

## **Contributions of Cathepsin A and Carboxylesterase 1 to the hydrolysis of Tenofovir Alafenamide in the Human Liver, and the Effect of CES1 Genetic Variation on Tenofovir Alafenamide Hydrolysis**

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**Abbreviations:**

AASLD: American Association for the Study of Liver Diseases

ACN: acetonitrile

BNPP: bis-(p-nitrophenyl) phosphate

CatA: cathepsin A

CES1: carboxylesterase 1

DIA: data-independent acquisition

FDA: Food and Drug Administration

HBV: hepatitis B virus

HINT1: histidine triad nucleotide-binding protein 1

HIV: human immunodeficiency virus

HLS9: human liver S9 fractions

LC-MS/MS: liquid chromatography–tandem mass spectrometry

MS: mass spectrometry

RDV: remdesivir

TAF: tenofovir alafenamide

TDF: tenofovir disoproxil

TFV: tenofovir

TFV-Ala: tenofovir-alanine

TFV-DP: tenofovir diphosphate

**Keywords:**

Carboxylesterase 1 (CES1); Cathepsin A (CatA); Hydrolases; Pharmacogenetics;  
Tenofovir alafenamide.

## Abstract

The prodrug tenofovir alafenamide (TAF) is a first-line antiviral agent for the treatment of chronic hepatitis B infection. TAF activation involves multiple steps, and the first step is an ester hydrolysis reaction catalyzed by hydrolases. This study was to determine the contributions of carboxylesterase 1 (CES1) and cathepsin A (CatA) to TAF hydrolysis in the human liver. Our *in vitro* incubation studies showed that both CatA and CES1 catalyzed TAF hydrolysis in a pH-dependent manner. At their physiological pH environment, the activity of CatA (pH 5.2) was approximately 1,000-fold higher than that of CES1 (pH 7.2). Given that the hepatic protein expression of CatA was approximately 200-fold lower than that of CES1, the contribution of CatA to TAF hydrolysis in the human liver was estimated to be much greater than that of CES1, which is contrary to the previous perception that CES1 is the primary hepatic enzyme hydrolyzing TAF. The findings were further supported by a TAF incubation study with the CatA inhibitor telaprevir and the CES1 inhibitor bis-(p-nitrophenyl) phosphate. Moreover, an *in vitro* study revealed that the CES1 variant G143E (rs71647871) is a loss-of-function variant for CES1-mediated TAF hydrolysis. In summary, our results suggest that CatA may play a more important role in the hepatic activation of TAF than CES1. Additionally, TAF activation in the liver could be affected by CES1 genetic variation, but the magnitude of impact appears to be limited due to the major contribution of CatA to hepatic TAF activation.

### **Significance statement**

Contrary to the general perception that carboxylesterase 1 (CES1) is the major enzyme responsible for tenofovir alafenamide (TAF) hydrolysis in the human liver, the present study demonstrated that cathepsin A (CatA) may play a more significant role in TAF hepatic hydrolysis. Furthermore, the CES1 variant G143E (rs71647871) was found to be a loss-of-function variant for CES1-mediated TAF hydrolysis.

## Introduction

Tenofovir (TFV) is an acyclic nucleotide phosphonate analog that potently inhibits the hepatitis B virus (HBV) DNA polymerase, a key enzyme required for viral genome replication (Trepo et al., 2014). However, the poor bioavailability of TFV is a significant limitation (Kearney et al., 2004). Tenofovir disoproxil (TDF), a bis-carbonate ester prodrug of TFV, was subsequently developed due to its enhanced stability and improved oral bioavailability (Rautio et al., 2008). Although TDF can achieve sustained viral suppression with a high barrier to drug resistance, its long-term use has been associated with renal toxicity and reduced bone mineral density because of its high plasma TFV level (Cooper et al., 2010; Buti et al., 2015; Bedimo et al., 2016). Consequently, a new TFV prodrug, tenofovir alafenamide (TAF), was developed for more efficient delivery into target tissues and cells. TAF exhibits a higher antiviral potency at ~10% of the TDF dose and an improved safety profile due to the reduced systemic exposure of TFV (Babuis et al., 2013; Agarwal et al., 2015; Murakami et al., 2015). TAF was listed as one of the four first-line antiviral agents in the latest American Association for the Study of Liver Diseases (AASLD) guidance for chronic hepatitis B treatment (2018) (Terrault et al., 2018). Despite these improvements, significant interindividual variability in both the pharmacokinetics (PK) and antiviral activity of TAF has been consistently observed in clinical studies (Markowitz et al., 2014; Agarwal et al., 2015; Custodio et al., 2016).

As a prodrug, the antiviral effect of TAF relies on multistep activation involving several enzymes (Figure 1) (Birkus et al., 2008b; Murakami et al., 2015). Therefore, identifying the key TAF activating enzymes is critical to understand the interindividual variability in the PK and pharmacodynamics (PD) of TAF therapy. Multiple hydrolases, including cathepsin A (CatA), carboxylesterase 1 (CES1), leukocyte elastase, pancreatic elastase 1, proteinase 3, and cathepsin H, have been shown to catalyze the cleavage of the ester group, forming the intermediate metabolite tenofovir-alanine (TFV-Ala), the first step in the TAF activation process (Birkus et al., 2008b; Birkus et al., 2016). Unsurprisingly, this step depends on the composition of hydrolases in different cells and tissues. For example, CatA was

reported as the primary enzyme hydrolyzing TAF in peripheral blood mononuclear cells, the target cells of the human immunodeficiency virus (HIV) (Birkus et al., 2007; Birkus et al., 2016); and CES1 was considered to be the major hydrolase catalyzing the first activation step of TAF in the human liver, the target tissue of HBV (Henningsson et al., 2005; Murakami et al., 2015). However, the contributions of individual hydrolases in TAF hydrolysis in the liver have not been fully evaluated because the expression profiles of hepatic hydrolases were not taken into consideration in the previous study (Murakami et al., 2015). In addition, the CES1 activity in the previous study was measured with porcine liver carboxylesterase (Birkus et al., 2008b), which may differ from human CES1 in terms of catalyzing TAF hydrolysis.

