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Efficacy of a novel metalloprotease inhibitor on botulinum neurotoxin B activity

Michael Adler[a,*], James D. Nicholson[b], Brennie E. Hackley Jr[c]

[a] *Neurotoxicology Branch, Pharmacology Division, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010, USA*

[b] *DAKKRO Corporation, Littleton, CO 80161, USA*

[c] *Office of the Commander, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010, USA*

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Abstract The novel inhibitor 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (ICD 1578) was tested for its ability to antagonize the zinc metalloprotease activity of botulinum toxin B (BoNT/B). The efficacy of this compound was tested in a cell-free system using a 50-mer synaptobrevin peptide as substrate. The peptide, designated as [Pya^{88}] S 39–88, had a fluorescent amino acid analog, L-pyrenylalanine (Pya), substituted for the normal Phe^{88} of synaptobrevin-2. Cleavage by BoNT light chain yielded fragments of 38 and 11 amino acids, respectively. The smaller fragment, containing the Pya fluorophore, was readily separated and quantified by fluorescence spectroscopy at 377 nm. In the presence of 7–200 μM ICD 1578, cleavage of [Pya^{88}] S 39–88 was progressively reduced (IC_{50} = 27.6 μM), and 100 μM ICD 1578 produced >95% inhibition. For comparison, captopril, a well-known zinc metalloprotease inhibitor, generated less than 10% inhibition at a concentration of 5 mM. ICD 1578 is the most potent antagonist of BoNT/B light chain thus far described.

Key words: Zinc metalloprotease inhibitor; Botulinum toxin B; Synaptobrevin-2

1. Introduction

Botulinum neurotoxin (BoNT) inhibits acetylcholine release from motor nerve terminals resulting in a flaccid paralysis of skeletal muscle [1]. The paralytic action of BoNT leads to a characteristic set of symptoms which generally include diplopia, dysphagia, generalized muscle weakness, muscle paralysis and death [1,2]. Each of the seven serotypes of BoNT consists of a light chain (LC) of ~50 kDa that has zinc metalloprotease activity and cleaves one of three proteins associated with transmitter release: synaptobrevin, SNAP-25 or syntaxin. The LC is linked by a single disulfide bond and noncovalent forces to a heavy chain of ~100 kDa that is responsible for the binding of the toxin to the nerve terminal and for internalization of the LC into the cytosol [1–3].

BoNT is a potential military threat and a significant public health problem [4]. Our current treatment of BoNT intoxication is largely symptomatic, directed at maintaining respiratory and cardiovascular function [5]. Since botulism leads to protracted paralysis with long-lasting consequences, it is important to develop effective pharmacological treatments for BoNT toxicity.

There are three potential targets for antagonism of BoNT action: the ectoacceptor on the surface of cholinergic nerve terminals, the exocytotic apparatus that mediates toxin internalization and the active site of the LC [2,6]. Blockade of the ectoacceptor has proved difficult, since this moiety is poorly characterized [1,2,7]. Inhibition of internalization has met with greater success, however, the agents thus far examined have only delayed but could not prevent muscle paralysis [8–10]. Attempts to inhibit the active site of the BoNT LC have been confined to the use of zinc chelators and the metalloprotease inhibitors, captopril and phosphoramidon. The chelators were found to be effective in vitro but highly toxic in vivo [11,12], while the available metalloprotease inhibitors were only marginally effective even under in vitro conditions [6,13,14]. The latter is not surprising, since these inhibitors are not optimized to interact with the active sites of the clostridial neurotoxin LCs.

The present effort represents a rational approach towards the use of selective metalloprotease inhibitors for BoNT/B; a task that is rendered difficult by the absence of X-ray crystallographic data on this BoNT serotype and by the lack of effective lead compounds. The inhibitor 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (ICD 1578) was originally designed to interact with matrix metalloproteases. For the current investigation, ICD 1578 was selected as a potential BoNT/B LC inhibitor since it shared structural similarities with a weakly active phosphoramidon analog [15]. ICD 1578 was assessed for its ability to inhibit the catalytic activity of BoNT/B LC using a sensitive fluorescence assay. The results indicate that ICD 1578 is the most potent inhibitor of BoNT/B thus far examined.

