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Impact of Genetic Polymorphisms and Drug-Drug Interactions Mediated by Carboxylesterase1 on Remimazolam Deactivation

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Running Title: Genetic Variants and DDIs Affecting Remimazolam Deactivation

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Non-standard Abbreviations

ACEIs , Angiotensin Converting Enzyme Inhibitors; AChE , Acetylcholinesterase; ACS, Acute Coronary Syndrome; ADHD, Attention-Deficit/Hyperactivity Disorder; ADP, Adenosine Diphosphate; ANOVA, Analysis of Variance; BChE, Butyrylcholinesterase; BCRP, Breast Cancer Resistance Protein; BNPP, Bis-para-nitrophenylphosphate; BSEP, Bile Salt Export Pump; CE, Collision Energy; CESs, Carboxylesterases; CES1, Carboxylesterase1; CES2, Carboxylesterase2; CV, Coefficient of Variation; CYP, Cytochrome P450; DDIs, Drug-drug Interactions; DMEM, Dulbecco's Modified Eagle Medium; EMA, European Medicines Agency; FBS, Fetal Bovine Serum; FDA, Food and Drug Administration; HIS9, Human Intestine S9 Fractions; HLS9, Human Liver S9 Fractions; HMG-CoA, Hydroxymethylglutaryl-Coenzyme A; IC_{50}, Half-maximal Inhibitory Concentration; ICU, Intensive Care Units; IS, Internal Standard; LC, Liquid Chromatography; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; LD, Linkage Disequilibrium; MAFs, Minor Allele Frequencies; MATE, Multidrug and Toxic

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Compound Extrusion; MDR, Multidrug Resistance Protein; nsSNP, Nonsynonymous Single Nuclear Polymorphism; OAT, Organic Anion Transporter; OATP, Organic Anion Transporting Polypeptide; OCT, Organic Cation Transporter; PBS, Phosphate-Buffered Saline; PD, Pharmacodynamics; PK, Pharmacokinetics; PONs, Paraoxonases; PRM, Parallel Reaction Monitoring; SNP, Single Nuclear Polymorphism; UPLC, Ultra Performance Liquid Chromatography; WT, Wild Type.

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Abstract

Remimazolam (Byfavo®), a recent FDA-approved ester-linked benzodiazepine, offers advantages in sedation, such as rapid onset and predictable duration, making it suitable for broad anesthesia applications. Its favorable pharmacological profile is primarily attributed to rapid hydrolysis, the primary metabolism pathway for its deactivation. Thus, understanding remimazolam hydrolysis determinants is essential for optimizing its clinical use. This study aimed to identify the enzyme(s) and tissue(s) responsible for remimazolam hydrolysis and to evaluate the influence of genetic polymorphisms and drug-drug interactions (DDIs) on its hydrolysis in the human liver. An initial incubation study with remimazolam and phosphate buffer saline (PBS), human serum, and the S9 fractions of human liver and intestine demonstrated that remimazolam was exclusively hydrolyzed by human liver S9 fractions. Subsequent incubation studies utilizing a Carboxylesterase inhibitor (Bis-para-nitrophenylphosphate, BNPP), recombinant human Carboxylesterase1 (CES1) and Carboxylesterase 2 (CES2) confirmed that remimazolam is specifically hydrolyzed by CES1 in human liver. Furthermore, *in vitro* studies with wild-type *CES1* and *CES1* variants transfected cells revealed that certain genetic polymorphisms significantly impair remimazolam deactivation. Notably, the impact of *CES1 G143E* was verified using individual human liver samples. Moreover, our evaluation of the DDIs between remimazolam and several other substrates/inhibitors of CES1-including simvastatin, enalapril, clopidogrel and sacubitril-found that clopidogrel significantly inhibited remimazolam hydrolysis at clinically relevant concentrations, with CES1 genetic variants potentially influencing the interactions. In summary, *CES1* genetic variants and its interacting drugs are crucial factors contributing

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to interindividual variability in remimazolam hepatic hydrolysis, holding the potential to serve as biomarkers for optimizing remimazolam use.

Significance

This investigation demonstrates that remimazolam is deactivated by CES1 in the human liver, with CES1 genetic variants and DDIs significantly influencing its metabolism. These findings emphasize the need to consider CES1 genetic variability and potential DDIs in remimazolam use, especially in personalized pharmacotherapy to achieve optimal anesthetic outcomes.

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Introduction

Approximately 40 million short diagnostic and therapeutic procedures requiring sedation are performed in the US annually (FDA 2012)(McMullan et al., 2017). However, a significant portion of the patients receiving anesthetics or sedatives experienced associated side effects or potential complications. These complications can lead to pain, discomfort, procedural failure or even additional health issues(Bellolio et al., 2016). Thus, the importance of precision anesthesia and sedation cannot be overstated, which ensures not only patient comfort and safety during interventions but also swift recovery, reducing monitoring needs and room occupancy time, consequently saving cost for healthcare system. Remimazolam (Byfavo®), a novel benzodiazepine ester derivative, was approved as a general anesthetic in Japan firstly in the world in Jan 2020. It received US FDA approval for induction and maintenance of procedural sedation in adults in July 2020(Keam, 2020). In comparison to current corner stone anesthetics and sedatives, like propofol and midazolam, remimazolam minimizes risk of respiratory depression and offers a smoother patient recovery(Wu et al., 2023; Zhao et al., 2023; Choe et al., 2024). Due to its promising profile, remimazolam is increasingly being considered for a wide range of surgical and medical procedures, such as general anesthesia, procedural sedation, intensive care unit (ICU) sedation, emergency medicine, outpatient procedures, cardiac interventions, and endoscopy(Goudra and Singh, 2014; Zhao et al., 2023; Dessai et al., 2024). However, its notable interindividual variability in patient responses poses a significant challenge, leading to patient distress and procedure failure(Kempenaers et al., 2023). Consequently, the extensive range of potential applications and common adverse effect underscore the critical need for the

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precise use of remimazolam, given its capacity to significantly enhance patient care across various medical settings(Goudra and Singh, 2014; Kim, 2022; Oue et al., 2023).

The favorably short and predictable action of remimazolam is largely attributed to its fast metabolism, primarily involving the hydrolysis of its ester group, which contributes to over 97% of its overall metabolic pathways(Schmalix et al., 2024). The ester linkage of remimazolam, connecting a small methyl alcohol to a big acyl group with a benodiazepine core, allows rapid hydrolysis to its inactive metabolite, remimazolam carboxylic acid (CNS7054). Notably, remimazolam exhibits approximately 300 times higher pharmacological activity than its hydrolytic metabolite CNS7054(Kilpatrick et al., 2007). It has been demonstrated that there are no significant interactions between remimazolam with CytochromP450s (CYPs), drug transporters, or plasma proteins(Petersen et al., 2024). Thus, the hydrolysis of remimazolam plays a crucial role in its disposition, enabling a precise control for its duration of sedation, which in turn significantly influences its overall efficacy and toxicity. However, the literature regarding remimazolam deactivation is marked by controversy(Kilpatrick et al., 2007; Antonik et al., 2012; Sneyd, 2012). Some studies suggest an involvement of organ-independent, nonspecific esterases in this process(Sneyd, 2012; Pambianco and Cash, 2016; Wesolowski et al., 2016; Noor et al., 2021; Kim, 2022), while others claimed Carboxylesterase1 (CES1) as the primary enzyme responsible for remimazolam deactivation(Freyer et al., 2019; Sneyd and Rigby-Jones, 2020; Ramamurthi and Hammer, 2023). This discrepancy highlights the need for further investigations focusing on the remimazolam deactivation in human, shedding light on its interindividual

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variability in response and potentially paving the way for precise anesthesia(Kilpatrick, 2021).

CES1 and carboxylesterase2 (CES2) are the major carboxylesterases, hydrolyzing various esters with distinct substrates preference. CES1 prefers the esters with larger acyl-group, whereas CES2 prefers esters with larger hydroxyl-group. These differences are due to variations in their catalytic triads—CES1 contains Ser221, Glu354, and His468, while CES2 has Ser228, Glu345, and His457. In addition to substrate specificity, these carboxylesterases exhibit tissue specificity. Although CES1 and CES2 coexist in human liver, CES1 is the primary hepatic esterase. In contrast, CES2, but not CES1, is found in human intestine(Laizure et al., 2013). As the primary hepatic hydrolase in humans, CES1 participates in the metabolism of numerous therapeutic agents. Significant interindividual variability in expression and activity of CES1 has been consistently reported(Her and Zhu, 2020). Our previous publications have demonstrated that several nonsynonymous *CES1* single nucleotide polymorphisms (nsSNPs), such as *CES1 G143E* (rs71647871), *E220G* (rs200707504), *L40Ter* (rs151291296), *Q169P* (rs143718310), *S75N* (rs2307240), and inhibitors are associated with significantly altered *in vitro* metabolism of several selective CES1 substrate drugs(Zhu et al., 2013; Cha et al., 2014; Shi et al., 2016a; Shi et al., 2016b; Wang et al., 2016; Wang et al., 2017). Notably, the genotypes of *G143E, E220G*, and *S75N* were associated with significantly altered pharmacokinetics (PK) and/or pharmacodynamics (PD) of CES1 substrate medications(Patrick et al., 2007; Zhu et al., 2008; Nemoda et al., 2009; Tarkiainen et al., 2012; Lewis et al., 2013; Tarkiainen et al., 2015a; Tarkiainen et al., 2015b; Xiao et al., 2017). Additionally, unexpected drug-drug interactions (DDIs) are

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another factor contributing to poor drug responses and severe adverse drug reactions, especially when the concurrently administered medications inhibit the metabolizing enzymes or transporters responsible for elimination or absorption(Magro et al., 2012; Bellosta and Corsini, 2018). In clinical practice, anesthetics are commonly applied to the patients who are already on multiple drugs. However, unlike the DDIs mediated by cytochrome P450s (CYPs) and Uridine 5'-diphospho-glucuronosyltransferases (UGTs) have been well documented in new drug application, those mediated by CES1 have often been overlooked as potential contributors to drug variability.

In the present study, we identified the enzyme and tissue that are responsible for remimazolam deactivation in human using human serum, s9 fractions prepared from human liver and intestine, carboxylesterases inhibitor, recombinant CES1 and Carboxylesterase2 (CES2) enzymes. We examined the impact of *CES1* nsSNPs on remimazolam deactivation using *WT CES1* and seven *CES1 variants* transfected cells and 54 individual human livers. Moreover, DDIs between remimazolam and other medications hydrolyzed by CES1 were also analyzed.

