Time-Dependent Inhibition of CYP3A4 by Azole Antifungals and Macrolide Antibiotics: Mechanistic Profiling Using Triazolam as a Probe Substrate

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Longyue Liu

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Advisor: David J Greenblatt, M.D.

Emmanuel Pothos, Ph.D.

Abstract

This study aims to characterize the time-dependent inhibition (TDI) potential across drug classes, specifically examining azole antifungals (posaconazole, voriconazole) and macrolide antibiotics (azithromycin, erythromycin, josamycin, clarithromycin), using human liver microsomes and triazolam as a CYP3A4 probe substrate. Posaconazole demonstrated robust TDI with a 5.2-fold IC_{50} shift (0.83 μM to 0.16 μM), indicative of irreversible enzyme inactivation similar to troleandomycin (positive control, 4.8-fold shift). Erythromycin showed moderate TDI (2.4-fold shift, 357.5 μM to 148.8 μM), whereas voriconazole exhibited borderline TDI (2.1-fold shift). Josamycin displayed minimal TDI (1.5-fold shift), and azithromycin showed no significant shift, underscoring its favorable safety profile. Clarithromycin demonstrated a 2.5-fold change in IC_{50} shift (244.5 μM to 96.5 μM), further highlighting variability within macrolides. Ketoconazole (negative control) maintained consistent IC_{50} values regardless of preincubation, validating the experimental approach for distinguishing reversible from irreversible inhibition. These findings emphasize the clinical relevance of accurately identifying TDI mechanisms to enhance drug safety and therapeutic efficacy, ultimately improving the predictability and management of drug-drug interactions.

Acknowledgments

I would like to sincerely thank my advisors, Dr. David J. Greenblatt and Dr. Emmanuel Pothos, for their invaluable guidance, support, and patience throughout this research. I am also grateful to my committee members and lab colleagues, including Yi Shan, Christopher Singleton, Dr. Md Amin Hossain, and Zhengzhe Yang, for their insightful discussions, assistance, and contributions to this experiment. Their support has been crucial throughout this journey.

Contributions

I am the sole contributor for all data, analyses, and chapters of the thesis. Yi Shan, Christopher Singleton, Dr. Md Amin Hossain, and Zhengzhe Yang contributed to the experiment design, data collection.

AI tools, including ChatGPT, were used for grammar correction, stylistic enhancements, and clarity improvements in the Introduction and Results sections.

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1. Introduction

1.1 Introduction to Time-Dependent Inhibition

Time-dependent inhibition (TDI) is a pharmacokinetic phenomenon characterized by progressive enzyme inactivation that intensifies with prolonged exposure to an inhibitor, leading to irreversible or quasi-irreversible loss of enzymatic activity [1]. Unlike classical reversible inhibition, where enzyme function rapidly recovers upon inhibitor removal, TDI involves covalent modification or stable metabolite-enzyme complex formation, resulting in prolonged suppression of metabolic activity even after the inhibitor is cleared. This persistence significantly elevates the risk of drug-drug interactions and adverse clinical outcomes, as it can extend the period of compromised metabolism beyond the presence of the inhibitor itself. Such extended enzyme inactivation poses particular dangers for patients receiving narrow-therapeutic-index medications, for which even minor alterations in plasma concentrations can produce substantial toxicity [2]. CYP3A4 exemplifies this concern, since it's responsible for 50% of the clinically used drugs [3], including sensitive substrates such as triazolam and midazolam. Among the

drug classes that are particularly relevant in the context of TDI are azole antifungals and macrolides. These drugs are widely prescribed for various infectious diseases and are well-known for their potential to inhibit CYP3A4. Azole antifungals such as posaconazole, voriconazole, and macrolides like erythromycin, josamycin and clarithromycin, have demonstrated significant TDI effects, particularly in patients with polypharmacy [4][5][6][7][8][9][10]. Understanding the extent and mechanisms of TDI with these drugs is crucial for preventing adverse drug interactions and optimizing clinical outcomes, especially in patients who are concurrently taking other CYP3A4 substrates.

1.2 Mechanisms of Time-Dependent Inhibition

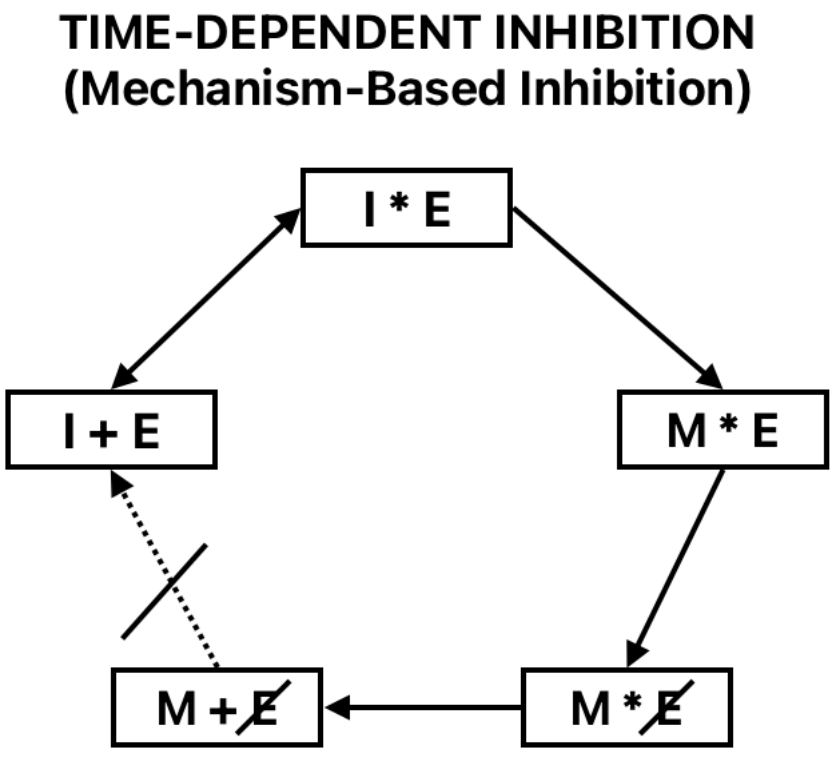

Figure 1. Mechanism-based inhibition of enzyme activity illustrating the biochemical pathways involved in time-dependent inhibition (TDI). Initially, the inhibitor (I) binds reversibly to the enzyme (E), forming an inhibitor-enzyme complex (I•E). Subsequent metabolic activation within this complex generates a reactive metabolite-enzyme intermediate (M•E). Depending on the inhibitor's chemistry and enzyme interaction, the metabolite-enzyme intermediate can covalently modify the enzyme's active site, resulting in irreversible inhibition. The dashed line indicates that once irreversible modification has occurred, the enzyme cannot readily return to its initial free form, thus significantly prolonging inhibition compared to classical reversible interactions.

