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

Authors: Hiroshi Miyoshi[1*&], Masahiro Otomo[2§&] and Kiyofumi Takahashi[2#&]

Affiliations: Department of [1]Microbiology and [2]Neuropsychiatry, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan.

[*]Corresponding author: Department of Microbiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan; Tel. +81 44 9778111; E-mail: hmiyoshi@marianna-u.ac.jp

[§] Present address: Shinkohkai izukan-nami hospital, 1694 Hirai, Kan-nami, Tagata, Shizuoka 419-0107 Japan; E-mail: otomo@shinkohkai.jp

[#] Present address: Research Organization for Nano and Life Innovation, Waseda University, Tokyo, Japan; E-mail: kiyofu-min@umin.org

[&] All authors contributed equally.

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 3-(9-chloro-5,6-dihydrobenzo[b][1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine (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 lipid species such as L-α-phosphatidyl-L-serine (PS) potentiate dynamin activity [8]. Dyn GTPase activities are inhibited by cationic surface-active compounds such as myristyl trimethyl ammonium bromide (MiTMAB). MiTMAB competes with 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 (1*S*)-*cis*-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-*N*-methyl-1-naphthalenamine (sertraline) is a competitive inhibitor of dynamin I GTPase 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 to create C-terminal-His_6-tagged Dyn isoform protein (Dyns-His_6) vectors. We expressed Dyns-His_6 from *E. coli* Rosetta2 (DE3) by transforming with those Dyn isoforms-coding pET21a expression plasmids. The expressed Dyns-His_6 were purified to homogeneity on a MonoQ column after TALON Metal Affinity Resin purification, as previously reported [15-17]. The purified proteins were confirmed by 15 %

SDS-PAGE.

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 suspended 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 curves using non-linear regression.

Blue native (BN)-PAGE

BN-PAGE was performed as previously reported [17]. Purified Dyn2-His_6 (2 µM) were incubated at room temperature for 30 min with 2 mM PS-liposomes in TBS, 2 mM PS-liposomes + 1% SDS in TBS, 1 mM clomipramine + 2 mM PS-liposomes in TBS, or 1 mM clomipramine + 2 mM PS-liposomes + 1% SDS in TBS, respectively. The incubation solutions were supplemented with 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 BN-PAGE and measured by western blotting with HRP–conjugated anti-Penta-His antibody.

Results

Dyns-His$_6$ was activated by PS-liposomes

We expressed and purified Dyns-His$_6$. Those purified Dyns-His$_6$ were confirmed by 15 % SDS-PAGE as shown in Fig. 1. 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. 2). 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. 2 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. 3 and Fig. 4). In the presence of PS-liposomes, clomipramine showed significant inhibition of GTPase activity (Fig.3). 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.

3A). 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. 3B). 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. 3C). In contrast to MiTMAB, clomipramine showed no overt inhibition of GTPase activity, similar to sertraline, in the absence of PS-liposomes (Fig. 4).

Clomipramine did not inhibit the oligomer formation of Dyn2-His_6

Dyn GTPase activity is increased by its adoption of helical oligomerization [18]. BN-PAGE was performed to analyse effect of clomipramine for 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 clomipramine (Fig. 5 Lanes 1 and 3). Moreover, those lanes showed similar band profiles in the higher molecular weight area than at Arrow III (Fig. 5 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 clomipramine treatment, (Fig. 5 Lanes 2 and 4, Arrow I).

Discussion

PS-liposomes had been previously reported to stimulate the GTPase activity of Dyn [19]. As shown in Fig. 2, PS-liposomes stimulated all purified Dyns-His_6 GTPase activities in this study. This meant that these purified Dyns-His_6 can be used to assess the inhibition by clomipramine with GTPase assays. In fact, The IC_{50} values of inhibitors (sertraline, MiTMAB and clomipramine) for Dyn1-His_6 obtained from Fig. 3A were not significantly different from those obtained in our previous study [15].