CES1 is one of the most abundant proteins in the human liver and has been considered the major contributor to the hepatic hydrolysis of many ester-containing drugs, including TAF (Imai, 2006; Murakami et al., 2015; Wang et al., 2020). CES1 function can be regulated by genetic variants, such as the loss-of-function nonsynonymous variant G143E (rs71647871) (Zhu et al., 2008; Her and Zhu, 2020). However, it remains unknown whether and to what extent the G143E variant may affect TAF activation.

Therefore, the purposes of this study were to 1) evaluate the contributions of CES1 and CatA to TAF hydrolysis in the human liver; and 2) determine the impact of the CES1 genetic polymorphism G143E on TAF hydrolysis. We performed an absolute quantitative proteomics analysis to determine the protein levels of CES1, CatA, and other hydrolases in human liver S9 fractions (HLS9). We also conducted an *in vitro* TAF hydrolysis study and found that the activities of CatA, CES1, and HLS9 on hydrolyzing TAF were pH-dependent. Moreover, we determined the kinetic parameters of CatA and CES1-catalyzed TAF hydrolysis. Based on the obtained proteomics and enzymatic activity data, it appears that, while both CatA and CES1 are capable of hydrolyzing TAF, the contribution of CatA in human hepatic hydrolysis of TAF could be substantially greater than that of CES1. Additionally, the G143E was found to be a loss-of-function variant for the CES1-mediated TAF hydrolysis.

## **Method**

### ***Materials***

TAF, remdesivir (RDV), bortezomib, and boceprevir were purchased from Cayman Chemical (Ann Arbor, MI). TFV and adefovir (AFV) were obtained from MP Biomedicals (Santa Ana, CA, USA). Recombinant human CatA (rhCatA), CES1 (rhCES1) were purchased from R&D Systems (Minneapolis, MN, USA); Bis-(p-nitrophenyl) phosphate (BNPP) and Tris-base (Trizma base) were purchased from Sigma-Aldrich (St. Louis, MO). Tris-HCl was purchased from Fisher Scientific (Fair lawn, NJ, USA). Pierce BCA protein assay kit and phosphate-buffered saline were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The MES [2-(N-morpholino) ethanesulfonic acid] buffer (0.2 M, pH 5.5 at 25°C and pH 5.2 at 37°C) was purchased from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and reagents were of analytical grade and commercially available. Pooled HLS9 was purchased from XenoTech LLC (Kansas City, KS).

### ***Cell S9 fractions preparation***

The Flp-In 293 cell lines stably expressing wild-type CES1 and the variant G143E were developed in our previous study, and CES1 protein expression levels are comparable between the two cell lines (Zhu et al., 2008). Cells were sonicated and centrifuged at 9,000g for 30 min at 4 °C. The supernatant (S9 fractions) was collected and stored at -80°C until use. The S9 fraction protein concentrations were measured using a Pierce BCA protein assay kit.

### ***rhCES1 and rhCatA activity assays***

The activity of rhCES1 and rhCatA on hydrolyzing TAF was measured using the method similar to that from a previous report (Murakami et al., 2010). A preliminary experiment was performed to determine the steady-state kinetic conditions for the enzymatic reactions (Supplemental Figure S1). The assay buffer for the rhCES1 and rhCatA activity assays was different in composition and pH. The assay buffer for rhCatA reactions was an MES buffer (0.2 M, pH 5.2 at 37°C), containing 100 mM NaCl, 1 mM DTT, and 0.1% Nonidet (Murakami et al.,



2010). The buffer for rhCES1 reactions was 50 mM Tris buffer, which was prepared by dissolving 6.06 g Tris-HCL and 1.39 g Tris-base in 1000 mL water (pH 7.2 at 37°C). TAF was stable in both buffers for at least 30 min at 37°C (Supplemental Figure S2).

The CatA assay was conducted in 50  $\mu$ L MES pH 5.2 assay buffer, containing the activated rhCatA (0.2 ng/ $\mu$ L) and various concentrations of TAF (10, 20, 50, and 100  $\mu$ M). The rhCatA assay mixtures were incubated at 37°C for 5 min. The reaction was terminated by adding a two-fold volume of acetonitrile (ACN) containing the internal standard RDV (5  $\mu$ M). The samples were then vortexed for 15 seconds and centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was collected and centrifuged again using the same condition to remove the precipitated proteins. The resulting supernatant was collected and diluted with a four-fold volume of 66.7% methanol (methanol: water, 2:1, v/v) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The rhCES1 activity study was conducted in 10  $\mu$ L Tris buffer (pH 7.2), containing 40 ng/ $\mu$ L rhCES1 and various concentrations (10, 20, 50, and 100  $\mu$ M) of TAF. The rhCES1 reaction mixtures were incubated at 37°C for 20 min. The reaction was terminated by adding a five-fold volume of ACN containing the internal standard RDV (2  $\mu$ M). The samples were then vortexed for 15 seconds and centrifuged at 15,000 rpm at 4°C for 10 min to remove the precipitated proteins. The centrifugation was repeated once, and the resulting supernatant was collected for LC-MS/MS analysis.

The TAF hydrolysis activity was calculated by dividing the reduction of TAF concentration after incubation by the protein concentrations of the recombinant enzymes or HLS9 and incubation time.

### ***pH-dependent TAF hydrolysis***

To evaluate the effect of pH on enzymatic activity, rhCES1 (40 ng/ $\mu$ L) and rhCatA (0.25 ng/ $\mu$ L) were incubated with 20  $\mu$ M TAF at 37°C in the MES assay buffer (pH 5.2) for 20 min and the Tris buffer (pH 7.2) for 10 min, respectively. Our preliminary study confirmed that TAF hydrolysis was in a linear relationship with the enzyme concentrations (CatA: 0.1 - 0.5 ng/ $\mu$ L; CES1: 20 - 40 ng/ $\mu$ L) and the

incubation duration (CatA: 0 - 5 min; CES1: 0 - 30 min). Moreover, TAF (20  $\mu$ M) was incubated with HLS9 (0.5 mg/mL HLS9 protein) at 37°C for 15 min in both MES pH 5.2 assay buffer and Tris pH 7.2 buffer. All reactions were performed on a shaker (Benchmark, Multi-Therm™) at 1,500 rpm and terminated by adding a two-fold volume of ACN containing the internal standard RDV (5  $\mu$ M). Samples were vortexed for 15 seconds and centrifuged twice at 15,000 rpm at 4°C to remove the precipitated proteins. The resulting supernatant was collected and diluted with a four-fold volume of 66.7% methanol. The remaining TAF after incubation was determined using an LC-MS/MS assay.