Time-dependent inhibition (TDI) occurs through two principal biochemical mechanisms: irreversible covalent modification and quasi-irreversible metabolite-intermediate complex (MIC) formation. Irreversible inactivation occurs when an inhibitor undergoes metabolic activation, generating reactive intermediates that form covalent bonds with the enzyme's active site. This process permanently disables catalytic function, as observed with certain macrolide antibiotics that alkylate the enzyme's heme or apoprotein. In contrast, quasi-irreversible inhibition involves the formation of stable, non-covalent complexes between the enzyme and a metabolic intermediate of the inhibitor. For example, some macrolides are metabolized to derivatives that coordinate tightly with the enzyme's heme iron, obstructing substrate access without forming covalent bonds. As illustrated in Figure 1, the TDI process initiates when an inhibitor (I) binds reversibly to the enzyme (E), forming an inhibitor-enzyme complex (I•E). Upon metabolic activation within this complex, a reactive metabolite (M•E) intermediate is generated, which can either form a stable, quasi-irreversible complex with the enzyme's active site or undergo covalent binding, irreversibly modifying the enzyme. The distinction between these mechanisms

lies in their reversibility: irreversible inactivation requires de novo enzyme synthesis to restore activity, while quasi-irreversible inhibition may partially resolve upon inhibitor clearance. Both mechanisms are governed by kinetic parameters that describe the efficiency of enzyme inactivation and the inhibitor's binding affinity, critical for accurately predicting clinical outcomes.

1.3 Types of Enzyme Inhibition: Reversible vs. Irreversible

Enzyme inhibition is broadly categorized into reversible and irreversible mechanisms, each with distinct pharmacological implications. Reversible inhibitors, such as ketoconazole, bind non-covalently to the enzyme's active site, causing transient inhibition that rapidly dissipates upon inhibitor clearance. In contrast, irreversible inhibitors, including time-dependent inhibitors (TDI) like erythromycin, progressively inactivate enzymes via covalent bonds or formation of stable metabolite-intermediate complexes (MICs). The distinction between these inhibition mechanisms critically affects clinical management: reversible inhibition can typically be addressed through dose adjustments, while irreversible TDI requires stricter contraindications due to prolonged inhibitory effects [22]. Accurate characterization of the time-course of enzyme inhibition is therefore essential, as reversible inhibition reaches equilibrium quickly (within minutes), while irreversible inhibition intensifies over extended incubation periods, often requiring longer monitoring. Ensuring accuracy and precision in experimental measurements of these temporal patterns directly influences the reliability of predicting clinical drug-drug interactions and the design of appropriate dosing strategies.

1.4 Role of Time-Dependent Inhibition in Drug Development

Time-dependent inhibition (TDI) of cytochrome P450 enzymes, particularly CYP3A4, is a critical consideration in drug development due to its potential to cause serious drug-drug interactions (DDIs) and adverse effects [1]. Undetected irreversible enzyme inhibition can lead to drug withdrawals or strict labeling restrictions, making early identification of TDI crucial. To improve predictions of TDI risks, researchers now employ advanced kinetic modeling tools, including physiologically based pharmacokinetic (PBPK) simulations. These models incorporate key parameters such as enzyme turnover rates (k_{deg}), inhibitor binding constants (K_I), and inactivation rates (k_{inact}) to more accurately predict clinical outcomes [11][12][13][14]. Dr. David Greenblatt and colleagues have contributed significantly to understanding TDI mechanisms [15], particularly in the context of drug-drug interactions involving natural products. Their work on grapefruit juice interactions with CYP3A substrates has elucidated the role of furanocoumarins, such as 6',7'-dihydroxybergamottin (DHB), in irreversibly inactivating intestinal CYP3A enzymes. This mechanism-based inhibition can lead to reduced first-pass metabolism and consequently higher plasma concentrations of affected drugs, potentially enhancing clinical effects or even producing toxicity [16]. The complexity of TDI profiles, as seen with drugs like troleandomycin, a macrolide antibiotic, emphasizes the need for comprehensive screening assays and sophisticated modeling approaches to fully capture and predict clinical implications. These advancements in TDI assessment are crucial for improving drug safety and efficacy in clinical practice [17].

1.5 Investigated Compounds and Controls

1.5.1 Posaconazole

Posaconazole is a triazole antifungal widely prescribed for prophylaxis and treatment of invasive fungal infections, particularly in immunocompromised patients. Due to its broad-spectrum activity, posaconazole accounts for approximately 10–20% of systemic antifungal use in high-risk clinical populations, such as hematopoietic stem cell transplant recipients and patients undergoing chemotherapy. It features an extended half-life of around 35 hours and a convenient once-daily dosing regimen, which enhances patient adherence compared to older azoles. However, its lipophilic nature contributes to variable bioavailability and necessitates careful therapeutic drug monitoring to optimize clinical efficacy and minimize adverse effects. Additionally, posaconazole exhibits prolonged inhibitory effects on CYP3A4, resulting in an extended washout period following discontinuation, which further underscores the importance of careful therapeutic monitoring and dose adjustment [18][19][20].

1.5.2 Voriconazole

Voriconazole, another widely used second-generation triazole antifungal, is primarily utilized for invasive fungal infections caused by Aspergillus species. It is frequently employed in patients that intolerant or refractory to other antifungal therapies. Metabolized predominantly by CYP2C19, voriconazole generates a sulfoxide metabolite that contributes significantly to interpatient pharmacokinetic variability. Additionally,

voriconazole inhibits CYP3A4, although less potently than other azoles such as ketoconazole, which contributes to clinically relevant drug-drug interactions. Studies indicate that voriconazole's inhibitory effects on CYP3A4 can impact the metabolism of co-administered drugs metabolized by this enzyme, necessitating careful therapeutic drug monitoring and potential dose adjustments. Due to this variability and the resulting pharmacokinetic complexities, approximately 30% of patients receiving voriconazole require therapeutic drug monitoring to maintain effective drug concentrations and prevent toxicity or therapeutic failure [21].

1.5.3 Azithromycin

Azithromycin, a 15-membered macrolide antibiotic, is extensively prescribed due to its broad-spectrum activity and favorable safety profile. Its prolonged half-life enables shorter dosing courses compared to older macrolides, enhancing patient adherence. Annually, azithromycin is among the most frequently prescribed antibiotics in the United States, primarily for respiratory tract infections. Although generally considered safe in terms of CYP3A4-mediated interactions, isolated studies suggest a potential for weak delayed inhibition via metabolite-intermediate complex formation under prolonged use conditions, warranting cautious use in polypharmacy settings [22][23].

1.5.4 Erythromycin

Erythromycin, a 14-membered macrolide antibiotic, remains clinically significant despite its reduced use due to gastrointestinal adverse effects. It continues to serve as a primary agent in pertussis prophylaxis and the treatment of certain bacterial infections such as those caused by Campylobacter species. With approximately two million prescriptions annually in the United States, erythromycin is notably associated with potent CYP3A4 inhibition via irreversible metabolite-intermediate complex formation, causing clinically significant drug-drug interactions. This mechanism notably increases plasma levels of statins and other narrow-therapeutic-index CYP3A4 substrates, significantly heightening toxicity risks [22][24].

1.5.5 Josamycin

Josamycin, a 16-membered macrolide antibiotic, is primarily prescribed in Europe and Asia, accounting for approximately 10–15% of macrolide antibiotic use in those regions. Its structural differences from erythromycin, particularly the absence of a reactive tertiary amine, contribute to lower gastrointestinal intolerance and less robust CYP3A4 inhibitory effects. The clinical implications of its weaker inhibition profile remain uncertain, suggesting that josamycin may represent a safer macrolide alternative in patients receiving concomitant CYP3A4 substrates, though further research is necessary to fully characterize its inhibitory potential [25].

1.5.6 Clarithromycin

Clarithromycin, a macrolide antibiotic, is a well-characterized reversible inhibitor of cytochrome P450 3A (CYP3A), contributing to clinically significant drug-drug interactions (DDIs) by suppressing the metabolism of co-administered CYP3A substrates such as midazolam [26][27][28].Unlike mechanism-based inhibitors, its inhibition is concentration-dependent and intermediate in potency, increasing substrate exposure by up to 6.5-fold (e.g., midazolam AUC), as demonstrated in pharmacokinetic studies [29]. While not a prototypical time-dependent inhibitor (TDI), clarithromycin's reversible binding still necessitates caution with narrow-therapeutic-index drugs due to prolonged enzyme suppression and toxicity risks.

