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# Thinking Beyond Cytochrome P450 Inactivation Kinetics: Enhanced Understanding of the Mechanism of Tucatinib Interaction with CYP3A4


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## Introduction

Tucatinib is a highly selective tyrosine kinase inhibitor targeting the HER2 receptor, showing significant improvements in efficacy and safety for treating adult patients with advanced, unresectable, or metastatic HER2+ breast cancer, including those with brain metastases. In vitro CYP3A inactivation kinetic studies with tucatinib in human liver microsomes demonstrated time-dependent inhibition (TDI) of CYP3A4, with a mean $k_{inact}$ value of 0.011 $min^{-1}$ and a $K_I$ value of 0.54 µM, indicating TDI potential, albeit with low inactivation efficiency. Despite the well characterized kinetic observations, a complete mechanistic understanding is still lacking. Thus, in vitro investigations were performed towards elucidating the mechanism of inactivation by tucatinib. Classic approaches including spectral characterization (CO binding and MI complex), reversibility with potassium ferricyanide, as well as high-resolution mass spectrometry to seek evidence for covalent modification of CYP3A4 apoprotein were employed.

Tucatinib → CYP3A4 → [oxidized metabolites] → CYP3A4 TDI

## Methods

To assess the potential for modification or destruction of the heme of CYP3A4 as a mechanism of inactivation, spectral evaluation by carbon monoxide (CO) binding and characterization of P450 heme loss using LC-MS following incubation with tucatinib were both performed. In addition, to assess the potential of quasi-irreversible inhibition via a metabolite-intermediate (MI) complex with the heme of CYP3A4, two distinct approaches were employed, including searching for spectral evidence of an MI complex (absorbance at 455 nm) and assessing chemical reversibility of inactivation following incubation with potassium ferricyanide. Lastly, targeted proteomics using high-resolution mass spectrometry following protein digest was used to investigate if the observed inactivation might be the result of covalent modification of the CYP3A4 apoprotein.

## Results

### Time-Dependent Inhibition of CYP3A4 by Tucatinib

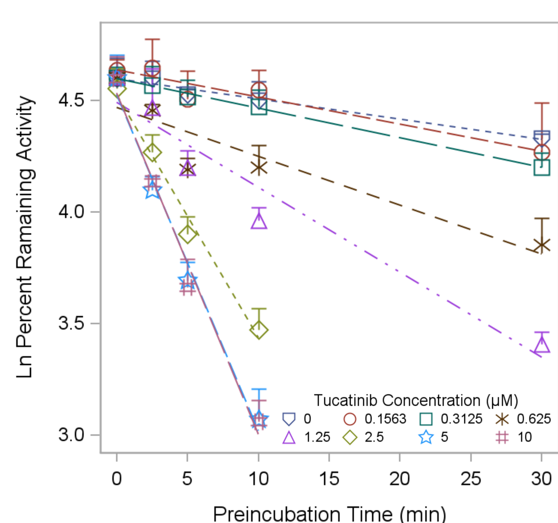


Fig 1A. Inactivation plot for the CYP3A4 time dependent inhibitor, tucatinib.

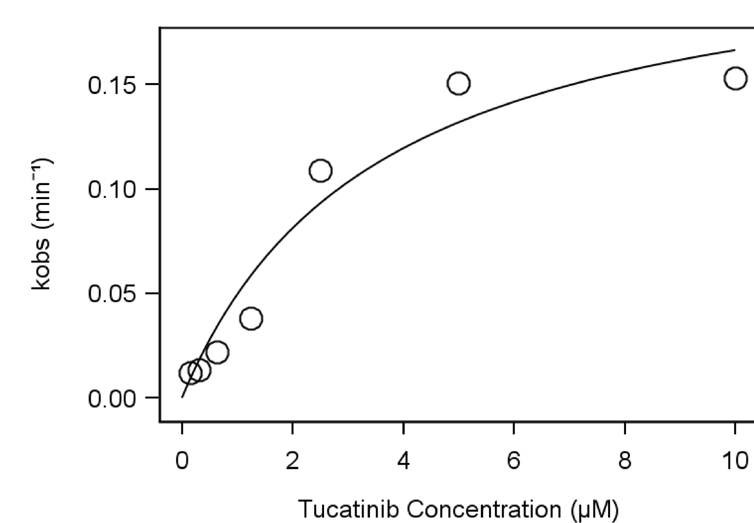


Fig 1B. Non-Linear regression analysis of tucatinib versus observed rate of tucatinib inactivation

