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# Assessment of Binding Affinity to 5-Hydroxytryptamine 2A (5-$HT_{2A}$) Receptor and Inverse Agonist Activity of Naftidrofuryl: Comparison With Those of Sarpogrelate

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**Abstract.** Naftidrofuryl is a peripheral vasodilator that has been clinically used in the treatment of intermittent claudication and dementia. It has 5-hydroxytryptamine 2 (5-$HT_2$) antiserotonergic activity and selectively binds with the 5-$HT_2$ receptor. The purpose of the present study is to assess the binding affinity and functional potency of naftidrofuryl to the 5-$HT_{2A}$ receptor, to find out the inverse agonist activity of this compound at a constitutively active mutant of 5-$HT_{2A}$ receptor, and finally to compare the findings with those of sarpogrelate. The investigation showed that the binding affinity ($pK_i$) of naftidrofuryl was decreased 25- or 50-fold compared to sarpogrelate in the wild-type 5-$HT_{2A}$ receptor or Cys322Lys mutant receptor, respectively. Moreover, the functional potency ($pK_b$) of naftidrofuryl was much lower compared to sarpogrelate at the 5-$HT_{2A}$ receptor. In addition, inverse agonist activity of naftidrofuryl was lower compared with sarpogrelate at the constitutively active mutant receptor. Thus, the data of the present study would be very important for the clarification of interaction sites of naftidrofuryl to 5-$HT_{2A}$ receptors and also may help to understand the mechanism of inverse agonist activity at the constitutively active mutant receptor.

***Keywords***: naftidrofuryl, inverse agonist activity, constitutively active mutant, sarpogrelate

## Introduction

Peripheral arterial disease (PAD), which is caused by atherosclerotic occlusion of the arteries to the legs, is an important manifestation of systemic atherosclerosis (1). Anti-platelet therapy is recommended to relieve claudication associated with PAD (2, 3). Among anti-platelet agents, sarpogrelate, a selective 5-hydroxytryptamine 2A (5-$HT_{2A}$)-receptor antagonist, has been approved for PAD and is in widely used in Japan, China, and the Republic of Korea (4). It also inhibits serotonin-induced platelet aggregation, vasoconstriction, and vascular smooth muscle cell proliferation (5, 6), which are mediated by 5-$HT_{2A}$ receptors and consequently reduces the ischemic symptoms associated with PAD. On the other hand, naftidrofuryl, which has been recommended for the pharmacotherapy of claudication, has been available in several European countries for more than 20 years but is not marketed in Asia (7 – 9). Moreover, several investigations have reported that sarpogrelate improves ischemic symptoms such as intermittent claudication, pain, and cold sensation of the lower extremities and objective indices such ankle-brachial pressure index (ABPI) (2, 10, 11). Thus, the present study is very important because it compares the binding affinity, functional potency, as well as inverse agonist activity of these two different chemical compounds.

In the 5-$HT_{2A}$ receptor, the substitution of cysteine 322 of the third intracellular loop to lysine stabilizes the active form and leads to a significant increase in the basal production of inositol phosphates (IPs) (12). In our previous study, we have demonstrated that sarpogrelate has moderate inverse agonist activity with the Cys322Lys constitutively active mutant of 5-$HT_{2A}$

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receptors (13). There has been no studies on the binding affinity, functional potency, and inverse agonist activity of naftidrofuryl towards 5-$HT_{2A}$ receptors. Although both sarpogrelate and naftidrofuryl have a similar pharmacological function to relieve intermittent claudication, there have been no determinations of the binding affinity, functional potency, and inverse agonist activity of naftidrofuryl.

We performed the present study to investigate the inverse agonist activity of naftidrofuryl on 5-$HT_{2A}$ receptors, determine if there is a correlation between binding affinity and functional potency of naftidrofuryl, and to compare the findings with those for sarpogrelate.

## Materials and Methods

*Materials*

Mianserin was obtained from RBI (Natick, MA, USA) and sarpogrelate, from Mitsubishi Tanabe Pharma (Tokyo). Di-methyl serotonin was synthesized at the department of Organic Chemistry, Niigata University of Pharmacy and Applied Life Sciences. Nafronyl oxalate (naftidrofuryl) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Serotonin was obtained from Merck (Darmstadt, Germany).