1.5.7 Controls

Troleandomycin (Positive Control)

Troleandomycin is a classical macrolide antibiotic and a well-established mechanism-based inhibitor of cytochrome P450 3A4 (CYP3A4). Its inhibition kinetics are characterized by irreversible enzyme inactivation, which is significantly enhanced by pre-incubation with microsomes prior to substrate addition. This property distinguishes troleandomycin from other inhibitors, such as ketoconazole, whose inhibition mechanism involves mixed reversible competitive–noncompetitive processes without enhancement by pre-incubation. The irreversible binding of troleandomycin to CYP3A4 provides a robust benchmark for validating experimental assays aimed at assessing time-dependent enzyme inactivation, ensuring methodological reliability [4].

Ketoconazole (Negative Control)

Ketoconazole, historically a first-line azole antifungal, is widely recognized as a potent reversible inhibitor of CYP3A4. Its inhibitory mechanism is characterized by a combination of noncompetitive and competitive binding, with a reported of approximately 0.011 to 0.045 μM (K_i, inhibition constant), and it does not exhibit time-dependent inhibition [4]. Despite its withdrawal from systemic therapy due to alleged hepatotoxicity, ketoconazole remains a critical reference inhibitor in drug interaction studies, particularly for differentiating reversible inhibition from irreversible enzyme modification. Greenblatt and colleagues extensively characterized ketoconazole's inhibitory profile, demonstrating its distinctly reversible binding compared to troleandomycin and erythromycin, which irreversibly inhibit CYP3A4 through metabolite-intermediate complex (MIC) formation. For example, ketoconazole's inhibition in human liver microsomes is immediate and fully reversible upon dilution, whereas erythromycin-induced inhibition persists due to irreversible enzyme modification. This property makes ketoconazole an essential tool in studies investigating CYP3A4 activity and drug-drug interactions [22].

2. Methods and Materials

2.1 Chemicals and Reagents

Ketoconazole, phenacetin, isocitric dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADPH) sodium salt hydrate, DL-isocitric acid trisodium salt hydrate, and

NADPH were purchased from Fisher Scientific (Waltham, MA). Human liver microsomes (HLMs) were sourced from XenoTech (Kansas City, KS). Ketoconazole, Phenacetin, Isocitric Dehydrogenase, Magnesium Chloride ($MgCl_2$), and all other analytical-grade reagents were prepared fresh. LC-MS grade acetonitrile and water were also obtained from Fisher Scientific.

2.2 Sample Preparation and Reaction Conditions for HPLC Analysis

Stock solutions were prepared in methanol, and incubation volumes were standardized at 200 μL per reaction, each containing 250 μM of the probe substrate triazolam. Ketoconazole was included at varying concentrations ranging from 0 to 0.005 μM and higher as appropriate for inhibition experiments. Organic solvents were evaporated to dryness prior to initiating incubations.

Two sets of tubes containing only triazolam (duplicates for each set) were prepared to assess whether preincubation time affected the IC_{50} values of the five tested drugs. Specifically, incubations with preincubation times of 0 minutes and 20 minutes were conducted. After preparation, the incubation buffer (containing human liver microsomes (HLMs), cofactor solution consisting of NADPH, isocitric acid trisodium salt, and magnesium chloride ($MgCl_2$, 3 mM) in 50 mM phosphate buffer) was preincubated at 37°C for 5 minutes. Subsequently, 1 mL of this buffer was transferred to tubes containing only inhibitors (positive control, negative control, and test drugs) and thoroughly mixed using pipettes. Immediately after mixing, 200 μL aliquots were transferred to tubes labeled "0 min preincubation" and placed into an incubator at 37°C. After 20 minutes,

additional 200 µL aliquots were transferred to tubes labeled "20 min preincubation" and returned to the incubator. Following incubation (30 minutes total for each set of preincubations), reactions were terminated by adding 200 µL of ice-cold acetonitrile containing phenacetin as the internal standard (2.3 mg/100 mL). Samples were then vortexed for 5 minutes and centrifuged at 12,000 rpm for 10 minutes to collect supernatants.

HPLC analyses were conducted using an Agilent 1290 HPLC system equipped with an ACQUITY UPLC HSS T3 1.8 µM 2.1 × 150 mm column (Waters). Analytes were separated using an isocratic mobile phase consisting of water and methanol: acetonitrile (50:50, v/v), each containing 0.1% formic acid. Column temperature was maintained at 50°C, with a flow rate of 0.5 mL/min. CYP3A4 activity was quantified by measuring residual triazolam metabolism based on retention times and peak areas relative to the internal standard phenacetin.

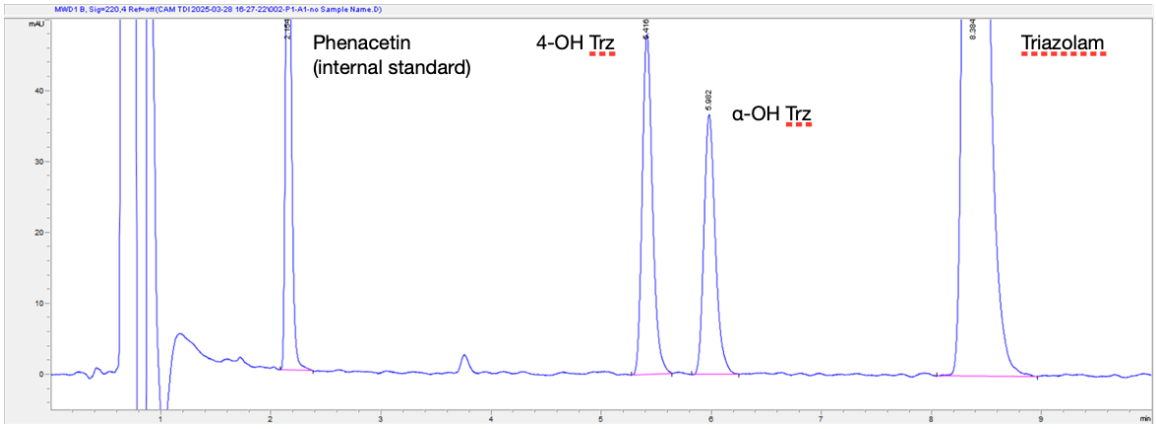

Figure 2. Liquid-Chromatography chromatogram showing the analysis of triazolam (Trz) metabolism in the absence of any inhibitor (control). The chromatogram displays the separation of triazolam and its hydroxylated metabolites, including α-OH triazolam (α-OH Trz) and 4-OH triazolam (4-OH Trz). Phenacetin was used as the internal standard (marked in the chromatogram). The peaks for α-OH Trz and

4-OH Trz indicate the primary metabolites formed during the incubation, and the triazolam peak serves as a reference for the residual unmodified substrate.

2.3 Data Analysis

Data analysis was conducted using GraphPad Prism software (GraphPad Software, La Jolla, CA). IC_{50} values were determined from concentration-response curves, assessing inhibition by ketoconazole and troleandomycin. IC_{50} shifts, indicative of time-dependent inhibition (TDI), were evaluated by comparing incubations performed with and without a preincubation period. The shifts were used to identify potential time-dependent inhibition effects.

