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RNA aptamer to thrombin binds anion-binding exosite-2 and alters protease inhibition by heparin-binding serpins

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Abstract We studied the RNA aptamer Toggle-25/thrombin interaction during inhibition by antithrombin (AT), heparin cofactor II (HCII) and protein C inhibitor (PCI). Thrombin inhibition was reduced 3-fold by Toggle-25 for AT and HCII, but it was slightly enhanced for PCI. In the presence of glycosaminoglycans, AT and PCI had significantly reduced thrombin inhibition with Toggle-25, but it was only reduced 3-fold for HCII. This suggested that the primary effect of aptamer binding was through the heparin-binding site of thrombin, anion-binding exosite-2 (exosite-2). We localized the Toggle-25 binding site to Arg 98, Glu 169, Lys 174, Asp 175, Arg 245, and Lys 248 of exosite-2. We conclude that a RNA aptamer to thrombin exosite-2 might provide an effective clinical reagent to control heparin's anticoagulant action.

Keywords: RNA aptamer; Thrombin; Serpin; Heparin

1. Introduction

Numerous proteases, protease inhibitors, cofactors and cells control the physiological process of hemostasis [1,2]. The hemostatic balance is disrupted either when an injury occurs or when a pathological process leads to underproduction (hemorrhage) or overproduction (thrombosis) of thrombin activity. Thrombosis accounts for significant morbidity and mortality in conditions such as deep venous thrombosis with pulmonary embolism, acute coronary syndrome, stroke, and peripheral vascular disease [3]. Hemostatic proteases are obvious targets for anticoagulants and antithrombotics. The anticoagulant heparin is believed to act by accelerating thrombin and factor Xa inhibition by binding to antithrombin (AT; systematic name *SERPINC1*) and other heparin-dependent serine protease inhibitors (serpins) [4,5]. However, heparin has potentially lethal side effects such as bleeding and heparin-induced thrombocytopenia, and although protamine sulfate can be used to reverse heparin's anticoagulant action, it also has associated toxicities [6]. Therefore, there is an interest for alternative therapeutic anticoagulants to substitute for heparin and for new substances to terminate heparin's action as a therapeutic anticoagulant.

Thrombin is a serine protease that possesses trypsin-like behavior in that it prefers to cleave its substrates after arginyl residues [7–10]. Unlike trypsin, thrombin has numerous surface subsites that influence macromolecular substrate interactions that make it a more discriminating protease [10]. One of these subsites is anion-binding exosite-1 (exosite-1). Thrombin exosite-1 has been implicated in binding of fibrinogen, leech anticoagulant hirudin, protease activated receptor-1, thrombomodulin, factors V and VIII, glycoprotein-1bα, and the acidic domain of the serpin heparin cofactor II (HCII; systematic name *SERPIND1*). Anion-binding exosite-2 (exosite-2) [10] is primarily responsible for thrombin's glycosaminoglycan binding abilities, which helps accelerate protease inhibition by the heparin-dependent serpins, AT, HCII, protein C inhibitor (PCI; also known as plasminogen activator inhibitor-3; systematic name *SERPINA5*), and protease nexin-1 [4,5]. The 60-insertion loop (60-loop) of thrombin forms a lid-like structure over the catalytic triad and active site region, and controls access of substrates and inhibitors to the active site [10]. These subsites offer unique possibilities to provide binding sites for ligands that might modulate thrombin's activity, give new understanding of structure-activity relationships, and generate novel anticoagulants.

DNA and RNA aptamers are one type of potential therapeutic agents [11,12]. RNA aptamers are made using an iterative in vitro selection process called SELEX (systematic evolution of ligands by exponential enrichment), which selects high affinity binding RNA molecule from a large pool of random-sequence single-stranded oligonucleotide populations [11–13]. Thrombin has been a frequent target for both DNA and RNA-specific aptamers, both for structure-function and therapeutic uses [14–17]. Toggle-25 is a RNA aptamer recently described by White et al. [16]. This RNA aptamer was selected by a technique of "toggling" between protein targets; thus, Toggle-25 binds both human and porcine thrombin. Toggle-25 is made from endonuclease-resistant nucleotides, which confer a half-life of many hours in vivo compared to minutes for unmodified RNA or DNA aptamers. We studied the effect of

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Abbreviations: Serpin, serine protease inhibitor; HCII, heparin cofactor II; AT, antithrombin; PCI, protein C inhibitor; exosite-1 and -2, anion-binding exosite-1 and anion-binding exosite-2

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the RNA aptamer–thrombin interaction on inhibition with AT, HCII, and PCI in the absence and presence of glycosaminoglycans, and we localized the RNA aptamer-binding site on the thrombin molecule. Based on the results of these experiments, we show that a RNA aptamer directed to exosite-2 of thrombin affords thrombin protection during inhibition by heparin-binding serpins.