Methods

Materials

Remimazolam, (S)-(+)-Clopidogrel hydrogensulfate and CES inhibitor bis(4-nitrophenyl) phosphate (BNPP) were purchased from Sigma-Aldrich (St. Louis, MO). The hydrolytic metabolite of remimazolam was obtained via incubation of the parent compound (200 μM) with the supernatant 9000 (s9) fraction (2 mg protein/mL) prepared from the transfected cells stably expressing wild-type CES1. The hydrolytic reaction was

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completed in 3 hours at 37°C. The completion of the hydrolysis was confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The internal standard (IS) rac-Clopidogrel-d4 carboxylic acid was acquired from Toronto Research Chemicals, Inc. (Toronto, Canada). The pooled human liver S9 (20 mg/mL) and the human intestine S9 (4 mg/mL) were procured from XenoTech (Kansas City, KS). The human CES1 and CES2 recombinant enzymes (1 mg protein) were obtained from BioIVT (Westbury, NY). Simvastatin (sodium salt), Simvastatin carboxylic acid (ammonium salt), Clopidogrel carboxylic acid (hydrochloride), Enalapril (maleate), Enalaprilat (hydrate) were ordered from Cayman Chemical (Ann Arbor, Michigan). Sacubitril and Sacubitrilat were purchased from MedChemexpress LLC. All other chemicals and reagents were of analytical grade and commercially available.

A total of 54 individual liver samples were obtained from the Cooperative Human Tissue Network (CHTN, Columbus, OH) and the XenoTech LLC (Kansas City, KS). Except for two livers' demographic information was unknown, other livers consisted of 30 females, and 22 males with ages ranging from 22 to 81 years (58.29 ± 13.98 years). The donors included 47 Caucasians, four African Americans, and one classified as others.

Preparation of S9 from CES1 transfected cells and individual livers

The S9 fractions from CES1 transfected cells and individual livers were prepared according to previous studies(Shi et al., 2016a; Shi et al., 2016b; Wang et al., 2016). In brief, human embryonic kidney cells (Flp-In-293; Invitrogen, Carlsbad, CA) stably expressing wild-type CES1 (*WT CES1*), and nonsynonymous CES1 variants (G143E, E220G, L40Ter, S75N, Q169P, T167S, and T290M) have been established and verified as described previously(Zhu et al., 2008; Wang et al., 2017). Cells were cultured in

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Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After reaching approximately 90% confluence, cells were rinsed, harvested and sonicated in phosphate buffered saline (PBS) buffer. Individual human livers were homogenized in cold PBS. Sonicated cells and individual liver homogenates were centrifuged at 9000g for 30 min at 4 ºC. The supernatant was collected as S9 fractions and stored at –80ºC until use.

Enzymatic activity assays

In vitro hydrolytic activities on remimazolam were assessed in PBS, human serum, S9 fractions from human liver (HLS9), intestine (HIS9), *wild-type CES1* (*WT CES1*), *CES1 nsSNPs* (*G143E, E220G, L40Ter, S75N, Q169P, T167S,* and *T290M*), and vector transfected cells (Vector), as well as recombinant CES1 and CES2. The hydrolytic reaction was initiated by mixing remimazolam with these s9 fractions or recombinant enzymes. The final concentration of remimazolam was 200 μM, and the final protein concentrations of human serum, HLS9, HIS9, *WT-CES1*-, *CES1 nsSNPs*- and vector-transfected cell s9 fractions were 0.2 mg/mL. A series of final protein concentrations at 5 ng/μL, 10 ng/μL, and 25 ng/μL were applied for the recombinant CES1 and CES2. After an incubation at 37 ºC for 20 minutes, remimazolam hydrolysis was terminated by adding four-fold of methanol containing internal standard (IS) of rac-Clopidogrel-d4 carboxylic acid at 100 ng/mL. Following a thorough vortex, the mixture was subject to centrifuge at the highest speed for 15 min at 4 ºC. The supernatant was transferred to a clean autosampler vial for LC-MS/MS assay.

Additionally, we included the selective CES1 substrate (sacubitril) and CES2 substrate (fluorescein diacetate), as positive controls to indicate CES1 and CES2 activities in

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these S9 fractions and recombinant enzymes, respectively. For the sacubitril and fluorescein diacetate incubation methods, we followed protocols described in our previous publication(Shi et al., 2016a) with some minor changes. Briefly, for sacubitril, the final concentrations of sacubitril and protein concentrations of human serum, HLS9 and HIS9 were 400 μM and 0.2 mg/mL, respectively. Sacubitril hydrolysis was terminated by adding four-fold of methanol containing IS of rac-Clopidogrel-d4 carboxylic acid at 100 ng/mL after a 10 min incubation. Following a vigorous vortex and a centrifuge at maximum speed as described above, the supernatant was transferred to a clean vial for LC-MS/MS analysis. For fluorescein diacetate, the final concentrations of fluorescein diacetate and protein concentrations of serum, HLS9 and HIS9 were 5 μM and 0.02 mg/mL, respectively. After a 10 min incubation, the formation of hydrolytic metabolite of fluorescein diacetate was measured using a SpectraMax M5 Microplate Reader (Molecular Devices Inc., San Jose, CA) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

For the enzymatic kinetic study, we incubated 0.2 mg/mL S9 fractions prepared from *WT CES1*, *CES1 G143E*, and *CES1 S75N* transfected cells with remimazolam at a series concentrations ranging from 0 to 500 μM. The rest incubation conditions were the same as the remimazolam hydrolytic activity assays described above.

Drug-drug interaction studies (DDIs)

In vitro DDIs between remimazolam and carboxylesterases inhibitor (BNPP), CES1 inhibitor and its metabolite (simvastatin and simvastatin acid), as well as CES1 substrates (clopidogrel, sacubitril, and enalapril) were evaluated using the S9 fractions prepared from pooled human liver (HLS9). To assess the impact of CES1 variants on

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DDIs, we compared the inhibitory effect of clopidogrel on remimazolam in the S9 fractions prepared from *WT CES1, CES1 G143E, and CES1 S75N* transfected cells.

Following a 20-minute pre-incubation of the S9 fractions (0.2 mg/mL) with the inhibitors, CES1 substrates were added to initiate the hydrolytic reaction. Specifically, the final concentrations of examined CES1 inhibitors, except for the BNPP (0 to 30 μM), ranged from 0 to 1000 μM. When applied as CES1 substrates, the final concentrations were 20 μM for remimazolam and 30 μM of clopidogrel, sacubitril, and enalapril. The co-incubation time for the substrate remimazolam was 20 minutes, while for other substrates including clopidogrel, sacubitril and enalapril, it was 10 minutes. The sample preparation procedures from the termination of hydrolysis were the same as described in the enzymatic assay section.

LC-MS/MS analysis

The LC-MS/MS analysis was performed on a Vanquish UPLC system (Thermo Fisher Scientific Inc., Waltham, MA) coupled with a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Analytes were separated on a Waters Acquity UPLC HSS T3 column (1.8 um, 2.1 × 100 mm) under 40 °C column temperature. The mobile phase consisted of 2 mM ammonium formate water solution containing 0.1% formic acid (v:v) as phase A, and 2 mM ammonium formate methanol solution containing 0.1% formic acid (v:v) as phase B, and was delivered at a flow rate of 0.3 mL/min. All analytes in remimazolam and sacubitril assays were successfully separated using the 10 min gradients (**Supplementary Table S1**). Parallel reaction monitoring (PRM) was applied to monitor all MS2 ions associated with respective MS1 ions under positive mode. Then the skyline software (University of Washington, Seattle,

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WA, USA) was used to extract specific transitions for quantifying the targeted analytes those were detected under respective collision energy (**Supplementary Table S2**).

Results

Remimazolam is specifically hydrolyzed by human hepatic CES1

We carried out a pilot study to determine the enzyme and tissue responsible for the hydrolysis of remimazolam using human liver (HLS9) and intestine S9 fractions (HIS9) and human serum. Remimazolam carboxylic acid (CNS7054) was rapidly formed after incubating remimazolam with human liver, hydrolysis rate = 1.25 ± 0.06 nmol/min/mg protein). In comparison, incubation with PBS, human serum and human intestine (HIS9) did not yield any appreciable metabolite, indicating remimazolam was specifically hydrolyzed by CES1 in human liver (**Figure1**). The same patterns were observed for the hydrolysis of a known CES1 substrate sacubitril. Fluorescein diacetate, an established CES2 substrate, was included in the study as a control indicating CES2-mediated hydrolysis. Fluorescein diacetate was metabolized by HLS9 and HIS9, where CES2 was expressed (**Figure1**).

We further examined the remimazolam hydrolysis in HLS9 samples with the presence of BNPP, a selective inhibitor of carboxylesterases (CES1 and CES2). BNPP showed strong inhibition on remimazolam hydrolysis with a IC_{50} of 102.1 nM, and the hydrolysis was completely inhibited at a low BNPP concentration of 1μM (**Figure 2**). Additionally, we verified the carboxylesterase that specifically hydrolyzes remimazolam using recombinant CES1 and CES2. As expected, remimazolam was only hydrolyzed/ deactivated by recombinant CES1 in an enzyme concentration dependent manner

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(**Figure 3**), confirming that remimazolam is selectively hydrolyzed/deactivated by CES1. Furthermore, we obtained the Michaelis-Menten curve of remimazolam hydrolysis in the s9 prepared from WT CES1 transfected cells. The V_{max} and K_m were determined as 7.25 nmol/mg protein/min and 73.20 μM, respectively (**Figure 4**).

The impact of *CES1* nonsynonymous variants on remimazolam hydrolysis in transfected cells

To study the impact of nonsynonymous *CES1* single nucleotide polymorphisms (nsSNPs) on the hydrolysis of remimazolam, we examined the remimazolam hydrolysis rates in the S9 fractions prepared from wild-type CES1 (*WT CES1*), *CES1* nonsynonymous variants- *(E220G*, *Q169P*, *S75N*, *T167S*, *T290M* and *G143E)* and vector- transfected cell lines (Vector). The minor allele frequencies (MAFs) and the relative activities of these *CES1* nsSNPs on remimazolam and sacubitril hydrolysis are listed in **Table 1**. Consistent with our previous reports for other CES1 substrates, remimazolam was efficiently hydrolyzed in *WT CES1* at a rate of 31.30 ± 2.52 nmol/min/mg protein, while no appreciable hydrolysis was detected in *L40Ter* or Vector groups. Significant impairments were observed in the *E220G* (V_{E220G}=9.98 $\pm$ 0.50 nmol/min/mg protein, P=0.0177), *Q169P* (V_{Q169P}=7.22 $\pm$ 0.29 nmol/min/mg protein, P=0.0116), and *G143E* groups (V_{G143E}=5.96 $\pm$ 0.05 nmol/min/mg protein, P=0.0097). In contrast, *S75N* significantly increased the remimazolam deactivation rate to nearly two-fold that of the *WT CES1* group (V_{S75N}=61.03$\pm$ 2.84 nmol/min/mg protein, P=0.0228). Sacubitril was included as positive control, and the impact of these *CES1* nonsynonymous SNPs on sacubitril activation was consistent with our previous study (**Figure 5 and Table 1**).

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Influence of *CES1* SNPs *G143E* and *S75N* on the enzymatic kinetics of remimazolam hydrolysis

Consistent with the activity assays for remimazolam hydrolysis in CES1 nonsynonymous variants, the S75N demonstrated the highest V_{max} at 22.15 nmol/mg protein/min, followed by the WT CES1 with a V_{max} of 7.25 nmol/mg protein/min, and the G143E variant with the lowest V_{max} of 1.46 nmol/mg protein/min. Interestingly, the K_m values did not align with the V_{max} results. WT CES1 exhibited highest affinity for remimazolam, with the lowest K_m of 73.2 μM, followed by G143E with the K_m of 122.3 μM, while S75N showed the lowest affinity with a K_m of 334.0 μM (**Figure 6**).