2.4 Data Processing

The concentration-response data were analyzed using nonlinear regression with GraphPad Prism software. Reaction velocities were expressed as percentages relative to control incubations without inhibitor (% control), and the resulting data were fitted to a four-parameter logistic model described by the following equation:

$$Y = 100 \times \left[1 - \frac{E_{\max} \times X^b}{X^b + (IC)^b}\right]$$

where Y represents the percent reaction velocity relative to control, X is the inhibitor concentration, E_{max} denotes the maximum inhibitory effect achievable, IC is the inhibitor concentration at the inflection point of the curve, and b is the Hill coefficient describing the steepness of the curve [4].

The half-maximal inhibitory concentration (IC_{50}) was subsequently calculated using the fitted parameters from the above equation, employing the following relationship:

$$IC_{50} = \frac{IC}{(2E_{\max} - 1)^{\frac{1}{b}}}$$

Nonlinear regression analysis generated best-fit curves that were utilized to determine the IC_{50} values, quantifying each inhibitor's potency. These IC_{50} values were graphically illustrated to facilitate comparisons among tested inhibitors and clearly represent the relationship between inhibitor concentration and CYP enzyme activity [4].

3. Results

3.1 Posaconazole

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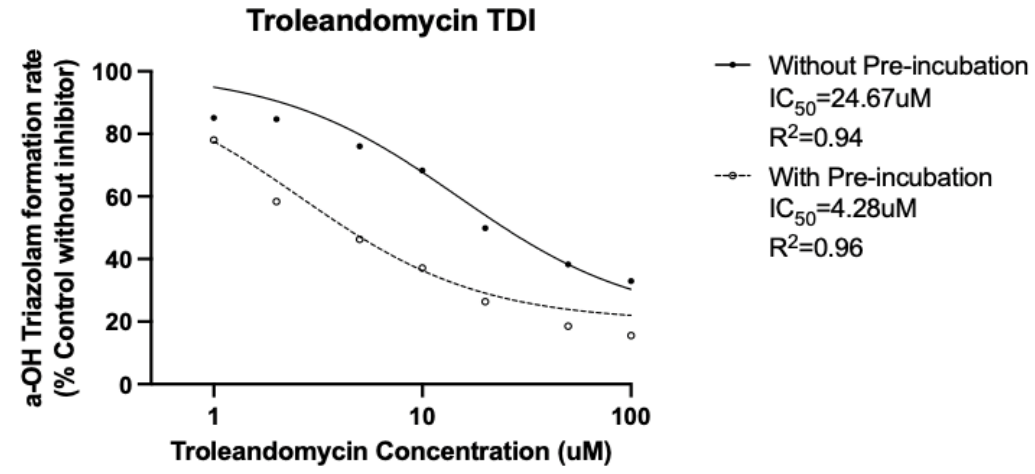


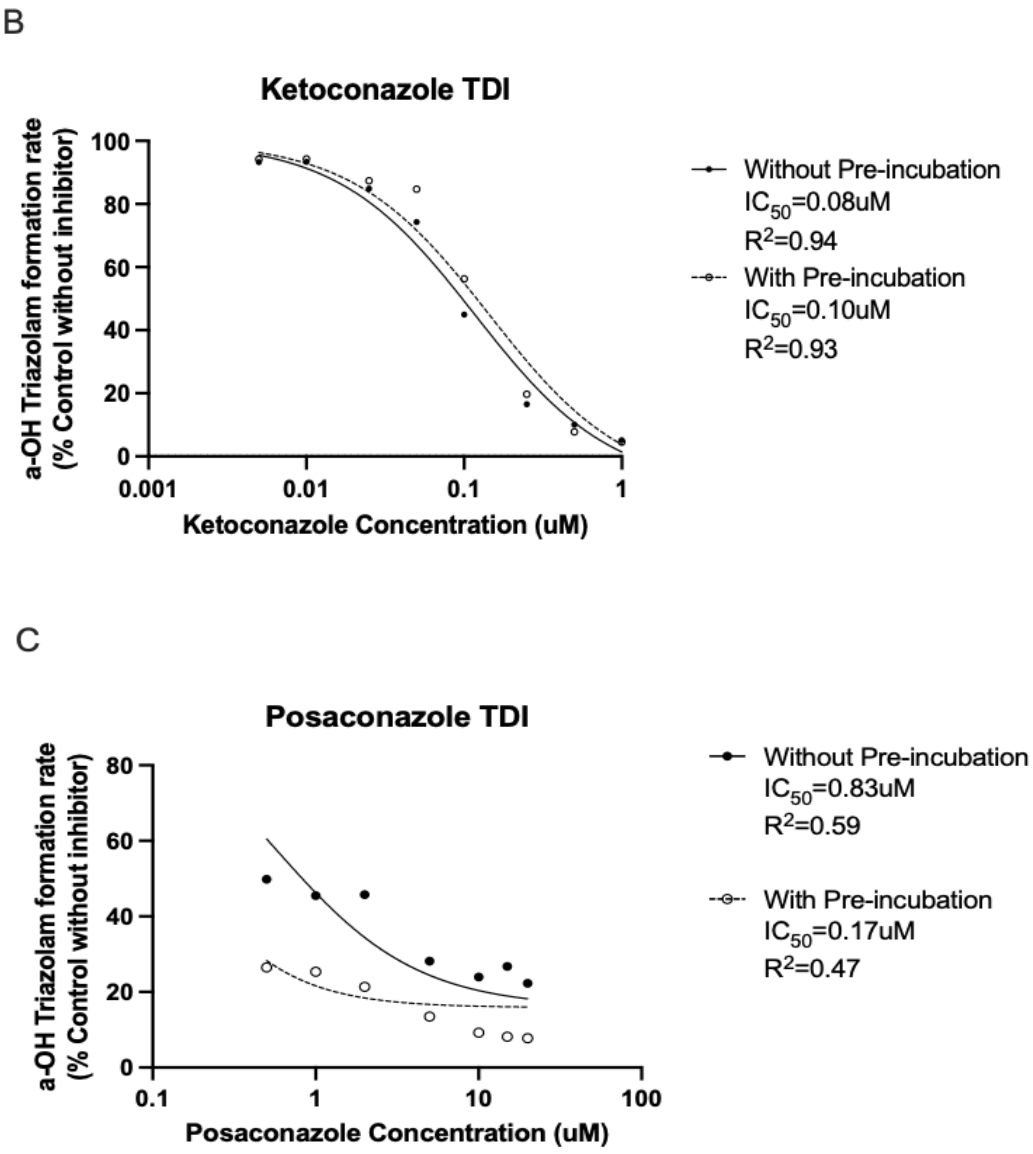


Figure 3. Inhibition of OH-Triazolam formation by (A) Troleandomycin, (B) Ketoconazole, and (C) Posaconazole with (dashed lines) and without (solid lines) preincubation. Graphs show concentration-dependent inhibition as a percentage of control. IC_{50} values (μM) without and with preincubation: Troleandomycin (24.67, 4.28), Ketoconazole (0.08, 0.10), Posaconazole (0.83, 0.17). Data points represent means of duplicate experiments.