*DNA constructs*

The cDNA clone encoding the human 5-$HT_{2A}$ receptor was generously provided by Dr. Stuart C. Sealfon (Department of Pharmacology and Neurology, Mount Sinai School of Medicine, NY, USA) (14). The insert was subcloned from pAlter (Promega, Madison, WI, USA) by SmaI-XbaI digestion. For expression, the insert was subcloned into the EcoRV and XbaI sites of pcDNA3 (Invitrogen, Carlsbad, CA, USA). The 5-$HT_{2A}$–receptor mutant was constructed with the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutation was confirmed by sequencing the mutation site in the expression vector.

*Cell culture and transfection*

HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. Stably expressing cell lines were constructed in HEK293 cells by transfecting with Lipofectamine™ 2000 reagent and selecting with 1.0 mg/ml G418–containing growth medium as detailed by the manufacturer's protocol (Invitrogen Life Technologies, Rockville, MD, USA).

*Cell harvesting and membrane fraction preparation*

Stably transfected HEK293 cells were grown in 10-cm plates. The confluent plates were washed twice with ice-cold phosphate-buffered saline (PBS), and then the cells were scraped-off with a rubber policeman. Cells were centrifuged at 3,000 × *g* for 5 min at 4°C. Cell pellets were homogenized in 1 ml of 50 mM Tris-HCl (pH 7.4) with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 35,000 × *g* for 15 min. The membrane pellets were resuspended in 50 mM Trish-HCl (pH 7.4) with a Teflon glass homogenizer. Protein content was measured by the method of Lowry et al. (15).

*Ligand binding assay*

Each binding incubation tube contained approximately 50 $\mu$g of membrane protein, [$^3$H]ketanserin (NEN Life Sciences, Boston, MA, USA), unlabeled drug as required, and binding buffer in a final volume of 250 $\mu$l. Both saturation and competition binding assays were carried out as described previously (16). Briefly, for the saturation binding studies, five concentrations (0.1 – 8 nM for wild type) of [$^3$H]ketanserin (specific activity of 76.5 Ci /mmol) were tested. For competition binding studies, the concentration of [$^3$H]ketanserin with wild-type receptors was 1.0 nM and 5 concentrations of competing ligands were used in duplicate. Nonspecific binding was defined with the use of 10 $\mu$M mianserin. For radioligand, incubations were carried out for 30 min at 37°C and were terminated by rapid filtration through Whatman GF/C filters that had been presoaked in 0.3% polyethyleneimine followed by washing with 10 ml ice-cold buffer. The radioactivity retained on the filters was quantitated by liquid scintillation spectrophotometry.

*Inositol phosphates (IP)-accumulation assay*

Accumulation of total [$^3$H]IP was assayed as described previously (18). Stably transfected HEK293 cells at about 90% confluence in 10-cm dishes were seeded into 24-well plates. Twenty-four hours after seeding, the cells were washed with serum-free DMEM and labeled with 1 $\mu$Ci/ml [$^3$H]myo-inositol in serum-free DMEM for 18 – 20 h. After labeling, the medium was replaced with the assay medium (Hank's buffered salt solution containing 20 mM HEPES and 20 mM LiCl, pH 7.4, 37°C). The cells were incubated for 15 min at 37°C by floating the plates in a temperature-controlled water bath. Both agonists and antagonists in assay medium were added to each well and incubation continued for additional 30 min. Assay medium was removed, and the reaction was stopped by adding 1 ml of 10 mM formic acid (previously stored at 4°C) to each well. The plates were stored at 4°C for 24 h, and cells were neutralized by adding 1 ml 500 mM KOH and 9 mM sodium tetraborate per well. The contents of each well were

extracted and centrifuged for 5 min at 3000 rpm and the upper layer loaded onto a 1-ml AG1-X8 resin (100 – 200 mesh; Assist Co., Tokyo) column. Columns were washed 2 times with 5 ml 60 mM sodium formate and 5 mM borax. Total IPs were eluted with 5 ml 1 M ammonium formate and 0.1 M formic acid. Radioactivity was measured by liquid scintillation spectrophotometry.

