Title: Clomipramine inhibits dynamin L-α-phosphatidyl-L-serine stimulated GTPase activity

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Abstract

Three dynamin isoforms play critical roles in clathrin-dependent endocytosis. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters host cells *via* clathrin-dependent endocytosis. We previously reported that clomipramine inhibits the GTPase activity of dynamin 1, which is in mainly neuron. Therefore, we investigated whether clomipramine inhibits the activity of other dynamin isoforms in this study. We found that, similar to its inhibitory effect on dynamin 1, clomipramine inhibited the L-α-phosphatidyl-L-serine-stimulated GTPase activity of dynamin 2, which is expressed ubiquitously, and dynamin 3, which is expressed in the lung. Inhibition of GTPase activity raises the possibility that clomipramine can suppress SARS-CoV-2 entry into host cells.

Keywords: clomipramine, SARS-CoV-2, dynamin, endocytosis, inhibition

Abbreviations: severe acute respiratory syndrome coronavirus 2, SARS-CoV-2; dynamin, Dyn; myristyl trimethyl ammonium bromide, MiTMAB; L-α-phosphatidyl-L-serine, PS; angiotensin-converting enzyme 2, ACE2; histidine, His; orthophosphate, Pi.

Introduction

Dynamin (Dyn) GTPase plays a critical role in clathrin- and caveolae-dependent endocytosis [1-3]. Mammals express three Dyn isoforms. Although Dyn 2 is expressed ubiquitously, Dyn 1 is assumed to be neuron specific. Dyn 3 is expressed mainly in the testis, brain and lung [4].

The essential function of Dyn GTPase activity is production of mechanical force to drive membrane fission during vesicle budding during endocytosis [1, 5]. Notably, endocytosis driven by Dyn GTPase activity is involved in the intracellular transportation of transferrin [6].

All Dyn isoforms carry four functional domains: a GTPase domain, pleckstrin homology domain, GTPase effector domain, and proline/arginine-rich domain.[1)] These functional domains act in concert during vesicle budding [7], and multivalent lipid species potentiate dynamin activity *via* electrostatic interactions [8]. Dyn GTPase activities are inhibited by cationic surface-active compounds such as myristyltrimethyl ammonium bromide (MiTMAB). MiTMAB competes with L-α-phosphatidyl-L-serine (PS) for binding to the pleckstrin homology domain in Dyn, but it does not compete with GTP to bind Dyn [9].

Cells can internalize many resources from the external cell environment via endocytosis, and non-enveloped viruses (e.g., hepatitis C virus) generally leverage related mechanisms to enter a cell following the same endocytic pathway as resources [10]. Many enveloped viruses, such as

severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), enter cells *via* endocytosis. The SARS-CoV-2 spike glycoprotein binds to host angiotensin-converting enzyme 2 (ACE2), thereby mediating membrane fusion and enabling virus entry into cells [11, 12].

Recently, Kato *et al.* reported that the tricyclic antidepressant clomipramine suppressed ACE2-mediated SARS-CoV-2 entry into host cells [13]. Clomipramine has been used in basic research as a reagent to inhibit endocytosis [14].

We previously reported that several antipsychotic treatments inhibited the GTPase activity of Dyn 1 and Dyn 2 and that the suppression of dynamin GTPase activity by those antipsychotic drugs led to inhibition of dynamin-driven endocytosis. Moreover, we explained that sertraline is a mixed-type inhibitor with respect to both GTP and PS [15, 16]. In a previous study, we also reported that clomipramine inhibited the GTPase activity of neuron-specific Dyn 1 [15]. However, the inhibition of the activity of lung-specific Dyn 3 and activity of ubiquitous Dyn 2 by clomipramine has not yet been investigated. Therefore, the inhibition of Dyn isoform GTPase activity by clomipramine was measured to understand the inhibitory mechanism.