Impact of *CES1* SNPs *G143E and S75N* on hydrolysis of remimazolam in individual human livers

We further confirmed the impact of two common *CES1* nsSNPs, including *CES1 G143E* and S75N, using 54 individual human livers. Remimazolam hydrolysis rates in five human livers from *G143E* carriers were compared to those in 49 non-carriers. Consistent with the results obtained from *CES1* variants transfected cells, the remimazolam deactivation rates in *G143E* carriers were significantly lower than those in the non-carriers (**Figure 7**, *G143E* carriers: 7.51 ± 3.14 *VS* non-carriers: 13.88 ± 6.53 nmol/mg protein/min, P=0.0164). For the S75N, we identified seven heterozygous carriers and 47 non-carriers in a total of 54 livers. Notably, none of the seven S75N carriers co-carried G143E. We observed a slight increase in the remimazolam deactivation rates in the S75N carriers compared to those non-carriers ((**Figure 7**, S75N carriers: 14.89 ± 6.41 *VS* non-carriers: 13.05 ± 6.60 nmol/mg protein/min), which was not statistically significant (P=0.4635).

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Drug-drug interactions between remimazolam and other known CES1 substrate/inhibitor medications in human liver S9

We investigated *in vitro* inhibitory effect of remimazolam on CES1 activities by assessing its impact on the hydrolysis of other CES1 substrates, including enalapril, sacubitril, and clopidogrel. Additionally, we examined the influence of CES1 inhibitors and substrates-including simvastatin, simvastatin acid, enalapril, clopidogrel and sacubitril-on remimazolam hydrolysis. Remimazolam inhibited CES1 activities on hydrolyzing sacubitril, clopidogrel, and enalapril, with IC_{50} values of 113.3 μM, 171.9 μM, and 214.8 μM, respectively (**Figure 8A**). Conversely, the hydrolysis of remimazolam was inhibited by the CES1 inhibitor simvastatin and its metabolite simvastatin acid, as well as the CES1 substrates clopidogrel, enalapril and sacubitril with IC_{50} values of 179.5 μM, 146.8 μM, 73.3 μM, 171.9 μM, 467.1 μM, respectively (**Figure 8B**).

Effect of *CES1* SNPs *G143E and S75N* on the inhibitory effect of clopidogrel on remimazolam hydrolysis

To evaluate the impact of *CES1* nonsynonymous variants on CES1- mediated DDIs, we compared the inhibitory effect of clopidogrel on remimazolam hydrolysis between *CES1* variants and the *WT CES1*. The S9 fractions prepared from *CES1 G143E* and *S75N* variants were used to represent the variants with decreased- and increased- CES1 activity, respectively. Clopidogrel exhibited varying inhibitory effects on Remimazolam across G143E, WT CES1, and S75N variants, with IC_{50} values of 119.0 μM, 151.5 μM, and 181.3 μM, respectively, ranked from lowest to highest (**Figure 9).**

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Discussion

The hydrolysis of remimazolam ester bond permits its rapid deactivation, resulting in a unique and favorable pharmacological profile, including short-acting and predictable duration of action. However, literature shows controversy regarding the enzymes responsible for its hydrolysis process(Kilpatrick, 2021). While some sources claim remimazolam is hydrolyzed via organ-independent esterases(Sneyd, 2012; Goudra and Singh, 2014; Wesolowski et al., 2016; Zhou et al., 2017; Noor et al., 2021; Hu et al., 2022; Kim, 2022; Lee, 2022; Chen et al., 2023), others suggest CES1 is the primary enzyme involved(Luo et al., 2024; Schmalix et al., 2024). An often-cited study linking CES1 to remimazolam hydrolysis did not examine the enzyme or tissue specificity but rather the impact of remimazolam on CES1 mRNA levels(Freyer et al., 2019).

To clarify remimazolam hydrolysis/deactivation process, we utilized various human tissues, a carboxylesterase inhibitor, and recombinant enzymes. Given the tissue-specific expression patterns of human esterases, we examined the PBS, human serum (lacking CES1 or CES2, but containing other esterases, such as butyrylcholinesterase (BChE), Paraoxonases (PONs), acetylcholinesterase (AChE) and albumin)(Yang et al., 2007; Hernández et al., 2009), HLS9 (CES1 >> CES2)(Taketani et al., 2007), and HIS9 (CES2-exclusive)(Taketani et al., 2007) to identify the tissue(s) and enzyme(s) involved in remimazolam hydrolysis. Our results showed no hydrolysis in PBS, human serum, or HIS9, indicating that common blood esterases or CES2 do not catalyze remimazolam hydrolysis (**Figure1**). In contrast, remimazolam was efficiently hydrolyzed in HLS9, suggesting hepatic CES1 as the specific enzyme (**Figure1**). Additionally, the selective

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carboxylesterases inhibitor, BNPP, completely inhibited remimazolam hydrolysis at 1 μM, confirming remimazolam is the substrate of carboxylesterases but no other esterases (**Figure2**). Recombinant CES1 and CES2 studies followed by Michalis-Menten kinetics of remimazolam by WT CES1 confirmed that remimazolam is selectively hydrolyzed by human CES1 rather than CES2 (**Figure3**). While preparing this manuscript, Schmalix et. al published a study on remimazolam metabolism, examining multiple tissues, including human serum, liver, brain, kidney and lung to identify the hydrolase responsible for remimazolam(Schmalix et al., 2024). In addition to human serum and HLS9, we included HIS9, BNPP, recombinant enzymes, transfected cells, which complements their work. Our results align well with theirs, confirming that remimazolam is specifically hydrolyzed by CES1.

CES1 is the most abundant enzyme expressed in human liver, contributing to 80%-95% of total hepatic hydrolytic activity(Imai, 2006). It is responsible for metabolizing numerous clinically important medications, such as clopidogrel(Zhu et al., 2013; Laizure et al., 2020), methylphenidate(Zhu et al., 2008; Lyauk et al., 2016), oseltamivir(Suzaki et al., 2013; Zhu and Markowitz, 2013), ACE inhibitors (ACEIs)(Wang et al., 2016), dabigatran(Laizure et al., 2014), and sacubitril(Shi et al., 2016a). Significant variability in CES1 expression and activity among individuals(Wang et al., 2017), as well as the association between *CES1* genetic polymorphisms and variability in the PK and PD of its substrate medications(Zhu et al., 2008; Johnson et al., 2013; Zhu et al., 2013; Zhu and Markowitz, 2013; Hoh et al., 2016; Shi et al., 2016a; Shi et al., 2016b; Wang et al., 2016; Stage et al., 2017a; Stage et al., 2017b; Stage et al., 2019; Her et al., 2021; Brown et al., 2022; Ikonnikova et al., 2022), has been constantly reported. We and Dr.

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Schmalix's group have demonstrated that remimazolam is a specific substrate of CES1 in human liver. However, the impact of CES1 genetic polymorphisms on remimazolam hydrolysis remains unexplored. As the first group to investigate this, we examined the remimazolam hydrolysis in the S9 fractions from *CES1*- and *CES1 nsSNPs*- transfected cells, as well as individual human livers. The results were largely consistent with previous reports on other substrates like clopidogrel, enalapril, and sacubitril(Wang et al., 2017), with minor variations (**Figure 5**).

Similar to our previous report (Wang et al., 2017), the L40Ter resulted in a premature stop codon, leading to early termination of protein translation and null mature CES1 expression and function (**Figure 5**). We were unable to verify this effect in human livers due to the extremely low MAF of L40Ter in our liver population (mostly Caucasian, MAF=0.08%, **Table1**).

In contrast, the Q169P and E220G did not completely block CES1 activity but significantly reduced remimazolam hydrolysis (**Figure 5**). The E220G variant, also known as c.662A>G, was predicted to reduce CES1 enzyme activity in an *in-silico* analysis, as it is located near the CES1 active site, which may alter enzyme-substrate binding(Nzabonimpa et al., 2016). Our previous *in vitro* studies showed that E220G significantly decreased CES1 activity and the metabolism of several substrates, including enalapril, clopidogrel, and sacubitril(Wang et al., 2017). E220G is found in 0.55% of East Asians but is rare in other populations. A clinical study in 20 healthy Korean volunteers found that E220G had a statistically insignificant effect on oseltamivir activation *in vivo*(Oh et al., 2017). Consistent with previous reports, our current transfected cell study found that remimazolam hydrolysis was significantly impaired in

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the E220G group compared to the WT control. Like the L40Ter, their effects were not validated in individual human liver samples due to their low MAF in our liver cohort (**Table 1**).

The *G143E* is located at the active center of CES1, which is the only clinically significant CES1 variant reported so far and is well known as a loss-of-function variant for most CES1 substrates. However, unlike those CES1 substrates such as methylphenidate(Zhu et al., 2008), clopidogrel(Zhu et al., 2013), enalapril(Wang et al., 2016), and sacubitril(Shi et al., 2016a), where G143E is a loss-of-function variant, it retained approximately 19% of the WT CES1 activity for remimazolam hydrolysis (**Figure 5**). Consistently, when comparing the enzymatic kinetics of remimazolam hydrolysis between *WT CES1* and *CES1 G143E,* the V_{max} in *CES1 G143E* was only 20% of that observed in the WT CES1 (**Figure 6**). Additionally, CES1 G143E exhibited reduced binding affinity for remimazolam, as indicated by K_m values that were 67% higher than those of WT CES1 (**Figure 6**). Similarly, G143E activity on oseltamivir activation was approximately 25% of that in the WT CES1 control(Zhu and Markowitz, 2013), indicating that the effect of G143E is substrate-dependent. We then verified the impact of *CES1* G143E on remimazolam hydrolysis in human livers. Consistent with the cell data, remimazolam hydrolysis was significantly impaired in human livers (**Figure 7**).

The S75N is one of the most common CES1 nonsynonymous SNPs, with MAFs ranging from 2% to 7% in different populations (**Table 1**). Unlike the previously discussed nsSNPs, which reduced CES1 function, S75N has been reported to be associated with increased CES1 activities, as observed in a retrospective analysis of clopidogrel therapy outcomes in coronary disease patients(Xiao et al., 2017). Several potential mechanistic

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hypotheses have been proposed to explain the increased activity of the S75N variant. One possibility is that the serine-to-asparagine substitution at position 75 may affect protein phosphorylation, which could alter CES1's catalytic function. Alternatively, S75N might impact the secondary structure of the CES1 protein, affecting its interaction with ligands. Another hypothesis is that S75N could influence CES1 expression by promoting exon skipping, though further research is needed to confirm this(Xiao et al., 2017). Notably, the findings regarding the effect of S75N has been inconsistent. Some other studies have reported that the S75N has no significant impact on CES1 function(Johnson et al., 2013). Our previous *in vitro* study demonstrated that the S75N variant was not associated with the expression and activity of CES1 on hydrolyzing enalapril, clopidogrel or sacubitril(Wang et al., 2017). A clinical study by Johnson et. al, found the S75N variant not associated with outcomes in ADHD children treated with methylphenidate(Johnson et al., 2013). In the current study, we found S75N significantly increased CES1 activities on remimazolam hydrolysis in transfected cells (**Figure 5**) but the increase was not significant in human livers (**Figure 7**). The Michaelis-Menten Kinetics revealed that S75N had a V_{max} approximately three times higher than WT CES1. However, the binding affinity of S75N was significantly lower, with Km value over four times higher than that of WT CES1 (**Figure 6**). Notably, all S75N carriers in our individual liver samples were heterozygotes, and due to the lower binding affinity of S75N for remimazolam, the impact of one S75N allele could be substantially diminished by the presence of the WT allele. Additionally, variability in the effects of CES1 S75N across independent studies might be attributed to other variants in high linkage disequilibrium (LD) with S75N and/or unknown confounding factors.

