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Chemical Structure of Retro-2, a Compound That Protects Cells against Ribosome-Inactivating Proteins

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Shiga-like toxins and ricin are ribosome-inactivating proteins (RIPs) that are lethal to mammals and pose a global health threat. No clinical vaccines or therapeutics currently exist to protect against these RIPs. Two small molecules (Retro-1 and Retro-2) were discovered with high-throughput screening and reported for their protection of cells against RIPs. Of great significance, Retro-2, reported as (*E*)-2-(((5-methylthiophen-2-yl)methylene)amino)-*N*-phenylbenzamide, fully protected mice from lethal nasal challenge with ricin. Herein, we report studies showing that the chemical structure of Retro-2 is (±)-2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1*H*)-one rather than (*E*)-2-(((5-methylthiophen-2-yl)methylene)amino)-*N*-phenylbenzamide. The latter is an achiral molecule that converts spontaneously to the former, which is a racemate and showed cell protection against RIPs. This calls for attention to (±)-2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1*H*)-one as a promising RIP inhibitor and for chemical characterization of drug leads obtained from high-throughput screens.

Shiga-like toxins (Stx1 and Stx2) produced by certain strains of *Escherichia coli* are potent ribosome-inactivating proteins (RIPs)[1] responsible for outbreaks of foodborne disease with significant morbidity and mortality[2]. Ricin, produced by the castor plant *Ricinus communis*, is another potent RIP that has been used for both bioterrorism and the targeted killing of cancerous cells[3]. No US Food and Drug Administration-approved vaccines or therapeutics currently exist to protect against ricin, Shiga-like toxins, or other RIPs.

Small-molecule inhibitors of ricin and Shiga-like toxins have been sought as potential therapeutics for pre- or post-exposure prophylaxis against RIP poisoning. Two small-molecule structures (**Retro-1** and **Retro-2**; Figure 1) have been discovered with high-throughput screening for their cell protection against RIPs[4]. Of great significance, **Retro-2** at a concentration of 200 mg/kg demonstrated full protection of mice against a dose of ricin that killed 90% of an unprotected control mouse population[4].

Using the doorstop approach in search of small-molecule RIP inhibitors that target the catalytic domain of the toxin[5], we synthesized **Retro-2** as a benchmark and found that the reported achiral **Retro-2** structure[4] is unstable. Herein, we report our chemical syntheses and cell-based assays showing that the chemical structure of the compound that conferred cell protection activity against RIPs is **Retro-2^{cycl}** (a racemic mixture; Figure 1). This raises a call for attention to **Retro-2^{cycl}** as a promising RIP inhibitor and for chemical characterization of small molecules to be used in biological studies.

Results

Chemical structure analysis of Retro-2. Reaction of 2-amino-*N*-phenylbenzamide with 4-chlorobenzaldehyde in ethanol at room temperature with a catalytic amount of *p*-toluenesulfonic acid reportedly yielded **IA4CL** (a close analog of **Retro-2**; Figure 1)[6]. No synthetic procedure has been reported for the commercially available **Retro-2**, and the vendor ChemBridge (San Diego, CA) provided **Retro-2** for the reported biological study[4]. We obtained **Retro-2^{cycl}** in 60% yield using the same reaction conditions reported for the synthesis of **IA4CL**, and found that these reaction conditions actually produced **A4CL** (a close analog of **Retro-2^{cycl}**; Figure 1). Evidence that **Retro-2^{cycl}** rather than **Retro-2** was the reaction product is found in the chemical shifts of two aliphatic carbon atoms (71.38 and 15.63 ppm) in the carbon NMR spectrum of **Retro-2^{cycl}** because **Retro-2** and **Retro-2^{cycl}** have one and two aliphatic carbon atoms, respectively.