*Data analyses*

Nonlinear regression analysis of saturation and competition binding assay were performed using GraphPad Prism software (San Diego, CA, USA). $pK_i$ is the negative logarithm of the equilibrium dissociation constant in nM. The $pK_b$ values were estimated from the inhibitory effects of the antagonists on the concentration-dependent total inositol phosphate–accumulation curve for serotonin according to Furchgott (17). $pK_b$ is the negative logarithm of the $K_b$ value, where $K_b$ value was determined using the following equation:

$$(EC_{50}\text{agonist} / EC_{50}\text{antagonist}) - 1 = [B] / K_b$$

, where $EC_{50}$agonist = concentration of agonist at which 50% of total IP is produced; $EC_{50}$antagonist = concentration of agonist in the presence of a particular concentration of antagonist ([B]) at which 50% of total IP is produced; [B] = concentration of antagonist, which is 100 times higher than the $K_i$ value; $K_b$ = Dissociation constant of antagonist. The agonist and antagonist $EC_{50}$ values were calculated by nonlinear analysis using GraphPad Prism software. Statistical analyses were performed by Student's *t*-test.

## Results

*Saturation binding analysis*

[$^3$H]Ketanserin was used to determine the dissociation constant ($K_d$) and maximum bound ($B_{max}$) values for the wild-type and mutant receptors. The wild-type and the 5-$HT_{2A}$–receptor Cys322Lys mutant showed similar binding affinity towards [$^3$H]ketanserin (Table 1 and Fig. 1) The protein expression ($B_{max}$) was determined 3 fold lower ($P$<0.05) for the mutant Cys322Lys compared to wild-type 5-$HT_{2A}$ receptor.

*Competition binding analysis*

The affinities ($pK_i$ values) of the 5-$HT_2$ antagonists sarpogrelate and naftidrofuryl for the wild-type receptors were determined in competition binding experiments for sites labeled with [$^3$H]ketanserin. Figure 2 shows representative competition curves, and all data are summarized in Table 2. Sarpogrelate had the highest binding affinity ($pK_i = 9.06 \pm 0.06$). The binding affinity of naftidrofuryl was decreased 3-fold ($P$<0.05) for the Cys322Lys mutant compared to the wild-type 5-$HT_{2A}$ receptor.

**Table 1.** Saturation analysis of [$^3$H]ketanserin-labeled wild-type $HT_{2A}$ receptor and Cys322Lys mutant $HT_{2A}$ receptor

| | Wild type | Cys322Lys |
|---|---|---|
| $K_d$ (nM) | 1.0 ± 0.01 | 1.1 ± 0.10 |
| $B_{max}$ (fmol/mg) | 1525 ± 94 | 529 ± 76 |
| | * | |

Each value is the mean ± S.E.M. of four experiments performed in duplicate. *$P$<0.05.