The present study evaluated the time-dependent inhibition (TDI) potential of posaconazole, a triazole antifungal, on CYP3A4 activity using human liver microsomes

(HLMs) and triazolam as a probe substrate. Troleandomycin and ketoconazole were included as positive (mechanism-based inactivator) and negative (reversible inhibitor) controls, respectively. Experimental data demonstrated that posaconazole induced a marked IC_{50} shift from 0.83 μM (without preincubation) to 0.17 μM (with preincubation), reflecting a 4.9-fold reduction, indicative of irreversible CYP3A4 inactivation. This TDI profile closely mirrored troleandomycin's behavior, which exhibited a 5.8-fold IC_{50} shift (from 24.67 μM to 4.28 μM), while ketoconazole showed no significant shift (from 0.08 μM to 0.10 μM), consistent with its transient inhibition mechanism.

Importantly, posaconazole has a long half-life, resulting in slow washout after discontinuation [19][20]. This characteristic leads to prolonged CYP3A4 inhibition, meaning drug-drug interactions (DDIs) may persist even after the antifungal has been discontinued. The inhibition patterns, as illustrated in Figure 1 (Panels A–C), underscore posaconazole's capacity to irreversibly inactivate CYP3A4, likely through metabolite-intermediate complex (MIC) formation—a mechanism shared with prototypical TDI agents such as troleandomycin. These findings have significant clinical implications: the prolonged CYP3A4 suppression by posaconazole may elevate systemic exposure to co-administered drugs metabolized by this enzyme, even after the discontinuation of the antifungal.

In conclusion, the robust TDI profile of posaconazole, coupled with its long half-life, necessitates stringent monitoring when prescribing it alongside CYP3A4 substrates. Further studies are needed to confirm its inactivation kinetics and assess the magnitude of clinical DDIs to optimize therapeutic safety [19][20].

3.2 Voriconazole

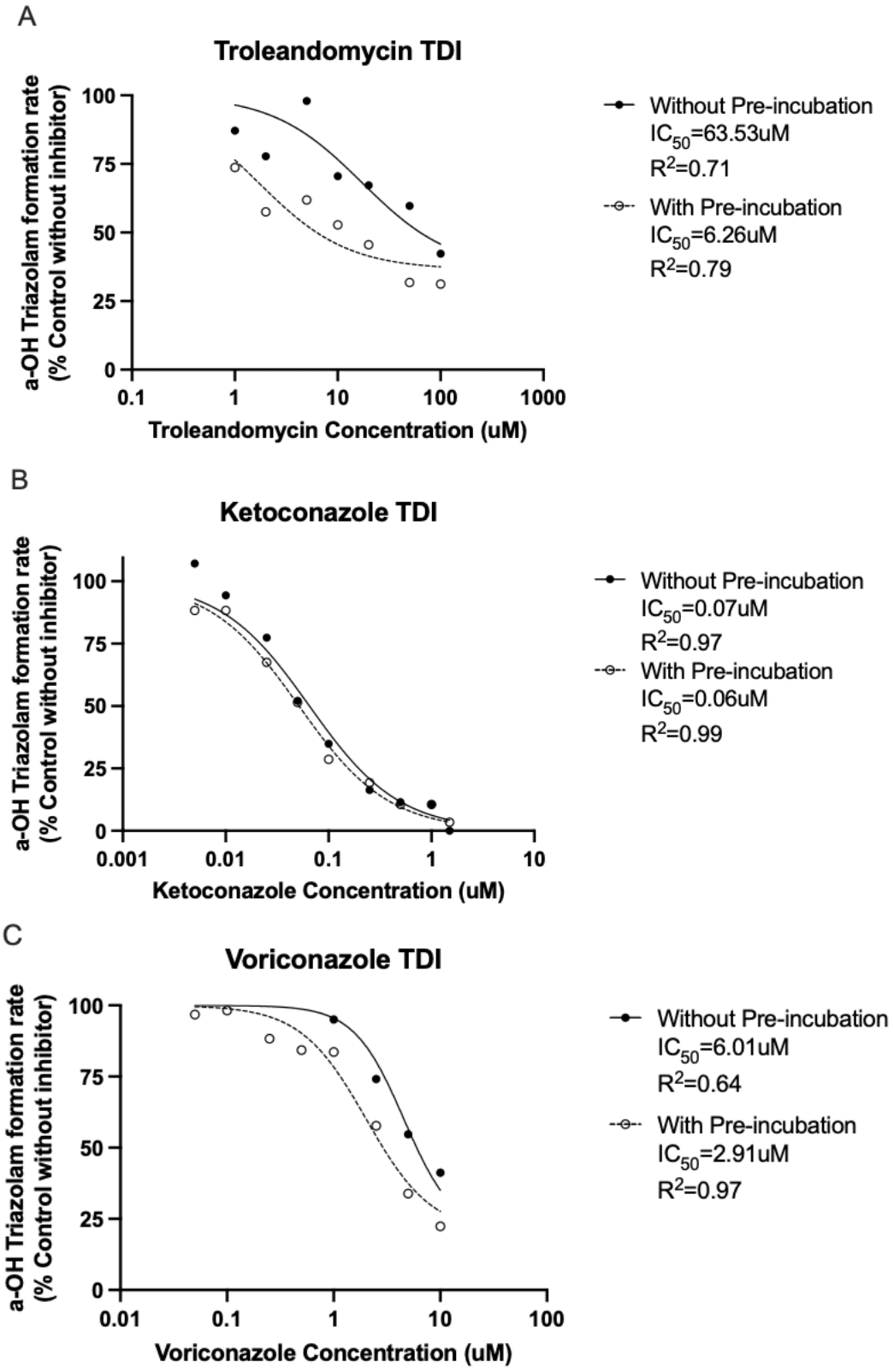


Figure 4. Inhibition of OH-Triazolam formation by (A) Troleandomycin, (B) Ketoconazole, and (C) Voriconazole with (dashed lines) and without (solid lines) preincubation. Graphs show concentration-dependent inhibition as a percentage of control. IC_{50} values (μM) without and with preincubation: Troleandomycin (63.53, 6.26), Ketoconazole (0.07, 0.06), Voriconazole (6.01, 2.91). Data points represent means of duplicate experiments.

Troleandomycin exhibited robust TDI, with its IC_{50} decreasing from 63.53 μM without preincubation to 6.26 μM after preincubation (a 10.1-fold shift), consistent with its classification as a potent mechanism-based inactivator. In contrast, ketoconazole demonstrated negligible TDI, as its IC_{50} values remained nearly unchanged (0.07 μM without preincubation vs. 0.06 μM with preincubation), aligning with its established reversible inhibition mechanism.

Voriconazole displayed a modest IC_{50} shift from 6.01 μM (without preincubation) to 2.91 μM (with preincubation), corresponding to a 2.1-fold decrease. This observation implies transient enzyme interactions, potentially through unstable metabolite-intermediate complex (MIC) formation or partial enzyme modification [5].Although voriconazole's TDI effect is less pronounced than that of troleandomycin, the observed shift approaches the threshold (≥2-fold) often flagged in regulatory assessments for potential clinical relevance. Such findings suggest that voriconazole may pose a risk for drug-drug interactions (DDIs) when administered with CYP3A4 substrates, particularly those with narrow therapeutic indices.