### ***CatA and CES1 Inhibition study***

An *in vitro* study was conducted to evaluate the effects of telaprevir (CatA inhibitor) and BNPP (CES1 inhibitor) on TAF hydrolysis. The remaining TAF concentration was determined after incubation of TAF (20  $\mu$ M) with pooled HLS9 at 37°C for 10 min in the absence or presence of various concentrations of telaprevir (0.5, 5, and 50  $\mu$ M) and BNPP (1, 10, and 50  $\mu$ M) in the MES pH 5.2 assay buffer and Tris pH 7.2 buffer. TAF was incubated with 0.5 mg/mL HLS9 for 15 min in the MES pH 5.2 buffer, whereas the TAF incubation in the Tris pH 7.2 buffer was 20 min with 1 mg/mL HLS9. The sample preparation procedure following incubation was identical to the HLS9 activity assay described above.

### ***Effect of CES1 variant G143E on TAF hydrolysis***

Another *in vitro* incubation study was conducted to assess the effect of the CES1 nonsynonymous variant G143E on TAF hydrolysis. TAF (100  $\mu$ M) was incubated with the S9 fractions (0.15 mg protein/mL) of Flp-In HEK 293 cells stably transfected with wild-type CES1, the CES1 variant G143E, or blank vector at 37°C for 30 min. CES1 protein levels are comparable between the wild-type CES1 and the G143E variant cell lines (Zhu et al., 2008). The reactions were terminated by adding a two-fold volume of ACN containing the internal standard AFV (20  $\mu$ M). The samples were then vortexed for 5 min and centrifuged at 17,000 rpm at 4°C for 10 min to remove the precipitated proteins. The resulting

supernatant was collected for analysis of the formed TFV using an LC-MS/MS assay.

### ***LC-MS/MS analysis of TAF and TFV and CES1 and CatA protein concentrations in HLS9***

TAF and TFV were quantified based on the previously reported methods with some modifications (King et al., 2006; Avataneo et al., 2020). The methods were detailed in Supplemental Materials. A proteomics study was conducted to quantify the proteomes of the pooled HLS9 using a DIA-MS-based proteomic method established in our laboratory (Wang et al., 2020). The study was carried out on a TripleTOF 5600 plus mass spectrometer (AB Sciex, Framingham, MA) coupled with an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). The proteomics data were analyzed using the Spectronaut Pulsar software (version 11.0; Biognosys AG, Schlieren, Switzerland) with its internal reference spectral library "Human - Liver (fractionated)". The absolute protein expressions of the hydrolases of interest were calculated using the MS2-based DIA-TPA algorithm (He et al., 2019).

### ***Data Analysis***

GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA) was used for generating graphs. IBM SPSS 28.0 (IBM Corp, Armonk, NY, USA) was used for statistical analysis. Student's *t*-test was used to analyze the differences in TAF hydrolysis activity between two pH conditions. The relative contributions of CatA and CES1 to TAF hydrolysis in HLS9 were calculated by multiplying the TAF hydrolytic activity of each purified enzyme with its protein expression level in HLS9. The analysis of variance (ANOVA) was used to analyze the differences in TAF hydrolysis between groups incubated with various concentrations of CES1 or CatA inhibitors and different TAF hydrolytic rates following the incubation with the vector, wild type CES1, and the G143E variant cell S9 fractions. A *P*-value less than 0.05 was considered statistically significant.

## Results

### ***CatA contributed more to TAF hydrolysis in HLS9 than CES1***

The activity of rhCatA and rhCES1 on TAF hydrolysis are summarized in Table 1. rhCatA activity was approximately 1,000-fold higher than rhCES1. Our DIA-based proteomics analysis showed that the protein concentrations of CatA and CES1 were 0.082 and 17.7  $\mu\text{g}$  enzyme/mg total protein, respectively, in the pooled HLS9. We estimated the contributions of CatA and CES1 to TAF hydrolysis in HLS9 by multiplying their activity with their protein abundance in HLS9. The results indicate that the contribution of CatA to TAF hydrolysis in HLS9 was approximately 3 to 5-fold of that of CES1 when TAF concentrations were 10-100  $\mu\text{M}$  (Table 1).

### ***Activity of rhCatA and rhCES1 and HLS9 on hydrolyzing TAF was affected by pH***

A significant pH-dependent effect was observed on TAF hydrolysis following incubation with rhCES1, rhCatA, and HLS9 (Figure 2). rhCES1 activity in its optimal pH condition (pH 7.2) was about 4-fold higher than that at pH 5.2 ( $6.10 \pm 1.11$  vs.  $1.19 \pm 0.74$  pmol/min/ $\mu\text{g}$  protein,  $n = 3$ ,  $P < 0.01$ , Figure 2a). On the contrary, the activity of rhCatA at pH 5.2 was over 1-fold higher than its activity at pH 7.2 ( $2,742.45 \pm 185.48$  vs.  $1,187.99 \pm 111.80$  pmol/min/ $\mu\text{g}$  protein,  $n = 3$ ,  $P < 0.01$ , Figure 2b). Moreover, TAF hydrolysis activity of HLS9 measured in the MES pH 5.2 buffer was about 3-fold higher than that in the Tris pH 7.2 buffer ( $1904.20 \pm 17.38$  vs.  $480.58 \pm 75.08$  pmol/min/mg protein,  $n = 3$ ,  $P < 0.01$ , Figure 3c). TAF was stable in both MES pH 5.2 and Tris pH 7.2 blank buffers at 37°C for 30 min.