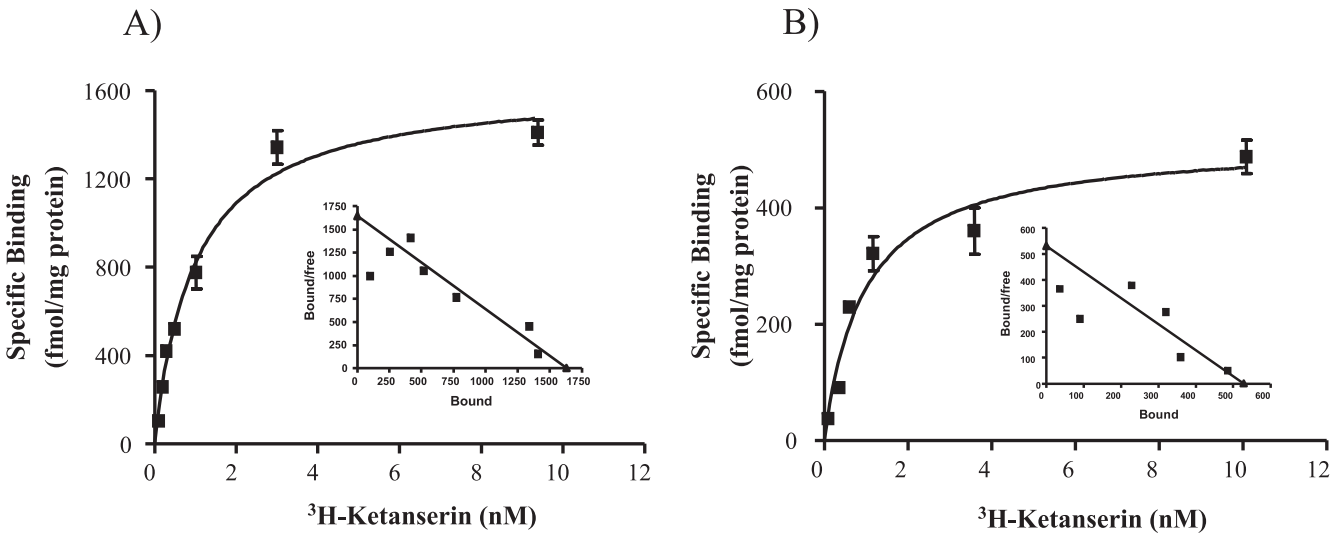



**Fig. 1.** Saturation binding curve and Scatchard plot of [$^3$H]ketanserin-labeled wild-type (A) and Cys322Lys mutant (B) 5-$HT_{2A}$ receptor. Each point represents the mean ± S.E.M. of four experiments performed in duplicate.

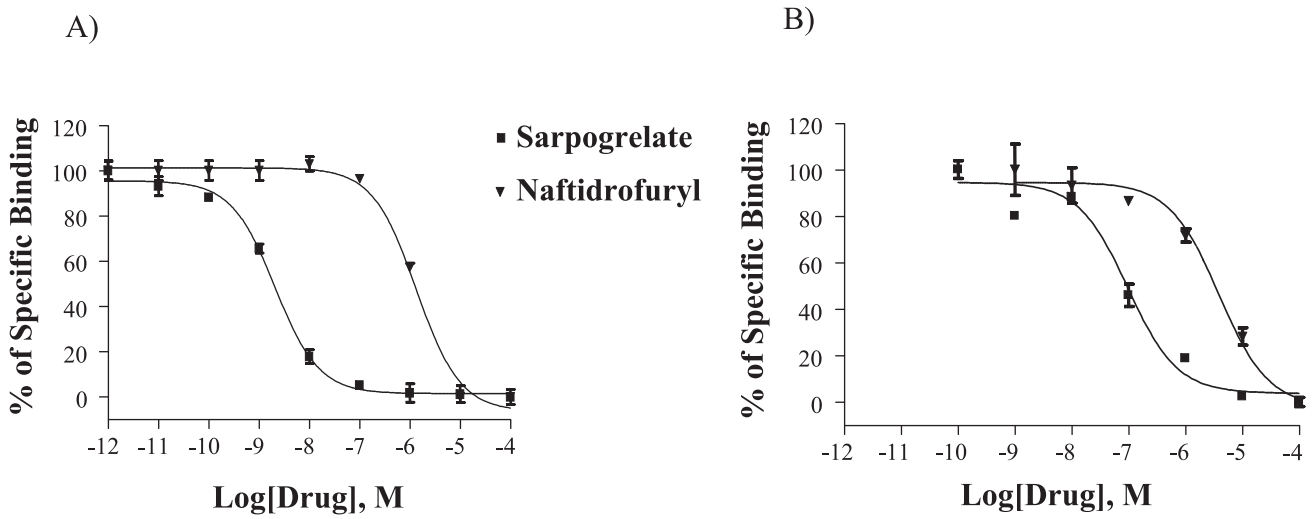



**Fig. 2.** Competition binding studies of naftidrofuryl and sarpogrelate for [$^3$H]ketanserin bound to wild-type (A) and Cys322Lys mutant (B) 5-HT$_{2A}$ receptors. The best fit of the competition data was monophasic and the Hill coefficient was near to unity.