Clomipramine exhibited inhibition of Dyn2 and Dyn3 GTPase activity similar to its effect on Dyn1 GTPase activity in the presence of PS-liposomes (Fig. 3). 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. 3A-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. 4A-C). Basal intrinsic GTPase activity tends to decrease after disassembly of the dynamin oligomer [20]. Therefore, it is possible that clomipramine does not affect oligomerization of Dyns-His_6. Accordingly, 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. 5, Lane 1 versus Lane 3). Taken together, these results demonstrate that inhibition of Dyns-His6 activity did not result from by clomipramine-induced destabilization of the oligomer. It is known that pleckstrin homology domain on dynamin binds to lipids [21]. Consequently, clomipramine appears to directly interact pleckstrin homology domain, so that appears to effect 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 [22]. Kato *et al.* mentioned that the tricyclic antidepressant clomipramine suppressed ACE2-mediated SARS-CoV-2 entry [13, 23]. 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 [23]. Our results reinforce the potential for the concomitant use of these drugs.

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Author contributions

HM contributed to data analysis and manuscript writing. MO and KT contributed to the collection of data and data analysis. All authors have read and approved the final manuscript.

Data availability

The data underlying this article are available in the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Purified Dyns-His$_6$ in this study.

The purified proteins were resolved by 15 % SDS-PAGE and stained with Coomassie Brilliant Blue.

Fig. 2 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. 3 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. 4 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. 5 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.

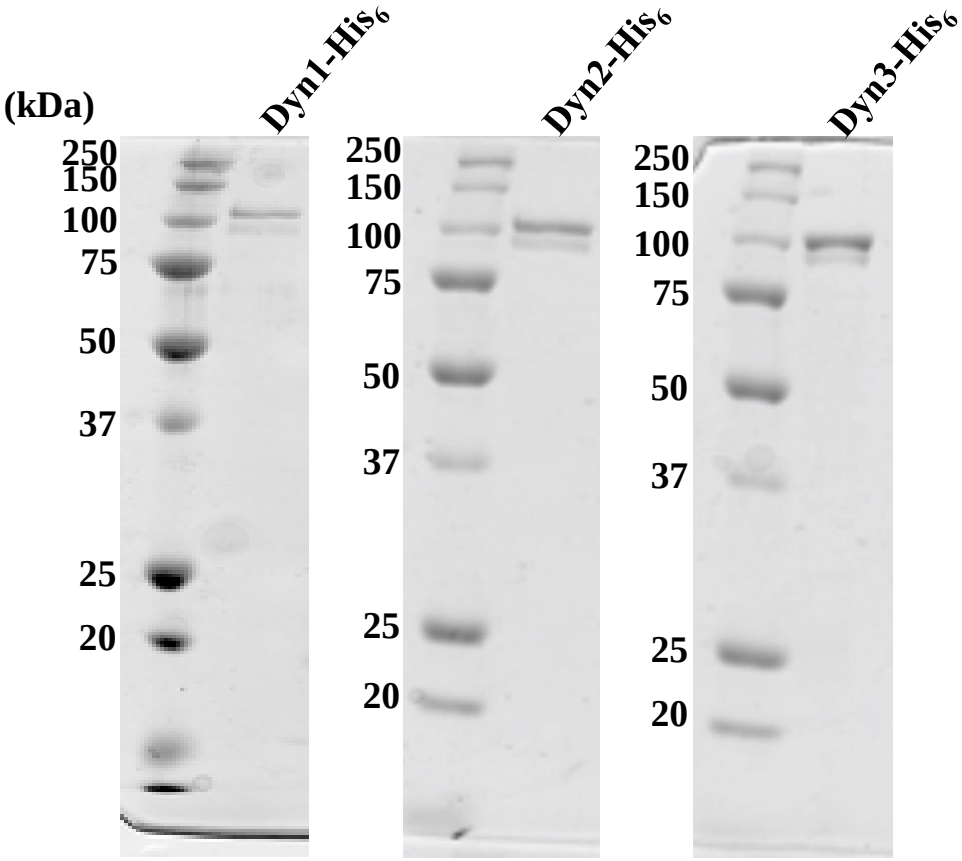
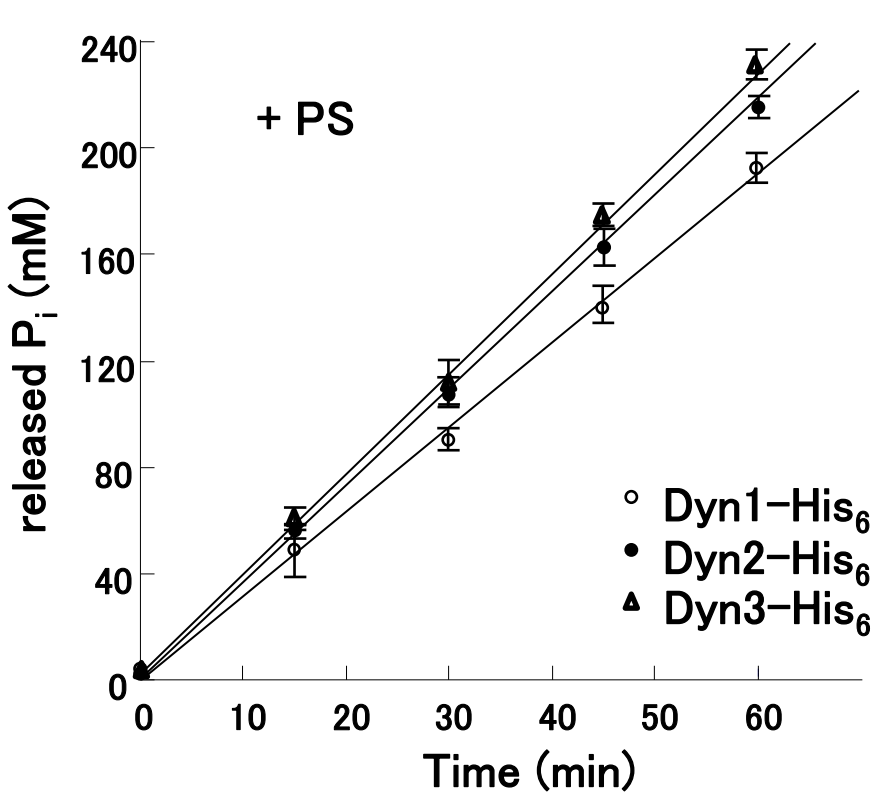