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The synergistic interaction between benzodiazepines, such as remimazolam, and opioid analgesics is well-documented and typically beneficial in procedural sedation and anesthesia settings(Bevans et al., 2017; Zhou et al., 2020; Kops et al., 2021; Zhou et al., 2021). Remimazolam distinguishes itself from other sedatives like propofol and midazolam by lacking CYP3A4-mediated drug-drug interactions, offering a more predictable and safer option(Wandel et al., 1994; Bauer et al., 1995; Hamaoka et al., 1999; Yuan et al., 1999). Specifically, remimazolam and its primary metabolite, CNS7054, do not inhibit or induce major cytochrome P450 enzymes (CYPs) such as CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, or 3A4, nor do they affect human drug transporters like OAT1, OAT3, OATP1B1, OATP1B3, OCT2, MATE1, MATE2-K, BCRP, BSEP, and MDR1, as reported by the Food and Drug Administration (FDA, https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/212295s000lbl.pdf) and the European Medicines Agency (EMA, https://www.ema.europa.eu/en/documents/product-information/byfavo-epar-product-information_en.pdf). Importantly, remimazolam is specifically hydrolyzed by CES1, and its exposure can increase by 1.2–2.1 times in a dose-dependent manner when co-administered with alcohol, a known CES1 inhibitor(Pesic et al., 2020). This suggests the potential for DDIs with other clinical drugs that interact with CES1.

To explore these DDIs further, we first examined the inhibitory effect of remimazolam on several commonly used CES1 substrate medications, including enalapril, clopidogrel and sacubitril. Remimazolam consistently displayed a mild inhibitory effect on these substrates, with IC_{50} values of 113.3 μM for sacubitril, 171.9 μM for clopidogrel, and 214.8 μM for enalapril, indicating a gradient of inhibition from strong to weak (**Figure**

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8A). At a clinically relevant concentration of approximately 1 μM(Schuttler et al., 2020), remimazolam resulted in less than 10% inhibition on the hydrolysis of these CES1 substrates. This suggests that remimazolam is unlikely to significantly interfere with the metabolism of other drugs catalyzed by CES1 at typical clinical concentrations.

Conversely, we evaluated the impact of CES1 inhibitors or substrates-specifically simvastatin, simvastatin acid, clopidogrel, enalapril, and sacubitril-on the hydrolysis of remimazolam. Among the tested compounds, clopidogrel exhibited the most potent inhibitory effect on remimazolam hydrolysis, with an IC_{50} of 73.3 μM (**Figure 8B**). As a prodrug, approximately 85% of clopidogrel undergoes hydrolysis via CES1, while the remainder is activated through two steps involving several CYP enzymes-including CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/5-to produce its active metabolite(Zhu et al., 2013). This active metabolite inhibits the platelet $P2Y_{12}$ adenosine diphosphate (ADP) receptor, decreasing platelet activation and aggregation. Clopidogrel is widely utilized for preventing thrombotic events in patients with acute coronary syndrome (ACS), the DDIs associated with clopidogrel have been extensively documented(Norgard et al., 2009; Bates Eric et al., 2011; Delavenne et al., 2013; Wang et al., 2015b; Xiao et al., 2017; Laizure et al., 2020). Research indicates that atorvastatin, omeprazole, and various other drugs can competitively inhibit or induce the CYP-mediated activation of clopidogrel, significantly affecting its responsiveness(Norgard et al., 2009; Bates Eric et al., 2011; Delavenne et al., 2013; Wang et al., 2015b). However, studies on clopidogrel's influence on other drugs are limited. Our *in vitro* study is the first to present evidence of its inhibitory effect on remimazolam hydrolysis. Since clopidogrel is a selective CES1 substrate, it binds to the

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enzyme's active site and is likely to inhibit remimazolam hydrolysis through competitive inhibition. Notably, clinical plasma concentrations of clopidogrel and its major metabolite, clopidogrel carboxylic acid, can reach plasma levels as high as 100 μM(Zhou et al., 2020), aligning with the observed IC_{50} for clopidogrel's effect on remimazolam hydrolysis. This finding suggests that at clinically relevant concentrations, clopidogrel may significantly inhibit the deactivation of remimazolam, potentially leading to elevated plasma drug concentrations. This could result in prolonged patient exposure to remimazolam, which could increase patients' risk of experiencing benzodiazepine related adverse effects.

Simvastatin, an HMG-CoA reductase inhibitor, is primarily used to lower lipid levels and reduce the risk of cardiovascular events. Given that approximately 6.9 million patients use simvastatin chronically, a substantial number of them may concurrently utilize sedative or anesthetic drugs. While simvastatin is generally well tolerated with a favorable safety profile, concerns regarding statin-associated adverse events, particularly muscle-related issues, have led to medication non-adherence and discontinuation(Silva et al., 2006; Joy and Hegele, 2009). Most studies examining DDIs involving simvastatin focus on cytochrome P450 enzymes (CYPs) and transporters(Rätz Bravo et al., 2005; Elsby et al., 2012; Elsby et al., 2019; Wojtyniak et al., 2021), while research on hydrolase-mediated interactions remains limited. Notably, simvastatin has been shown to inhibit CES1-mediated clopidogrel hydrolysis, with an IC_{50} of 18.3 μM(Wang et al., 2015a). In contrast, simvastatin exhibited significantly lower inhibition of remimazolam hydrolysis according to our data. Both simvastatin and its active metabolite, simvastatin acid, exhibited mild inhibitory effect on remimazolam

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hydrolysis, with IC_{50} values of 179.5 μM and 146.8 μM, respectively (**Figure 8B**), considerably exceeding their typical clinical concentrations(Barrett et al., 2006). Enalapril, an ACEI prodrug widely employed for managing hypertension and heart failure, similarly demonstrated mild inhibition of remimazolam hydrolysis, with an IC_{50} of 171.9 μM (**Figure 8B**). At a clinically relevant concentration of roughly 0.1 μM(MacFadyen et al., 1993; Arafat et al., 2005), enalapril has minimal impact on the hydrolysis of remimazolam. Sacubitril, a neprilysin inhibitor often combined with valsartan for heart failure treatment, exhibited minimal inhibition of remimazolam hydrolysis, with a high IC_{50} value of 467.1 μM (**Figure 8B**). Thus, sacubitril is unlikely to cause significant inhibition on remimazolam hydrolysis at clinical concentrations below 5 μM(Kobalava et al., 2016).

Moreover, this is the first study to reveal the genetic impact on CES1-mediated drug-drug interactions (DDIs), offering new insights into the interplay between genetic polymorphisms and DDIs. We compared the inhibitory effect of clopidogrel on remimazolam hydrolysis using *CES1 G143E* and *CES1 S75N* to represent the variants causing reduced and increased CES1 activities, respectively, with *CES1 WT* as the control. Our findings demonstrate that *CES1* genetic variants with various enzymatic activities could affect the DDIs involving remimazolam. Specifically, the *S75N* variant, which exhibited the highest CES1 activity (V_{max} = 22.15 nmol/mg protein/min), required higher concentrations of the inhibitor clopidogrel (IC_{50} = 181.3 μM) to achieve the same inhibitory effect compared to variants with lower CES1 activity, such as *WT CES1* (V_{max} = 7.25 nmol/mg protein/min, IC_{50} = 151.5 μM) and *G143E* (V_{max} = 1.46 nmol/mg protein/min, IC_{50} = 119.0 μM). The inhibitory effect on remimazolam hydrolysis by

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clopidogrel at the same concentration can vary significantly between genetic variants, such as *CES1 G143E* and *CES1 S75N* (**Figure9**). While these *in vitro* results require further clinical validate, they underscore the importance of considering genetic polymorphisms when evaluating DDIs, which may extend to other drugs and drug-metabolizing genes.

Besides its favorable pharmacological profile, a key advantage of remimazolam as a sedative/anesthetic is its availability of an antidote, flumazenil, a specific benzodiazepine antagonist that can reverse its sedative effects(Toyota et al., 2023). However, the presence of an antidote does not eliminate the possibility of all adverse or side effects(Chen et al., 2020). While flumazenil can counteract the sedation, it may not address other complications or interactions that could lead to cardiovascular or respiratory issues, especially in special populations(Heard and Fletcher, 2011). Moreover, flumazenil has contraindications, such as in patients with epilepsy, hypersensitivity to benzodiazepines, or tricyclic antidepressant overdose. In these cases, administering flumazenil may expose the patients to severe adverse effects, potentially complicating their conditions(Penninga et al., 2016). Therefore, it is essential to understand the potential factors that may influence remimazolam use and remain vigilant regarding both genetic and non-genetic factors. A thorough evaluation of remimazolam pharmacogenetics and potential DDIs is imperative to ensure the safety and efficacy of remimazolam in clinical settings.

In summary, our investigation demonstrated that remimazolam is specifically deactivated by CES1 in the human liver. Multiple *CES1* genetic variants can significantly affect the hydrolysis of remimazolam, as shown in the assessments using the s9

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fractions prepared from WT and variant *CES1*-transfected cells. Notably, the *CES1* G143E variant has been confirmed to significantly impair remimazolam hydrolysis in individual human livers, highlighting the potential impact of genetic polymorphisms on remimazolam metabolism and treatment outcomes. Furthermore, our study identified a significant *in vitro* DDI between remimazolam and clopidogrel at clinically relevant concentrations, which could be influenced by certain *CES1* genetic variants. These results highlight the clinical importance of considering CES1 genetic variability and potential DDIs in the safe and effective use of remimazolam, particularly in personalized pharmacotherapy where patient-specific factors can be critical to optimal therapeutic outcomes.

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Authorship Contribution

Participated in research design: X.W. and J.A.B.

Conducted experiments: Z.W., L.L., Y.A., and Z.M.

Performed data analysis: X.W., Z.W., L.L., and D.T.

Contributed new reagents or analytic tools: H-J Z. and X.S.Z.

Wrote and contributed to the writing of the manuscript: X.W., Z.W., D.T., Y.A., Z.M., H-J Z., X.S.Z., and J.A.B.

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Dataset/Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

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Footnote

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Figure Legends

Figure 1. Hydrolysis of remimazolam, sacubitril (specific CES1 substrate), and fluorescein diacetate (specific CES2 substrate) in human liver s9 fractions (HLS9), human intestine s9 fractions (HIS9), human serum and PBS. The metabolites were determined after incubating the substrates at 37°C for 20 min. The final protein concentrations of HLS9, HIS9, and serum were 0.2 mg/mL for remimazolam and sacubitril and 0.02 mg/mL for fluorescein diacetate incubations. The final concentrations of remimazolam, sacubitril, and fluorescein diacetate were 200 μM, 400 μM, and 5 μM, respectively. Data are expressed as the formation rate of hydrolytic metabolites (mean ± S.D., n=3).

