Protection from Anthrax Toxin-Mediated Killing of Macrophages by the Combined Effects of Furin Inhibitors and Chloroquine

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Bacillus anthracis secretes three proteins involved in pathogenesis: protective antigen (PA), lethal factor (LF), and edema factor (EF) (8, 32). PA binds to a ubiquitous cellular receptor, anthrax toxin receptor (ATR), and mediates entry of toxin enzymes LF and EF into the target cells (6). On the macrophage cell surface, full-length, receptor-bound PA (83 kDa; PA83) is thought to be cleaved by furin or furin family proteases (37) at the sequence RKKR167 of cleaved PA (63 kDa; PA63) forms a heptameric prepore on which one to three LF binding sites become accessible (31, 35). Assembled prepore-toxin complexes bound to ATR redistribute to glycosphingolipid/cholesterol-rich lipid domains and undergo endocytosis, preferentially via a clathrin-dependent mechanism (1, 5). Acidification of the endosomal compartment converts the prepore to a pore through which LF, a Zn2+ metalloprotease, is translocated into the cytosol of the macrophage. LF cleaves mitogen-activated protein kinase kinases at their amino termini (11), initiating a cascade of cellular events resulting in cell death (9).

Previously, it was shown that blocking proteolytic processing of PA83 by mutation of the furin cleavage site blocked prepore formation and endocytosis (5). Ammonium chloride and chloroquine block the toxic effects of LF and EF, presumably by impairing translocation into the cytosol by neutralizing endosomal pH (14, 17). Here we show that three high-affinity inhibitors of furin efficiently block killing of murine J774A.1 macrophages by recombinant protective antigen plus lethal factor: RRD-eglin and RRDG-eglin, developed by engineering the protein protease inhibitor eglin c, and the peptide boronic acid inhibitor acetyl-Arg-Glu-Lys-boroArg pinanediol. Inhibition of killing was dose dependent and correlated with prevention of protective antigen processing. Previous studies have shown that weak bases, such as chloroquine, which neutralize acidic compartments, also interfere with toxin-dependent killing. Here we show that combining furin inhibitors and chloroquine strongly augments the inhibition of toxin-dependent killing, suggesting that combined use of antifurin drugs and chloroquine might provide enhanced therapeutic benefits. Reversible furin inhibitors protected against anthrax toxin killing for at least 5 h, but by 8 h, toxin-dependent killing resumed even though furin inhibitors were still active. An irreversible chloromethylketone inhibitor did not exhibit this loss of protection.

MATERIALS AND METHODS

Materials. Standard reagents were from Sigma, Aldrich, or Fisher. Chloroquine was from Sigma. Nitrocellulose membrane was from Schleicher and Schuell (Keene, NH). Monoclonal antibody against B. anthracis PA was from Abcam (Cambridge, MA). Pefabloc SC was purchased from Roche (Indianapolis, IN). Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-cmk) was from Bachem Bioscience (King of Prussia, PA). Acetyl-Arg-Ala-Arg-Tyr-Arg-Arg-MCA (Ac-RARYR-MCA) was synthesized as described previously (28). Other methylcoumarinamide substrates were from Bachem Bioscience (San Diego, CA). Recombinant PA and LF were kindly provided by R. J. Collier (Harvard Medical School). Secreted, soluble furin (herein, “furin”) was expressed and purified as described previously (26). Acetyl-Arg-Lys-Val-Lys-boroArg pinanediol (Ac-REKboroR), which functions as a boronic acid inhibitor in aqueous solutions as described previously (21, 23), was generously provided for furin inhibition by Charles Kettner (DuPont Pharmaceutical Co., Wilmington, DE). Eglin c containing the wild-type reactive site loop (WT-eglin) and eglin c variant Tyr49Asp-R4R1-eglin (RRDG-eglin) were prepared as described previously (27).

RRDG-eglin. The three-dimensional structure of the complex of the Kex2 catalytic domain with acetyl-Ala-Lys-boroArg (20) was superimposed onto the coordinates of the thermitase-eglinc complex (18) using the catalytic Asp, His, and Ser residues as reference points. The superimposition identified eglin residue Val16 as a potential, novel adventitious contact (27) between Kex2 and RRD-eglin. The codon for Val16 was randomized in the vector encoding RRD-eglin, and the resulting mutant library was screened to identify improved furin

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inhibitors, as described previously (27). VdLys-Gly-RRD-eglin (RRDG-eglin) was identified as an improved inhibitor and was purified as described previously (26).

