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## HIV-Protease Inhibitors Block the Enzymatic Activity of Purified Ste24p

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### Abstract

We reported that several HIV protease inhibitors (HIV-PIs) interfere with the endoproteolytic processing of two farnesylated proteins, yeast  $\alpha$ -factor and mammalian prelamin A. We proposed that these drugs interfere with prelamin A processing by blocking ZMPSTE24, an integral membrane zinc metalloproteinase known to play a critical role in its processing. However, because all of the drug inhibition studies were performed with cultured fibroblasts or crude membrane fractions rather than on purified enzyme preparations, no definitive conclusions could be drawn. Here, we purified Ste24p, the yeast ortholog of ZMPSTE24, and showed that its enzymatic activity was blocked by three HIV-PIs (lopinavir, ritonavir, and tipranavir). A newer HIV-PI, darunavir, had little effect on Ste24p activity. None of the HIV-PIs had dramatic effects on the enzymatic activity of purified Ste14p, the prenylprotein methyltransferase. These studies strongly support our hypothesis that HIV-PIs block prelamin A processing by directly affecting the enzymatic activity of ZMPSTE24, and in this way they may contribute to lipodystrophy in individuals undergoing HIV-PI treatment.

### Keywords

ZMPSTE24; Ste24p; HIV-PI; lamins; Ste14p; lipodystrophy; protease; methyltransferase

Many eukaryotic proteins, such as the mating pheromone  $\alpha$ -factor in *Saccharomyces cerevisiae*, the Ras proteins in both yeast and mammals, and the nuclear lamins in mammals, terminate with a CaaX motif (in which the “C” is cysteine, “a” is often an aliphatic amino acid, and “X” is one of several different amino acids) [1]. The CaaX motif triggers a series of posttranslational modifications. First, the cysteine is farnesylated or geranylgeranylated by cytosolic protein prenyltransferases. Second, the last three amino acids of the protein (*i.e.*, the “aaX”) are clipped off and released. This reaction is generally carried out by RCE1, a membrane endoprotease of the endoplasmic reticulum (ER); however, in the case of  $\alpha$ -factor in yeast and

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prelamin A in mammals, this step can be carried out by both RCE1 and a membrane zinc metalloproteinase of the ER, designated Ste24p in yeast and ZMPSTE24 in mammals [2–4]. Finally, the newly exposed isoprenylcysteine is methylated by an integral membrane methyltransferase, designated Ste14p in yeast and ICMT in mammals [5,6]. These reactions leave the protein with a carboxyl-terminal prenylcysteine  $\alpha$ -carboxyl methyl ester.

At least two farnesylated proteins, **a**-factor in yeast and prelamin A in mammals, undergo additional endoproteolytic processing. In the case of **a**-factor, the first seven amino acids of the protein are removed by Ste24p, which render the protein susceptible to a final endoproteolytic processing reaction by Ax11p [2,7,8]. In the case of prelamin A, ZMPSTE24 cleaves off the last 15 amino acids of the protein, including the farnesylcysteine methyl ester, releasing mature lamin A [9]. ZMPSTE24 and Ste24p are orthologs. Mammalian ZMPSTE24 faithfully carries out both the carboxyl- and amino-terminal endoproteolytic processing steps in the maturation of yeast **a**-factor, and the two enzymes exhibit high degrees of amino acid identity/similarity and share the classic features of a zinc metalloprotease [10–12].

Several mutations in *LMNA*, the gene encoding prelamin A and lamin C, cause lipodystrophy, and a similar phenotype has been noted in patients treated with HIV-PIs [13,14]. Furthermore, individuals with mandibuloacral dysplasia (MAD), which is characterized in part by lipodystrophy, contain mutations in ZMPSTE24 [11]. Recently, several groups have shown that HIV-PIs can lead to accumulation of prelamin A in fibroblasts, suggesting that these drugs interfere with the conversion of prelamin A to mature lamin A [15–17]. Coffinier *et al.* [16, 17] found that the prelamin A that accumulated is farnesylated, and additional studies with crude membrane fractions suggested that HIV-PIs might inhibit ZMPSTE24. This conclusion was tentative, however, since no studies were performed with purified enzyme preparations. Moreover, one could have argued that an effect of HIV-PIs on ZMPSTE24 was implausible, given that the HIV-protease is an aspartyl protease and HIV-PIs have never been reported to inhibit a zinc metalloproteinase. For these reasons, we considered it important to examine the effects of HIV-PIs on purified enzyme preparations.