2. Materials and methods

2.1. Proteins and reagents

Plasma derived human α-thrombin, AT, and HCII were prepared and purified to homogeneity as described previously [18–20]. Human wild-type recombinant PCI was expressed in *Escherichia coli* [strain BL21(DE3)pLysS] using the Qiagen pET15b expression vector (with a N-terminal polyhistidine sequence), and purified to homogeneity by Ni^{2+}-chelate chromatography (Réhault, S.M. et al., 2004, unpublished). Recombinant wild-type thrombin and exosite-2 thrombin mutants were prepared as described [21]. The following thrombin exosite-2 mutants were studied: R89A/R93A/E94A, R98A, D122A/R123A/E124A, E169A/K174A/D175A, R178A/R180A/D183A, R245A/K248A/Q251A, R245A, K248A, and Q251A (residue numbers according to the thrombin numbering system[3]). Unbleached crude heparin was obtained from Diosynth (Oss, The Netherlands). Dermatan sulfate (Calbiochem) was treated with nitrous acid to remove contaminating heparin/heparan sulfate as detailed [22]. Toggle-25 (5′-GGGAACAAAGCUGAAGUACUUACCC-3′) was synthesized, purified and deprotected as described previously [16]. The control aptamer BulgeUs is a point mutant at nucleotides 20 and 21 (changing UU to AA) that has no measurable affinity for thrombin [16]. Both aptamers contain a 3′-inverted deoxythymidine cap to prevent exonuclease degradation, and all Cs and Us contain 2′-deoxyfluoro sugars [16]. Both RNA aptamers were diluted to a final concentration of 50 μM in 20 mM HEPES, 150 mM NaCl, and 2 mM $CaCl_2$ at pH 7.4.

2.2. Serpin/thrombin inhibition assays

All assays were performed at room temperature in 96-well microtiter plates previously coated with 2 mg/mL ovalbumin essentially as described previously [23,24]. In the absence of glycosaminoglycan, 100–500 nM serpin (AT, HCII, or PCI) was incubated with 0.5 nM α-thrombin in 20 mM HEPES, 150 mM NaCl, and 2 mM $CaCl_2$ at pH 7.4, in the absence of RNA aptamer and with 10 nM Toggle-25 or BulgeUs, all in a final reaction volume of 50 μL. At varied time points (5–120 min), residual thrombin activity was measured by adding 50 μL of 450 μM tosGly-Pro-Arg-*p*-nitroanilide and 30 μg/mL Polybrene in the HEPES buffer at pH 7.4. Substrate cleavage was measured by color development at 405 nm on a V_{max} Kinetic Microplate Reader (Molecular Devices).

In the presence of glycosaminoglycans (heparin or dermatan sulfate), 5–20 nM serpin (AT, HCII or PCI) was incubated with 0.1–2000 μg/mL heparin or dermatan sulfate with 0.5–1 nM thrombin in 20 mM HEPES, 150 mM NaCl, and 2 mM $CaCl_2$ at pH 7.4, in the absence of RNA aptamer or 10 nM Toggle-25 or BulgeUs in a final reaction volume of 50 μL. In all assays, the RNA aptamer was incubated for 60 s with thrombin prior to the addition of the other reactants. At varied time incubations (15 s–25 min), 50 μL of 450 μM tosGly-Pro-Arg-*p*-nitroanilide and 1–5 mg/mL Polybrene (final concentration to immediately neutralize glycosaminoglycan) was added to measure residual thrombin activity. After allowing tosGly-Pro-Arg-*p*-nitroanilide cleavage by the remaining thrombin, the reaction was stopped by adding a 50% acetic acid solution. The plates were then spun at $1350 \times g$ for 15 min to remove the glycosaminoglycan/Polybrene precipitate that forms at high glycosaminoglycan concentrations. After transferring 100 μL of the supernatant of each well to a new plate, substrate cleavage was measured by color development at 405 nm on a V_{max} Kinetic Microplate Reader.