Fig. 1


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

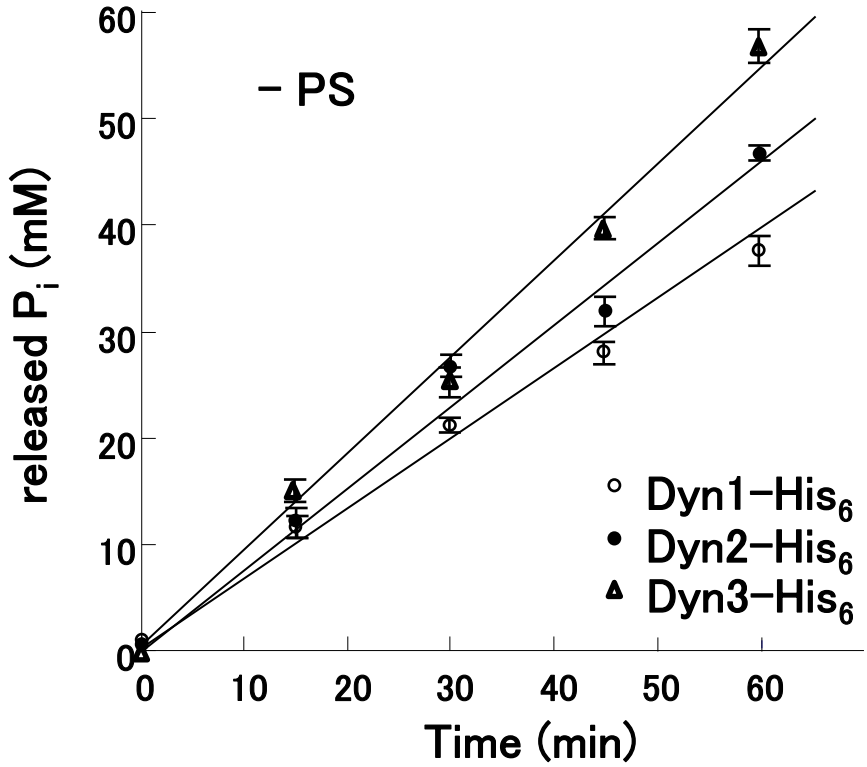

B
− PS
released P_i (mM)
Time (min)
Dyn1-His$_6$
Dyn2-His$_6$
Dyn3-His$_6$
0
10
20
30
40
50
60
0
10
20
30
40
50
60

