

IN VITRO ANALYSIS OF AKR1D1 INTERACTIONS WITH CLOPIDOGREL: EFFECTS ON ENZYME ACTIVITY AND GENE EXPRESSION

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ABSTRACT

Clopidogrel, a P2Y₁₂ receptor antagonist, is widely used to prevent cardiovascular events, but significant variability in its efficacy persists among patients. AKR1D1, involved in bile acid synthesis and regulation of CYP enzymes, may contribute to this variability. This study aims to investigate whether clopidogrel and its inactive metabolite, 2-oxoclopidogrel, interact with AKR1D1 at the enzymatic or transcriptional level. Enzymatic activity assays demonstrated that neither clopidogrel nor 2-oxoclopidogrel acts as a substrate or inhibitor of AKR1D1. Expression studies in HepG2 cells further revealed no significant changes in *AKR1D1* mRNA levels following treatment with these compounds. These findings indicate that clopidogrel does not directly influence AKR1D1's metabolic functions, including bile acid synthesis, steroid hormone clearance, or the production of 5 β -reduced steroids, which regulate CYP enzyme expression. From a physiological perspective, the absence of interaction minimizes the risk of adverse effects on CYP-mediated drug metabolism, nutrient absorption, lipid digestion, and the absorption of lipophilic drugs. Future research should explore AKR1D1's broader substrate specificity, particularly focusing on non-steroidal compounds, and investigate the clinical implications of AKR1D1 polymorphisms in clopidogrel-treated patients to enhance personalized therapeutic strategies.

Keywords: AKR1D1, clopidogrel, drug metabolism, enzyme interaction, pharmacogenomics, gene expression.

INTRODUCTION

Clopidogrel, a P2Y₁₂ receptor antagonist, is a cornerstone of dual antiplatelet therapy alongside aspirin, widely used to prevent major cardiovascular events in patients with acute coronary syndromes or undergoing percutaneous coronary interventions (1, 2). While clopidogrel has been shown to be more effective than aspirin in reducing the risks of myocardial infarction, ischemic stroke, and vascular death, there remains significant variability in treatment outcomes among patients (3, 4). This variability can range from reduced efficacy or resistance to therapy, affecting 5–44% of patients, to increased risk of bleeding due to excessive antiplatelet activity (5, 6). The phenomenon of “clopidogrel resistance” presents a major clinical challenge, particularly because the underlying mechanisms are not fully understood (7–9).

The metabolism of clopidogrel is complex. As a pro-drug, it undergoes two primary processes: hydrolysis by carboxylesterase 1 (CES1), which inactivates the majority of the drug, and a two-step activation mediated by several cytochrome P450 (CYP) enzymes, which convert clopidogrel to its active form (10, 11). Among these, CYP2C19 is the most crucial, with polymorphisms in this enzyme (*CYP2C19**2, *3, and *17) being major determinants of clopidogrel's variable pharmacokinetics and treatment outcomes (12–14). However, these genetic variations explain only about 12% of the observed variability, leaving much of the interindividual differences unexplained (12, 15).

The variability in CYP enzyme activity and its effects on drug metabolism extend beyond clopidogrel, as CYP enzymes play a pivotal role in the biotransformation of many medications (16). Aldo-Keto Reductase 1D1 (AKR1D1), an enzyme critical in bile acid synthesis and steroid clearance (17–19), has been identified by Chaudhry et al. (2013) as a key trans-regulator of the CYP enzyme network, suggesting a broader regulatory

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mechanism. *AKR1D1* regulates *CYP2C19*, *CYP3A4*, and *CYP2C9* expression via its metabolic products. Specifically, the 5 β -reduced steroids generated by AKR1D1 activity act as ligands for nuclear receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR), which in turn regulate CYP enzyme expression (20, 21). The *AKR1D1**36 polymorphism (*rs1872930*), in particular, leads to increased expression of *AKR1D1*, resulting in the upregulation of these CYP enzymes (21).

Kapedanovska et al. (2019) explored this hypothesis in the context of clopidogrel and demonstrated that the *AKR1D1**36 allele is associated with an increased risk of major adverse cardiovascular and cerebrovascular events (MACCE) in patients treated with clopidogrel, establishing *AKR1D1* as an independent risk factor (22).

