findings collectively hinted that the salient discrepancies in MBI potencies between the CYP3A isoforms might be alluded to the propensity for PEM to interact more favorably with a conserved serine residue in position 312 of CYP3A4 as compared with CYP3A5. The involvement of serine here is not unexpected as the implicated iminium ion species is a hard electrophile and will preferentially react with a nucleophile of comparable "hardness" such as the hydroxyl groups of serine residues (LoPachin and DeCaprio, 2005). Additionally, the possibility of this serine residue being implicated in its MBI is further reinforced by a recent study which identified Ser312 in CYP3A4 as an important binding site residue implicated in ligand inhibitory interactions (Kiani et al., 2019).

In conclusion, our findings established that PEM is an archetypal MBI of CYP3A. Furthermore, we also illuminated the direct and sequential bioactivation pathways of PEM and its major *O*-desmethylated metabolite to reactive iminium ion species that are likely to be implicated in the irreversible covalent modification of CYP3A. Due to the profound pharmacokinetic and toxicological liabilities associated with hard electrophiles, we advocate that reactive metabolite screens should routinely monitor for both types of soft and hard electrophilic species.

#### Authorship Contributions

*Participated in research design:* Tang, Wei, Verma, Fan, Chan.

*Conducted experiments:* Tang, Wei, Verma, Koh.

*Contributed new reagents or analytical tools:* Koh, Zhou, Fan.

*Performed data analysis:* Tang, Wei, Verma, Fan, Chan.

*Wrote or contributed to the writing of the manuscript:* Tang, Wei, Verma, Fan, Chan.

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