Fig. 2

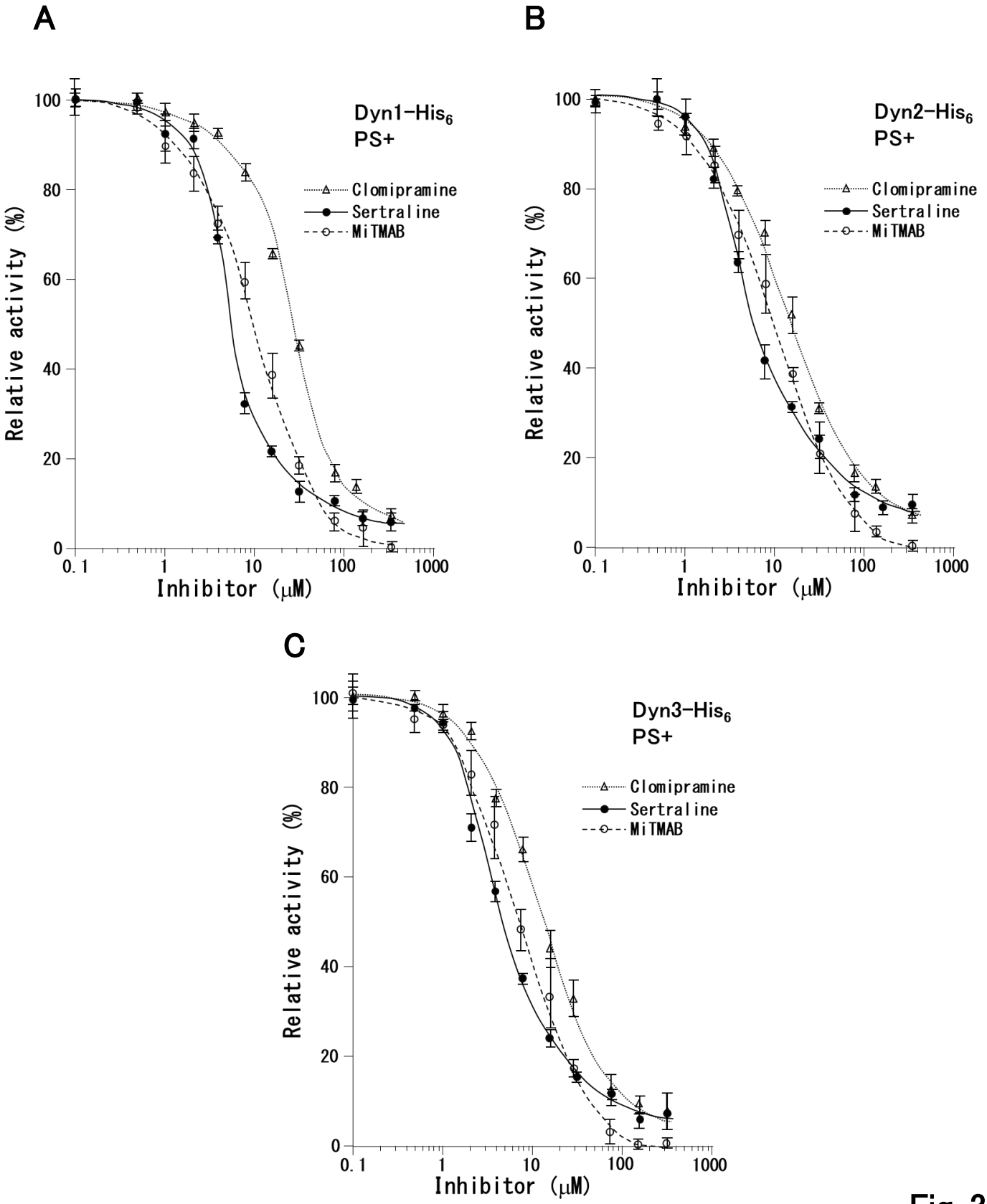

A
Dyn1-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)
B
Dyn2-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)
C
Dyn3-His6
PS+
Clomipramine
Sertraline
MiTMAB
Relative activity (%)
Inhibitor (μM)