## Materials and Methods

### Materials

Lopinavir (LPV), ritonavir (RTV), and tipranavir (TPV) were obtained from the NIH AIDS Research and Reference program, Division of AIDS, NIAID, NIH. Darunavir (DRV) was a gift from Dr. Arun Ghosh (Department of Chemistry, Purdue University). n-Dodecyl- $\beta$ -D-maltopyranoside (DDM) was obtained from Anatrace (Maumee, OH). A farnesylated **a**-factor peptide (YIIKGVFWDPA(farnesyl)CVIA) was synthesized and purchased from either California Peptide Research (San Diego, CA) or EZBiolab (Westfield, IN). *E. coli* polar lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL). S-Adenosyl-L-[<sup>14</sup>C-methyl]-methionine (SAM) was purchased from GE Healthcare (Piscataway, NJ). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

### Yeast Strains and Plasmids

A *Δste24Δrce1* strain of *S. cerevisiae* (SM3614) was transformed with pSM1282, a plasmid encoding a His<sub>10</sub>HA<sub>3</sub>N-terminally-tagged version of Ste24p or pCHH10m3N-Ste14, a plasmid encoding a His<sub>10</sub>myc<sub>3</sub>N-terminally-tagged version of Ste14p [12,18,19]. These strains were designated CH2771 and CH2733, respectively. Both proteins were under the control of the constitutive 3'-phosphoglycerate kinase promoter.

## Purification of Ste24p and Ste14p

Microsomes were isolated as previously described [18]. Briefly, CH2733 and CH2771 cells were grown in synthetic complete drop out media (minus uracil) and harvested at 2 OD<sub>600</sub>. Cell pellets were washed with 50 mM NaN<sub>3</sub> and centrifuged for 5 min at 5000 × g. Cells were resuspended in lysis buffer plus inhibitors (300 mM sorbitol, 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 7.5, 1% aprotinin, 2 mM AEBSF-HCl, 1 mM DTT) and incubated for 15 min on ice. Cells were twice frozen on liquid nitrogen and thawed. Cells were then lysed twice by passing the cells through a French press at 18,000 psi. After removing cellular debris by centrifugation at 500 × g, the supernatant fluid was incubated with micrococcal nuclease (Worthington Biochemical, Lakewood, NJ) (50 units/per ml lysate). The lysate was then centrifuged at 150,000 × g for 1 h at 4 °C. Membrane fraction pellets were resuspended in lysis buffer containing inhibitors and 10% glycerol.

Ste14p and Ste24p are multispinning membrane proteins and were purified by methods similar to those described by Anderson *et al.* [18]. Briefly, CH2733 or CH2771 membranes (125 mg) were solubilized at a final concentration of 5 mg/mL for 1 h at 4 °C in 1% (w:v) DDM and 20 mM imidazole in buffer S containing 300 mM sorbitol, 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 7.5, 1% aprotinin, 2 mM AEBSF-HCl, 1 mM DTT, and 10% glycerol. Nonsolubilized membranes were removed by centrifugation (30 min at 300,000 × g at 4 °C). The supernatant fluid was mixed with Talon metal affinity resin beads (500 µL of a 50% v:v slurry equilibrated with buffer S) (Clontech, Palo Alto, CA) for 1 h at 4 °C. After binding, the beads were washed twice with 5 mL buffer A (buffer S, 1% DDM, 40 mM imidazole), 5 mL of buffer B once (buffer A and 0.5 M KCl), and 5 mL buffer C once (buffer S, 0.1% DDM, 0.5 M KCl, 40 mM imidazole). The protein was then eluted from the beads with 3 mL elution buffer (buffer S containing 1 M imidazole and 0.1% (w:v) DDM). The eluate was added to an Amicon Ultra 30,000 MWCO concentrator and centrifuged 15–20 min at 5000 × g at 4 °C. Protein concentration was determined with the amido black protein assay [20]. Ste24p and Ste14p were reconstituted in liposomes composed of *E. coli* polar lipid extract by rapidly diluting the proteins 33-fold in 100 mM MES, pH 7.0 containing a ~500-fold lipid:protein ratio (w:w) [18].