### ***Effects of CES1 and CatA inhibitors on TAF hydrolysis in HLS9***

To confirm the role of CatA and CES1 in hydrolyzing TAF in human livers, we further investigated the effects of the CatA inhibitor telaprevir and the CES1 inhibitor BNPP on TAF hydrolysis in HLS9. As shown in Figure 3. in the pH 5.2 condition, BNPP (1 - 50  $\mu\text{M}$ ) showed an inappreciable effect on TAF hydrolysis by

HLS9, while telaprevir at only 0.5  $\mu\text{M}$  could significantly inhibit TAF hydrolysis ( $P < 0.01$ ). Of note, telaprevir at 50  $\mu\text{M}$  could nearly abolish the TAF hydrolysis activity in HLS9. In the pH 7.2 condition, telaprevir (0.5 - 50  $\mu\text{M}$ ) significantly inhibited the TAF hydrolysis in HLS9, while for BNPP, no significant inhibitory effect on TAF hydrolysis was observed even when the BNPP concentration reached 50  $\mu\text{M}$  ( $P > 0.05$ ).

***CES1-mediated TAF hydrolysis was significantly impaired by the genetic polymorphism G143E***

TAF was incubated with the S9 fractions of Flp-In HEK 293 cells stably expressing wild-type CES1 and the G143E variant. The S9 fractions of the wild-type CES1 cells efficiently hydrolyzed TAF to the intermediate metabolite TFV ( $2.45 \pm 0.30$  nmol/mg S9 protein/min). The catalytic activity was reduced by 64% in the S9 fractions of both the G143E and the blank vector-transfected cells (Figure 4), suggesting that the G143E is a loss-of-function variant for CES1-mediated TAF hydrolysis.

## Discussion

Identifying key hepatic enzymes in TAF activation is essential to understand the interindividual variability in the PK and anti-HBV efficacy of TAF therapy. A previous study suggested that CES1 was the primary enzyme responsible for TAF hydrolysis in the human liver (Murakami et al., 2015), and the information has been included in the FDA label of TAF (Vemlidy®). This conclusion was based on an *in vitro* inhibitory study in primary human hepatocytes (Murakami et al., 2015), which showed that the CatA inhibitors telaprevir and boceprevir did not significantly affect TAF activation, whereas the CES1 inhibitor BNPP significantly reduced the formation of the TAF active metabolite TFV-DP. Birkus *et al.* reported that six hydrolases, namely porcine liver carboxylesterase (a close homolog of human CES1 (Lange et al., 2001)), CatA, leukocyte elastase, pancreatic elastase 1, proteinase 3, and cathepsin H, out of the 16 tested enzymes showed catalytic activity towards TAF hydrolysis (Birkus et al., 2008b; Birkus et al., 2016). Specifically, CatA exhibited the highest hydrolytic activity (31,000 pmol/μg enzyme/min), which was approximately 35- to 1,148-fold higher than other hydrolases, when TAF was incubated at a single concentration of 100 μM. In the present study, the rhCatA activity was found to be  $33,918 \pm 1,185$  pmol/l/μg enzyme/min when TAF concentration was 100 μM. Of note, Birkus and colleagues performed the CatA activity assay at pH 6.5 while we conducted the CatA activity study at pH 5.2 to mimic the acidic condition in the lysosomes. The enzyme manufacture also recommended using an acidic reaction buffer (~pH 5.5) to determine the CatA catalytic activity. One of the aims of this study was to determine the contributions of CES1 and CatA the TAF hydrolysis in the liver based on the hepatic protein levels and the enzymatic efficiencies on catalyzing TAF hydrolysis of the two enzymes. Our DIA proteomics analysis showed that the protein abundances of CES1 and CatA in the pooled HLS9 were 17.7 and 0.082 μg/mg HLS9 protein, respectively. A recent study reported that the CES1 and CatA were  $34 \pm 1$  and  $0.23 \pm 0.11$  μg/mg total protein, respectively, in pooled HLS9 (Li et al., 2021). The differences may be due to the different HLS9 resources and the protein quantification methods used in the two studies (i.e., DIA

proteomics vs. Western Blot). In addition, a previous proteomics study profiled the proteomes of primary hepatocytes from six donors and found that CES1 and CatA were  $13.7 \pm 3.0$  and  $0.059 \pm 0.014$   $\mu\text{g}/\text{mg}$  total protein, respectively (Wiśniewski et al., 2016). Interestingly, despite the slight differences in the absolute values, all studies demonstrated that hepatic CES1 protein expression was approximately 200-fold of the CatA protein level in the human liver. Our study revealed that the activity of CatA on hydrolyzing TAF was approximately 1,000-fold higher than that of CES1 when TAF concentrations were within 10 and 100  $\mu\text{M}$  (Table 1). Taking both activity and protein abundance into consideration, we estimated that the contribution of CatA to TAF hydrolysis in HLS9 was about 3 to 5-fold of that of CES1. Of note, although hepatic TAF concentration in humans is unknown, plasma TAF mean  $C_{\text{max}}$  was found to be approximately 4  $\mu\text{M}$  in patients who received a single dose of 120 mg TAF (Markowitz et al., 2014); thus, the TAF concentrations utilized in the *in vitro* incubation study are clinically relevant. Birkus *et al.* reported that, besides CES1 and CatA, leukocyte elastase, pancreatic elastase 1, proteinase 3, and cathepsin H also showed catalytic activity towards TAF hydrolysis (Birkus et al., 2008b; Birkus et al., 2016). Nevertheless, their contributions appear to be negligible due to their very low activity as well as very low protein expression in the human liver.

Given that the subcellular locations of CatA and CES1 are different (i.e., CatA: lysosome (pH  $\sim 4.7$ ) and CES1: endoplasmic reticulum lumen/cytoplasm (pH 7.1-7.4) (Casey et al., 2010), the *in vitro* incubation studies were conducted in two different buffers: MES buffer (pH 5.2) and Tris buffer (pH 7.2). A significant pH-dependent effect was observed for the activity of both CES1 and CatA. It is generally accepted that CatA is an acidic pH-dependent serine protease, and the acidic condition is critical for its catalytic functions. For example, chloroquine, a common agent capable of increasing lysosomal pH, was found to inhibit the intracellular activation of GS-9191, another ester prodrug hydrolyzed by CatA (Birkus et al., 2011). The activity of CES1 was decreased at pH 5.2 compared with that at pH 7.2, which was consistent with the previous report that the CES1 activity was stable in pH 6.5-7.5 (Inoue et al., 1980). Thus, the activity measured

in pH 5.2 for rhCatA and pH 7.2 for rhCES1 should reasonably reflect their *in situ* physiological catalytic activities.