1'-Hydroxymidazolam formation was used to measure remaining enzyme activity in the activity assay mixture with a 1:20 (CYP3A4) dilution of the pre-incubation mixture
$K_{obs}$ was determined by linear fitting of natural log remaining of enzyme activity vs. pre-incubation time; $K_I$ and $k_{inact}$ were determined by fitting the equation: $k_{obs} = k_{inact} \times [I]/(K_I + [I])$

### Inactivation by Tucatinib Partially Disrupts Carbon Monoxide Binding

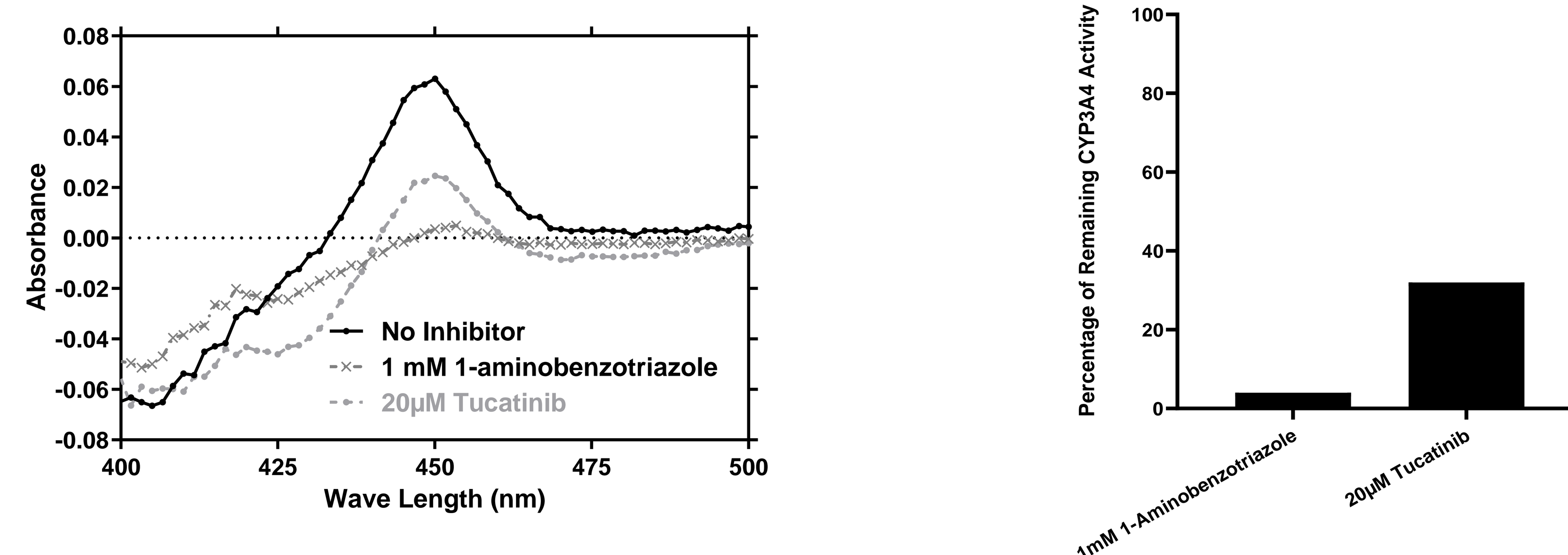


Fig 2. CO binding absorbance after incubation with 20 µM tucatinib, with 1mM 1-aminobenzotriazole (ABT) as a positive control. Loss of CYP3A4 activity was also confirmed using midazolam 1`-hydroxylation.