## Silver Staining and Immunoblot Analysis

Proteins (1 µg for silver staining and 0.1 µg for immunoblotting) were size-fractionated on a 10% SDS-polyacrylamide gel, and either stained with silver or transferred to a 0.2-µm nitrocellulose membrane (Whatman, Florham Park, NJ) for immunoblotting. For silver staining, the gel was fixed in 45% ethanol/9% glacial acetic acid, incubated in 0.125% AgNO<sub>3</sub> and developed in 3% NaCO<sub>3</sub>/1.5% formaldehyde. Percent purity was estimated with ImageJ (National Institutes of Health, Bethesda, MD). Nitrocellulose membranes were blocked with nonfat powdered milk (20% (w:v) in PBS containing 0.05% TWEEN-20) and probed with primary polyclonal antibodies (1:1500 anti-Ste24p or 1:1000 anti-Ste14p) in 5% nonfat powdered milk (w:v) in PBS containing 0.05% TWEEN-20. After washing the membrane, antibody binding was detected with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:10,000) in 5% (w:v) nonfat powdered milk in PBS containing 0.05% TWEEN-20. Signal was detected using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

## In Vitro Proteolysis Methyltransferase Coupled Vapor Diffusion Activity Assay

Ste24p activity was measured with a coupled proteolysis-methylation activity assay in the presence of excess Ste14p [16,19,21]. Activity assays were assembled on ice. Purified proteins were incubated with lipid and varying concentrations of HIV-PI or vehicle (DMSO) for 5 min. After adding the **a**-factor peptide substrate, a mixture of MES pH 7.0/[<sup>14</sup>C]SAM was added to a final concentration of 100 mM MES and 20 µM [<sup>14</sup>C]SAM. This rapidly diluted sample (130

μl) was then divided into two 60 μl reactions that contained 0.09 μg Ste24p, 0.19 μg Ste14p, and 5 μM of the peptide substrate. After incubating the reactions for 30 min at 30 °C, reactions were terminated by adding 50 μl 1 M NaOH/1% SDS. The samples were spotted onto filter paper and lodged into the neck of a scintillation vial containing 10 ml BioSafe II scintillation fluid (RPI, Mount Prospect, IL), and [<sup>14</sup>C]methanol was allowed to diffuse into the fluid for 2.5 h at room temperature. Radioactivity was quantified in a Packard Tri-Carb 1600CA liquid scintillation analyzer. Base-releasable [<sup>14</sup>C]methanol in reactions containing the HIV-PI was reported as a percent of the DMSO control. The apparent IC<sub>50</sub>max was calculated with GraphPad Prism 4.0 (San Diego, CA).

Ste14p activity was measured with the same methods except that 40 μM N-acetyl-S-farnesyl-L-cysteine (provided by Dr. Richard A. Gibbs, Purdue University) was used as the substrate.

## Results and Discussion

Ste14p and Ste24p, both multispanning integral membrane proteins, were solubilized from crude yeast membrane preparations with 1% (w:v) DDM and isolated by cobalt metal affinity chromatography after removal of the nonsolubilized fraction by ultracentrifugation. Following binding of the solubilized material to the resin and washing as described in Materials and Methods, proteins were eluted from the resin in 0.1% DDM buffer containing 1 M imidazole, pH 8.0. The identity of the purified proteins was established by immunoblotting (Figure 1A), and the purity was assessed on silver stained SDS-polyacrylamide gels (Figure 1B). Both proteins were >90% pure, as judged by ImageJ analysis.