Materials and Methods

Materials

The following materials were obtained from the cited sources and used in this study: Pfu Ultra II Fusion HS DNA Polymerase (Agilent Technologies); restriction enzymes and DNA ligation kit ver.2 (Takara Bio); pET21a and *E. coli* Rosetta2 (DE3) (Merck); TALON Metal Affinity Resin (Clontech); Mono Q 5/50 GL (GE Healthcare); L-α-phosphatidyl-L-serine (Sigma Aldrich); clomipramine (Sigma); and HRP–conjugated anti Penta-His antibody (Qiagen). Other chemicals used in this study were of an analytical grade or higher. Clomipramine, sertraline and MiTMAB were prepared in stock solutions consisting of 50% (v/v) DMSO and 30 mM Tris-HCl pH 7.4 and diluted in 30 mM Tris-HCl, pH 7.4, prior to use in the assay.

Plasmid construction, protein expression and purification of Dyn isoforms

All Dyn isoform genes were amplified by PCR as described previously [15, 16]. These Dyn genes were cloned into separate pET21a plasmids (Merck, Germany) to create C-terminal-His_6-tagged Dyn isoform protein (Dyns-His_6) vectors. The expression and purification of Dyns-His_6 were reported previously [15-17].

Preparation of PS-liposomes and performance of a GTPase assay with purified Dyns-His6

Forty microlitres of PS (10 mg ml^{-1}) in chloroform/methanol (95:5) was evaporated to a final 5-μl volume, and then PS was resuspended in 1 ml of 30 mM Tris-HCl, pH 7.4, and sonicated for 2 min on ice to yield a working solution of 400 μg ml^{-1}. GTPase assays with the purified Dyns-His_6 were performed via sensitive malachite green colorimetric detection of orthophosphate (Pi), as described previously [15-17]. Phosphate release was quantified and the levels were compared against a standard curve of sodium dihydrogen orthophosphate monohydrate after each experiment. KaleidaGraph 4.0 (Synergy Software) was used to plot and analyse data points.

Blue native (BN)-PAGE

BN-PAGE was performed as previously reported [17]. Purified Dyn2-His_6 (2 μM) was incubated at room temperature for 30 min with 2 mM PS-liposomes in TBS, 1 mM clomipramine + 2 mM PS-liposomes in TBS, or 1% SDS in TBS. The incubation solutions were supplemented with 10X BN-PAGE sample buffer (5% Coomassie Brilliant Blue G-250; 500 mM 6-aminocaproic acid; 100 mM Bis–Tris-HCl, pH 7.0; and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 5 min. All samples were resolved in 4 to 16% gels by Native-PAGE and measured by western blotting with HRP–conjugated anti-Penta-His antibody.

Results

Dyns-His$_6$ was activated by PS liposomes

To confirm the GTPase activities of purified Dyns-His$_6$, malachite green colorimetric detection of released orthophosphate was performed to confirm the GTPase activity levels of purified Dyns-His$_6$ with/without PS liposome treatment. Purified Dyns-His$_6$ at all concentrations hydrolysed GTP to release Pi in a time-dependent manner, and the GTPase activity was stimulated by the PS liposomes (Fig. 1). The addition of PS liposomes increased Dyn1-His$_6$ activity by 5.1-fold, Dyn2-His$_6$ activity by 4.7-fold, and Dyn3-His$_6$ activity by 5.7-fold (Fig. 1 A versus B, 60 min).