Fig. 3

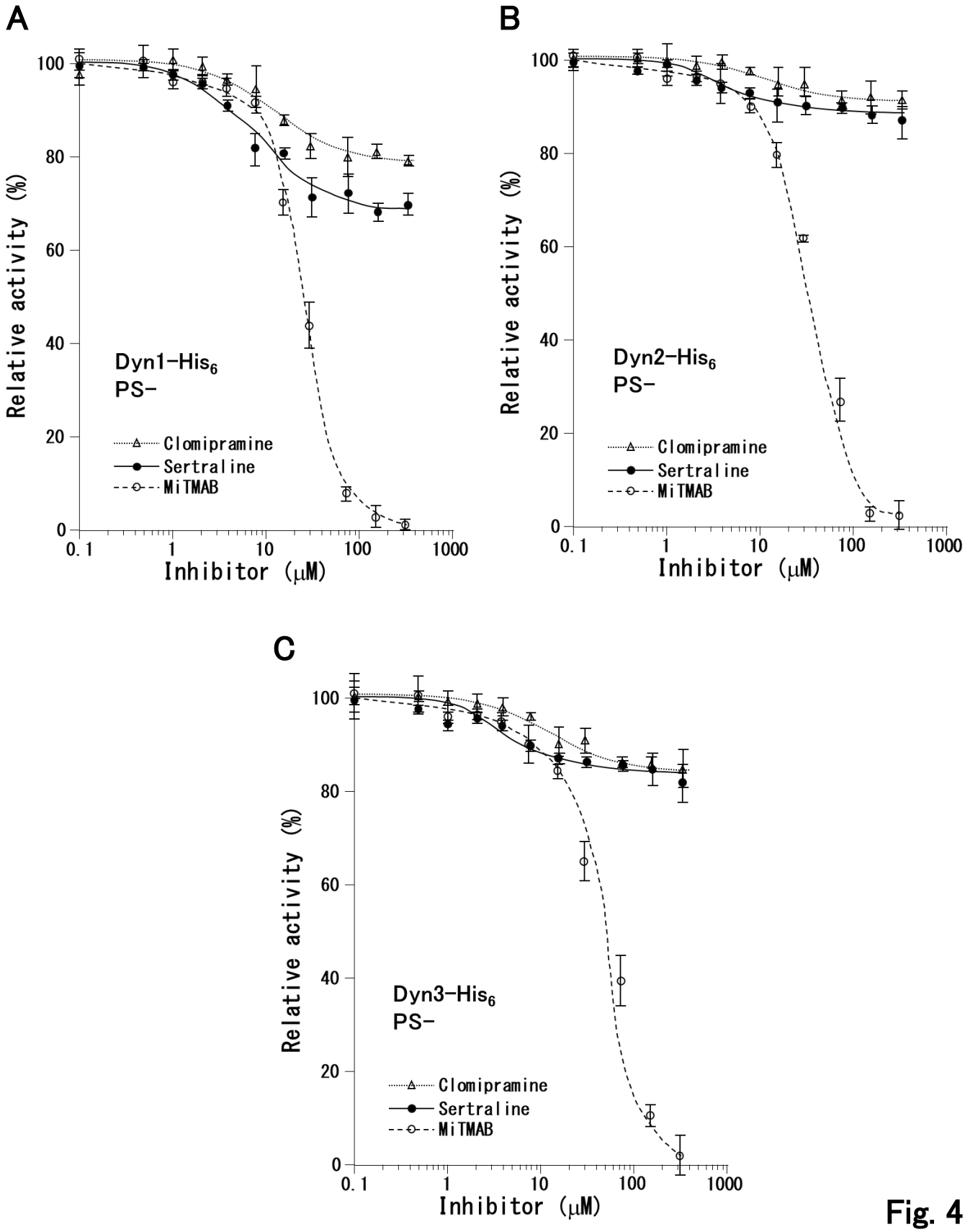

A
Relative activity (%)
Inhibitor (μM)
Dyn1-His6
PS-
Clomipramine
Sertraline
MiTMAB
B
Relative activity (%)
Inhibitor (μM)
Dyn2-His6
PS-
Clomipramine
Sertraline
MiTMAB
C