Building on previous findings linking *AKR1D1**36 to the regulation of the cytochrome P450 enzyme network, this study seeks to elucidate the potential role of AKR1D1 in clopidogrel metabolism. Specifically, the research aims to evaluate whether clopidogrel and its inactive metabolite, 2-oxoclopidogrel, act as substrates or inhibitors of the AKR1D1 enzyme, thereby elucidating potential interactions at the protein level. Furthermore, the study investigates the influence of clopidogrel and its metabolite on *AKR1D1* gene expression in HepG2 cells to explore potential mechanisms that could modulate clopidogrel metabolism. These investigations are intended to provide a deeper understanding of AKR1D1's involvement in drug metabolism and its broader implications for pharmacological research.

MATERIALS AND METHODS

Reagents and Chemicals

– Molecular Cloning Reagents:

- Full-length *AKR1D1* cDNA (OriGene, Rockville, MD, USA; Cat# RC223056): Used for amplifying and cloning the *AKR1D1* gene into an expression vector.
- pET28b+ vector (Novagen (Merck KGaA), Darmstadt, Germany; Cat# 69865-3): Expression vector used for recombinant protein production.

– Enzyme Assay Reagents:

- Testosterone (Sigma-Aldrich, St. Louis, MO, USA; Cat# T1500): Substrate for AKR1D1 activity assays.
- Clopidogrel Bisulfate (Sigma-Aldrich, St. Louis, MO, USA; Cat# C0612): Evaluated for interactions with AKR1D1 at both enzymatic and transcriptional levels.

- 2-oxoclopidogrel (Cayman Chemical, Ann Arbor, MI, USA; Cat# 20394): Metabolite of clopidogrel tested for interactions with AKR1D1 at both enzymatic and transcriptional levels.

– Cell Culture Reagents:

- HepG2 Hepatocyte Cell Line (ATCC, Manassas, VA, USA; Cat# HB-8065): An in vitro model system used to study AKR1D1 expression and hepatic metabolism.
- Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA; Cat# D6429): Basal medium for HepG2 cell maintenance.
- Fetal Bovine Serum (FBS) (Capricorn Scientific, Ebsdorfergrund, Germany; Cat# FBS-12A): Supplemented in DMEM to support cell growth and viability.
- Trypsin-EDTA Solution (Lonza, Walkersville, MD, USA; Cat# CC-5012): Used for cell detachment during subculturing.

– MTT Assay Reagents:

- MTT Solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA; Cat# M2128): Used to evaluate cell viability through formazan crystal formation.
- Dimethyl Sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA; Cat# D12345): Used to dissolve formazan crystals for spectrophotometric analysis.
- NADPH (Sigma-Aldrich, St. Louis, MO, USA; Cat# N7505): Essential cofactor for spectrofluorometric enzyme activity assays.

– Expression Assay Reagents

- HpaI Restriction Enzyme (New England Biolabs, Ipswich, MA, USA; Cat# R0105S): Used for linearizing the *AKR1D1* plasmid to generate a calibration curve for qRT-PCR quantification.
- Shrimp Alkaline Phosphatase (Takara Bio, Shiga, Japan; Cat# 2650A): Used for dephosphorylating the linearized plasmid DNA to prevent self-ligation.
- TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA; Cat# T9424): Used for total RNA extraction from HepG2 cells.
- ProtoScript® II Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA; Cat# M0368): Used to synthesize complementary DNA (cDNA) from isolated RNA.
- XCEED qPCR SG 2x Mix Lo-ROX (Institute of Applied Biotechnologies, Prague, Czech Republic; Cat# QR0100): Used for quantitative real-time PCR analysis of *AKR1D1* gene expression.

- Forward and Reverse Primers (GeneriBiotech, Hradec Králové, Czech Republic): Specific primers designed for the amplification of *AKR1D1* during quantitative real-time PCR.

METHODS

Cloning, Expression, and Purification of AKR1D1

The coding sequence for the wild-type *AKR1D1* (*AKR1D1*1*) was amplified and subcloned into the pET28b+ vector using standard molecular cloning techniques (23,24). *E. coli* BL21 (D3) cells were transformed with the prepared pET28b+ *AKR1D1* vector, and over-expression of the enzyme was induced using isopropyl β -D-1-thiogalactopyranoside (IPTG). The enzyme was subsequently purified using the NGC™ Discover™ 100 Pro chromatography system (Bio Rad, USA). The cloning, expression, and purification methods for *AKR1D1*, including optimization steps, have been comprehensively described in a recent publication (25).