Clomipramine inhibits GTPase activity of all dynamin isoforms in the presence of PS-liposomes

GTPase assay was performed to evaluate the inhibition of Dyns-His$_6$ GTPase activity by clomipramine, and the activity levels were compared with those of sertraline and MiTMAB (Fig. 2 and Fig.3). In the presence of PS liposomes, clomipramine showed significant inhibition of GTPase activity (Fig.2). The IC_{50} values for Dyn1-His$_6$ were 5.5 ± 1.2 μM (sertraline), 8.1 ± 3.7 μM (MiTMAB) and 22.0 ± 2.2 μM μM (clomipramine) in the presence of PS liposomes (Fig. 2A). For Dyn2-His$_6$, the IC_{50} values were 4.9 ± 0.6 μM (sertraline), 9.2 ± 2.5 μM (MiTMAB) and

12.8 ± 3.2 μM (clomipramine) in the presence of PS liposomes (Fig. 2B). The IC_{50} values for Dyn3-His_6 were 4.4 ± 0.5 μM (sertraline), 7.8 ± 3.0 μM (MiTMAB) and 11.3 ± 2.9 (clomipramine) in the presence of PS liposomes (Fig. 2C). In contrast to MiTMAB, clomipramine showed no overt inhibition of GTPase activity, similar to sertraline, in the absence of PS liposomes (Fig. 3).

Clomipramine did not inhibit the oligomer formation of Dyn2-His_6

BN-PAGE was performed to analyse oligomerization of Dyn2-His_6. Several bands corresponding to Dyn2-His_6 oligomers were observed at approximately 300 kDa (Arrow II) and approximately 600 kDa (Arrow III) after BN-PAGE of Dyn2-His_6 treated with/without PS liposomes (Fig. 4 Lanes 1 and 3). Moreover, the lanes showed similar band profiles in the higher molecular weight area than at Arrow III (Fig. 4 Lanes 1 versus 3). In the presence of 1% SDS, a denaturant, a single band of approximately 140 kDa, corresponding to the Dyn2-His_6 monomer, was observed for Dyn2-His_6 with or without PS liposome treatment, (Fig. 4 Lanes 2 and 4, Arrow I).

Discussion

PS-liposomes had been previously reported to stimulate the GTPase activity of Dyn [18]. PS liposomes stimulated all purified Dyns-His_6 GTPase activities in this study, as shown in Fig. 1. This meant that purified Dyns-His_6 with GTPase assays performed *via* malachite green colorimetric Pi detection can be used to assess the inhibition of clomipramine.

Clomipramine exhibited inhibition of Dyn2 and Dyn3 GTPase activity similar to its effect on Dyn1 GTPase activity in the presence of PS liposomes (Fig. 2). The IC_{50} values for Dyn1-His_6 were 5.5 ± 1.2 μM (sertraline), 8.1 ± 3.7 μM (MiTMAB) and 22.0 ± 2.2 μM μM (clomipramine), as shown in Fig. 2A. These IC_{50} values were not significantly different from those obtained in our previous study [15].

Although Dyns-His_6 GTPase activity in the presence of PS liposomes was completely lost after 320 μM MiTMAB treatment, Dyns-His_6 GTPase activity in the addition of clomipramine and sertraline (320 μM) was incompletely lost (Fig. 2 A-C). The residual activity is thought to result from GTPase activity in the absence of phospholipids, that is, the so-called basal intrinsic GTPase activity. In fact, these observations agree with the observation that clomipramine did not strongly inhibit basal intrinsic GTPase activity (Fig. 3 A-C). Basal intrinsic GTPase activity tends to decrease after disassembly of the dynamin oligomer [19]. Thus, it is possible that clomipramine does not affect oligomerization of Dyns-His_6. Therefore, we performed BN-PAGE to examine

whether clomipramine affects the stabilization of the Dyn2-His_6 oligomer. The addition of clomipramine did not affect any bands corresponding to Dyns-His_6 oligomers (Fig. 4, Lane 1 versus Lane 3). Taken together, these results demonstrate that inhibition of Dyns-His_6 activity did not result from by clomipramine-induced destabilization of the oligomer. Clomipramine appears to directly effect to interaction between PS-liposome and dynamin.