We conducted an *in vitro* inhibition study to further elucidate the involvement of CatA and CES1 in hepatic TAF hydrolysis. It has been well documented that telaprevir and BNPP are potent CatA and CES1 inhibitors, respectively (Murakami et al., 2010; Li et al., 2021). Moreover, at the tested concentrations (1 - 50  $\mu$ M), telaprevir did not impact the CES1 activity, whereas BNPP had no effect on the activity of CatA (Murakami et al., 2010; Li et al., 2021). Telaprevir at 0.5  $\mu$ M could markedly inhibit TAF hydrolysis in HLS9 at pH 5.2 and pH 7.2, while BNPP only showed a weak inhibitory effect at 50  $\mu$ M under the pH 7.2 condition. It should be noted that 50  $\mu$ M BNPP could fully impair CES1 activity (Fujiyama et al., 2010); however, a significant portion of TAF hydrolysis activity remained intact after incubation with 50  $\mu$ M BNPP. Thus, the inhibitory study further supports a more significant role of CatA in hydrolyzing TAF in HLS9 relative to CES1.

Interestingly, Murakami *et al.* did not observe a significant inhibitory effect of the protease inhibitors telaprevir and boceprevir on TAF metabolism in primary human hepatocytes (Murakami et al., 2015). This discrepancy might be due to the limited intracellular accumulation of these protease inhibitors since both telaprevir and boceprevir are the substrates of the efflux transporter P-gp, which is highly expressed in hepatocytes (Chu et al., 2013; Fujita et al., 2013; Weiss et al., 2014; Murakami et al., 2015). The lack of inhibitory effects of telaprevir and boceprevir in primary human hepatocytes could also be caused by the potential reduction of CatA expression in hepatocytes under *in vitro* culture conditions (Richert et al., 2006). In the present study, we performed an *in vitro* inhibition study using HLS9 samples instead of hepatocytes to avoid the abovementioned two potential limitations and demonstrated a significant inhibitory effect of telaprevir on hepatic TAF hydrolysis.

Taken together, our conclusion that CatA may play a more important role than CES1 in hydrolyzing TAF in the liver is supported by the following three observations: 1) a greater contribution of CatA to TAF hydrolysis in HLS9 than



CES1 based on the activity data and the hepatic protein abundances of the two enzymes; 2) a substantial increase of TAF hydrolysis in HLS9 when the incubation study was conducted at the optimal pH for CatA (i.e., pH 5.2); 3) the CatA inhibitor telaprevir exhibited a more potent inhibitory effect on TAF hydrolysis in HLS9 than the CES1 inhibitor BNPP.

The CES1 genetic polymorphism G143E is a loss-of-function variant for many CES1 substrates (Her and Zhu, 2020). Its minor allele frequencies are approximately 3.7%, 2.0%, and 4.3% in Caucasian, African American, and Hispanic populations, respectively, but the variant is extremely rare in Asian populations (Zhu et al., 2008; Suzaki et al., 2013). The G143E variant abolishes CES1 catalytic activity and subsequently affect the PK and PD of various CES1 substrate medications, such as methylphenidate, clopidogrel, dabigatran etexilate, and angiotensin-converting enzyme inhibitors (Zhu et al., 2008; Lewis et al., 2013; Tarkiainen et al., 2015a; Tarkiainen et al., 2015b; Shi et al., 2016a; Shi et al., 2016b; Shi et al., 2016c; Wang et al., 2016; Stage et al., 2017). To investigate the impact of the CES1 genetic polymorphism G143E on the hydrolysis of TAF, we conducted a TAF incubation study using Flp-In HEK 293 cells stably expressing wild-type CES1 and the G143E variant. We found that the G143E variant markedly reduced TAF hydrolysis to a level similar to that in the blank vector-transfected cells (Figure 4), indicating that the G143E is a loss-of-function variant for the CES1-mediated TAF hydrolysis. However, given that CES1 was estimated to only account for a small portion of the TAF hydrolysis in the liver, the impact of the G143E variant on hepatic TAF activation is expected to be less significant compared with its impact on other selective CES1 substrates, such as methylphenidate and clopidogrel (Zhu et al., 2008; Zhu et al., 2013). We also observed considerable TAF hydrolysis activity in the G143E and the blank vector cells (Figure 4), which is likely attributed to CatA.

This study has several potential limitations. One limitation is that, due to the limited TAF solubility, we were unable to conduct a kinetic study to determine the kinetic parameters (i.e.,  $K_m$  and  $V_{max}$ ) of the two enzymes because of the inability to include high substrate concentrations at levels that could saturate the enzymes.

Another limitation is that the estimated contributions of CatA and CES1 to hepatic TAF hydrolysis are based on the assumption that CatA and CES1 proteins found in HLS9 are reflective of their levels in the liver. CES1 is present in both endoplasmic reticulum lumen and cytoplasm, and CatA is exclusively expressed in the lysosomes. It has been reported that the HLS9 preparation could not completely extract the endoplasmic reticulum luminal fractions and the lysosomes (Xu et al., 2018). However, the recovery rates of endoplasmic reticulum lumen (~85-95%) and lysosomal lumen (~85%) in the S9 fractions were comparable (Xu et al., 2018), and thus, the incomplete extraction should not affect our estimation of the relative contributions of CatA and CES1 to hepatic TAF hydrolysis. A further limitation of this study is that the HLS9 system is unable to evaluate the effect of the partition of a drug in cell organelles (e.g., lysosomes) on drug metabolism. Since CatA is a lysosomal protein, TAF needs to enter the lysosomes to be hydrolyzed by CatA. Although it remains unexplored regarding how efficient and to what extent TAF can be accumulated in the lysosomes, TAF appears to readily enter into the lysosomes according to a previous report, which showed that TAF was effectively hydrolyzed by CatA in peripheral blood mononuclear (PBMC) cells (Birkus et al., 2007). Moreover, lysosomes have been identified as the organelle involving TAF hydrolysis in MT-2 and PBMC cells using organelle marker assays (Birkus et al., 2008a).