3.3 Azithromycin

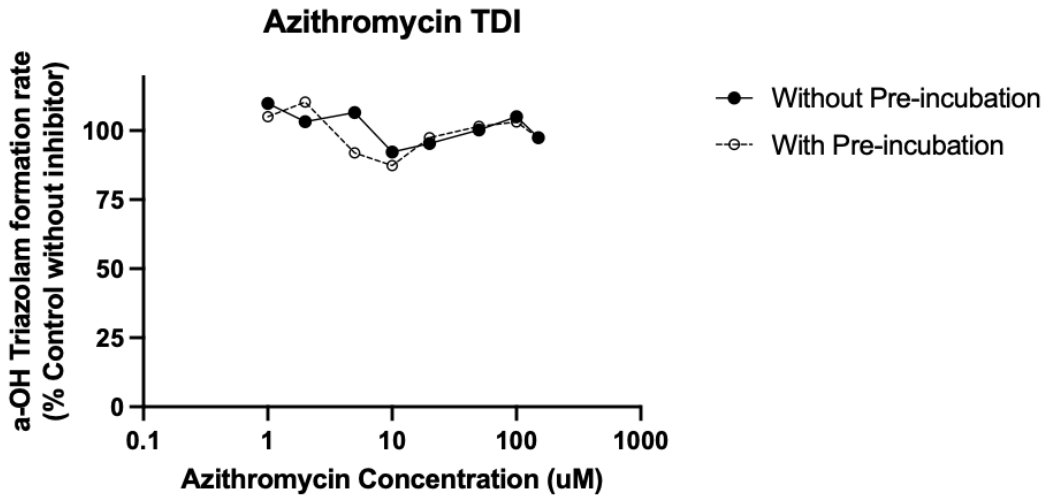
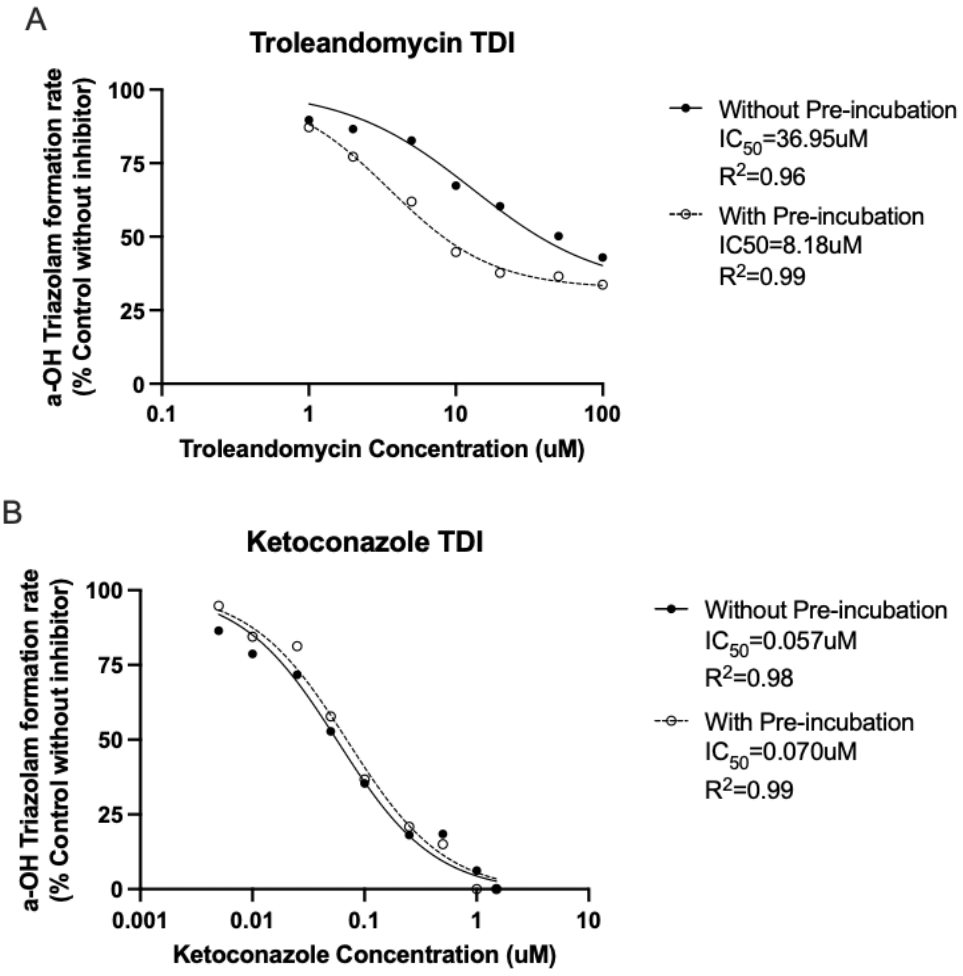


Figure 5. Evaluation of azithromycin for time-dependent inhibition (TDI) of CYP3A4. Reaction velocities of α-OH triazolam formation were measured in human liver microsomes in the presence of varying concentrations of azithromycin, either with or without a pre-incubation step. Data points represent the mean percentage relative to control reactions conducted without inhibitor. No significant differences were observed between the pre-incubation and no pre-incubation groups, consistent with the known pharmacological profile of azithromycin as neither a potent nor time-dependent inhibitor of CYP3A4. Consequently, azithromycin serves as a negative control, highlighting the specificity of the assay for detecting genuine time-dependent CYP3A4 inhibitors.

Azithromycin, a widely prescribed 15-membered macrolide antibiotic, is commonly used for respiratory infections, sexually transmitted diseases, and various bacterial infections due to its broad-spectrum activity, convenient dosing regimen, and well-established safety profile. Unlike other macrolides, such as erythromycin and clarithromycin, azithromycin is structurally distinct, resulting in markedly lower affinity for the cytochrome P450 3A4 (CYP3A4) enzyme. Azithromycin was evaluated in the current study for its potential to cause time-dependent inhibition (TDI) of CYP3A4 using human liver microsomes and triazolam as a probe substrate. The results, shown in Figure 3, demonstrate that azithromycin does not exhibit TDI characteristics, as evidenced by

nearly identical inhibition curves with and without preincubation and no significant IC_{50} shift. This observation aligns with previous clinical studies showing minimal CYP3A4-related drug-drug interactions involving azithromycin [24]. The absence of TDI further confirms azithromycin's lack of metabolite-intermediate complex formation or irreversible enzyme modification, supporting its favorable clinical pharmacokinetic profile and relatively low potential for interactions when co-administered with CYP3A4 substrates.

3.4 Erythromycin



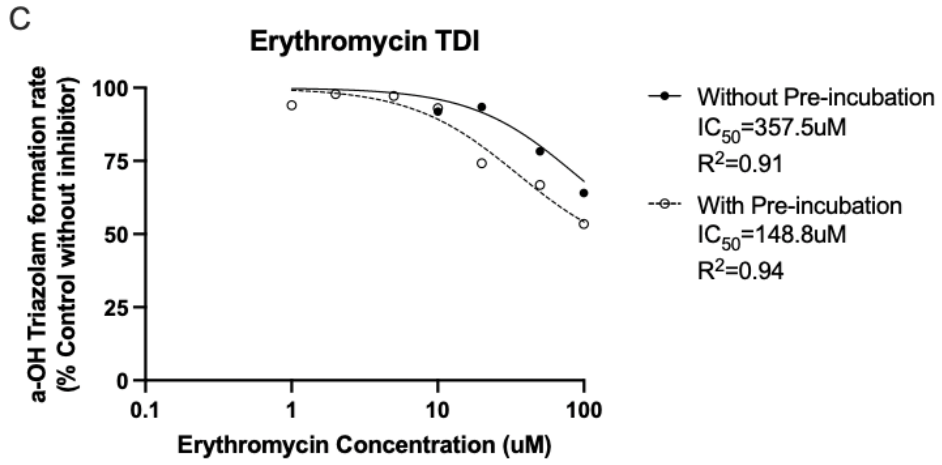


Figure 6. Inhibition of CYP3A4 activity by (A) Troleandomycin, (B) Ketoconazole, and (C) Erythromycin with (dashed lines) and without (solid lines) preincubation. Graphs show concentration-dependent inhibition as a percentage of control. IC_{50} values (μM) without and with preincubation: Troleandomycin (36.95, 8.18), Ketoconazole (0.057, 0.070), Erythromycin (357.5, 148.8). Data points represent means of duplicate experiments.