**Table 2.** Binding affinities (pK$_i$) of the 5-HT$_{2A}$ agonist and antagonists to wild-type and Cys322Lys mutant of 5-HT$_{2A}$ receptors

| | pK$_i$ value | |
|---|---|---|
| | Wild type | Cys322Lys |
| 5-HT | 7.1 ± 0.04 | 9.3 ± 0.04 |
| Sarpogrelate | 7.6 ± 0.06 | 7.4 ± 0.30 |
| Naftidrofuryl | 6.2 ± 0.12 | 5.7 ± 0.01 |

pK$_i$ values were determined using [$^3$H]ketanserin in cell membranes from stably transfected HEK293 cells as described in Materials and Methods. Each value is the mean ± S.E.M. of four independent experiments, each performed in duplicate. *$P$<0.05, **$P$<0.001.

**Table 3.** Analysis of functional affinity (pK$_b$) of antagonists for wild-type and Cys322Lys mutant 5-HT$_{2A}$ receptors

| | pK$_b$ value | |
|---|---|---|
| | Wild type | Cys322Lys |
| Sarpogrelate | 8.8 ± 0.11 | 7.8 ± 0.40 |
| Naftidrofuryl | 6.3 ± 0.01 | 6.7 ± 0.20 |

Each value is the mean ± S.E.M. of four independent experiments, each performed in duplicate. *$P$<0.05, **$P$<0.001.

*Effect of antagonists on receptor-mediated IP accumulation*

The C322K mutant receptors were expressed in stably transfected HEK293 cells, and the concentration–response curves for serotonin stimulated IP accumulation was determined. 5-HT had EC$_{50}$ values of 3.5 ± 0.6 and 51 ± 11 nM for the mutant and the wild-type receptors, respectively. The data of inhibition of 5-HT–stimulated IP formation by naftidrofuryl is summarized in Table 3. The pK$_b$ values for sarpogrelate and naftidrofuryl showed that they had similar function potency for both wild-type and mutant receptor (Table 3).

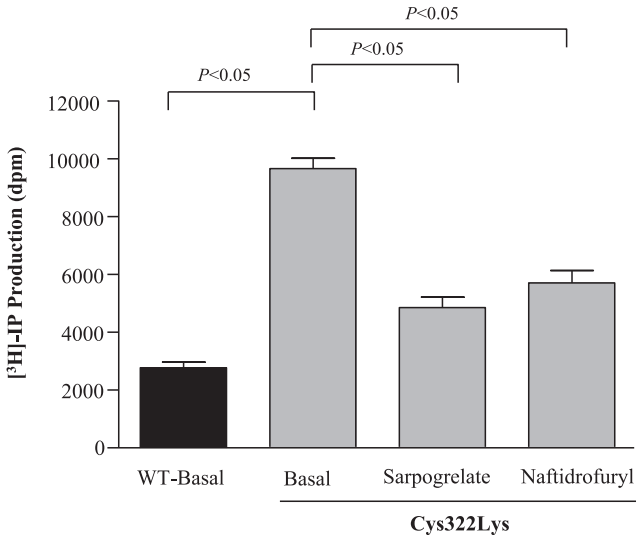



**Fig. 3.** Inverse agonist activity of sarpogrelate and naftidrofuryl with the Cys322Lys mutant of 5-HT$_{2A}$ receptors.

Naftidrofuryl and sarpogrelate significantly inhibited the basal IP production at the constitutively active mutant (Cys322Lys) receptor (Fig. 3). However, naftidrofuryl inhibited the basal IP production less than sarpogrelate did.

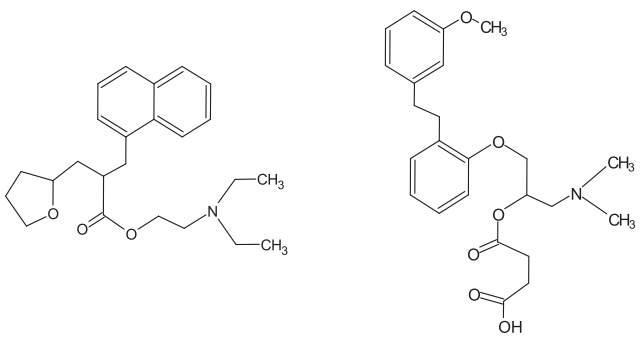
## Discussion

Naftidrofuryl is a 5-HT$_2$–receptor antagonist with

vasodilator effects, and it has been used for treatment of intermittent claudication, dementia, and glaucoma (18). Our previous reports demonstrated that sarpogrelate, a selective 5-$HT_{2A}$ antagonist, has inverse agonist activity at a constitutively active 5-$HT_{2A}$–receptor mutant (13). From the results of clinical trials, Matsuo et al. (19) reported that sarpogrelate may be used for the treatment of intermittent caludication. This is our first report determining the binding affinity, functional potency, and inverse agonist activity of naftidrofuryl at the 5-$HT_{2A}$ receptor, in comparison to those of sarpogrelate.