Standard Spectrofluorometric Assay and Enzyme Studies

Substrate Evaluation: The enzymatic reduction of testosterone to 5 β -dihydrotestosterone (5 β -DHT) was monitored using a spectrofluorometric assay. The reaction mixture contained *AKR1D1* (7.8 μ M), NADPH (15 μ M), and testosterone (10 μ M) in 100 mM potassium phosphate buffer (pH 6.0). The reduction was followed by measuring NADPH fluorescence, with an excitation wavelength of 340 nm and an emission wavelength of 460 nm, on an Infinite M200 Tecan spectrophotometer (Tecan, Switzerland) over a 45-minute period. For substrate evaluation, testosterone was replaced with either clopidogrel or 2-oxoclopidogrel at a final concentration of 50 μ M.

Inhibition Studies: For inhibition studies, *AKR1D1* (7.8 μ M) was pre-incubated with clopidogrel or 2-oxoclopidogrel (10 μ M or 50 μ M) in the same reaction conditions as described above. Reactions were initiated by the addition of NADPH, and fluorescence changes were monitored over 45 minutes.

The concentrations of clopidogrel and 2-oxoclopidogrel (10 μ M and 50 μ M) were chosen based on established practices in enzymatic studies involving related *AKR1C* enzymes (26). The 50 μ M concentration was selected to ensure sufficient substrate availability for detecting potential enzymatic activity, while 10 μ M and 50 μ M were used in inhibition studies to observe potential dose-dependent effects. This approach allows for exploratory assessment of *AKR1D1* interactions with these compounds under conditions aligned with prior enzymatic studies.

Cell Culture Preparation and Viability Assessment

HepG2 cells, chosen for their similarity to *AKR1D1* expression in primary human liver cells (27), were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere.

An MTT assay was performed to determine non-toxic concentrations of clopidogrel and 2-oxoclopidogrel for expression studies. HepG2 cells were treated with concentrations ranging from 0.1 μ M to 50 μ M for 48 hours, and cell viability remained >90% at all tested concentrations. Based on these findings 5 μ M was selected as a safe and physiologically relevant concentration for further experiments.

Expression Studies

HepG2 cells were seeded into 12-well plates (1,600,000 cells per well) and allowed to adhere for 24 hours. Cells were then treated with 5 μ M clopidogrel or 2-oxoclopidogrel for 24 hours. Following treatment, total RNA was extracted using TRI Reagent®, and RNA quality and concentration were assessed spectrophotometrically. Complementary DNA (cDNA) was synthesized using reverse transcriptase, and 80 ng of cDNA was utilized for quantitative real-time PCR (qRT-PCR) analysis.

qRT-PCR was conducted in triplicate to quantify *AKR1D1* expression, with amplification specificity validated by dissociation curve analysis. Absolute quantification was achieved using a calibration curve generated from serial dilutions of linearized *AKR1D1* cDNA.

To prepare the plasmid for the calibration curve, the *AKR1D1* plasmid was linearized using the *HpaI* restriction enzyme and subsequently dephosphorylated with shrimp alkaline phosphatase to prevent self-ligation.

The concentration of 5 μ M aligns with previous studies involving *AKR1D1*-related compounds, such as anabolic steroids, where 5 μ M approximates intracellular levels after cellular uptake (28). While plasma concentrations of clopidogrel and its metabolites are in the nanomolar range due to rapid metabolism (12), the chosen concentration ensures sufficient exposure to detect potential regulatory effects on *AKR1D1* expression in vitro (29).

Statistical Analysis

Statistical comparisons between treated and control groups were conducted using a two-tailed Student's t-test. Data are presented as mean $\pm$ standard deviation (SD), with significance set at $p < 0.05$. All experiments were performed in triplicate unless otherwise stated.