2. Materials and methods

2.1. Fluorescence assay

A quantitative fluorescent assay was used for evaluating the efficacy of BoNT/B LC inhibitors. The assay is based on monitoring reductions in the rate of cleavage of a 50-mer peptide that spans residues 39 to 87 of human synaptobrevin-2 and contains a modified tyrosine (pyrenylalanine) fluorophore in position 88 that is normally occupied by phenylalanine. This substrate, designated as [Pya^{88}] S 39–88, was described in detail by Soleihac et al. [16].

The assay was carried out at 22°C in a reaction volume of 100 μl. Due to the light sensitivity of the pyrenylalanine fluorophore, all operations were performed with subdued incandescent lighting. First, 60 nM of pure BoNT/B LC was preincubated with 50 μM $ZnSO_4$,

*Corresponding author. Fax: +1 (410) 671 1960.

Abbreviations: ICD 1578, 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin; BoNT/B, botulinum neurotoxin B; Pya, pyrenylalanine; TFA, trifluoroacetic acid; LC, light chain

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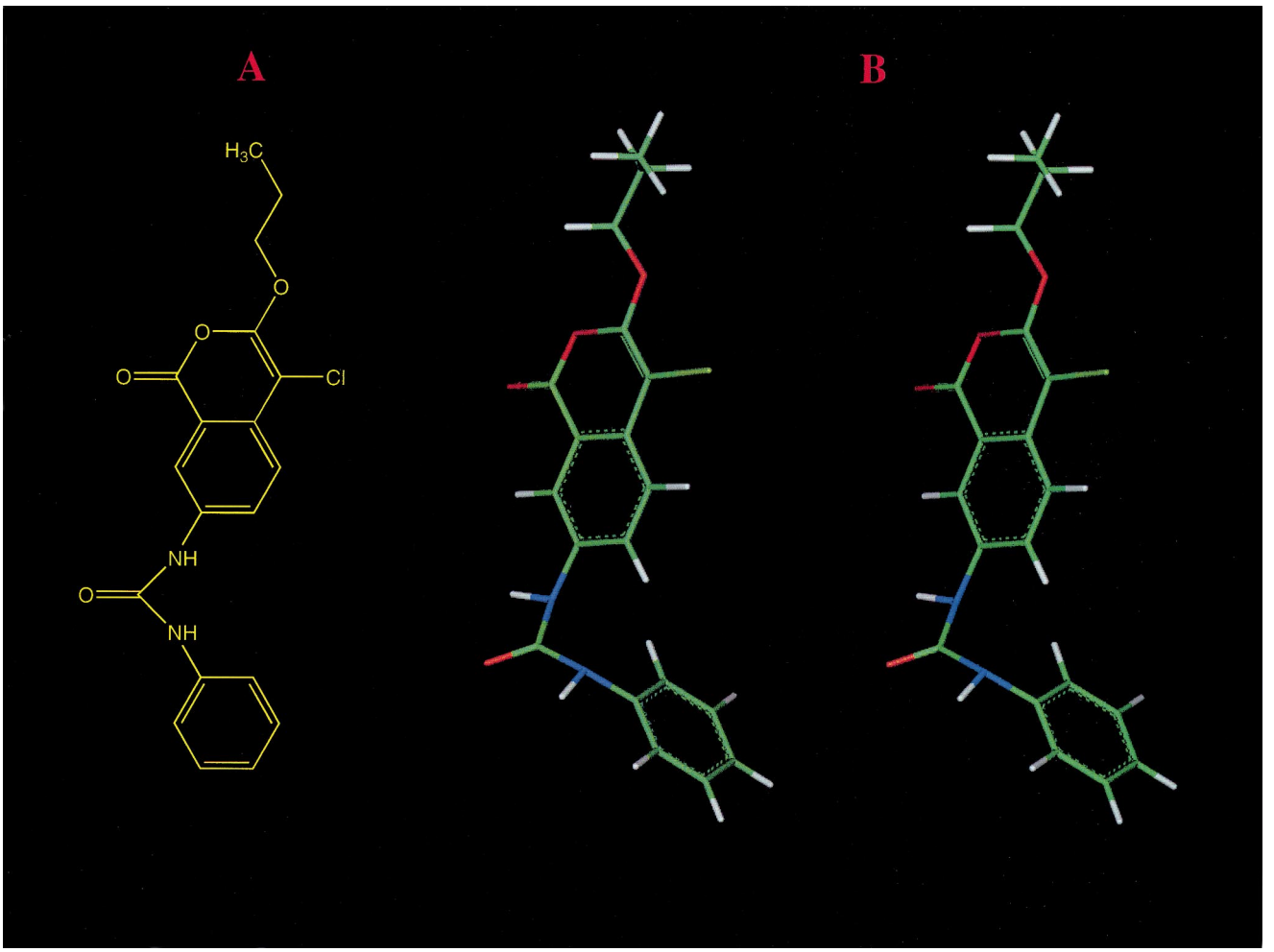