In sum, our study suggested that CatA may play a major role in hydrolyzing TAF in the human liver. For the first time, the activities of CES1, CatA, and HLS9 on TAF hydrolysis were found to be highly pH-dependent, highlighting the importance of using appropriate pH in *in vitro* drug metabolism studies. Moreover, we showed that the CES1 genetic polymorphism G143E is a loss-of-function variant for CES1-mediated TAF hydrolysis. However, we expect that the impact of this variant on the anti-HBV therapy of TAF is modest because of the significant involvement of CatA in hepatic TAF hydrolysis. Further investigations are warranted to confirm the significance of CatA in activating TAF in human livers using *in vivo* models.

## **Authorship Contributions**

Participated in research design: Jiapeng Li, Jian Shi, and Hao-Jie Zhu.

Conducted experiments: Jiapeng Li, Jian Shi, Lana Tran, Jingcheng Xiao.

Performed data analysis: Jiapeng Li, Jian Shi, Lana Tran, Xinwen Wang, Jingcheng Xiao, and Hao-Jie Zhu.

Wrote or contributed to the writing of the manuscript: Jiapeng Li, Jian Shi, Lana Tran, Xinwen Wang, and Hao-Jie Zhu.

## **Conflict of Interest**

The authors have declared no conflict of interest. Lana Tran's affiliation at the time of involvement is with the University of Michigan College of Pharmacy and therefore declares no conflict of interest.

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## Footnotes

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## Figure legends

**Figure 1.** Activation pathway of TAF in the human liver. TAF was first hydrolyzed to tenofovir-alanine (TFV-Ala) by hepatic hydrolases, then converted to TFV via histidine triad nucleotide-binding protein 1 (HINT1)-mediated hydrolysis. TFV was further phosphorylated to the active metabolite tenofovir diphosphate (TFV-DP) by native kinases.

**Figure 2.** TAF hydrolysis activity of rhCES1 (a), rhCatA (b), and HLS9 (c) in MES pH 5.2 assay buffer and Tris pH 7.2 buffer. 40 ng/ $\mu$ L rhCES1, 0.25 ng/ $\mu$ L rhCatA, and 0.5 mg/mL HLS9 were incubated with 20  $\mu$ M TAF at 37°C for 20 min, 10 min, and 15 min, respectively. Bars are mean values ( $\pm$  S.D.) of at least three independent experiments ( $n \geq 3$ ). \*\* $P < 0.01$ .

**Figure 3.** Effect of telaprevir and BNPP on TAF hydrolysis in HLS9 in MES pH 5.2 assay buffer (a) and Tris pH 7.2 buffer (b). Data are shown as the remaining TAF (mean  $\pm$  S.D.,  $n = 3$ ) following incubation. \*\* $P < 0.01$  compared to the vehicle control. In the MES pH 5.2 assay buffer, 20  $\mu$ M TAF were incubated with 0.5 mg/mL HLS9 at 37°C for 15 min; in the Tris pH 7.5 buffer, 20  $\mu$ M TAF were incubated with 1 mg/mL HLS9 at 37°C for 20 min.

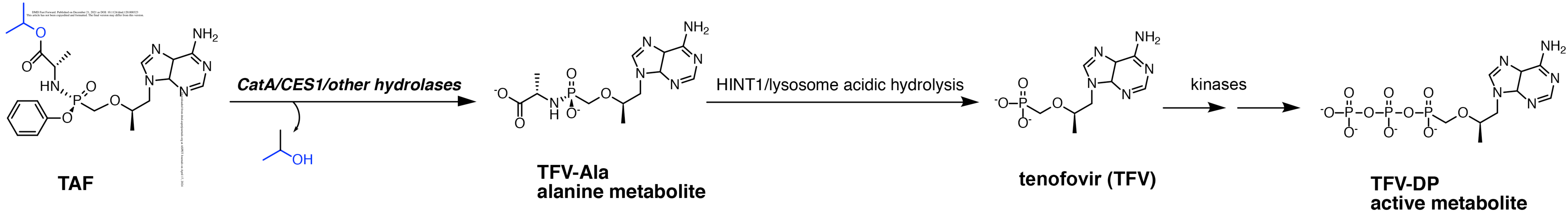
**Figure 4.** Effect of the CES1 genetic polymorphism G143E on TAF activation in the S9 fractions of the transfected Flp-In HEK 293 cells. Data are shown as the formation rates of TFV (mean  $\pm$  S.D.,  $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$  compared to the wild-type HEK 293 cells.

**Table 1.** Catalytic activity of recombinant human CatA and CES1 on hydrolyzing TAF and the estimated relative contributions of CatA and CES1 to TAF hydrolysis in HLS9.

TAF ( $\mu\text{M}$ )	rhCatA activity (pmol/min/ $\mu\text{g}$ protein)*	rhCES1 activity (pmol/min/ $\mu\text{g}$ protein)*	rhCatA to rhCES1 Activity ratio	CatA abundance in HLS9 ( $\mu\text{g}/\text{mg}$ total protein)	CES1 abundance in HLS9 ( $\mu\text{g}/\text{mg}$ total protein)	Contribution to TAF hydrolysis in HLS9 (CatA:CES1)
10	3328.7 $\pm$ 162.1	4.8 $\pm$ 0.4	693 : 1	0.082	17.70	3.2 : 1
20	6817.3 $\pm$ 1030.5	6.7 $\pm$ 1.1	1048 : 1			4.9 : 1
50	16221.0 $\pm$ 2564.3	16.2 $\pm$ 4.0	1061 : 1			4.9 : 1
100	33918.0 $\pm$ 6175.3	28.6 $\pm$ 4.2	1203 : 1			5.6 : 1

\* Data were obtained from three independent experiments (n = 3)