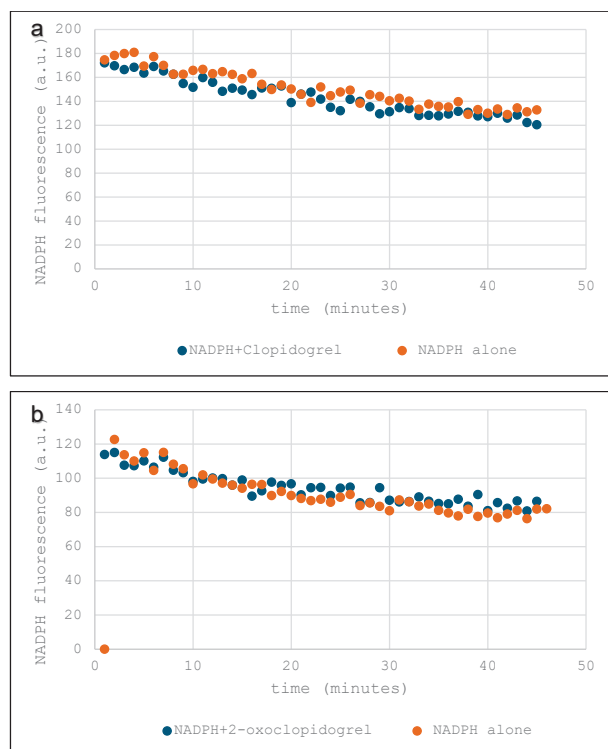


Figure 1. Evaluation of AKR1D1 substrate specificity using NADPH fluorescence assays for: (a) clopidogrel (50 μ M) and (b) 2-oxoclopidogrel (50 μ M). Changes in NADPH fluorescence over time were measured in the presence (blue) and absence (orange) of the substrate candidates. No significant differences were observed ($p > 0.05$).

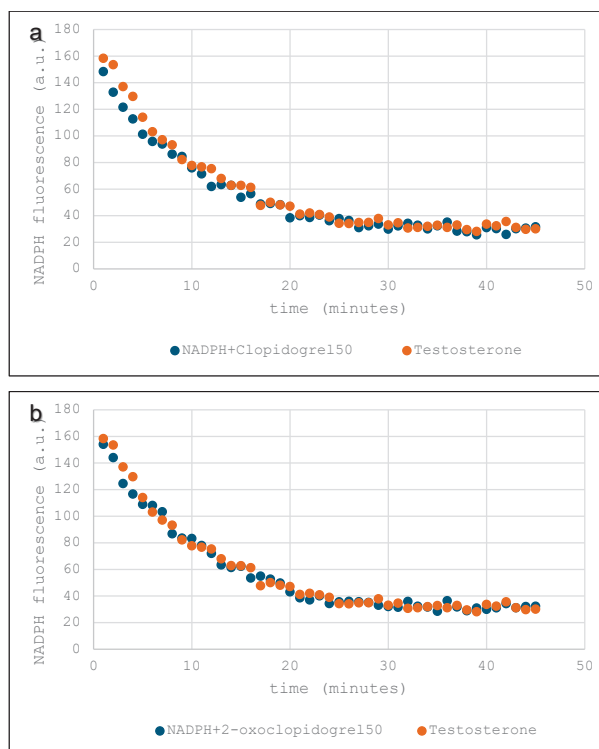


Figure 2. Evaluation of the inhibitory activity of (a) clopidogrel (50 μ M) and (b) 2-oxoclopidogrel (50 μ M) on AKR1D1. NADPH fluorescence was measured in the presence of the candidate inhibitor and testosterone (blue) and compared to the response to testosterone alone (orange). No significant differences were observed ($p > 0.05$).

RESULTS

Enzyme Substrate and Inhibition Studies

The potential role of clopidogrel and 2-oxoclopidogrel as substrates or inhibitors of AKR1D1 was investigated through a set of enzymatic activity assays using spectrofluorometric measurements. The enzymatic activity was monitored by measuring NADPH fluorescence, and no significant changes were observed in the presence of either clopidogrel or 2-oxoclopidogrel, suggesting that neither compound acts as a substrate (Fig. 1) or an inhibitor (Fig. 2) of AKR1D1. The fluorescence remained unchanged across all treatment groups, supporting the conclusion that these compounds do not interact directly with AKR1D1 ($p > 0.05$).

Expression Studies

The impact of clopidogrel and 2-oxoclopidogrel on AKR1D1 expression was assessed by conducting qRT-PCR on HepG2 cells treated with each compound. The absolute quantification approach, employing a calibration curve generated from linearized AKR1D1, revealed

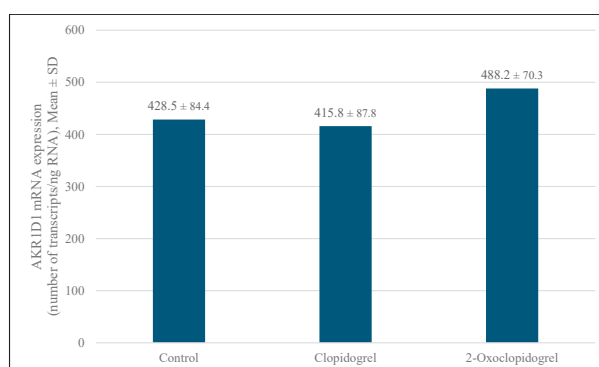


Figure 3. AKR1D1 mRNA expression levels (mean $\pm$ SD) in HepG2 cells following treatment with clopidogrel and 2-oxoclopidogrel (5 μ M each). No significant changes were observed compared to the control group ($p > 0.05$).

no statistically significant changes in AKR1D1 mRNA levels in response to treatment with either clopidogrel or 2-oxoclopidogrel compared to control samples ($p > 0.05$; Fig. 3). These results suggest that these compounds do not induce or repress AKR1D1 expression at the transcriptional level in HepG2 cells.