Dyn plays a critical role in clathrin-dependent endocytosis, and SARS-CoV-2 enter into host cells *via* clathrin-dependent endocytosis [20]. Kato *et al.* mentioned that the tricyclic antidepressant clomipramine suppressed ACE2-mediated SARS-CoV-2 entry [13, 21]. In this study, we found that clomipramine inhibited the PS-liposome-stimulated GTPase activity of Dyn 2, which was ubiquitously expressed, and Dyn 3, which was expressed in the lung [4]. Taken together, inhibition of PS-liposome-stimulated GTPase activity by clomipramine may participate in suppression of ACE2-mediated SARS-CoV-2 entry.

Kato *et al.* also mentioned that the inhibition mechanism by which clomipramine inhibited the entry of SARS-CoV-2 differed from that of other antiviral drugs, such as remdesivir, and suggested the potential for the concomitant use of clomipramine and drugs with other mechanistic effects for treating COVID-19 patients [21]. Our results reinforce the potential for the concomitant use of these drugs.

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Figure legends

Fig. 1 All Dyn-His$_6$ isoforms released orthophosphate *via* GTP hydrolysis in a time-dependent manner.

The GTPase activities of Dyn1-His$_6$ (hollow circles), Dyn2-His$_6$ (solid circles), and Dyn3-His$_6$ (triangles) were measured as the concentration of released Pi. (A) GTPase activities of Dyns-His$_6$ isomers in the presence of 5.0 μM PS liposomes. The GTPase activities of 20 nM Dyns-His$_6$ isomers were measured at 30 °C. (B) GTPase activities of Dyns-His$_6$ isomers in the absence of PS liposomes. The GTPase activities of 20 nM Dyns-His$_6$ were measured at 30 °C.

Fig. 2 Clomipramine inhibits dynamin GTPase activity by PS-liposome stimulation.

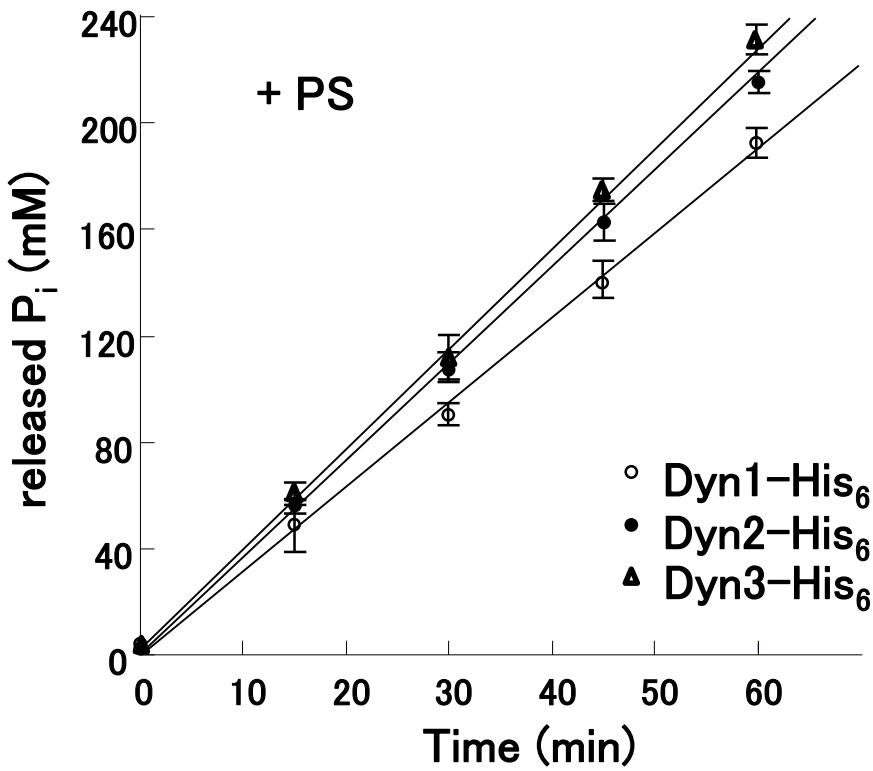
The GTPase activity of purified Dyns-His$_6$ (20 nM) was determined in the presence of various concentrations of MiTMAB (hollow circles), sertraline (solid circles) or clomipramine (triangles). All results are representative of at least 3 independent experiments. (A) GTPase activity of Dyn1-His$_6$ in the presence of 5.0 μM PS liposomes. (B) GTPase activity of Dyn2-His$_6$ in the presence of 5.0 μM PS liposomes. (C) GTPase activity of Dyn3-His$_6$ in the presence of 5.0 μM PS liposomes.