All assays were performed at least three times with duplicate or triplicate data points for all of the proteins except the AT time course inhibition studies with the thrombin exosite-2 mutants, which were done two times in triplicate. The second order rate constants of inhibition were calculated as described previously [23,24].

[3] In parentheses are the thrombin residues using the numbering system with respect to bovine chymotrypsinogen [7,10]: R89A/R93A/E94A (R93A/R97A/E97aA), R98A (R101A), D122A/R123A/E124A (D125A/R126A/E127A), E169A/K174A/D175A (E164A/K169A/D170A), R178A/R180A/D183A (R173A/R175A/D178A), R245A/K248A/Q251A (R233A/K236A/Q239A), R245A (R233A), K248A (K236A), and Q251A (Q239A).

3. Results

3.1. Effect of RNA aptamer to thrombin on serpin–thrombin inhibition in the absence of glycosaminoglycans

In the absence of glycosaminoglycans, the addition of Toggle-25 resulted in a significant decrease (~3-fold) in thrombin inhibition by both AT and HCII, but a slight increase (1.4-fold) in thrombin inhibition by PCI (Table 1). There was no significant change in thrombin inhibition by AT, HCII and PCI in the presence of the control RNA aptamer BulgeUs (Table 1). These results suggest that Toggle-25 ($M_r \sim 9000$) binding to thrombin, possibly by steric hindrance, alters inhibition by serpins.

3.2. Effect of RNA aptamer to thrombin on serpin–thrombin inhibition in the presence of glycosaminoglycans

It is likely that aptamers will be generated to protein regions with substantial charge, such as exosite-1 or exosite-2. To investigate the possibility that Toggle-25 binds to exosite-2, we studied glycosaminoglycan-accelerated thrombin inhibition by AT, HCII and PCI. In the presence of heparin, the addition of Toggle-25 resulted in a substantial loss of inhibitory activity for the AT–heparin and PCI–heparin inhibition reactions (Fig. 1 and Table 1). There was only a slight decrease in inhibitory activity in the presence of Toggle-25 both with HCII–heparin and with HCII–dermatan sulfate, consistent with the fold-change in activity found in the absence of glycosaminoglycan (Fig. 1 and Table 1). The control RNA aptamer BulgeUs had no effect on thrombin inhibition by serpin–glycosaminoglycans (Fig. 1 and Table 1). To further evaluate Toggle-25, we titrated in increasing amounts of the RNA aptamer (from 0.01 to 10 nM) to thrombin (0.5 nM) and measured the rate of inhibition by AT (5 nM)–heparin (500 ng/mL, ~0.1 units/ml, which is slightly less than the clinical range of 0.3–0.7 units/ml). There was a 50% loss in the rate of thrombin inhibition at 2 nM Toggle-25 (data not shown), which is similar to the K_d reported previously for Toggle-25/thrombin binding [16]. These results imply that Toggle-25 may interact with the heparin-binding region of thrombin to reduce serpin–glycosaminoglycan–thrombin complex formation, thereby protecting thrombin from inhibition. They also imply that Toggle-25 might neutralize heparin activity at relevant therapeutic dosages.

3.3. Mapping the binding site of the RNA aptamer to thrombin exosite-2

Our results above implied that Toggle-25 might be interacting with residues in the glycosaminoglycan-binding site of thrombin (exosite-2). Therefore, a series of exosite-2 Ala-scanned thrombin mutants were used in an attempt to localize the Toggle-25 binding site. The rationale was that if specific amino acids were responsible for Toggle-25 binding, these thrombin mutants would be "resistant" to the RNA aptamer and show no additional loss of activity. As the exosite-2

Table 1
Effect of Toggle-25 during thrombin inhibition by serpins in the absence and presence of glycosaminoglycans