This study assessed the time-dependent inhibition (TDI) potential of erythromycin, a macrolide antibiotic, on CYP3A4 activity using human liver microsomes (HLMs) and triazolam as a probe substrate. As illustrated in Figure 4C, erythromycin exhibited notable TDI, with its IC_{50} decreasing from 357.5 μM without preincubation to 148.8 μM following preincubation—a 2.4-fold reduction. This shift suggests irreversible enzyme inactivation, likely mediated by metabolite-intermediate complex (MIC) formation, a mechanism well-documented for erythromycin's CYP3A4 inhibition.

For comparison, the positive control troleandomycin displayed a more pronounced TDI effect, as expected, with a 4.5-fold IC_{50} shift, consistent with its role as a prototypical mechanism-based inactivator. In contrast, ketoconazole, the negative control, showed minimal IC_{50} alteration, reaffirming its reversible inhibition profile. These results

corroborate prior evidence of erythromycin's capacity to induce clinically relevant drug-drug interactions (DDIs) when administered alongside CYP3A4 substrates, such as certain anticoagulants or immunosuppressants.

3.5 Josamycin

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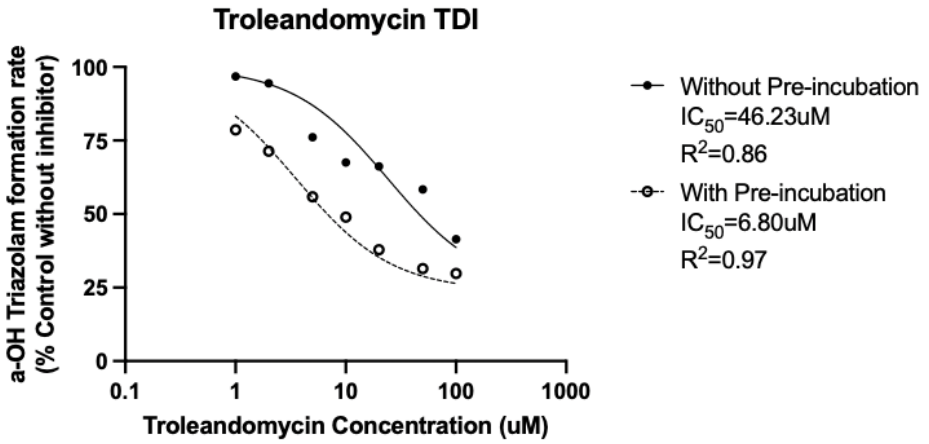

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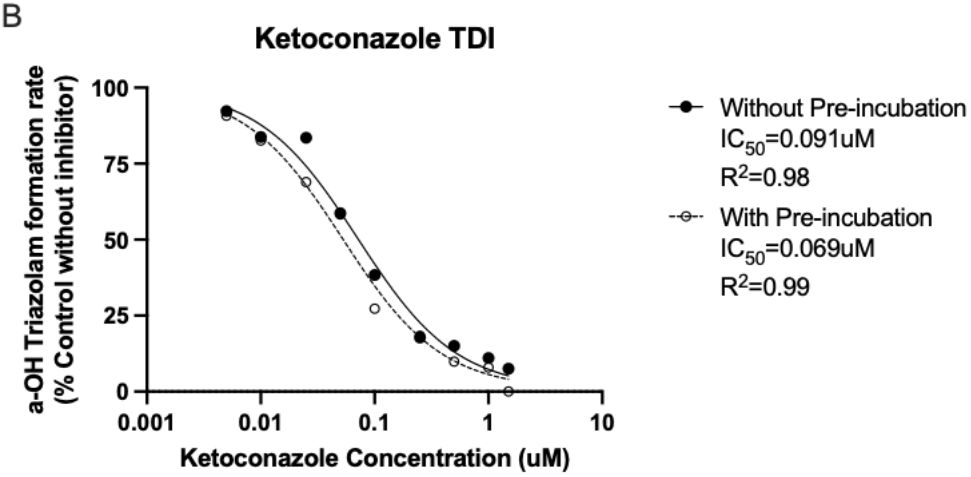

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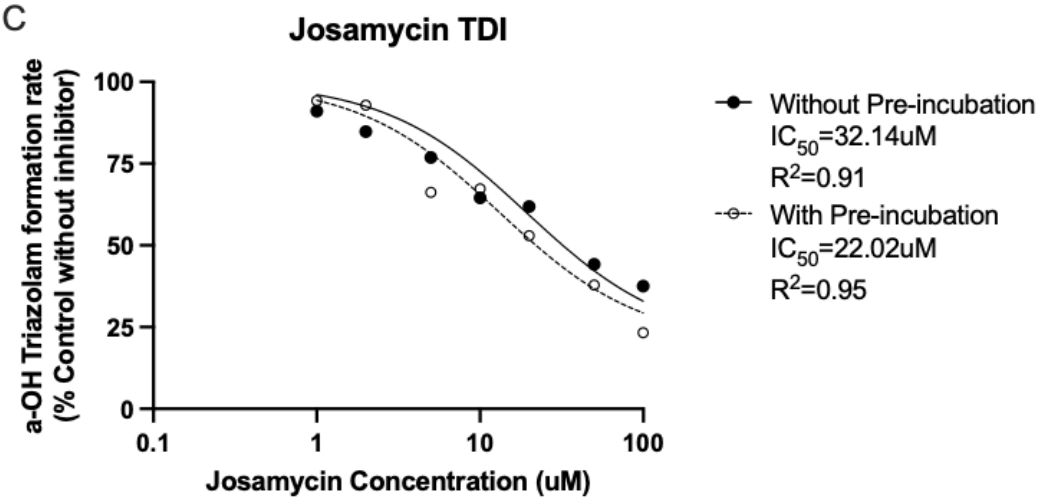

Figure 7. Inhibition of CYP3A4 activity by (A) Troleandomycin, (B) Ketoconazole, and (C) Josamycin with (dashed lines) and without (solid lines) preincubation. Graphs show concentration-dependent inhibition as a percentage of control. IC_{50} values (μM) without and with preincubation: Troleandomycin (46.23, 6.80), Ketoconazole (0.091, 0.069), Josamycin (32.14, 22.02). Data points represent means of duplicate experiments.

The study investigated the time-dependent inhibition (TDI) potential of josamycin on CYP3A4 activity, using troleandomycin as a positive control (mechanism-based inactivator) and ketoconazole as a negative control (reversible inhibitor). Troleandomycin demonstrated a marked reduction in IC_{50} from 46.23 μM without preincubation to 6.80 μM after preincubation, reflecting a 6.8-fold shift—consistent with its known irreversible inactivation of CYP3A4 via metabolite-intermediate complex formation. In contrast, ketoconazole exhibited negligible TDI, with IC_{50} values remaining nearly unchanged (0.09 μM without preincubation vs. 0.069 μM with preincubation), aligning with its reversible inhibition mechanism. Josamycin displayed a modest IC_{50} shift from 32.14 μM (without preincubation) to 22.02 μM (with preincubation), corresponding to a 1.5-fold decrease. This marginal shift, while indicative of weak time-dependent effects, falls near the threshold (1.5–2-fold) often considered biologically relevant in regulatory assessments. The inhibition curves (Figure 5) further support this observation: unlike troleandomycin's steep post-preincubation curve, josamycin's inhibition profile showed only slight enhancement, suggesting transient or partial enzyme interactions rather than robust mechanism-based inactivation. These findings imply that while josamycin exhibits limited TDI potential compared to troleandomycin, its borderline IC_{50} shift warrants cautious evaluation in clinical settings, particularly when co-administered with narrow-therapeutic-index CYP3A4 substrates.