### Inactivation by Tucatinib: No Evidence for Heme Modification

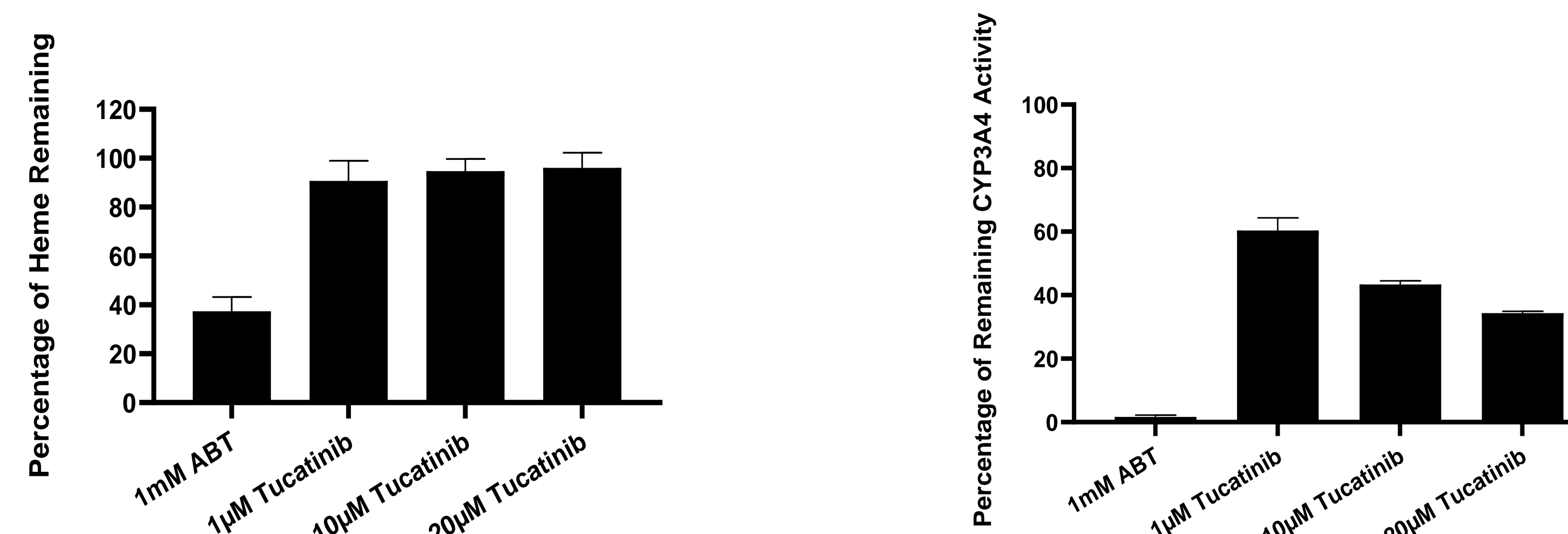


Fig 3A. Assessment of intact Protoporphyrin IX loss after 0.1 µM recombinant CYP3A4 treatment with 1, 10, and 20 µM tucatinib, or 1 mM ABT.

Fig 3B. Quantitative assessment of remaining activity of 0.1 µM recombinant CYP3A4 after treatment with 1, 10, and 20 µM tucatinib, or 1 mM ABT.