Figure 2. The impact of BNPP, inhibitor of carboxylesterases (CES1 and CES2), on remimazolam hydrolysis in HLS9. The BNPP was pre-incubated with HLS9 for 20 min at 37°C, and the remimazolam was added for an additional 20 min co-incubation. The final concentrations for BNPP, remimazolam and HLS9 were 0-30 μM, 20 μM, and 0.2 mg/mL, respectively. Data are expressed as the formation rate of remimazolam carboxylic acid (mean ± S.D., n=3).

Figure 3. Remimazolam deactivation in recombinant CES1 and CES2 enzymes with protein concentrations ranging from 5 to 25 ng/μL. The final concentration of remimazolam was 200 μM. The metabolites were determined after incubating the substrates at 37°C for 20 min. Data are expressed as the formation of remimazolam carboxylic acid (mean ± S.D., n=3).

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Figure 4. Enzymatic kinetics of remimazolam hydrolysis in S9 fractions prepared from wild-type *CES1* (*WT CES1*) transfected cells. The final concentrations of *WT CES1* S9 and remimazolam were 0.2 mg/mL and 0 to 500 μM, respectively. The hydrolytic metabolite remimazolam carboxylic acid was determined after a 20 min incubation at 37°C. Data are expressed as the formation rates of remimazolam carboxylic acid (mean ± S.D., n=3).

Figure 5. Remimazolam hydrolysis was assessed in the S9 fractions prepared from the cells transfected with *WT CES1*, vector and seven nonsynonymous *CES1* single nucleotide polymorphisms (nsSNPs), including *CES1 L40Ter*, *E220G*, *Q169P*, *S75N*, *T167S*, *T290M*, and *G143E*. The final concentrations of the S9 fractions and remimazolam were 0.2 mg/mL and 200 μM, respectively. Incubation at 37°C lasted for 20 minutes. Data are expressed as the formation rates of remimazolam carboxylic acid (mean ± S.D., n=3). ANOVA test with *Dunnett correction* for multiple comparisons was performed to compare remimazolam hydrolytic rates in nsSNPs to *WT CES1* (* adjusted $P<0.05$, ** adjusted $P<0.01$).

Figure 6. Michalis-Menten kinetics of remimazolam hydrolysis in S9 fractions prepared from wild-type *CES1* (*WT CES1*), *CES1 G143E*, and *CES1 S75N* transfected cells. The final concentrations of *S*9 fractions and remimazolam were 0.2 mg/mL and 0 to 500 μM, respectively. The hydrolytic metabolite remimazolam carboxylic acid was determined after a 20 min incubation at 37°C. Data are expressed as the formation rates of remimazolam carboxylic acid (mean ± S.D., n=3).

Figure 7. Remimazolam hydrolysis was assessed in 54 individual liver S9 fractions (HLS9). The subjects were genotyped and grouped as 49 G143E non-carriers and five

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G143E carriers, as well as 47 S75N non-carriers and 7 S75N carriers. The final concentrations of the S9 fractions and remimazolam were 0.2 mg/mL and 200 μM, respectively. Incubations were conducted at 37°C for 20 minutes. Data are expressed as the formation rate of remimazolam carboxylic acid (Mann-Witney test, n=3, * $P<0.05$).

Figure 8. Assessment of the inhibitory effect of remimazolam on CES1 activities in hydrolyzing its substrates sacubitril, enalapril, and clopidogrel in HLS9 (**A**), and the inhibition of CES1 substrates and inhibitors on remimazolam hydrolysis (**B**). Final concentrations of examined inhibitors ranged from 0 to 1000 μM. Final concentrations of remimazolam, HLS9, and established CES1 substrates, including sacubitril, enalapril, and clopidogrel, were 20 μM, 0.2 mg/mL, and 30 μM, respectively. The pre-incubation time was 20 min, and the co-incubation times were 20 min for remimazolam and 10 min for other CES1 substrates, including sacubitril, enalapril, and clopidogrel. Data are expressed as the formation rate of respective hydrolytic metabolites relative to the no-inhibitor control group (%, n=3).

Figure 9. Evaluation of clopidogrel's inhibitory effect on remimazolam hydrolysis in S9 fractions prepared from *WT CES1*, *CES1 G143E* and *CES1 S75N* transfected cells. Final clopidogrel concentrations ranged from 0 to 1000 μM, while the final concentrations of the S9 fraction and remimazolam were set as 0.2 mg/mL and 20 μM, respectively. Pre-incubation of clopidogrel with the S9 fraction and co-incubation after the addition of remimazolam lasted for 20 minutes each. Data are expressed as the formation rate of remimazolam hydrolytic metabolites relative to no-inhibitor group (%, n=3).

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Table 1. CES1 nsSNPs Analyzed in This Study: MAFs Across Various Ethnicities and Activity Levels Compared to WT CES1 (%).

			Races/Ethnicities				CES1 Activities relative to WT (%)	
rs numbers	Protein residue changes	Nucleotide changes	White/Caucasian	Black/African American	Asian	Other	Remimazolam	Sacubitril
rs151291296	L40Ter	T227G	0.08%	0.37%	0.00%	0.04%	0	0
rs71647871	G143E	G428A	1.49%	0.27%	0.00%	1.72%	19	0
rs200707504	E220G	A767G	0.01%	0.00%	0.00%	0.00%	32	13
rs2307240	S75N	G332A	7.74%	3.90%	15.20%	5.05%	195	132
rs143718310	Q169P	A614C	0.00%	0.00%	0.00%	0.00%	23	34
rs147694791	T167S	C608G	0.00%	4.56%	0.00%	0.21%	67	66
rs202001817	T290M	C977T	0.15%	0.10%	0.00%	0.00%	120	84

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Figure 1

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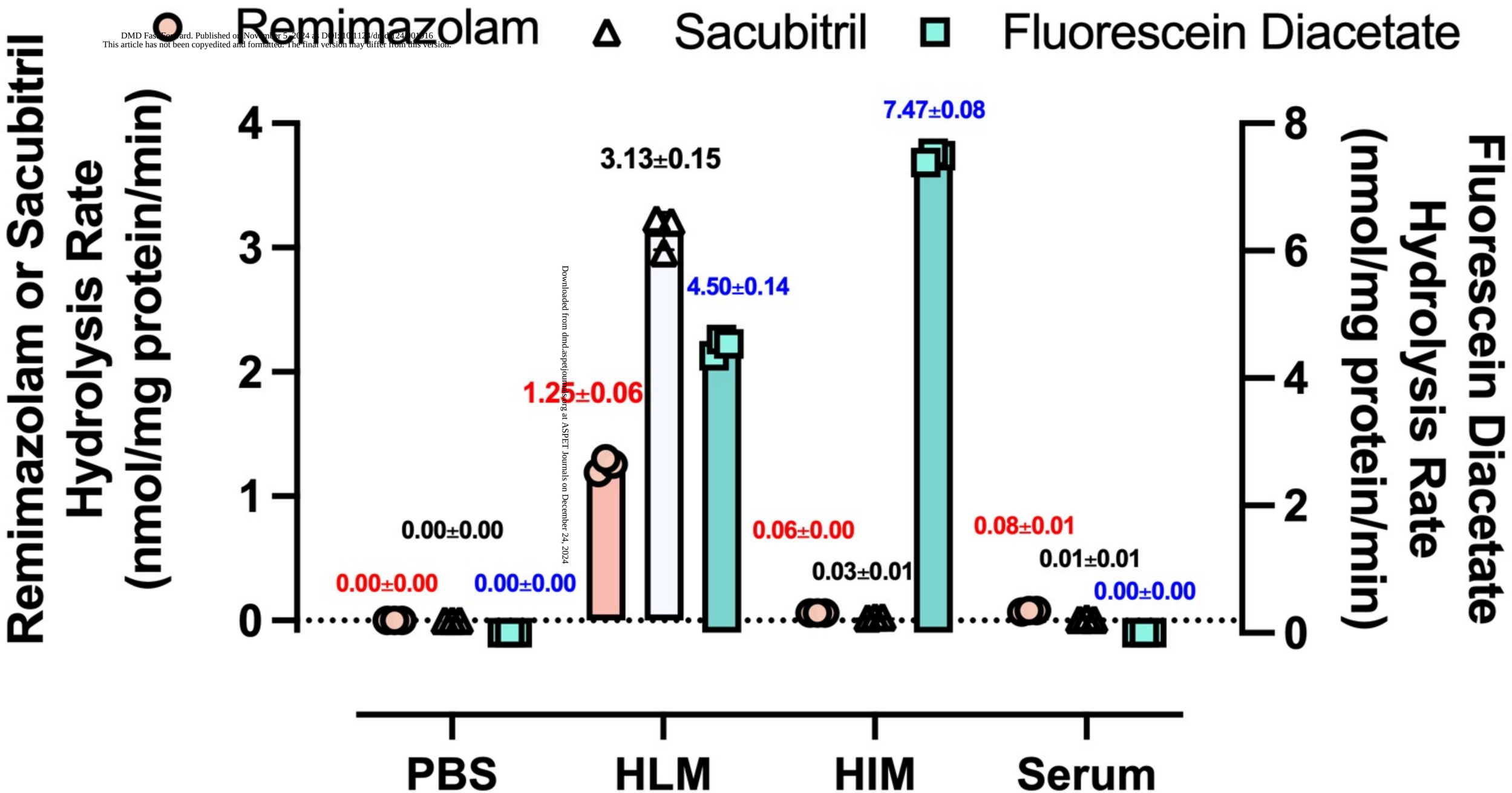