**Cytoxicity assays.** J774.A1 murine macrophages (3 × 10^6 to 6 × 10^6 cells/well) were plated onto 96-well tissue culture plates (CorningCostar) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and were cultured overnight at 37°C in a humidified incubator containing 5% CO₂. Cells were washed once with modified Ringer's buffer (RB*: 155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, pH 7.2), and subsequent incubations were carried out in 100 μl RB* for 2.5 to 8 h in a humidified incubator at 37°C. Unless otherwise indicated, recombinant P₄ₓ was 12 nM and recombinant LF was 1 nM, both diluted in RB*. Furin inhibitors and chloroquine were also added in RB*. Initial experiments to examine protection against killing by PA plus LF were carried out using propidium iodide staining as an assay (5). However, this assay was tedious (requiring cell fixation, staining, and cell counting by fluorescence microscopy) and variable (variable cell loss during washing steps) and exhibited a high, variable background. Instead, we followed cell killing by measuring release of lactate dehydrogenase (LDH), which was both easily quantified and highly reproducible, using the CytoTox 96 cytotoxicity assay (Promega, Madison, WI), following the manufacturer's protocol. Released LDH was monitored by measuring absorbance at 490 nm on an Envision precision microplate reader (Molecular Devices). RB* was used in all incubations because the presence of LDH in the serum component of Dulbecco’s modified Eagle’s medium produced a high background in the LDH assay. When RB* was used, the background of the LDH assay was extremely low. In the absence of PA plus LF, LDH release was 6.2% ± 0.5% of the total LDH released from cells lysed with 0.5% Triton X-100 prior to addition of LDH substrates. This background value represents the mean for all control data, i.e., with incubation times ranging from 2.5 to 8 h. There was no significant difference between the background release seen at 2.5 h and that seen at 8 h of incubation in RB*. Both experimental and control assays were carried out in triplicate or quadruplicate; average values are reported with error bars corresponding to standard errors of the mean.

**Characterization of furin inhibition.** Furin was titrated with Ac-REKboroR in the furin assay buffer, 20 mM Na-MES (pH 7) containing 1 mM CaCl₂ and 0.1% Triton X-100, by using boc-RVRR-MCA as substrate (2 μM) using the method of Angliker et al. (3). Furin concentration determined by titration with Ac-REKboroR was in agreement with the concentration determined by the rapid quench flow method (7). Affinity of RRDG-eglin for furin was determined as described previously (26). To determine the affinity of Ac-REKboroR for furin, furin (0.43 nM) was incubated with Ac-REKboroR (0 to 1 nM) for 1 h at room temperature and Kᵣ was determined using a sensitive substrate, Ac-RARYRR-MCA (0.66 μM) as described previously (26). The association rate, kₐ, for binding of Ac-REKboroR to furin was determined by recording inhibition progress curves, measuring hydrolysis of Ac-RARYRR-MCA (1.3 μM) by furin (0.4 nM) in the presence of inhibitor with [I]/[E] ratios ranging from 10 to 50. Reactions were monitored for 70 min at 30°C using an f-max fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Progress curves were graphically analyzed by fitting fluorescence intensity (F) of released 7-amino-4-methylcoumarin to equations 1 and 2 using KaleidaGraph (Synergy Software) (25), where F₀ is the initial uninhibited rate, Fₑ is the final steady-state rate, and kₑ is the observed relaxation rate constant. Because the hexapeptidyl substrate exhibits substrate inhibition at high substrate concentration (28), the kᵣ of furin for Ac-RARYRR-MCA (1.24 μM) was obtained from the slope of the linear portion of an Eadie-Hofstee plot (F₀ versus Fₑ) (13) using substrate concentrations lower than 0.8 μM.

\[ F = F₀ + (Fₑ - F₀)(1 - e^{-kₑt})kᵣ + Fₑ \]  
(1)  
\[ kᵣ = kᵣ/Kᵣ(1 + S/Kᵣ) \]  
(2)

The effect of chloroquine on furin activity was examined by incubating furin with chloroquine for 1 h in furin assay buffer and then measuring hydrolysis of boc-RVRR-MCA.