Relative activity (%)
Inhibitor (μM)
Dyn3-His6
PS-
Clomipramine
Sertraline
MiTMAB

Fig. 4

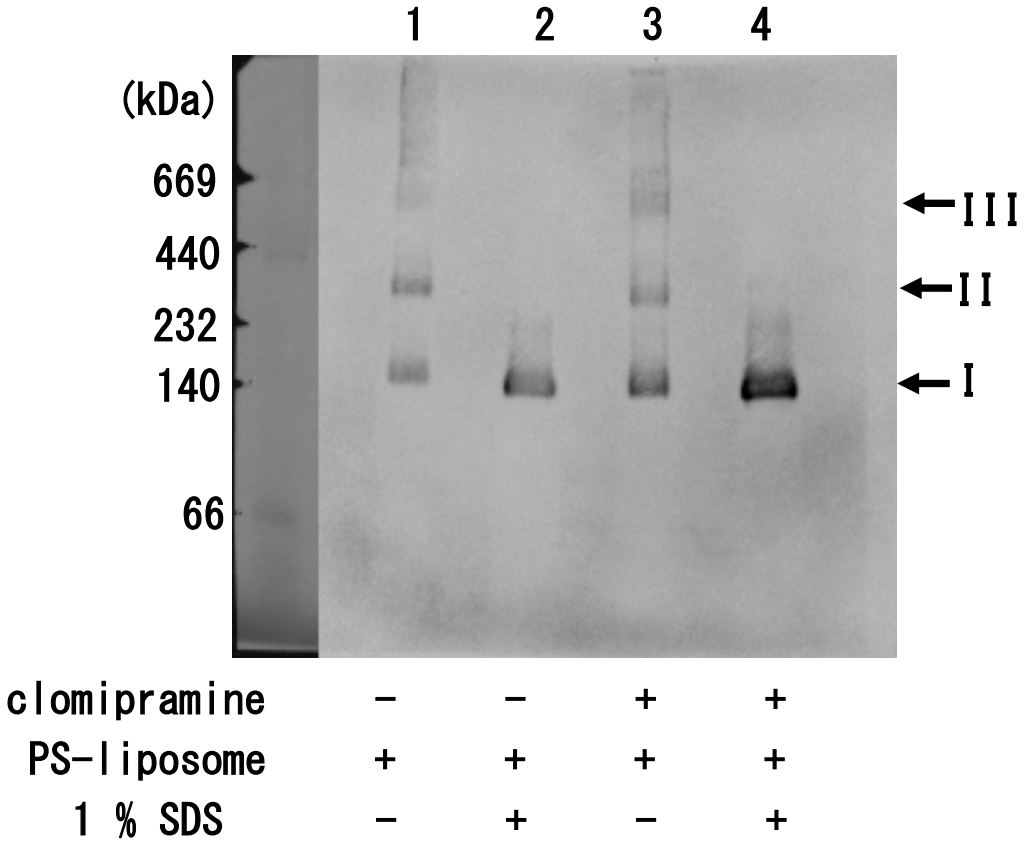

1
2
3
4
(kDa)
669
440
232
140
66
III
II
I
clomipramine − − + +
PS-liposome + + + +
1 % SDS − + − +

Fig. 5