The binding affinity ($pK_i$) of naftidrofuryl was decreased 25- or 50-fold compared to sarpogrelate in the wild-type 5-$HT_{2A}$ receptor or the Cys322Lys mutant receptor. Our previous reports (20) suggested that dimethyl amine and carboxyl groups of sarpogrelate are very important functional groups for binding with wild-type 5-$HT_{2A}$ receptor. The molecular modeling data (21) suggested that the dimethyl amine of sarpogrelate binds with aspartic acid at 152 and tryptophan at 156 in the transmembarne domain (TMD) III, and carboxyl groups of sarpogrelate bind with tryptophan at position 337 in TMD VI of 5-$HT_{2A}$ receptors. Mutagenesis of these amino acid produced a mutant receptor with a dramatic change in sarpogrelate binding. On the other hand, diethyl amine and furan ring of naftidrofuryl makes its structure different from that of sarpogrelate. Thus the results of the present study may be due to different chemical structures of naftidrofuryl and sarpogrelate (Fig. 4).

In addition, the binding affinity of sarpogrelate was not decreased in the Cys322Lys mutant in comparison with the wild-type 5-$HT_{2A}$ receptor. However, the binding affinity of naftidrofuryl was decreased significantly ($P<0.05$) in the Cys322Lys mutant in comparison with the wild-type 5-$HT_{2A}$ receptor. The present data revealed that binding affinity of naftidrofuryl was lower in comparison with sarpogrelate due to constrain different three dimensional structures or the different chemical structure of naftidrofuryl.




Naftidrofuryl Sarpogrelate

**Fig. 4.** Chemical structures of naftidrofuryl and sarpogrelate.

Naftidrofuryl is a compound that has been successfully used for the treatment of vascular diseases and constitutively active mutant receptor found in the disease state. The present study has established new drugs showing inverse agonist activity on the constitutively active mutant receptors or 5-HT receptors in the disease state. It has been shown in the context of several GPCRs that certain molecules could act as inverse agonists and reduce the levels of spontaneous activity and functional cellular responses (22 – 25). Inverse agonism is very common among GPCR antagonists (26). At the $\alpha_{1A}$-adrenergic receptor, histamine $H_1$–receptor, and also the 5-$HT_{2A}$ receptor, the majority of the known antagonists are actually inverse agonists (27 – 29). Considering the therapeutic implications, it is suggested that all new antagonists should be routinely tested for their potential inverse agonistic activity in future drug development programs (30, 31).

Our previous reports also suggested that sarpogrelate showed moderate binding affinity ($pK_i$) to the wild-type receptor and the constitutively active mutant receptor (13). Sarpogrelate showed inverse agonist activity, causing a decrease in the basal IP production at the constitutively active mutant receptor. The present study revealed that naftidrofuryl inhibited the basal IP production at constitutively active mutant receptor (Fig. 3). The results demonstrated that inhibition of basal IP production by naftidrofuryl was less than that by saprogrelate, which may be due to the higher potency of sarpogrelate. It is clear that naftidrofuryl showed almost a similar extent of inverse agonism as sarpogrelate at the constitutively active mutant receptor. However, the mechanism of inverse agonist activity of naftidrofuryl is still not known.

In conclusion, it is apparent from the present study that although naftidrofuryl and sarprogrelate have similar pharmacological function at the 5-HT receptors, sarpogrelate has moderate binding affinity, functional potency, and inverse agonist activity on the 5-$HT_{2A}$ receptor, whereas naftidrofuryl showed low binding affinity and functional potency and inverse agonist activity.

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