**Evaluation of PA processing.** J774.A1 macrophages (5 × 10⁶) were incubated in 10 ml RB* with PA (12 nM) and either RRD-eglin (200 nM), Ac-REKboroR (100 nM), or no addition (control) for 2.5 or 8 h. Macrophages were rinsed twice with phosphate-buffered saline and scraped in the presence of ice-cold 5% (wt/vol) trichloroacetic acid (TCA) (1 ml). Cell suspensions were homogenized on ice with 20 strokes of a ground-glass homogenizer. Samples (500 μl) of cell lysate were kept on ice for 30 min, and protein precipitates were collected by centrifugation. On ice, pellets were washed once with 5% TCA and twice with a 1:1 (vol/vol) mixture of ethanol and acetone. Precipitated proteins were dissolved in sodium dodecyl sulfate (SDS) sample buffer (1% SDS and 30 mM dithiothreitol), heated at 96°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide separating gel). The gel was blotted using nitrocellulose membrane, and the membrane was probed with monoclonal anti-PA antibody (Abcam) using SuperSignal West Pico chemiluminescence detection (Pierce). As a control, PA₄₀ was generated by digesting PA₃₀ with secreted, soluble furin.

**RESULTS**

Inhibition of furin with eglin c variants and Ac-REKboroR. Ac-REKboroR, previously used in structural analysis of the furin homologue Kex2p (19), was a potent furin inhibitor with a Kᵣ of 28 pM (Fig. 1A). A newly developed eglin c variant,
was calculated to be $k_d$ of 0.17 nM, compared to $K_i$ values for RRD-eglin of 0.33 nM and for WT-eglin c of 11 μM (26, 27). The $k_{on}$ value for the peptide boroarginine inhibitor was $1.8 \times 10^6$ s$^{-1}$, determined from the slope of a plot of $k_{obs}$ versus inhibitor concentration (Fig. 1B). The $k_{diss}$ value for the peptide boroarginine inhibitor was calculated to be $5 \times 10^{-5}$ s$^{-1}$ from the relationship $k_{diss} = k_{on} \times K_i$. 

**Inhibition of macrophage killing by furin inhibitors.** Anthrax toxin-mediated killing was assayed as LDH activity released from J774A.1 macrophages incubated with PA and LF in protein-free buffer relative to that released by detergent treatment, as described in Materials and Methods. By this measure, addition of 1 μg/ml PA (12 nM) and 0.1 μg/ml LF (1.1 nM) resulted in essentially 100% lysis of macrophages (3 × 10$^4$ to 6 × 10$^4$ cells per well) after 2.5 h of incubation at 37°C (Fig. 2A and B, zero inhibitor). Under these conditions, the ratio of PA molecules to cells was $\sim$10$^7$ and the ratio of PA to ATR can be estimated at $\sim$10$^6$, based on the number of ATR molecules per cell (15). Moreover, because the $K_D$ of ATR for PA is $\sim$1 nM, PA is present at a saturating concentration. Despite the high levels of PA and LF used, killing depended absolutely on the presence of both molecules: omitting either PA or LF resulted in no LDH release above background (data not shown). This assay therefore provided a stringent test of the ability of furin inhibitors to block toxin-dependent killing. When RRD-eglin and Ac-REKboroR were added to J774A.1 macrophages at the same time as PA and LF, concentration-dependent inhibition of cell killing was observed (Fig. 2A). At 200 nM, RRD-eglin (Fig. 2A) and RRDg-eglin (not shown) blocked anthrax toxin lethality almost completely. Ac-REKboroR exhibited near-complete protection at 100 nM (Fig. 2A). Titration of WT-eglin c, a poor inhibitor of furin, resulted in a slight (15%), apparent protection from toxin lethality at the highest concentration tested (Fig. 2A), underlining the specificity of protection afforded by the furin inhibitors but also suggesting the possible existence of a minor pathway of PA activation.