# Figure 1



**Figure 2**

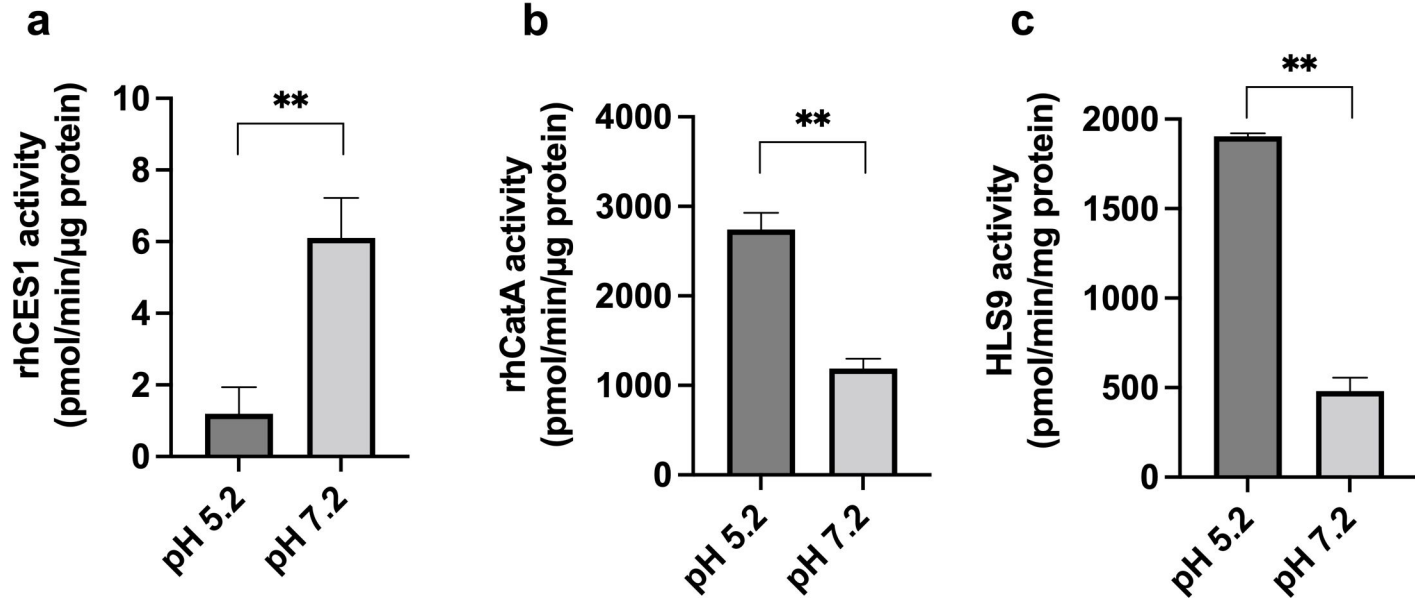
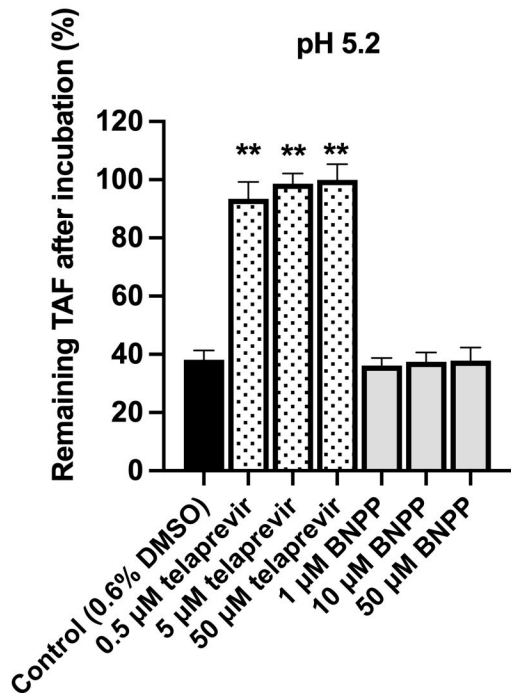


Figure 3

a



b

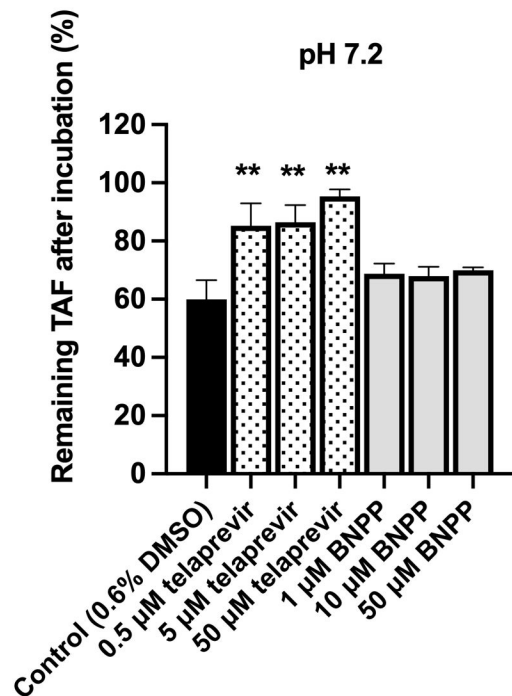
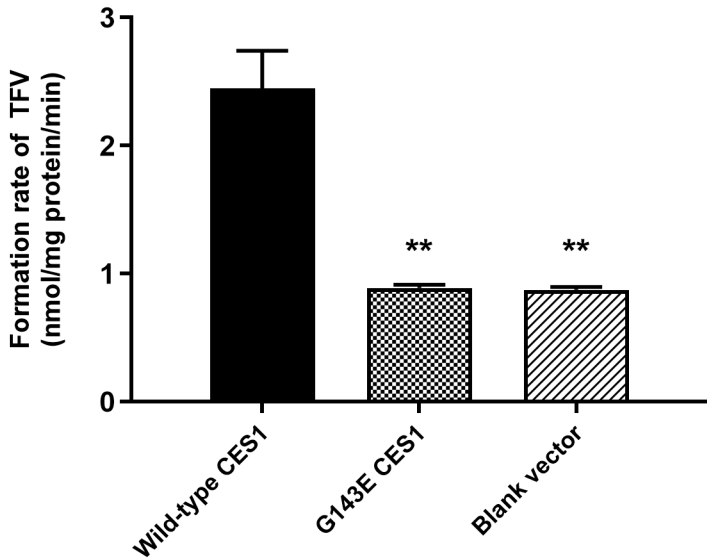


Figure 4



## Supplemental Materials

### **Contributions of Cathepsin A and Carboxylesterase 1 to the hydrolysis of Tenofovir Alafenamide in the Human Liver, and the Effect of CES1 Genetic Variation on Tenofovir Alafenamide Hydrolysis**

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<sup>1</sup> The authors contributed equally to the project.