We also found that reacting 2-amino-*N*-phenylbenzamide with 5-methylthiophene-2-carbaldehyde in acetic acid at room temperature produced **Retro-2^{cycl}** exclusively in 88% yield. Interestingly, we found that stirring the

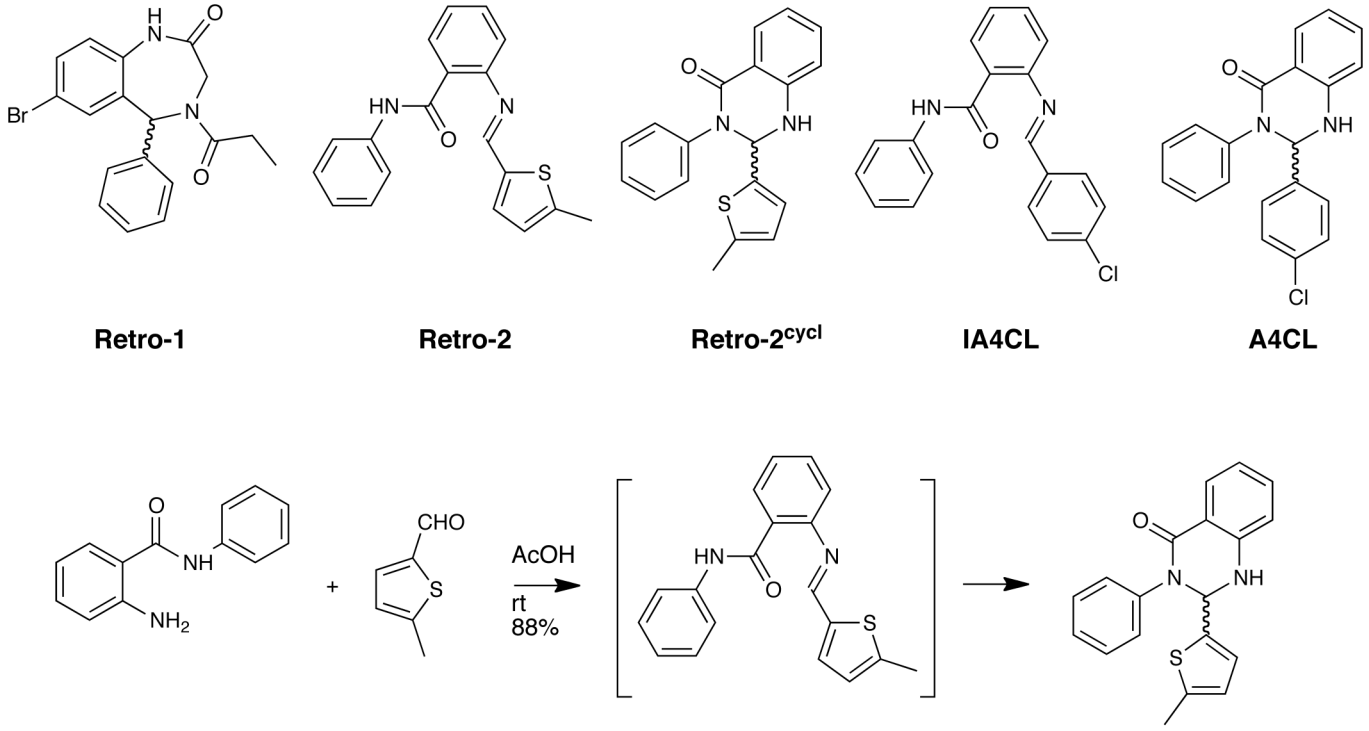

Figure 1 | **Chemical structures of Retro-2 and its analogs and a related synthetic scheme.**