**Combined effects of chloroquine and furin inhibitors.** The lysosomotropic weak base chloroquine inhibited anthrax toxin lethality in the assay with a concentration dependence similar to that found in previous studies (14, 17), with near-complete protection at 100 μM (Fig. 2B). NH$_4$Cl (10 mM) also inhibited toxin lethality, but with lower efficiency (data not shown), and so was not pursued further.

When furin inhibitors were titrated into toxin assay mixtures containing 10 μM chloroquine, substantial augmentation of protection was seen (Fig. 3). Addition of 10 μM chloroquine alone resulted in $\sim$37% protection from toxin (Fig. 3A to C). Chloroquine (10 μM) lowered the concentration of RRD-eglin necessary for 50% protection from 75 nM to less than 5 nM and reduced the concentration of RRD-eglin required for 95% protection from $\sim$200 nM in the absence of chloroquine to less than 125 nM (Fig. 3A). Addition of 10 μM chloroquine lowered the concentration of Ac-REKboroR necessary for 50% protection from 50 nM in the absence of chloroquine to less than 10 nM and reduced the concentration of Ac-REKboroR required for 95% protection in the absence of chloroquine from 110 nM to less than 60 nM. Similar augmentation with chloroquine was also seen for RRDG-eglin (Fig. 4; also data not shown). Chloroquine even exhibited a mild augmentative effect with WT-eglin c (Fig. 3C). Chloroquine and the protease inhibitors are expected to function at distinct, sequential steps in killing by PA plus LF. If so, then the inhibition of killing afforded by combining the inhibitors would be expected to be...
at least the product of the fractional inhibition obtained with each inhibitor alone. The dotted line plotted in Fig. 3A to C represents the product of inhibition seen with 10 μM chloroquine inhibition and inhibition seen with each protease inhibitor alone. In each case, the combination of the protease inhibitor with chloroquine resulted in inhibition of lysis greater than that of the product, arguing that chloroquine and the protease inhibitors do in fact block independent steps and that there is an added benefit of the combination of inhibitors. This was even true of the modest inhibition of killing seen with wild-type eglin c.

**Durability of inhibition.** Cell killing assays described above utilized a uniform 2.5-h incubation. To test the “durability” of inhibition, we measured the degree of protection afforded by furin inhibitors during 5-h and 8-h incubations with PA plus LF at 37°C. There was no increase in background release of LDH during these extended incubations in RB*: LDH released from untreated cells was equivalent to the level of spontaneous release without incubation (data not shown). RRD-eglin (200 nM), RRDG-eglin (200 nM), and the boroarginine inhibitor (100 nM) exhibited persistent protection of the macrophages from toxin-mediated killing for 5 h (Fig. 4A, gray bars). However, at 8 h, inhibition of toxin lethality by protease inhibitors was substantially decreased, with the effect almost completely abated in the case of the eglin variants, and with the boroarginine inhibitor exhibiting only 50% protection from toxin at that time point (Fig. 4A, black bars).

Even under these conditions of “escape” from protection by furin inhibitors, addition of chloroquine (15 μM) substantially increased protection at the 8-h time point (Fig. 4A). Combination of the eglin variants (200 nM) with chloroquine blocked 50% of the anthrax toxin lethality at 8 h compared to less than 20% protection with protease inhibitors alone (Fig. 4A). Ac-REKboroR (100 nM) plus chloroquine (15 μM) provided ≥70% protection at 8 h compared to ~50% protection with Ac-REKboroR alone.

Repetitive addition of RRD-eglin (200 nM) or Ac-REKboroR (100 nM) at 2-hour intervals during 8-h incubations did not significantly improve protection (data not shown), suggesting that escape from protection did not involve inactivation of inhibitors. To test this directly, macrophages were incubated with RRD-eglin (200 nM) or Ac-REKboroR (100 nM) for 8 h in the presence of PA plus LF and residual inhibitory activity in the RB* medium was measured by titration using purified secreted, soluble furin. Both inhibitors retained ~95% of inhibitory activities after 8 h of incubation at 37°C with macrophages and PA plus LF (data not shown). Furthermore, when the RB* medium from incubations containing RRD-eglin was precipitated with 10% TCA and analyzed by SDS-PAGE, the intact Coomassie blue-stained RRD-eglin band was undiminished relative to the unincubated control (data not shown), demonstrating that macrophages were lysed by the PA plus LF by 8 h even though intact RRD-eglin persisted.