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Figure 2

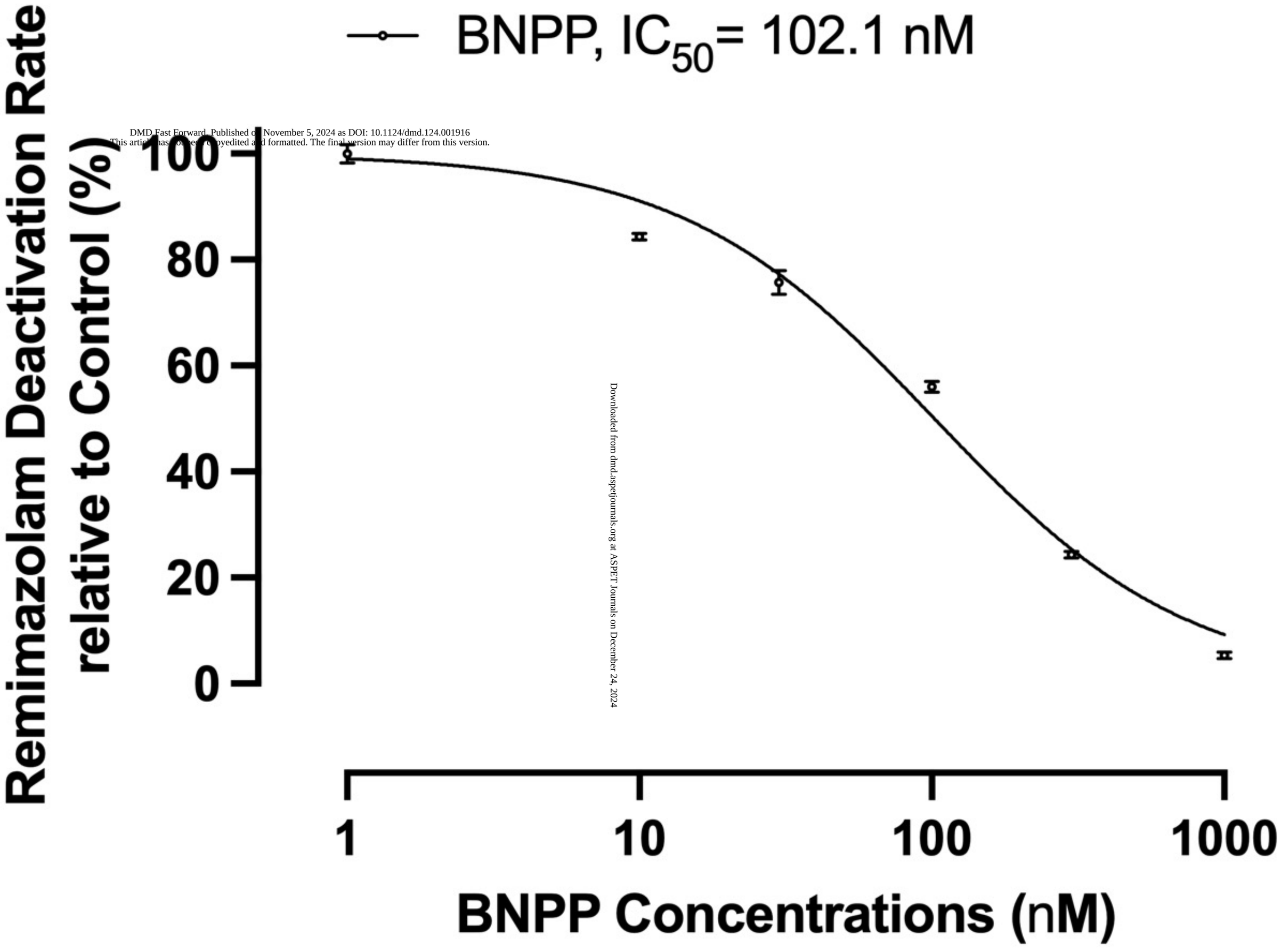

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Figure 3

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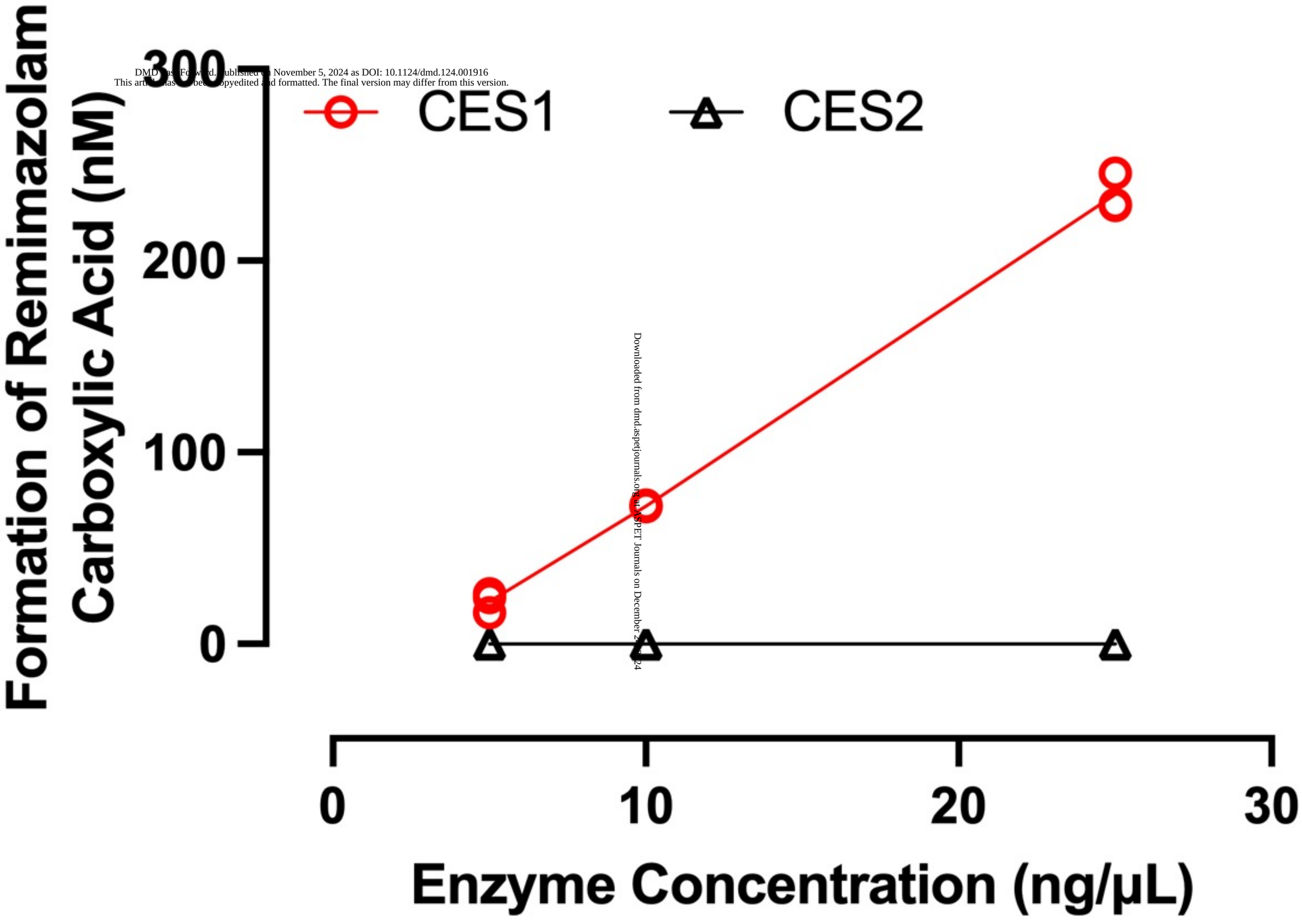

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Figure 4

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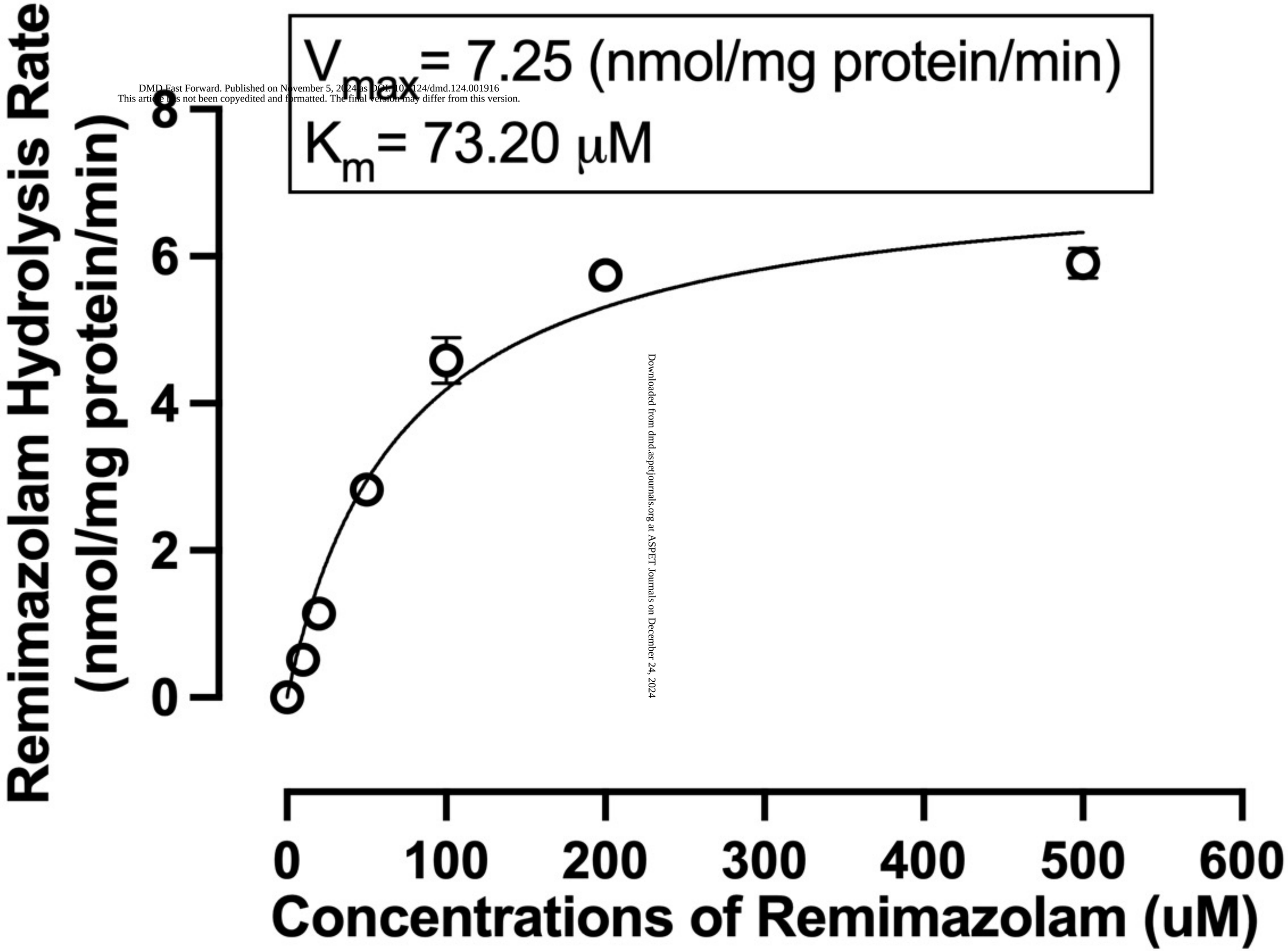