Ste24p activity was measured using a coupled endoproteolysis-methylation assay with an **a**-factor substrate containing the C-terminal *-aaX* sequence (YIIKGVFWDPA(farnesyl)CVIA) in the presence of an excess of purified Ste14p [19]. In this assay, the purified Ste24p releases the last three amino acids from the farnesylated peptide, exposing a farnesylcysteine that subsequently undergoes carboxymethylation by Ste14p. Methylation was assessed by quantifying base-releasable [<sup>14</sup>C]methanol in a vapor diffusion assay [18,22]. In order for the purified proteins to exhibit their maximal enzymatic activities, they needed to be reconstituted into *E. coli* polar lipid extract by rapid dilution. Upon reconstitution in *E. coli* lipid, the protein activity was enhanced (Figure 1C).

Ste24p activity was inhibited by several commonly prescribed HIV-PIs (Figure 2A). Because the inhibition curves do not approach a specific activity of zero, perhaps due to the presence of excess lipid, an apparent IC<sub>50</sub>max was calculated by defining the bottom of each inhibition curve as zero. Under the conditions used in these assays, the extent of inhibition was greatest with tipranavir (apparent IC<sub>50</sub>max of 33.3 ± 3.4 μM), followed by ritonavir (apparent IC<sub>50</sub>max of 93.6 ± 12.6 μM) and lopinavir (apparent IC<sub>50</sub>max of 125.6 ± 11.4 μM) (Figure 2A). Darunavir had little effect on Ste24p activity (Figure 2A). Assessing the effects of the HIV-PIs on Ste24p activity with the coupled endoprotease-methylation assay requires that these drugs have little effect on Ste14p activity. Indeed, none of the drugs tested had any effect on Ste14p activity, except TPV, which showed some inhibition at 100 μM (Figure 2B).

Purification of both Ste24p and Ste14p in the same detergent made it possible to create an ideal assay for Ste24p activity. When using crude membrane extracts, one is forced to add a large excess of membranes to make sure that all of the prenylprotein substrates are methylated. When one uses pure proteins, the amount of Ste14p and its enzymatic activity are known. Most importantly, the use of highly purified enzymes reduces the chance that the peptide substrate will be cleaved by another protein in the crude membrane extract.

Our protocol used to purify Ste14p and Ste24p improved upon an earlier procedure by the use of cobalt *versus* nickel affinity resin and the addition of an ultracentrifugation step to remove

nonsolubilized membranes [19]. Thus, the purified material that we generated was not contaminated with yeast membranes containing active Ste24p. The older protocol also used a 1% (w:v) DDM elution buffer, which reduced the ability to dilute the protein below the critical micellar concentration. In our revised protocol, we use a 0.1% (w:v) DDM solution to elute the purified protein. Another improvement in the current protocol was the use of 30,000 MWCO concentrators, allowing us to generate 100-fold more concentrated enzyme preparations. The final change we made was to add a step in which we reconstituted the concentrated proteins into liposomes by rapid dilution, thereby returning the enzymes to a lipid environment and increasing their activity.

In early studies using crude membrane fractions, we suggested that HIV-PIs might inhibit ZMPSTE24 activity [16,17]. However, the possibility that the observed inhibition in the coupled proteolysis methylation assay was due to effects on another enzyme remained. The current studies with purified enzymes established that these drugs block Ste24p activity (Figure 2A). These results were used to compare the inhibition data of pure Ste24p to that of ZMPSTE24 contained in crude membrane fractions [16,17]. Importantly, the order of potency of the different HIV-PIs was similar for mouse ZMPSTE24 in the crude membrane system and purified Ste24p in the reconstituted yeast assay system. TPV was the best inhibitor, LPV and RTV had intermediate inhibitory effects and DRV did not inhibit in either enzyme assay system. We emphasize that the actual amounts of the inhibitor available to the enzyme in both crude membranes and the reconstituted enzyme system are not known, and thus the calculated  $IC_{50}$  and  $IC_{50max}$  values are estimates and specific to each experimental system. Importantly, LPV, RTV and DRV did not affect the activity of Ste14p, the prenylprotein methyltransferase, and TPV inhibited Ste14p to a relatively small extent. Together, the current studies confirm our previous results suggesting a direct effect of HIV-PIs on ZMPSTE24 activity. LPV, RTV, and TPV all blocked endoproteolysis by purified Ste24p, and the data correlated well with results obtained with ZMPSTE24 in crude membranes [16,17].