Fig. 3 Clomipramine do not inhibit basal intrinsic dynamin GTPase activity.

The GTPase activity of purified Dyns-His_6 (20 nM) was determined in the presence of various concentrations of MiTMAB (hollow circles), sertraline (solid circles) or clomipramine (triangles). All results are representative of at least 3 independent experiments. (A) GTPase activity of Dyn1-His_6 in the absence of PS liposomes. (B) GTPase activity of Dyn2-His_6 in the absence of PS liposomes. (C) GTPase activity of Dyn3-His_6 in the absence of PS liposomes.

Fig. 4 Clomipramine did not affect the oligomerization of Dyn2-His_6

The effect of clomipramine on the oligomerization of Dyn2-His_6 was analysed by BN-PAGE. Lane 1, Dyn2-His_6 + PS-liposomes; Lane 2, Dyn2-His_6 + PS-liposomes + 1% SDS; Lane 3, Dyn2-His_6 + PS-liposomes + clomipramine; Lane 4, Dyn2-His_6 + PS-liposomes + clomipramine + 1% SDS. Arrow I corresponds to a dynamin monomer. Arrows II and III correspond to dynamin oligomers.


A
+ PS
released P_i (mM)
Time (min)
Dyn1-His$_6$
Dyn2-His$_6$
Dyn3-His$_6$
0
40
80
120
160
200
240
0
10
20
30
40
50
60

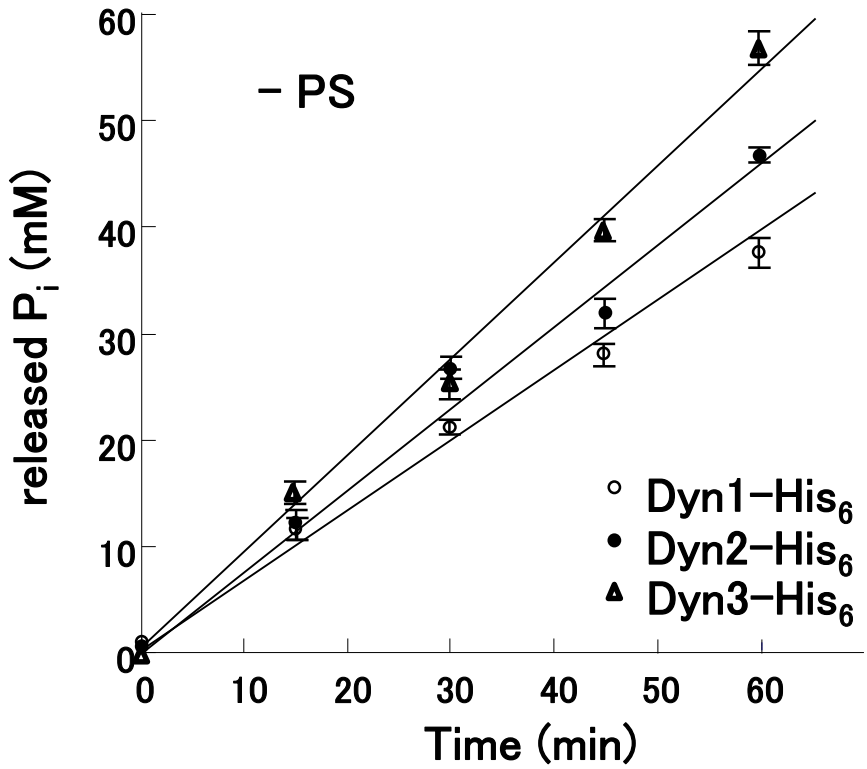

B
− PS
released P_i (mM)
Time (min)
Dyn1-His$_6$
Dyn2-His$_6$
Dyn3-His$_6$
0
10
20
30
40
50
60
0
10
20
30
40
50
60