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Figure 5

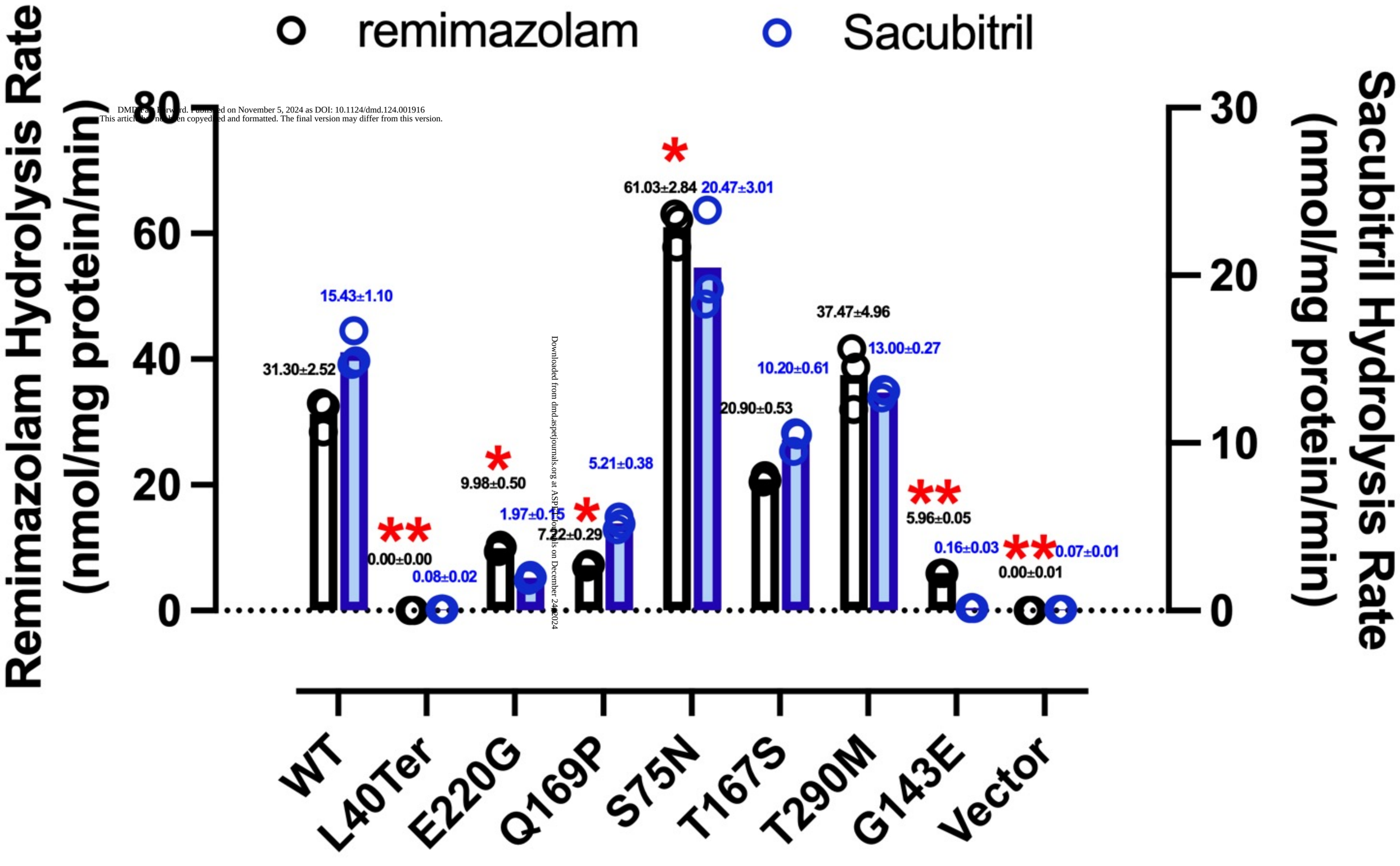

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Figure 6

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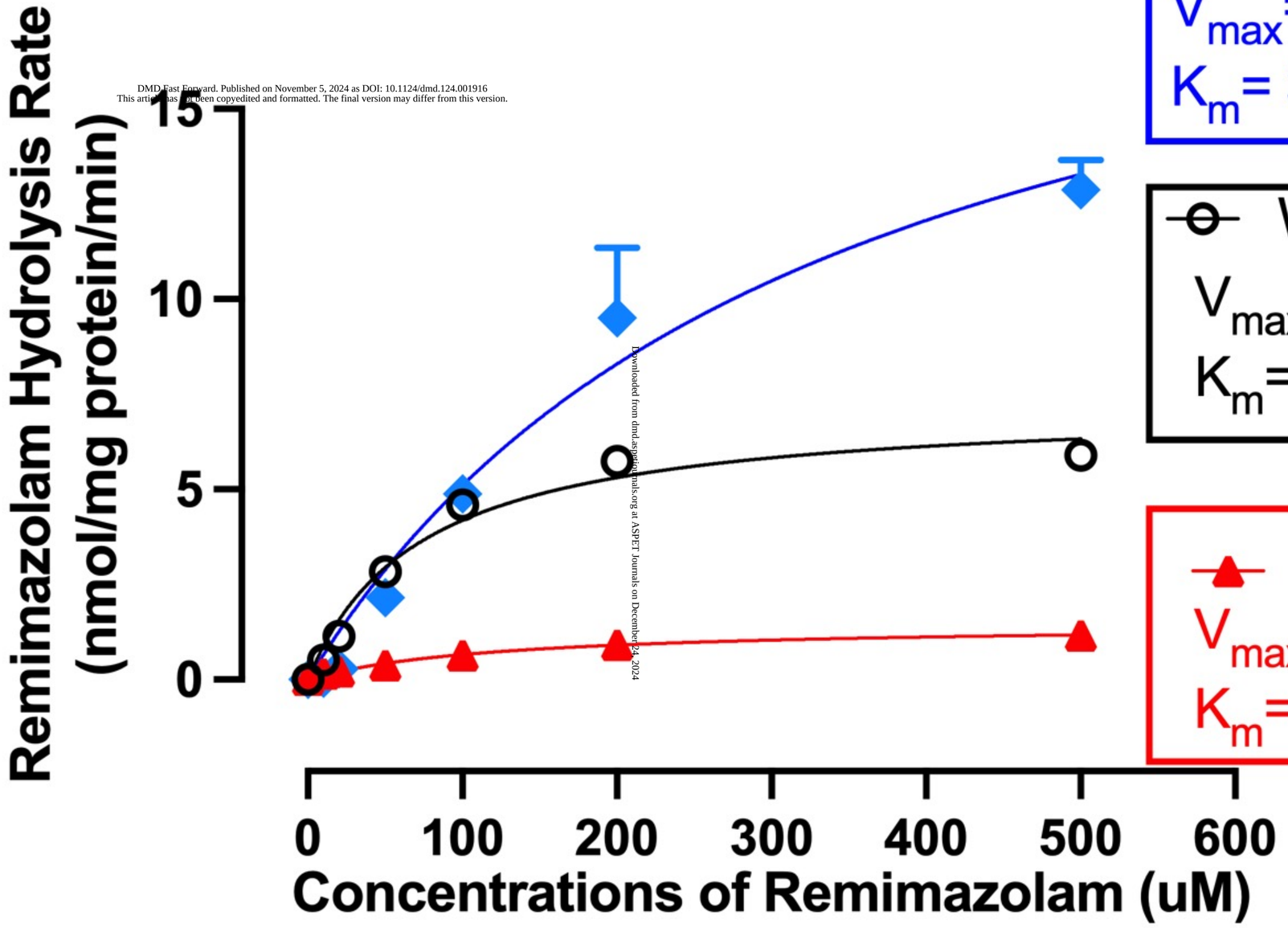

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S75N
V_{max}= 22.15 (nmol/mg protein/min)
K_m= 334.0 μM