In humans and mouse, prelamin A is generally efficiently cleaved by ZMPSTE24 to yield mature lamin A. When cleavage is blocked, farnesylated and carboxylmethylated prelamin A accumulates and causes progeroid phenotypes, prominent among which are lipodystrophy. This problem occurs for individuals with Hutchinson-Gilford Progeria Syndrome (HGPS) who have a mutant form of lamin A deleted for the ZMPSTE24 cleavage site. Similarly individuals with recessive mutations that diminish ZMPSTE24 activity have the disorder MAD, also characterized by lipodystrophy [11,23,24]. Lipodystrophy is also known to occur in some HIV patients undergoing long-term treatment with HIV-PIs [14,25]. The work presented here and elsewhere strongly suggests that this side effect is at least in part likely due to the direct inhibition of ZMPSTE24 proteolytic activity by some HIV-PIs [15–17]. Notably DRV is not inhibitory, warranting consideration of additional non-inhibitory HIV-PIs for use in therapeutic cocktails for HIV [17]. The reason that HIV-PIs, which are aspartyl protease inhibitors, potentially inhibit the zinc metalloproteinase ZMPSTE24 is not understood and remains to be further investigated.

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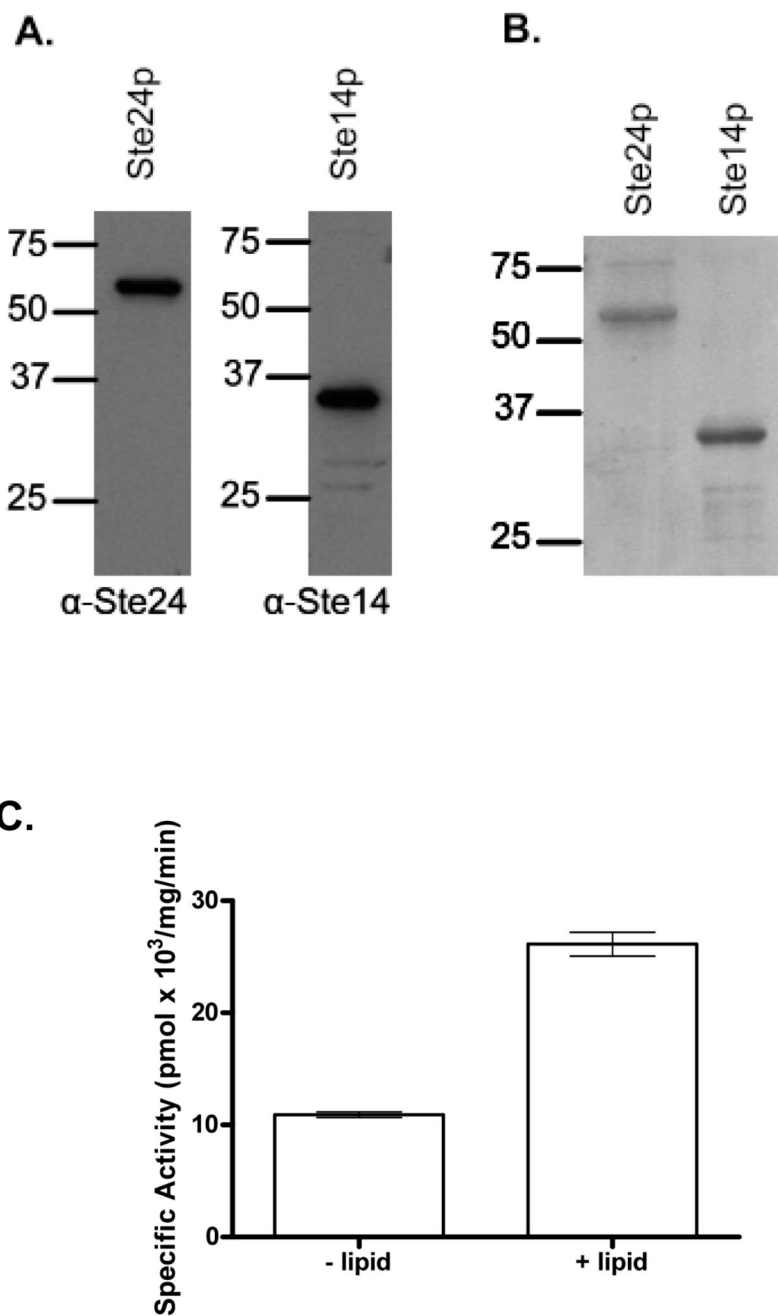