Fig. 1

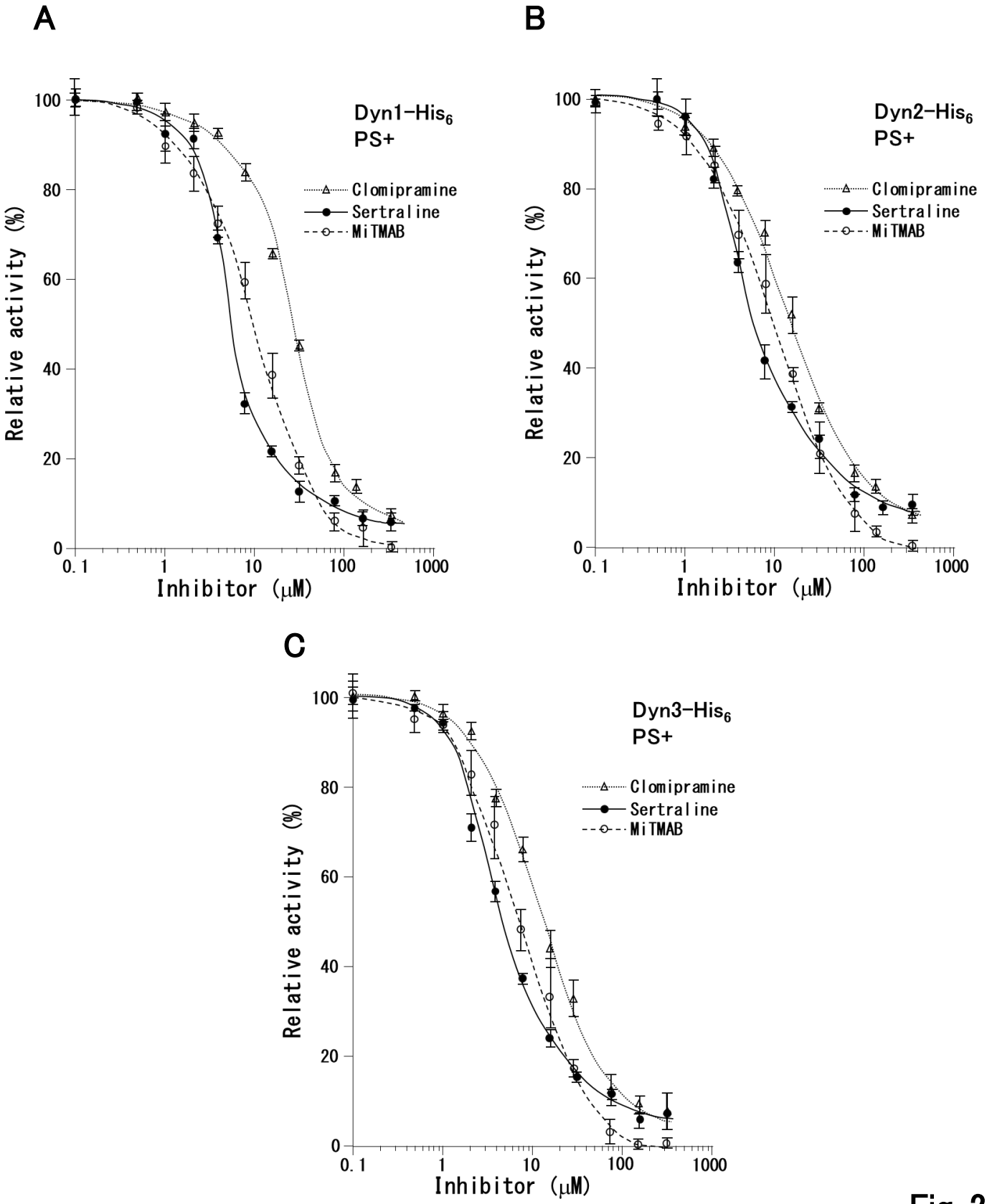

A
Dyn1-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)
B
Dyn2-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)
C
Dyn3-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)