Fig. 1. Molecular structure of ICD 1578. A is a conventional structural diagram; B shows a wall-eyed stereo view of an energy minimized structure. The molecular weight of ICD 1578 is 372.45. The identity and purity of the compound were confirmed by NMR analysis. Note that ICD 1578 is predicted to be semi-rigid due to the fused isocoumarin ring and the limited degrees of rotational freedom between isocoumarin and phenyl rings.

1 mM dithiothreitol and 20 mM HEPES (pH 7.1) for 30 min. This was followed by a 30 min incubation with 10 μM of the substrate [Pya^{88}] S 39–88. The reaction was stopped after 30 min by addition of 0.9 ml of 0.1% trifluoroacetic acid (TFA) in 72% methanol. The reaction blank consisted of equivalent concentrations of HEPES and [Pya^{88}] S 39–88 but without BoNT LC, dithiothreitol or added $ZnSO_4$. Stock solutions of the inhibitors ICD 1578 or captopril were prepared in 20 mM HEPES (adjusted to pH 7.1) and added to the preincubation mixture.

During incubation, BoNT/B LC cleaves the peptide bond between Gln^{76} and Phe^{77}, resulting in two fragments: a 38-mer peptide (S 39–76) and a smaller 12-mer peptide ([Pya^{88}] S 77–88) that contains the pyrenylalanine fluorophore. Isolation of the latter was accomplished by using C18 Sep-Pak Vac syringe body cartridges (Waters Corp., Milford, MA). The C18 matrix retains the hydrophobic parent peptide and larger S 39–76 cleavage product while eluting the hydrophilic [Pya^{88}] S 77–88 fragment [16]. Sep-Pak cartridges were mounted on a Baker-10 (Phillipsburg, NJ) solid phase extraction vacuum manifold equipped with stopcock valves to control flow through individual cartridges. Prior to separation, cartridges were conditioned by three 5 ml washes containing, respectively, 0.1% TFA in methanol, pure methanol and 0.1% TFA in 65% methanol: these solutions were subsequently discarded. To achieve separation, 1 ml sample volumes containing reaction products and uncleaved substrate were loaded on cartridges, and the hydrophilic C-terminal peptide fragment was eluted through the C18 matrix at ~1 ml per min. Cartridges were then washed with 3 ml of 0.1% TFA in 65% methanol to remove residual [Pya^{88}] S 77–88 cleavage product. The eluate and 2 ml of the final wash solution were collected and used for fluorometric analysis.

For quantitative determinations, the [Pya^{88}] S 77–88-containing solution was transferred to a quartz cuvette, and monitored for fluorescence using a Spex Fluoromax (Edison, NJ) with a 90° fluorescence cell. The excitation wavelength was set to 353 nm (5 nm slit) and emission wavelength was adjusted to scan between 360–460 nm (7 nm slit). Fractional inhibition was calculated at 377 nm using the formula 1−(I−B)/(C−B), where I, B and C are emissions for inhibited, blank and control samples, respectively.