Serpin/cofactor	$k_2 \times (M^{-1}\ min^{-1})$[a]		
	No aptamer[b]	BulgeUs (control aptamer)	Toggle-25 (thrombin aptamer)
AT	$2.16 \pm 0.01 \times 10^5$	$2.23 \pm 0.16 \times 10^5$	$0.71 \pm 0.04 \times 10^5$
AT/Heparin	$1.95 \pm 0.11 \times 10^8$ (900)	$1.97 \pm 0.08 \times 10^8$(880)	$0.04 \pm 0.003 \times 10^8$ (60)
HCII	$1.99 \pm 0.11 \times 10^4$	$1.90 \pm 0.10 \times 10^4$	$0.71 \pm 0.09 \times 10^4$
HCII/Heparin	$1.57 \pm 0.31 \times 10^8$ (7890)	$1.52 \pm 0.28 \times 10^8$ (8000)	$0.54 \pm 0.07 \times 10^8$ (7600)
HCII/DS	$4.17 \pm 0.52 \times 10^8$ (20 950)	$3.96 \pm 0.40 \times 10^8$ (20 840)	$2.57 \pm 0.23 \times 10^8$ (36 200)
PCI	$2.94 \pm 0.12 \times 10^5$	$3.13 \pm 0.03 \times 10^5$	$4.21 \pm 0.11 \times 10^5$
PCI/Heparin	$5.85 \pm 0.33 \times 10^6$ (20)	$5.95 \pm 0.09 \times 10^6$(19)	$0.60 \pm 0.21 \times 10^6$ (1.4)

Inhibition of thrombin by AT, HCII and PCI was measured in the absence and in the presence of heparin or dermatan sulfate (DS), in the absence and presence of Toggle-25 and control BulgeUs RNA aptamers. In the presence of glycosaminoglycans, the maximal inhibition of each curve was used in the calculation of the average inhibition rate. Values are expressed as means ± S.D. All assays were performed as described in Section 2.
[a] Rate constants are the mean values of at least three separate determinations performed in duplicate or triplicate.
[b] Values in parentheses are the maximum fold-stimulation with glycosaminoglycan for AT-(5 μg/mL heparin), HCII-(200 μg/mL heparin and 500 μg/mL dermatan sulfate) and PCI-(5 μg/mL heparin) thrombin inhibition reactions under the inhibition reactions condition used in Section 2.

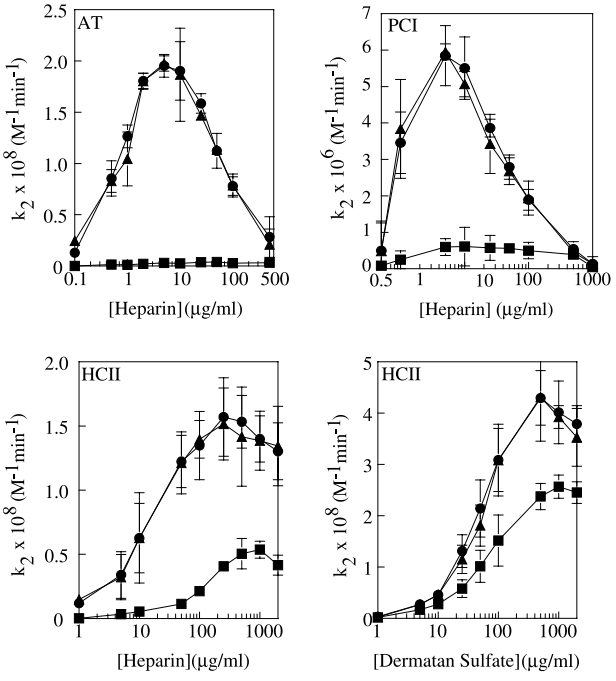

Fig. 1. Inhibition of α-thrombin by heparin-binding serpins in the presence of the RNA thrombin aptamer and in the presence of glycosaminoglycans. Thrombin was incubated with AT (upper left panel), PCI (upper right panel) and HCII (lower left and right panels) in the presence of various amounts of glycosaminoglycans (heparin or dermatan sulfate) in the absence of aptamer (●), and in the presence of 10 nM Toggle-25 aptamer (■) or control BulgeUs aptamer (▲) as described under Section 2. The data represent an average of at least three separate assays performed in triplicate.