Fig. 2

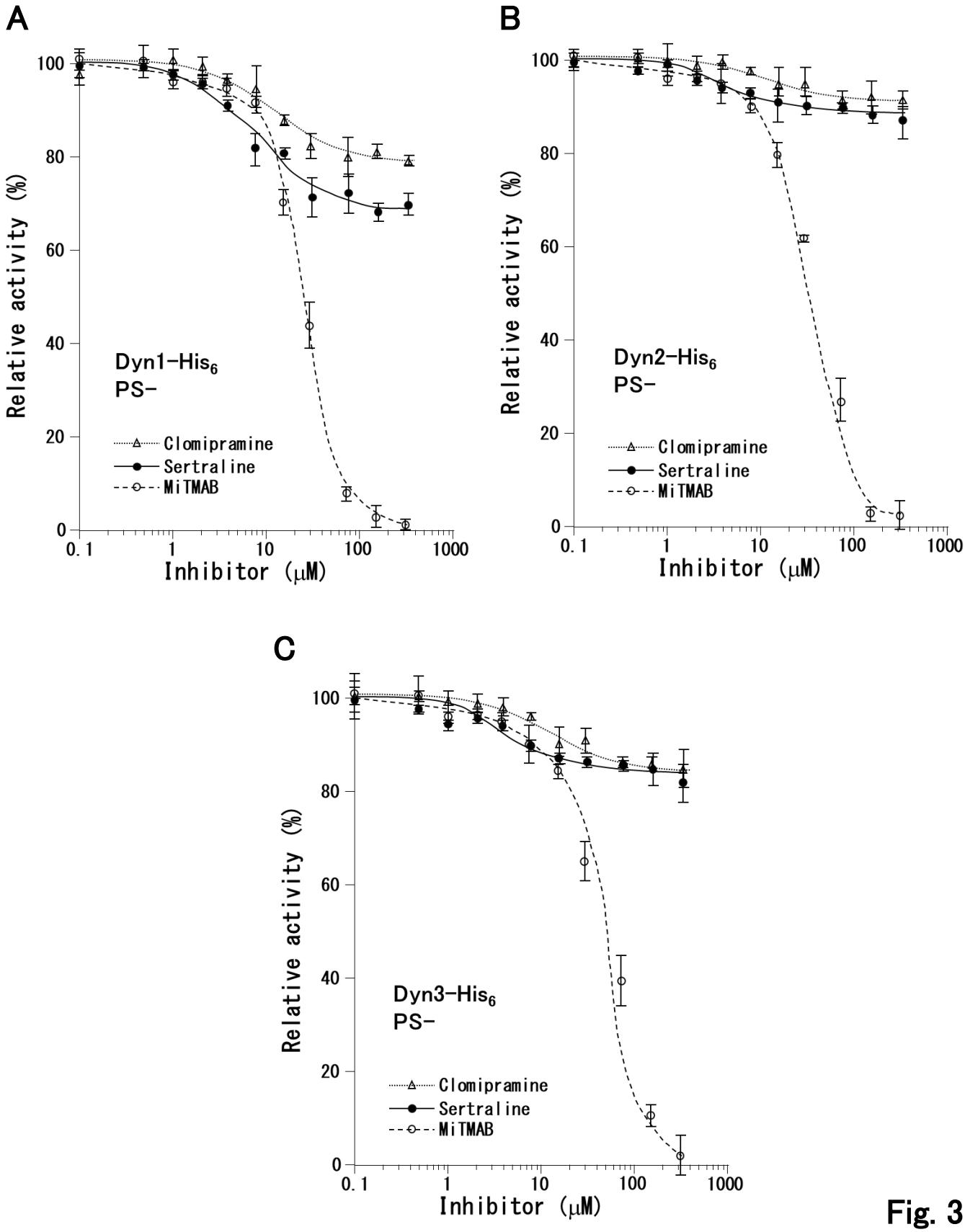
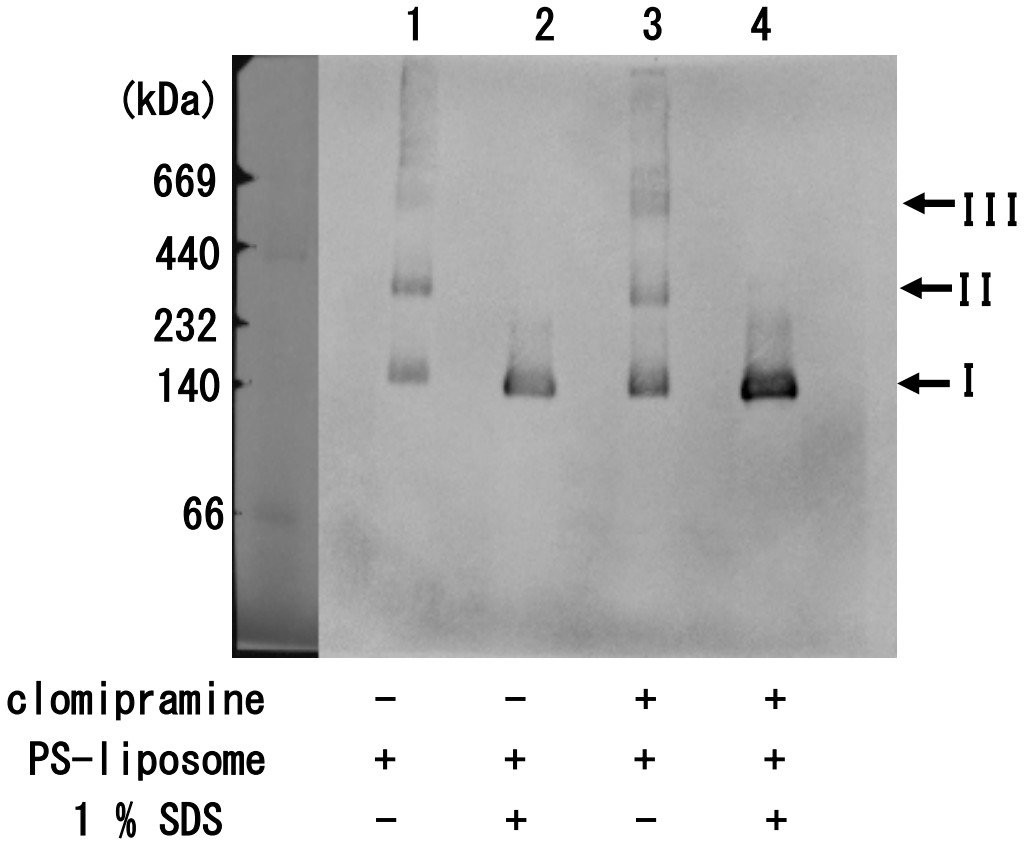

A
Relative activity (%)
Inhibitor (μM)
Dyn1-His6
PS-
Clomipramine
Sertraline
MiTMAB
B
Relative activity (%)
Inhibitor (μM)
Dyn2-His6
PS-
Clomipramine
Sertraline
MiTMAB
C
Relative activity (%)
Inhibitor (μM)
Dyn3-His6
PS-
Clomipramine
Sertraline
MiTMAB

Fig. 3


1
2
3
4
(kDa)
669
440
232
140
66
III
II
I
clomipramine − − + +
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1 % SDS − + − +

Fig. 4