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## Abbreviations

HIV, human immunodeficiency virus  
PI, protease inhibitor  
DRV, darunavir  
LPV, lopinavir  
RTV, ritonavir  
TPV, tipranavir  
ICMT, isoprenylcysteine carboxyl methyltransferase  
DDM, N-dodecyl- $\beta$ -D-maltopyranoside  
MES, 2-(N-morpholino)-ethanesulfonic acid  
PBS, phosphate buffered saline  
AFC, N-acetyl-S-farnesyl-L-cysteine  
MAD, mandibuloacryl dysplasia

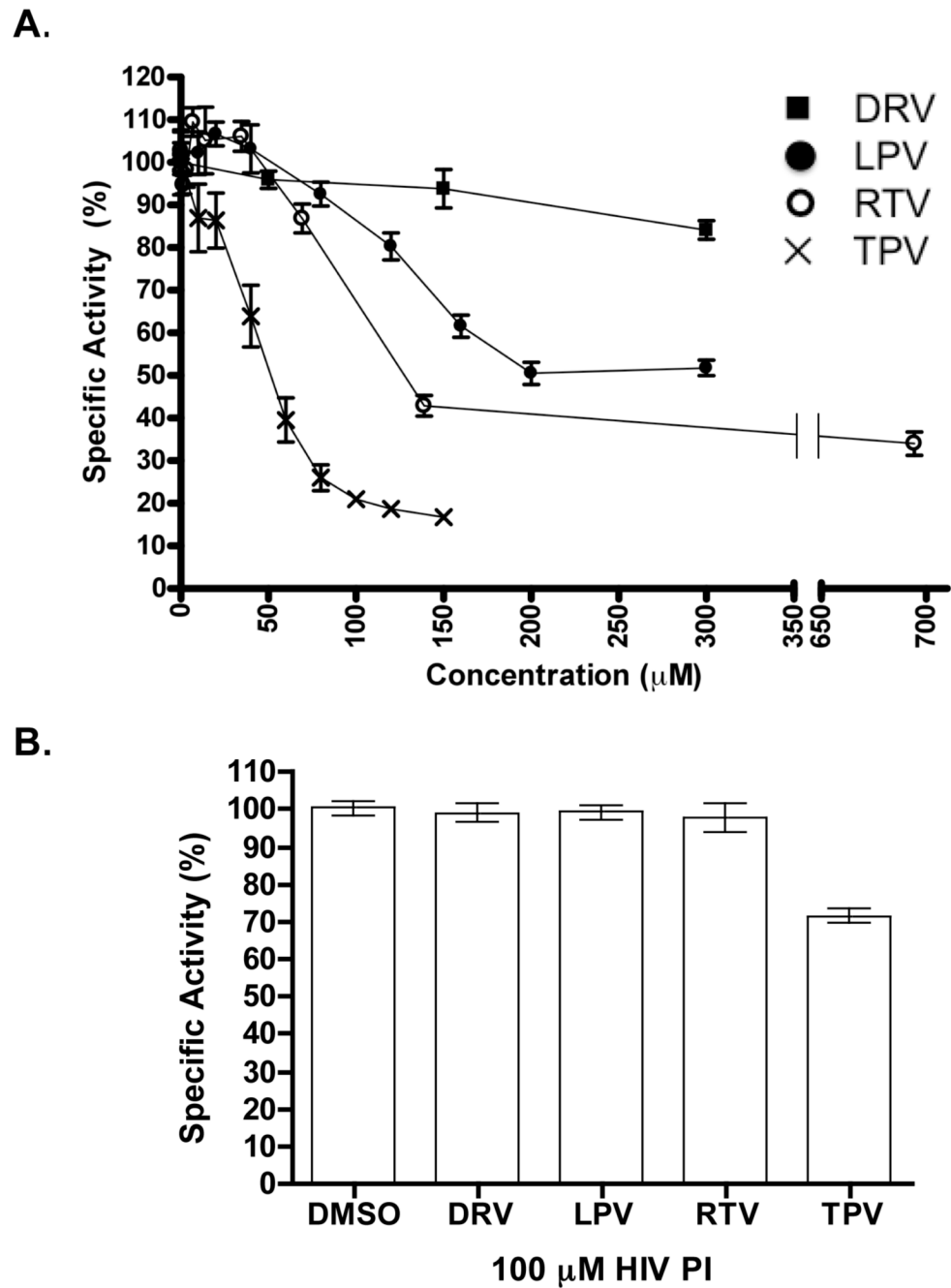


**Figure 1. Purification of Ste24p and Ste14p in 1% DDM**

A. Immunoblots of the purified proteins with antibodies specific for Ste24p and Ste14p. Ste24p was detected with the rabbit polyclonal antibody SM215 specific for amino acids 223–306 of Ste24p (1:1500 dilution) [8]; Ste14p was detected with the rabbit polyclonal antibody SM168 that recognizes amino acids 197–239 of Ste14p (1:1000) [26]. B. Silver-stained SDS-polyacrylamide gel of purified Ste24p (1  $\mu$ g) and Ste14p (1  $\mu$ g). Both were >90% pure, as judged by ImageJ analysis. C. Effect of reconstitution by rapid dilution in polar lipid extract on the activity of Ste24p. Ste24p and Ste14p (0.09  $\mu$ g and 0.19  $\mu$ g, respectively) were either rapidly diluted 33-fold in 100 mM MES, pH 7.0 (- lipid) or reconstituted in 150  $\mu$ g *E. coli* polar lipid extract by 33-fold rapid dilution (+ lipid) and the specific activity was determined



by the *in vitro* coupled endoproteolysis-methylation vapor-diffusion activity assay. Data is the result of three independent experiments, with each point performed in duplicate. Error bars represent SEM.



**Figure 2. Effect of HIV-PIs on the enzymatic activity of pure Ste24p and Ste14p**

**A.** Effect of HIV-PIs on purified Ste24p activity. HIV-PIs darunavir (■) (0–300 µM), lopinavir (●) (0–300 µM), ritonavir (○) (0–695 µM), and tipranavir (×) (0–150 µM) were each incubated with Ste24p and Ste14p for 5 min before adding the peptide substrate and reconstituting the enzymatic activities by rapid dilution in lipid. Activity was detected using the coupled endoproteolysis-methylation vapor-diffusion activity assay. Data is reported as the percent of vehicle activity (DMSO). Each curve displays the data obtained from 3–5 independent experiments. Error bars show SEM. **B.** Effects of HIV-PIs on purified Ste14p activity. Ste14p was incubated with each HIV-PI (100 µM) for 5 min before adding AFC and reconstitution by rapid dilution in the presence of lipid. Activity is detected by the vapor diffusion activity assay

and is reported as percent of the activity found in the DMSO control. The data shown is the result of three independent experiments (mean  $\pm$  SEM).