2.2. Molecular modeling

Models of ICD 1578 were constructed using Insight II software and simulated under the 'electrostatic force field' using Discover software on a Silicon Graphics computer. The ICD 1578 molecule was placed in a solvent cell with periodic boundary conditions. Explicit water molecules were then added to the unit cell to approximate a solvated molecule. After energy minimization, 15 000 time steps of dynamics with a step size of 1 fs were performed. An average structure was then deduced by examining the trajectory files and averaging bond lengths and rotation angles after the structures appeared to have stabilized.

2.3. Materials

ICD 1578 (Fig. 1) was designed and synthesized by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA) under contract to the Department of the Army. Pure BoNT/B LC was obtained from Drs. Eric Johnson and Michael Goodnough (Food Research Institute, Madison, WI). [Pya^{88}] S 39–88 was a generous gift from

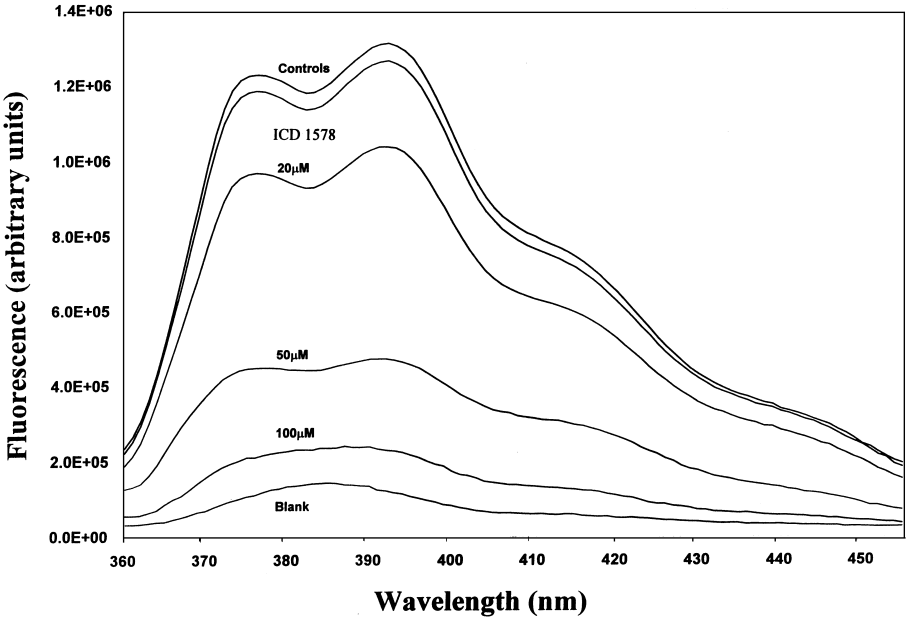

Fig. 2. Representative fluorescence spectra of the 12-mer [Pya^{88}] S 77–88 cleavage product in the absence or presence of ICD 1578. The spectra represent fluorescence intensity in arbitrary units at the indicated wavelengths. The peak at 377 nm was used for quantification in conformity with Soleihac et al. [16].

Drs. Fabrice Cornille and Bernard Roques (Université Rene Descartes, Paris, France). Methanol (optimal spectrophotometric grade) was purchased form Fisher Scientific Co. (Pittsburgh, PA). TFA (peptide synthesis grade) was obtained from Millipore, Inc. (Bedford, MA). Captopril, ultrapure $ZnSO_4$ and HEPES were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or higher.

2.4. Statistical analysis

Concentration-response data were fit by a nonlinear regression program (GraphPad Prism v 2.01, San Diego, CA). Statistical significance was tested by a one-way ANOVA with Dunnett's post tests.

3. Results

3.1. Effect of ICD 1578 on BoNT/B-mediated substrate cleavage

Fluorescence spectra of the 12-mer [Pya^{88}] S 77–88 cleavage product in the absence and presence of ICD 1578 are illustrated in Fig. 2. The upper traces, labeled control, represent cleavage of the substrate [Pya^{88}] S 39–88 by the pure BoNT/B LC prior to addition of inhibitor. Under this condition, fluorescence signals were observed with peaks at 377 and 392 nm corresponding to normal BoNT/B proteolytic activity. The control spectra shown in Fig. 2 were obtained from two separate reactions and demonstrate the reproducibility of the assay.