WT
V_{max}= 7.25 (nmol/mg protein/min)
K_m= 73.20 μM

G143E
V_{max}= 1.46 (nmol/mg protein/min)
K_m= 122.3 μM

Figure 7

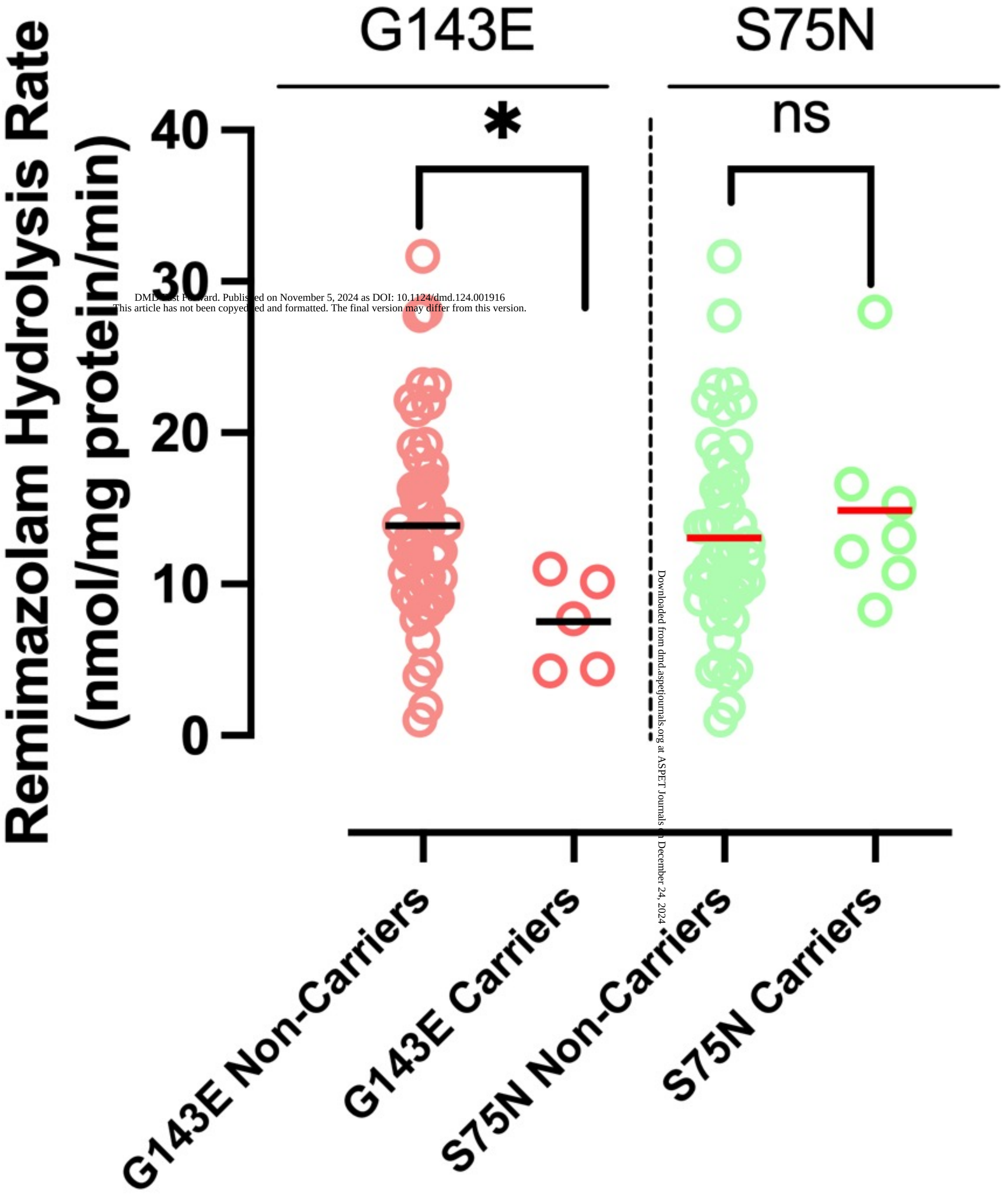

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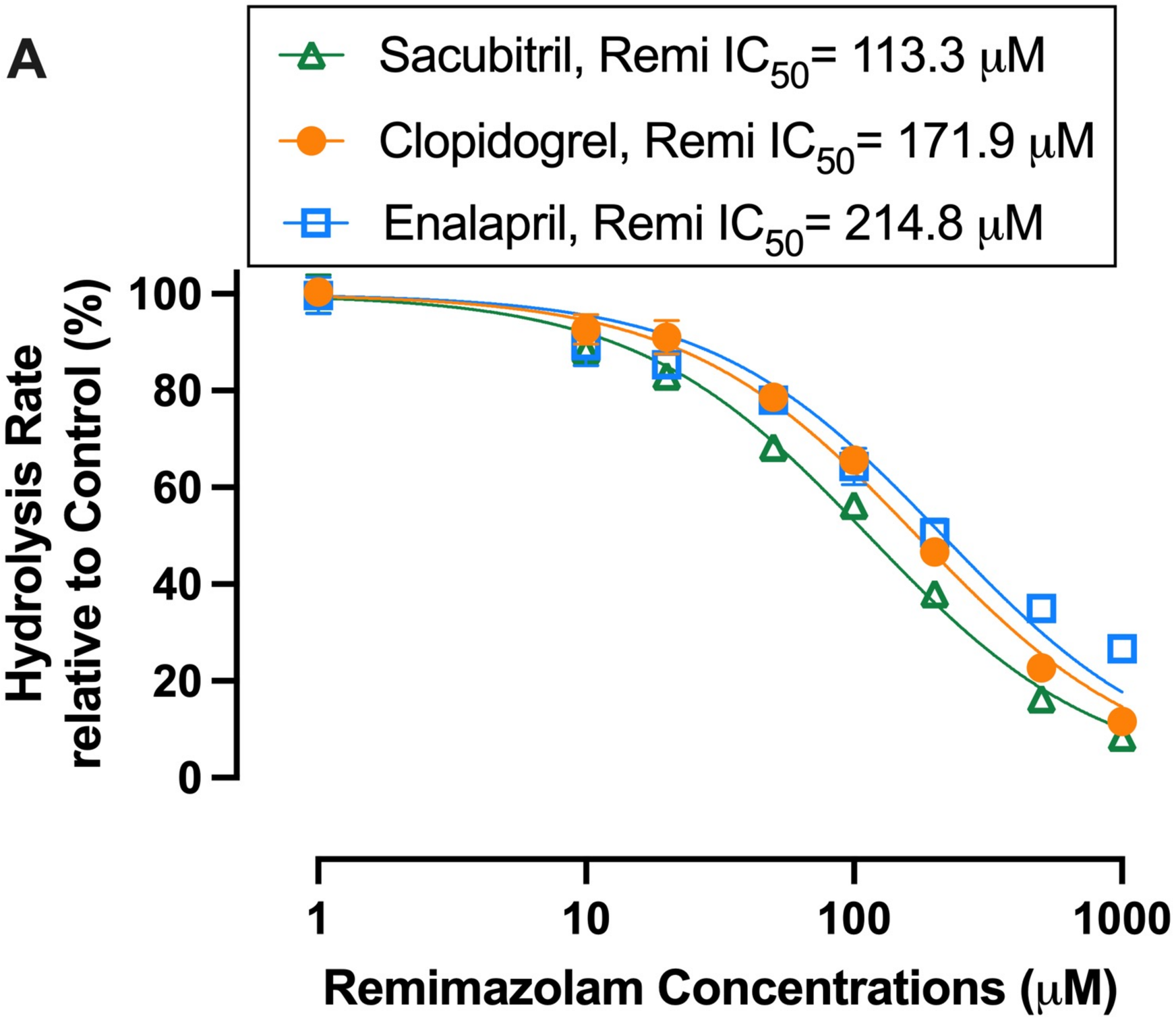
Figure 8



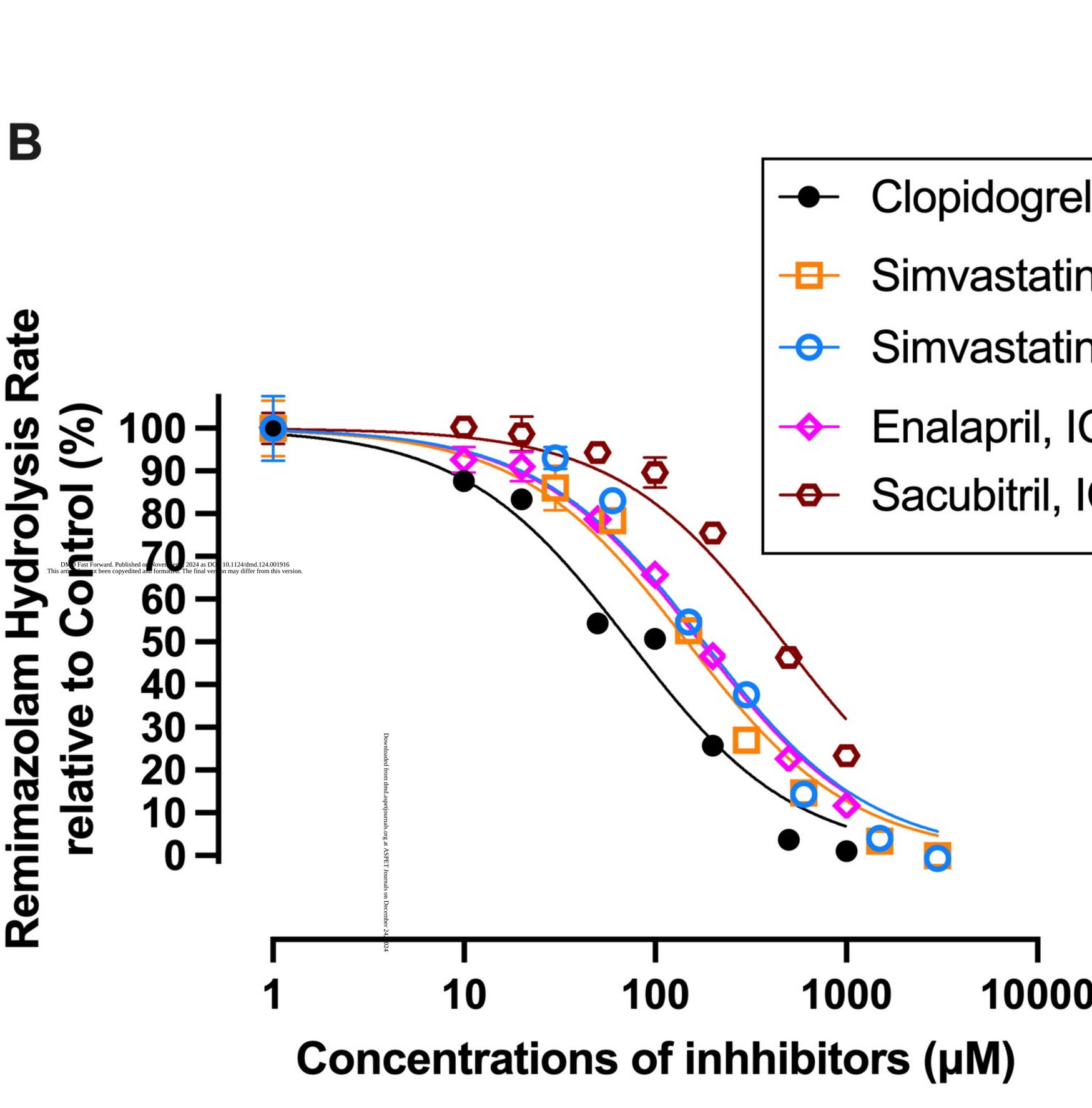


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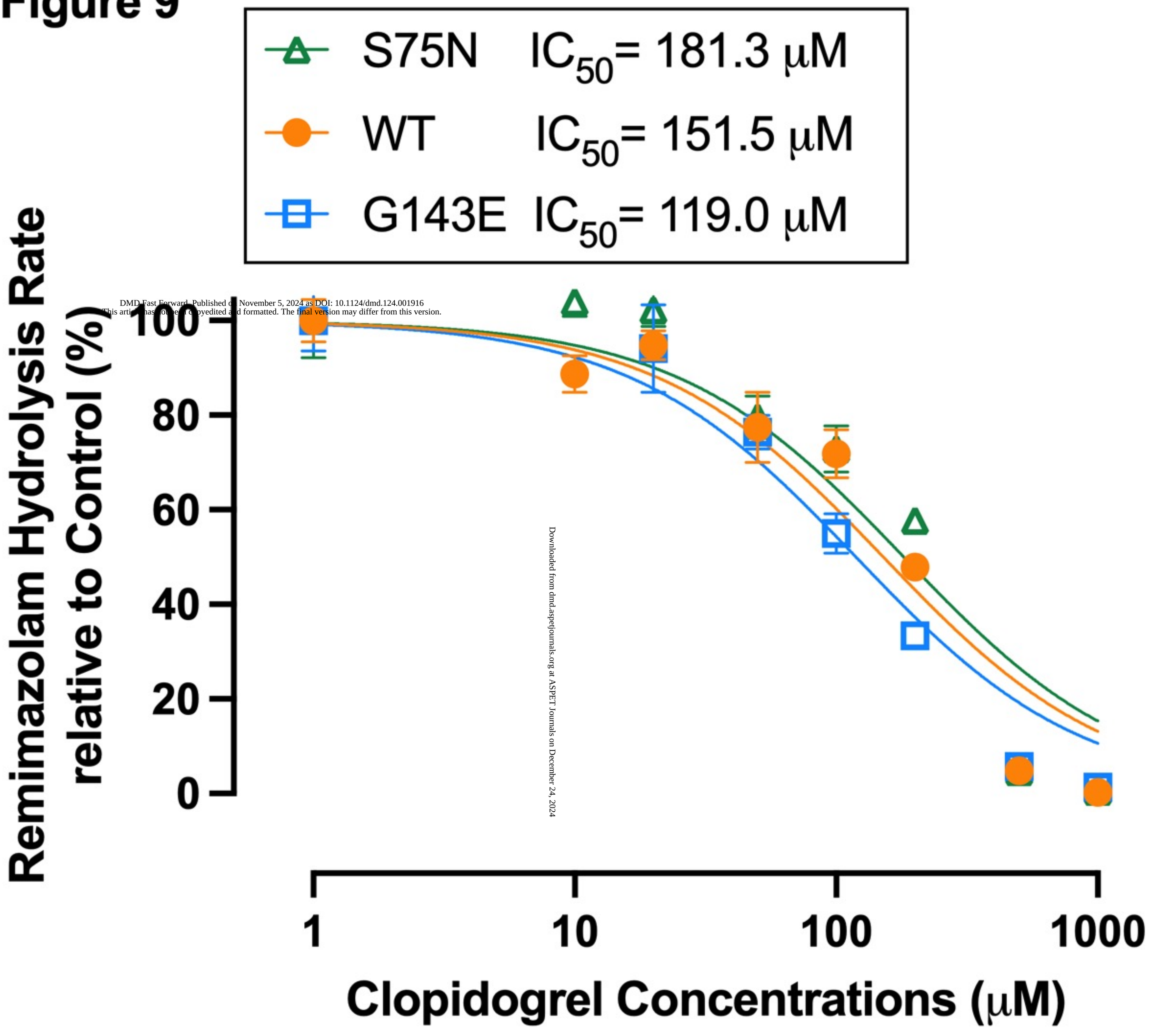
Figure 9



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