thrombin mutants used have been shown to not bind to heparin, we only studied the effect of Toggle-25 on the AT-thrombin inhibition reaction in the absence of added heparin. Of the nine exosite-2 thrombin mutants examined, five mutants (R98A, E169A/K174A/D175A, R245A/K248A/Q251A, R245A, and K248A) were resistant to Toggle-25, two mutants (D122A/R123A/E124A and Q251A) resembled wild-type thrombin and were altered by Toggle-25, and two thrombin mutants (R89A/R93A/E94A and R178A/R180A/D183A) gave equivocal results, and while they may have had some resistance to the aptamer there was substantial change even in the absence of Toggle-25 (data not included). We then gathered more detailed AT-thrombin inhibition data for the exosite-2 thrombin mutants in the presence and absence of Toggle-25 to further implicate specific residues (for instance, compare R245A/K248A/Q251A to R245A and Q251A) (Table 2). These results suggest that Toggle-25 binds at or near Arg 98, Glu 169, Lys 174, Asp 175, Arg 245, and Lys 248 in thrombin exosite-2 (Table 2), which corresponds to residues in the glycosaminoglycan-binding site of thrombin.

4. Discussion

The specificity of thrombin aptamers provides a novel biomedical/biochemical tool to probe thrombin's interactions with macromolecular substrates. The thrombin aptamer described by Bock and co-workers [25–27] is a potent antithrombotic with affinity to exosite-1, both in vitro and in vivo. Aptamers have been prepared to both exosite-1 [25,26] and exosite-2 [28] of thrombin. Toggle-25, with a K_D of ~2 nM, was created to bind both human and porcine thrombin [16].

Table 2
Effect of Toggle-25 during antithrombin inhibition of exosite-2 thrombin mutants

Thrombin mutant	No aptamer[a] $k_2 \times (M^{-1}\ min^{-1})$	Plus Toggle-25[a] $k_2 \times (M^{-1}\ min^{-1})$	Aptamer (k_2)/no aptamer (k_2) (%)
Wild-type	1.97×10^5	0.64×10^5	32
R98A	2.10×10^5	2.01×10^5	96
E169A/K174A/D175A	1.60×10^5	1.42×10^5	89
R245A/K248A/Q251A	2.49×10^5	2.55×10^5	102
R245A	2.26×10^5	2.21×10^5	98
Q251A	2.05×10^5	0.58×10^5	28

Inhibition of thrombin and the exosite-2 thrombin mutants by AT was measured in the absence of heparin as described in Section 2.
[a] Rate constants are the mean values of two separate determinations performed in triplicate.

We sought to further characterize the Toggle-25/thrombin interaction using heparin-binding serpins, which are important regulators of thrombin activity in vivo. Using a set of Ala-scanned thrombin mutants [21,29,30], our results suggest that thrombin exosite-2 is involved in Toggle-25 binding (Fig. 2).

Another result of this study was the verification of the critical need for exosite-2 for AT–heparin (900-fold versus 60-fold acceleration in the absence and presence of Toggle-25, respectively) and PCI–heparin (20-fold versus 1.4-fold acceleration in the absence and presence of Toggle-25, respectively) thrombin inhibition reactions, and a less important role for exosite-2 during HCII–heparin (7900-fold versus 7600-fold acceleration in the absence and presence of Toggle-25, respectively) or HCII–dermatan sulfate (21 000-fold versus 36 000-fold acceleration in the absence and presence of Toggle-25, respectively) interactions. These observations reinforce other recent studies probing serpin–glycosaminoglycan/thrombin interactions [31–35].