The effect of potential inhibitors on this reaction was evaluated by pretreating the BoNT/B LC for 30 min with the presumptive inhibitor prior to incubation of the BoNT/B LC-inhibitor complex with [Pya^{88}] S 39–88. In the presence of ICD 1578, there was a marked and progressive decrease in fluorescence intensity due to inhibition of BoNT/B-mediated proteolysis.

Concentration-response data for ICD 1578 over the range 7–200 μM from 3–4 such experiments are shown in Fig. 3. The lowest concentration of ICD 1578 examined was 7 μM, which failed to inhibit the BoNT/B-mediated cleavage significantly. Higher concentrations of ICD 1578, however, produced a significant, concentration-dependent inhibition of BoNT/B proteolysis, with nearly complete inhibition occurring at 100 μM. From the sigmoid curve fitted to the data, the IC_{50} was determined to be 27.6 μM. In the absence of BoNT/B LC, ICD 1578 did not quench the fluorescence of [Pya^{88}] S 39–88, indicating that the decrease in fluorescence in Figs. 2 and 3 was due to inhibition of BoNT/B LC.

3.2. Effects of captopril on BoNT/B-mediated cleavage of [Pya^{88}] S 39–88

Since ICD 1578 is a new protease inhibitor, it was of interest to compare its activity with that of the well-characterized metalloprotease inhibitor, captopril. Fluorescence spectra for the inhibitory actions of captopril on BoNT-mediated cleav-

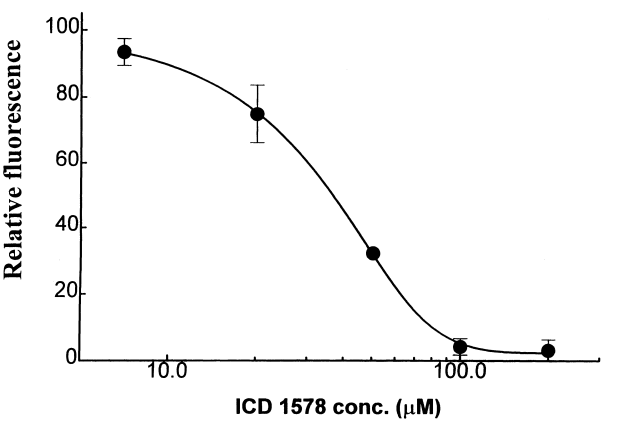

Fig. 3. Concentration-response curves for ICD 1578. The symbols represent the mean ± S.E. of data obtained from 3–4 replicate samples. The curve was fit by a nonlinear regression analysis and yielded an IC_{50} of 27.6 μM. All points ≥ 20 μM were found to differ significantly from control using a one-way ANOVA with Dunnett's post test ($P < 0.01$).