### Inactivation by Tucatinib: No Evidence for Formation of a Metabolite-Intermediate (MI) Complex with the Heme

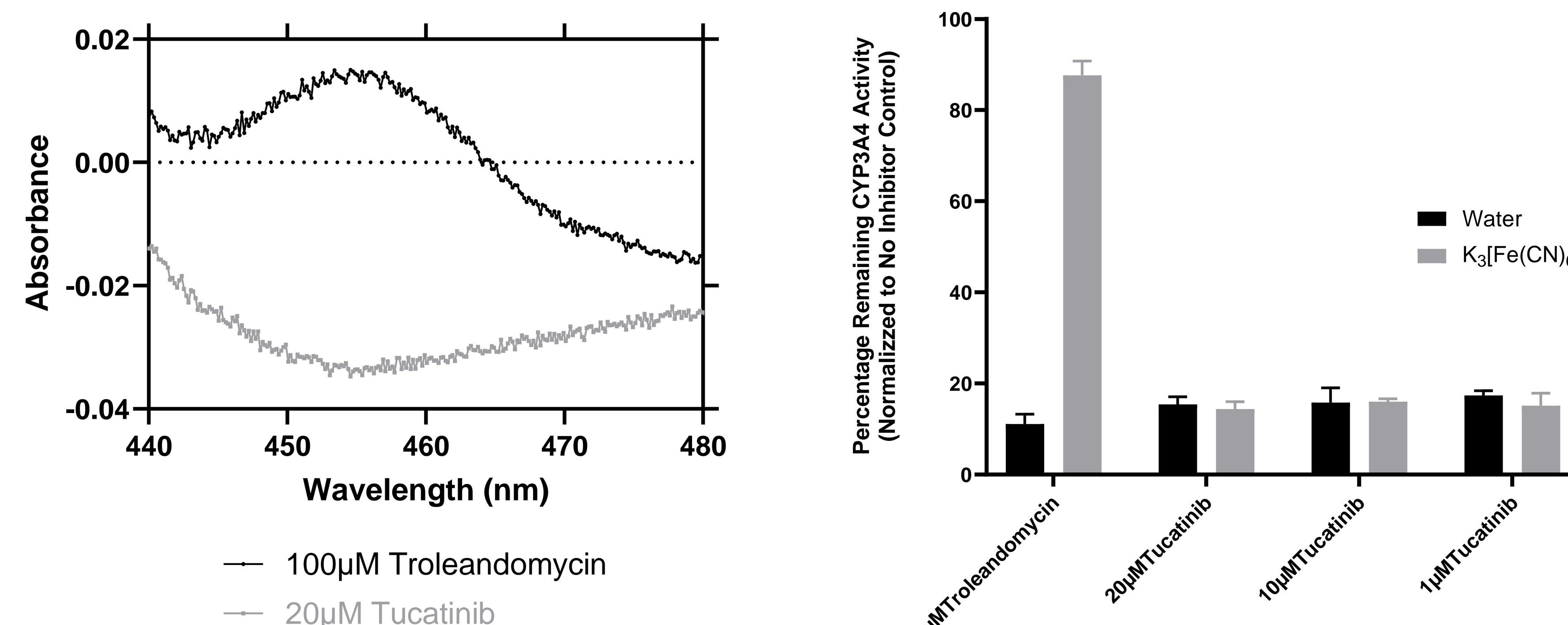


Fig 4A. MI complex spectrum of 0.5 µM recombinant CYP3A4 after incubation with 20 µM tucatinib or 100 µM troleandomycin.

Fig 4B. The inactivation of CYP3A4 was reversed by 2 mM $K_3[Fe(CN)_6]$ with 100 µM troleandomycin but not with 1, 10, and 20 µM tucatinib.