A second possible mechanism for escape is that incubation with the protease inhibitors induced a novel inhibitor-resistant activity capable of processing PA. To test this, macrophages were incubated with furin inhibitors for 8 h prior to addition of PA (12 nM) plus LF (1.2 nM) and LDH release assays were performed after an additional 2.5-h incubation. As shown in Fig. 4B, preincubation for 8 h induced no significant effect on the efficiency of protection from toxin-mediated killing by protease inhibitors either alone or in combination with chloroquine. As a control, the effects of prolonged incubation with furin inhibitors and chloroquine on cell viability in the absence of anthrax toxin were examined (Fig. 4C). None of the inhibitors tested, added in the absence of PA plus LF, exhibited cytotoxicity under the conditions of the assay.

From the above experiment, it appeared that incubation of cells with furin inhibitors for 8 h did not induce a bypass...
pathway for activation of PA. Rather, prolonged incubation of cells with toxins was required for escape from inhibitor protection. This led us to consider the possibility that escape might result from processed PA produced either by a low level of free (i.e., uninhibited) furin or another furin family protease or by a different enzymatic pathway. This mechanism might depend on levels of PA in the incubation. To test the dependence of escape on PA plus LF concentrations, we examined cell killing at 8 h in incubation mixtures containing PA concentrations reduced to 1.2 nM and 0.4 nM, with concentrations of LF decreased proportionately to maintain a 10:1 molar ratio of PA to LF. Although these decreased levels of PA plus LF still resulted in essentially complete cell killing in the absence of furin inhibitors, escape from inhibitor protection was reduced in the case of each of the protease inhibitors, as shown in Fig. 4A (yellow bars, 1.2 nM PA; green bars, 0.4 nM PA). Although the eglin derivatives and Ac-REKboroR are tight binding inhibitors, in both cases binding is reversible. To reduce levels of free furin/furin-like activity further, an irreversible furin inhibitor, Dec-RVKR-cmk (25 μM) (2), was incubated with macrophages in the presence of 12 nM PA and 1.2 nM LF for 8 h. This inhibitor did not show escape (Fig. 4D), implying that...
variable amounts of slow-migrating PA were seen after 2.5-h incubations with PA plus protease inhibitors and these aggregates could not be definitively identified as PA$_{63}$ heptamers. However, in multiple experiments, the PA$_{63}$ monomeric band was consistently seen in cells incubated with PA in the absence of protease inhibitors for 2.5 or 8 h, whereas, it was never seen in cells incubated with PA for 2.5 or 8 h in the presence of protease inhibitors. It should be noted that a PA-independent pathway of killing at 8 h was ruled out because 8-h incubations with LF alone resulted in no cell killing (data not shown).

**DISCUSSION**

Here we have shown that eglin c variants, a boroargininyl peptide, and a commercially available peptidyl chloromethylketone, all of which exhibit high-affinity inhibition of the pro-protein-processing protease furin, block anthrax toxin-mediated killing of murine macrophage cells by blocking cleavage of PA. This is in accord with recent studies showing that oligo-d-arginine-based furin inhibitors provide protection against anthrax toxin lethality (34, 38). Furthermore, we have shown that the combination of furin family inhibitors with the weak base chloroquine substantially augmented protection from toxin lethality. Examination of the temporal course of protection by both furin inhibitors and chloroquine revealed that, upon prolonged incubation with PA and LF, escape from inhibition of killing was observed with the eglin c variants and the peptidyl boronic acid inhibitor but not with Dec-RVKR-cmk or with chloroquine. It is important to note that, because of similarities in specificity of furin and other widely expressed homologous proprotein convertases such as PACE4, PC5/6 A and B, and PC7 (37), the effects of the protease inhibitors used in this study may to some degree also reflect inhibition of these other enzymes.