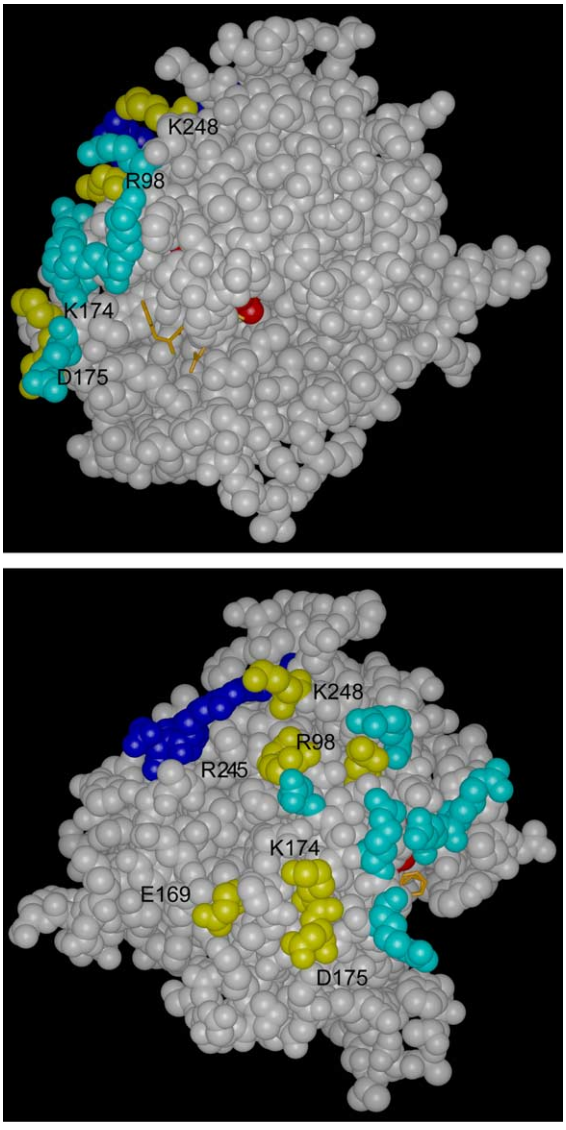

Fig. 2. Space-filled molecular model of α-thrombin. The figure is a CPK representation of the refined 1.9 Å crystal structure of human α-thrombin (PDB file 1PPB*) and produced using Insight II software (Accelrys, Inc., San Diego, CA). Upper panel: "traditional" front view; lower panel: 180° rotation to the right of traditional view. As identified in the assays performed (see Fig. 2 and Table 2), the residues involved in Toggle-25 binding are shown in yellow, residues that were equivocal are shown in light blue and the residues not involved are highlighted in dark blue. The thrombin active site residues are seen in red and the Phe-Pro-Arg-chloromethyl ketone is shown in orange.

As Toggle-25 alters the AT– and HCII–thrombin inhibition reactions in the absence of added glycosaminoglycan, the aptamer must alter thrombin's active site recognition of the AT- and HCII-reactive site loop region. It is interesting to note that the global nature of HCII and AT revealed by X-ray crystallography is quite similar [36,37], and they both had the same ~3-fold loss of activity in the presence of Toggle-25/thrombin (in the absence of glycosaminoglycan). The slight increase in thrombin inhibition activity by PCI (with no added heparin) with Toggle-25 is reminiscent of that seen with the R89A/R93A/R98A thrombin mutant [38], and may represent a subtle difference in the PCI-thrombin inhibition mechanism compared to AT and HCII. A monoclonal antibody isolated from a patient with multiple myeloma was recently shown to be a potent anticoagulant [39,40]. This IgG's anticoagulant activity was partly due to an enhancing effect during AT– and HCII–thrombin inhibition reactions (in the absence of heparin), and the antibody's binding site was mapped to thrombin exosite-2 [39,40]. Thus, ligand binding (in this case, a RNA aptamer compared to an IgG that binds exosite-2) to thrombin at or near the same site can differentially promote conformations that influence protease activity.

Aptamers (RNA or DNA) are a promising new group of compounds that have already found some utility and use in regulating blood coagulation in vitro and in vivo [14–17,26,41,42,43]. The objective of anticoagulant/antithrombotic therapy in cardiovascular disease is to prevent further fibrin deposition and platelet aggregation, halting current and future ischemic episodes [6]. While the most commonly used agents (heparin, coumadin and aspirin) have well established biological actions, there are some limitations to their effectiveness and this has led to the development of new antithrombotic compounds [6]. The results described here suggest that specific RNA aptamers directed at exosite-2 of thrombin may be used for many different facets of therapeutic regulation of thrombin. We have shown that Toggle-25 can reduce the effect of heparin on glycosaminoglycan-dependent thrombin inhibition by serpins and could serve to regulate the anticoagulant effect of heparin in vivo. The notion of therapeutically using an aptamer like Toggle-25 becomes especially attractive when used in conjunction with an antidote RNA aptamer [17], that can be specifically targeted to Toggle-25 to reverse its action.

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