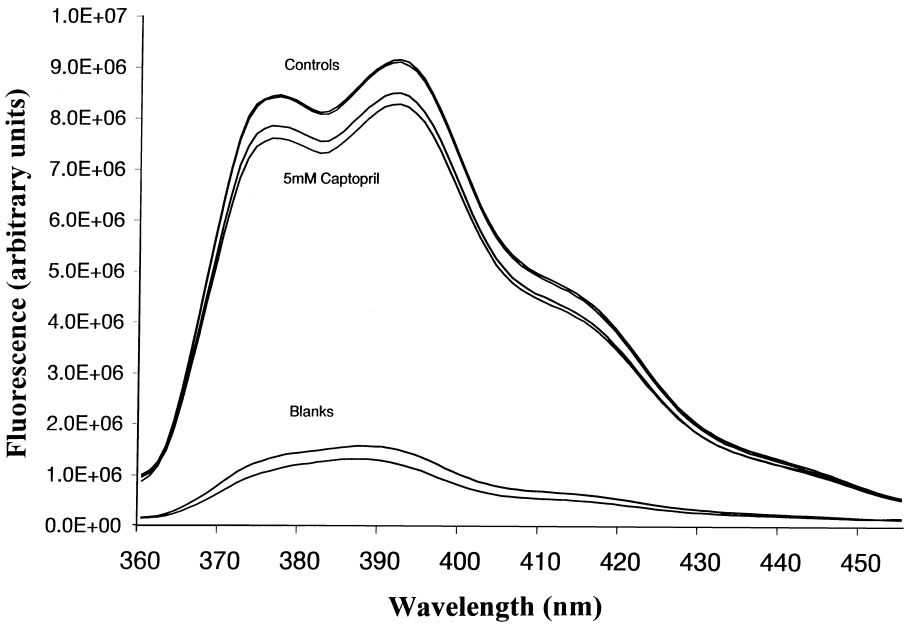

Fig. 4. Representative fluorescence spectra of the 12-mer [Pya^{88}] S 77–88 cleavage product in the absence or presence of captopril. The spectra represent fluorescence intensity in arbitrary units at the indicated wavelengths. Each condition was performed in duplicate. Note that even 5 mM captopril causes only a slight inhibition of BoNT/B activity.

age of [Pya^{88}] S 39–88 are depicted in Fig. 4. Experiments were performed in the absence of inhibitor and 30 min after preincubation of BoNT/B LC with 5 mM captopril. Even at this very high concentration, captopril produced only a marginal inhibition of BoNT/B activity. These results confirm those obtained earlier using a recombinant synaptobrevin peptide as substrate [14].

4. Discussion

The results of this investigation indicate that ICD 1578 is a potent antagonist of BoNT/B LC, and the first organic inhibitor for this site that is effective in the sub-millimolar range; ICD 1578 was able to inhibit the proteolytic activity of BoNT/B with an $IC_{50} < 30$ μM. Previous investigators have suggested that captopril, a widely prescribed antihypertensive agent, may be useful for treatment of BoNT/B intoxication [17]. Although captopril is effective in inhibiting angiotensin converting enzyme, its intended target, it had little efficacy in antagonizing BoNT in either cell-free [14] or isolated muscle preparations [6,13]. Similarly, the naturally occurring thermolysin inhibitor, phosphoramidon, has been found to have only marginal efficacy in inhibiting BoNT/B activity [13,14].

Using phosphoramidon as a template, we recently designed and synthesized structural analogs in which the dipeptide Leu-Trp of phosphoramidon was replaced by Phe-Glu: amino acids that are present at the BoNT/B cleavage site of synaptobrevin-2 [15]. In addition, the rhamnose ring of phosphoramidon was replaced by a methyl, ethyl or a phenyl group in different analogs. When tested on [Pya^{88}] S 39–88, only the phenyl derivative was found to have appreciable activity. From these results, the minimal requirement for efficacy against BoNT/B LC appeared to be a pair of aromatic rings flanking a central polarizable group oriented to interact with the active site zinc [13]. Molecular modeling studies with metalloproteases of known active site structure (e.g. *Pseudomonas aeruginosa* elastase) suggest that the zinc binding moiety in ICD 1578 may be the carbonyl oxygen situated between the phenyl and isocoumarin rings, the ketone oxygen in position 1 of isocoumarin or both (Fig. 1). In addition to the zinc binding group, ICD 1578 also has several other oxygens and two nitrogens that can participate in hydrogen bonding, thus stabilizing the molecule in the active site. From the energy minimized conformation of ICD 1578, the length of the molecule is ~17 Å. This is consistent with the relatively large active site for serotype A recently reported from X-ray crystallography data [18].

Although originally designed as a human leukocyte elastase inhibitor, ICD 1578 conformed to the general criteria for activity against BoNT/B LC and was therefore selected for detailed examination. This compound was found to be sufficiently potent to be considered as a lead candidate for further drug development. Discovery of therapeutic agents for BoNT intoxication will be difficult since, in addition to the customary safety and efficacy issues, the drug must be able to penetrate biological membranes and reside in the nerve terminal for as long as BoNT LC remains active. This study represents the first step to that eventual goal.

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