3.6 Clarithromycin

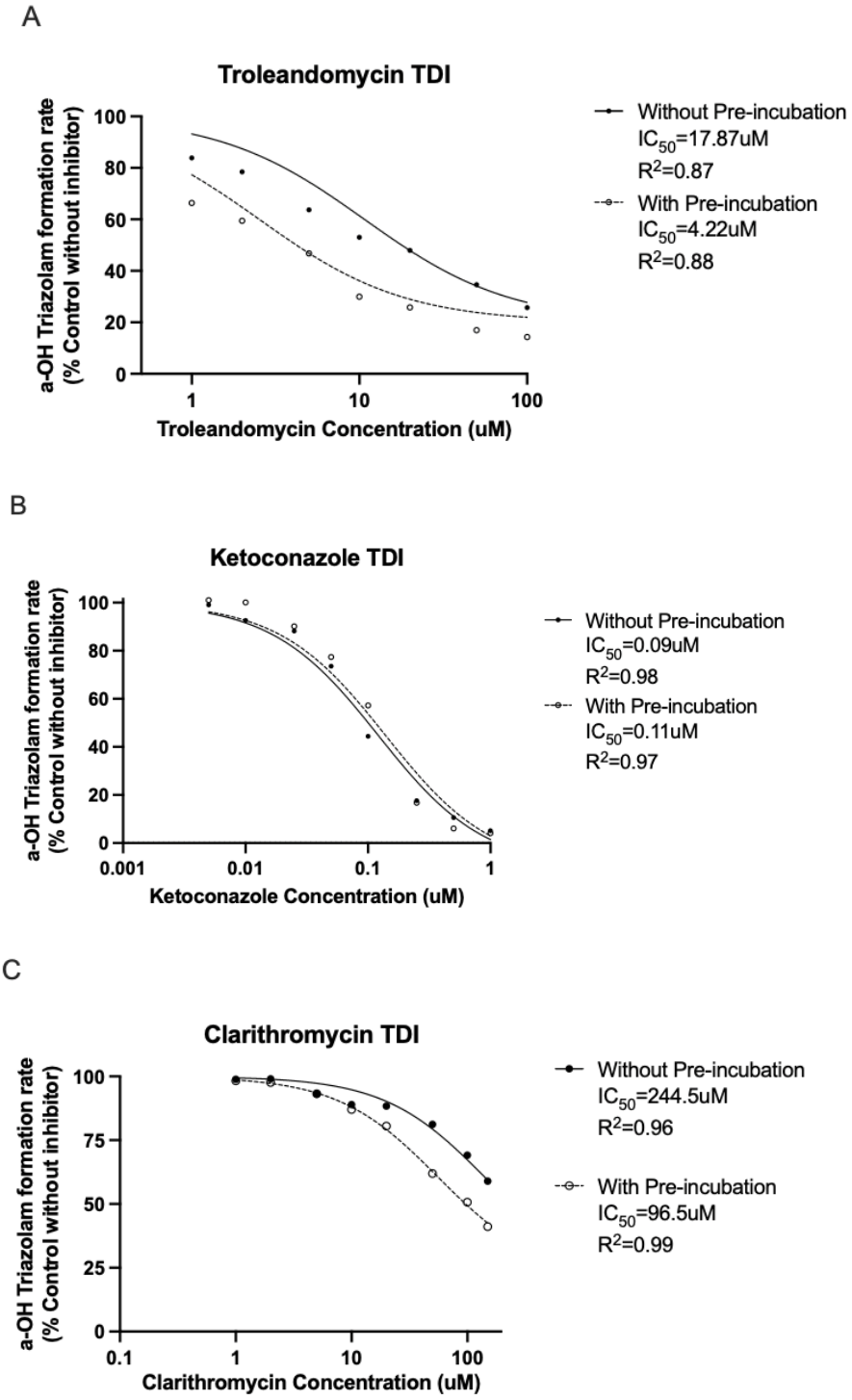

Figure 8. Inhibition of OH-Triazolam formation by (A) Troleandomycin, (B) Ketoconazole, and (C) Clarithromycin with (dashed lines) and without (solid lines) preincubation. Graphs show concentration-

dependent inhibition as a percentage of control. IC_{50} values (μM) without and with preincubation: Troleandomycin (17.87, 4.22), Ketoconazole (0.09, 0.11), Clarithromycin (244.5, 96.5). Data points represent means of duplicate experiments.

The results indicate moderate TDI, with clarithromycin's IC_{50} decreasing from 244.5 μM without preincubation to 96.5 μM following preincubation, a shift of approximately 2.5-fold. This shift suggests irreversible or quasi-irreversible enzyme inhibition through mechanisms such as metabolite-intermediate complex (MIC) formation. Clarithromycin's moderate inhibitory potency is clinically significant because it is widely prescribed for respiratory and gastrointestinal infections, often in patients with comorbidities and polypharmacy. Clinically, this TDI potential can lead to elevated plasma concentrations of co-administered CYP3A4 substrates, including drugs with narrow therapeutic windows such as statins, immunosuppressants (e.g., cyclosporine), and benzodiazepines (e.g., midazolam, triazolam). Consequently, careful therapeutic monitoring and dose adjustments may be required when clarithromycin is co-administered with these sensitive substrates.

4. Discussion

Time-dependent inhibition (TDI) of CYP3A4 is a critical pharmacokinetic concern during drug development due to its potential to prolong drug-drug interactions beyond inhibitor elimination [31]. The clinical importance of TDI arises from persistent enzyme inactivation, significantly impacting the metabolism of co-administered drugs, especially those with narrow therapeutic indices such as triazolam and statins. Thus, accurately

characterizing inhibitory mechanisms is essential for safer prescribing practices and reducing adverse drug reactions.

In this study, posaconazole demonstrated pronounced TDI, reflected by a 5.4-fold IC_{50} shift following preincubation. This strong inhibitory effect likely occurs via irreversible metabolite-intermediate complex (MIC) formation, resulting in persistent inhibition beyond systemic drug clearance and necessitating enzyme resynthesis for recovery of metabolic function. Erythromycin exhibited moderate TDI (2.4-fold shift), consistent with its established clinical interaction profile. Voriconazole showed borderline TDI (2.1-fold shift), suggesting possible interaction risks mainly for sensitive CYP3A4 substrates. In contrast, azithromycin displayed no significant TDI, aligning with its favorable safety profile and minimal interaction potential in polypharmacy settings. Differences observed among macrolide antibiotics emphasize structural factors that govern their TDI potential.

These findings highlight the necessity of complementary analytical approaches, such as physiologically based pharmacokinetic modeling, to better predict clinical interactions—particularly for compounds exhibiting borderline TDI like josamycin (1.5-fold shift). Future research should also quantify enzyme recovery kinetics following TDI exposure and investigate structural modifications that mitigate mechanism-based enzyme inactivation, thereby promoting safer therapeutic outcomes.

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