DISCUSSION

The study provides valuable insights into the interaction of clopidogrel and its metabolite, 2-oxoclopidogrel, with the enzyme AKR1D1. The enzymatic assays demonstrate that neither clopidogrel nor its metabolite, 2-oxoclopidogrel, acts as a substrate or inhibitor of AKR1D1. This aligns with the known specificity of AKR1D1, which predominantly interacts with steroidal compounds and not with non-steroidal drugs like clopidogrel. The absence of inhibition is significant because it suggests that clopidogrel does not interfere with AKR1D1's essential role in bile acid synthesis and steroid hormone clearance, thus preserving critical metabolic processes (17, 18, 20, 30). Disruption of this processes could impair digestion, absorption, and drug solubilization, which further emphasizes the physiological importance of AKR1D1's functional integrity.

While AKR1D1's substrate specificity appears to favor steroidal compounds, its structural homology with AKR1C enzymes - which can interact with both steroidal and non-steroidal substrates - raises the possibility that AKR1D1 could be flexible under certain conditions (19, 30, 31). Although studies have demonstrated that certain non-steroidal compounds, including indomethacin, mefenamic acid, and 4-benzoylbenzoic acid, which are potent inhibitors of AKR1C enzymes, do not inhibit AKR1D1 (31), only three non-steroidal compounds have been evaluated to date. Given the limited scope of these investigations, the possibility remains that AKR1D1 could interact with other non-steroidal substrates or inhibitors, warranting further exploration.

The potential flexibility in AKR1D1's substrate specificity is an intriguing aspect of the enzyme's functionality, particularly when considered in the context of genetic polymorphisms, such as the *AKR1D1**36 variant that has been associated with an increased risk of major adverse cardiovascular and cerebrovascular events (MACCE) in patients treated with clopidogrel (22). While the *AKR1D1**36 polymorphism has been linked to altered expression levels and downstream effects on CYP enzyme regulation (21), its direct impact on enzymatic function and substrate specificity remains unexplored. By contrast, engineered mutations, such as the E120H substitution, have demonstrated that single amino acid changes can significantly alter AKR1D1's enzymatic activity and substrate specificity (32). These findings highlight the potential for naturally occurring genetic variants, like *AKR1D1**36, to similarly influence enzyme functionality, warranting further investigation.

Gene expression analyses further demonstrated that clopidogrel and 2-oxoclopidogrel do not modulate *AKR1D1* expression at the transcriptional level. This finding implies that clopidogrel does not interfere with the

production of 5 β -reduced steroids, metabolic products of AKR1D1 that regulate CYP enzyme expression via nuclear receptor pathways such as FXR, CAR, and PXR (21). Consequently, the lack of *AKR1D1* modulation by clopidogrel reduces the likelihood of indirect effects on CYP enzyme activity, which could otherwise alter the metabolism of co-administered drugs.

This study's findings should be interpreted with caution regarding clinical relevance. In vivo systems involve a complex interplay of metabolic factors that may not be fully represented in the in vitro model used here. Therefore, further research is necessary to validate these observations and assess the broader implications of AKR1D1 and its genetic variations on drug metabolism as well as their potential impact on clinical outcomes particularly in the context of personalized medicine.

CONCLUSION

This study provides foundational insights into AKR1D1's interactions with clopidogrel and its metabolite, suggesting that they do not directly influence AKR1D1 activity or expression. However, further research, particularly in vivo and genetic studies, is necessary to fully elucidate AKR1D1's role in drug metabolism and its potential implications for clinical practice.

ACKNOWLEDGMENTS

This research would not have been possible without the tremendous support of the Faculty of Pharmacy, Charles University in Prague, specifically at the Department of Biochemical Sciences. The authors wish to express their deepest gratitude to Professor Ing. Vladimír Wsól, Ph.D, Head of the Department, for providing the invaluable opportunity to conduct this research and for his guidance throughout the project. Special thanks are also extended to RNDr. Bc. Eva Novotná, Ph.D, whose expertise, patience, and support were instrumental in the successful completion of this work. The authors are profoundly grateful to the entire team in Prague for their hospitality, mentorship, and collaborative spirit, which significantly contributed to the advancement of this research. Their contributions were truly instrumental, and the authors hold the utmost respect for their professionalism and generosity.

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