### ***LC-MS/MS assays for TAF and TFV quantification***

TAF was quantified based on a previously reported method with some modifications (Avataneo et al., 2020). The LC-MS/MS system consisted of a PE SCIEX API 3000 spectrometer and a Shimadzu UFLC system (Shimadzu, Tokyo, Japan).

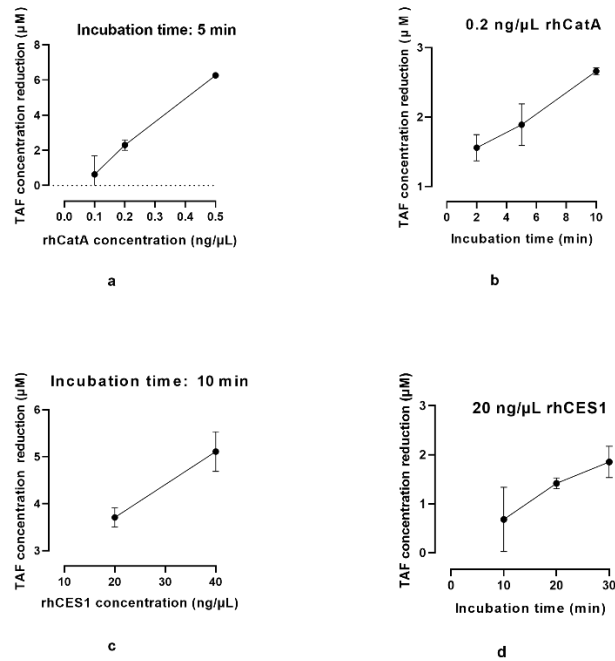
Chromatographic separation was obtained on a RESTEK Ultra II C18 column (5  $\mu\text{m}$ , 50  $\times$  2.1 mm, Bellefonte, PA) protected by a RESTEK UltraShield UHPLC precolumn filter (0.2  $\mu\text{m}$  frit, Bellefonte, PA) at 45  $^{\circ}\text{C}$ . The mobile phase flow rate was 0.5 mL/min. The injection volume was 1  $\mu\text{L}$ . The gradient elution was obtained using water containing 0.1% (v/v) formic acid (phase A) and ACN containing 0.1% (v/v) formic acid (phase B) with the following time program: mobile phase B was 2% for the first 0.1 min, increased from 2% to 90% during the time period of 0.1–3 min, maintained at 90% for 0.5 min, then returned to 2% at 3.6 min, and maintained at 2% until the end of the gradient at 5 min. The mass spectrometer was operated in a positive ion mode using turbo electrospray ionization. The following parameters were used for the MS analysis: nebulizer gas: 15 psi; curtain gas: 15 psi; collision gas: 6 psi; ionspray voltage: 5500 V; source temperature: 550  $^{\circ}\text{C}$ ; declustering potential: 30 V; focusing potential: 40 V; entrance potential: 10 V; collision energy: 50 V; collision cell exit potential: 15 V. The following transitions were monitored in a multiple reaction monitoring (MRM) mode: TAF,  $m/z$  477.20 > 176.10; RDV,  $m/z$  603.20 > 200.10, scan time: 0.25 second. Quantifications were based on the peak area ratios of TAF to RDV. The regression coefficients of calibration curves were greater than 0.99.

A previously reported method was utilized for TFV quantification with some modifications (King et al., 2006). The LC-MS/MS analysis was performed on an Applied Biosystems API 4000 triple quadrupole/linear ion trap (QTRAP) mass spectrometer (Foster City, CA, USA). Analytes were separated using a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) on a Shimadzu VP-ODS column (5  $\mu\text{m}$ , 150  $\times$  2.0 mm, Shimadzu, Tokyo, Japan). The mobile phase consisted of water with 1% formic acid (phase A) and methanol containing 0.05% formic acid (phase B), and was delivered at a flow rate of 0.2 ml/min. The time program of the gradient elution was as follows: mobile phase B was 2% for the first 3 min, increased from 2% to 88% during the time period of 3–8 min, maintained at 88% for 1 min, then returned to 2% at 9.5 min, and maintained

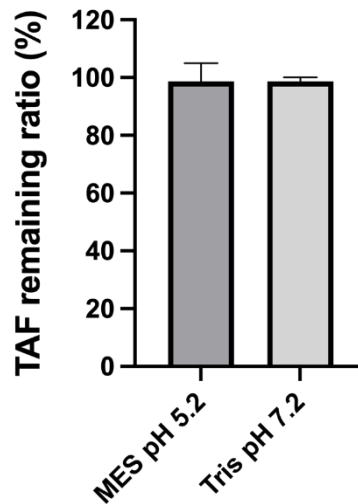


at 2% until the end of the gradient at 12 min. MS was operated in a positive ion mode using turbo electrospray ionization with the following parameters: curtain gas: 30 psi; ion source gas 1: 40 psi; ion source gas 2: 60 psi; ionspray voltage: 5500 V; source temperature: 500 °C; entrance potential: 10 V; dwell time: 100 ms; collision cell exit potential: 11 V; declustering potential: 60 V; and collision energy: 40 V. The following transitions were monitored in an MRM mode: TFV,  $m/z$  288.2 > 176.3; AFV,  $m/z$  274.6 > 162.4. Quantifications were based on the peak area ratios of TFV to AFV. The regression coefficients of calibration curves were greater than 0.99. The accuracy and precision of both TAF and TFV assays met the requirements in the FDA bioanalytical method validation guidance.

**Supplementary Figure 1.** Time- and rhCES1 and rhCatA concentration-dependent TAF hydrolysis. TAF (10  $\mu\text{M}$ ) was incubated with (a) rhCatA (0.1, 0.2, 0.5  $\text{ng}/\mu\text{L}$ ) for 5 min; (b) 0.2  $\text{ng}/\mu\text{L}$  rhCatA for 2, 5, and 10 min); (c) rhCES1 (20, 40  $\text{ng}/\mu\text{L}$ ) for 10 min; d. 20  $\text{ng}/\mu\text{L}$  rhCatA for 10, 20, 30 min. Data are shown as the reduced TAF concentration after incubation (mean  $\pm$  S.D.,  $n = 3$ ).



**Supplementary Figure S2.** TAF (20  $\mu\text{M}$ ) stability following incubation in MES pH 5.2 buffer and Tris pH 7.2 buffer at 37°C for 30 min.



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