### Inactivation by Tucatinib: Covalent Modification of CYP3A4 Apoprotein by Tucatinib

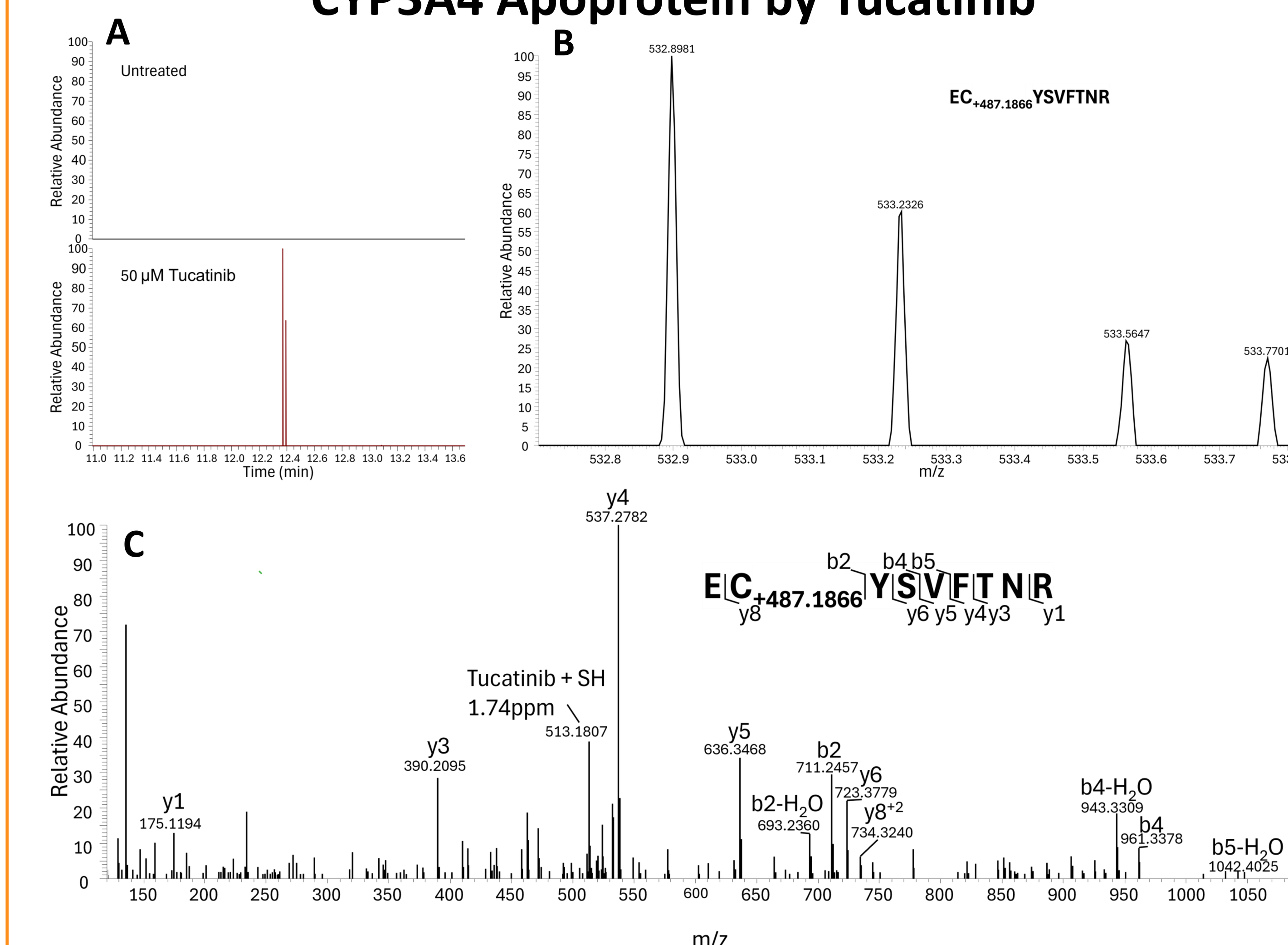


Fig 5. Identification of covalent modification of CYP3A4 by tucatinib.
A) Chromatogram of *m/z* 532.8982, corresponding to the mass of ECYSVFTNR with the addition of a tucatinib modification. A peak is present in the 50 µM tucatinib treated sample but not in the untreated sample. B) MS1 spectrum of *m/z* 532.8982. The mass corresponds to the +3 charge state of the ECYSVFTNR peptide modified by tucatinib with a mass error of 0.2 ppm. C) MS2 spectrum of the ECYSVFTNR peptide demonstrating modification of Cys98. An ion of *m/z* 513.1807 corresponds to a fragment ion consisting of tucatinib with the thiol from Cys98.

## Conclusions

•The inactivation of CYP3A4 by tucatinib cannot be attributed to heme modification, as no loss of intact heme (Fig. 2) or formation of a distinct covalent heme adduct (Fig. 3) was observed following inactivation.

•No evidence of a classic MI complex spectrum was detected, as tucatinib-induced inactivation could not be reversed by potassium ferricyanide treatment, and no MI complex spectrum was observed (Fig. 4).

•Targeted proteomics and high-resolution mass spectrometry revealed the formation of a covalent tucatinib adduct to Cys98 (Fig. 5).

•Future work will explore the covalent modification of other Cys residues on CYP3A4, as well as the potential mechanism of bioactivation of tucatinib.

## References


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