Unlike biosynthetic processing of host cell proproteins or viral envelope glycoproteins, which occurs in the trans-Golgi network or post-trans-Golgi network endosomal compartments, processing of anthrax protective antigen by furin occurs obligatorily at the plasma membrane. Inhibition of biosynthetic processing of von Willebrand factor by RRD-eglin or of cytomegalovirus glycoprotein processing with a1-PDX in cell culture required μM concentrations of the inhibitors even though the $K_I$ values for both inhibitors are in the nM range (22, 27). In contrast, furin inhibitors do not need to cross membranes in order to interfere with maturation of PA, which is processed from PA$_{63}$ to PA$_{63}$ at the plasma membrane. This study was undertaken in part to test the hypothesis that inhibition of furin processing of PA at the plasma membrane would require much lower concentrations of inhibitors. This expectation was borne out by the finding that PA processing was blocked by concentrations of inhibitors 25-fold lower than those needed to block processing of pro-von Willebrand factor in tissue culture cells (27).

Inhibitors of furin and endosomal acidification exhibit augmentation in blocking anthrax toxin lethality. This phenomenon was expected because the two inhibitors target sequential and essential steps in toxin entry into the cell. Other combinatorial approaches to anthrax toxin lethality may also be advantageous, such as the combination of furin inhibition with inhibition of lethal factor (33, 42). Chloroquine itself, however,
may be particularly useful as part of a cocktail of inhibitors because of the extensive clinical experience with its use as an antimalarial drug and prophylactic, because chloroquine is tolerated at relatively high doses with low toxicity (40), and because chloroquine by itself has been shown to enhance survival of BALB/c mice treated with PA plus LF (4). Substantial augmentation of the effects of furin inhibitors was seen with 10 \mu M chloroquine, close to the typical plasma concentrations (2 to 5 \mu M) seen after a therapeutic dose (10 mg/kg body weight) of chloroquine (10, 30, 41).

The temporal profile of protection from anthrax toxin lethality provided by furin inhibition was probed by determining the “durability” of inhibition. Surprisingly, between 5 and 8 h, protection by reversible furin inhibitors, but not by Dec-RVKR-cmk, was lost. We showed that this phenomenon was not due to inactivation of either the eglin or peptide borocarboxylate inhibitor over the period of incubation. Moreover, preincubation of cells for 8 h with (or without) furin inhibitors did not cause the cells to undergo rapid toxin killing upon addition of PA plus LF, ruling out the induction of an alternative processing pathway resistant to furin inhibitors. A previous study that showed residual cleavage of wild-type PA but not an RAAR cleavage site mutant by a furin-deficient CHO cell line suggests that other proteases can participate in PA maturation (16). However, because these enzymes are likely to be blocked by the eglin and boroArg inhibitors, escape is not likely to be explained by the action of these other proteases. Reducing the concentrations of PA plus LF substantially reduced escape from inhibition at 8 h, suggesting that high levels of toxin components drive the escape phenomenon. Although both the eglin c-based inhibitors and the peptide borocarboxylate inhibitor bind tightly to furin and furin-like enzymes, the binding in both cases is reversible. The half-life of inhibitor-furin complexes is estimated to be about 1 h for eglin c variants and 4 h for the peptide borocarboxylate inhibitor from the dissociation rates of the eglin c variants (>3 \times 10^{-5} \text{ s}^{-1} [26]) and the peptide borocarboxylate inhibitor (≈5 \times 10^{-5} \text{ s}^{-1} [this work]). These values taken together with the \( K_d \) values for these inhibitors predict that a low level of free furin will exist even in the presence of high concentrations of these inhibitors during long incubation times. High concentrations of PA may increase the likelihood of PA cleavage by the low amount of free enzyme present. Although no cleavage of PA was seen in 8-h incubations in the presence of furin inhibitors (Fig. 5B), low levels of processing over time might result in formation of active heptamers (likely present in the aggregated species seen in Fig. 5B). The onset of cell death between 5 and 8 h may indicate that a threshold level of heptamer formation and LF translocation has been reached in that interval. The irreversible furin inhibitor Dec-RVKR-cmk did not permit escape at 8 h. Other irreversible inhibitors, such as those based on metal chelates (36), may have an advantage in blocking the deleterious effects of anthrax toxin in vivo.

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