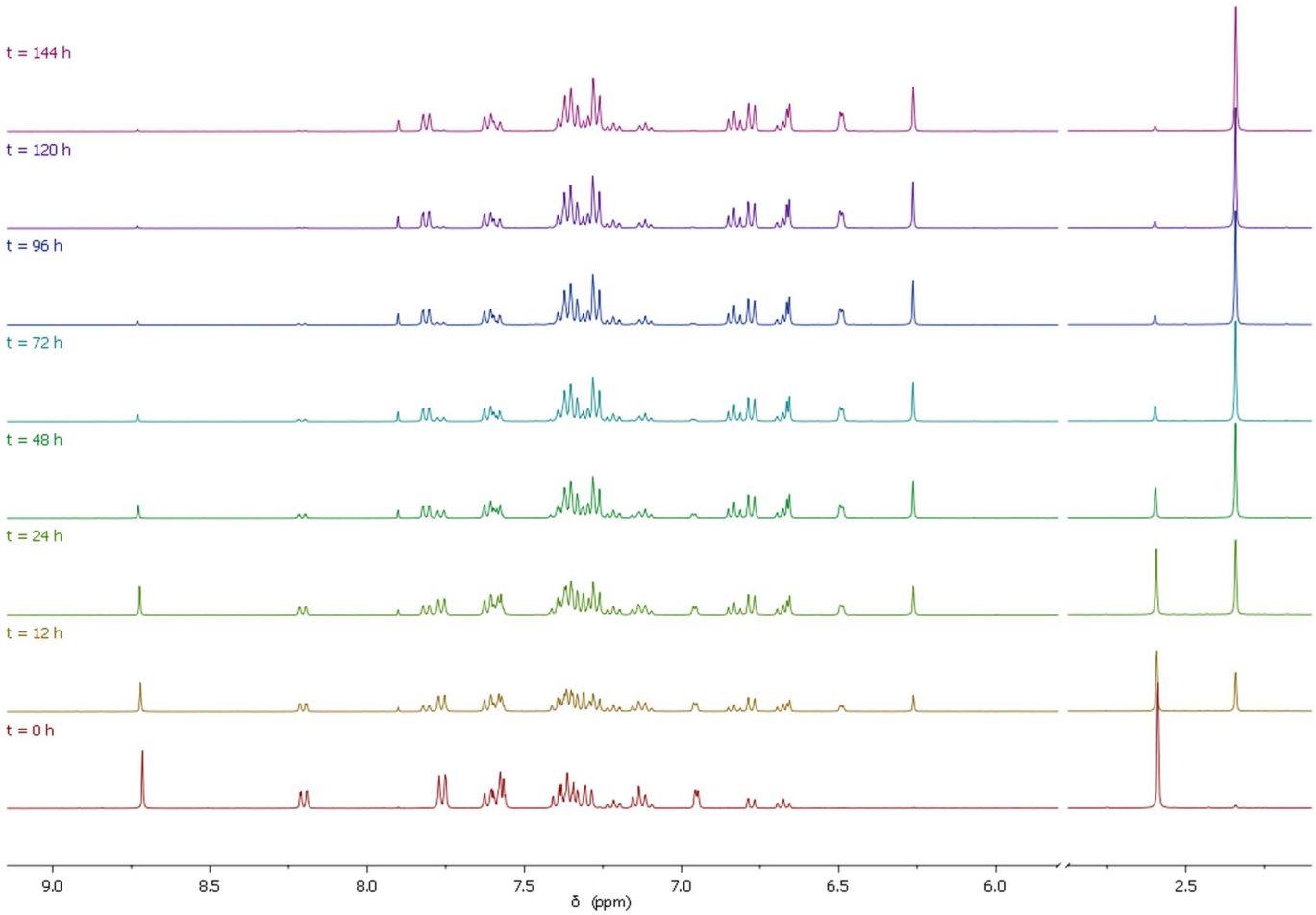

Figure 2 | **Proton NMR spectra showing spontaneous conversion of Retro-2 to Retro-2^{cycl} in neat deuterated methanol over 144 hours.**

two reactants in methanol for 1.5 hours at room temperature yielded **Retro-2^{cycl}** and **Retro-2**. The latter has a chemical shift of only one aliphatic carbon atom (16.49 ppm) in the carbon NMR spectrum. Our proton NMR spectroscopic study showed that **Retro-2** spontaneously converted to **Retro-2^{cycl}** in neat deuterated methanol over a period of 144 hours, as indicated in Figure 2 by the gradual disappearance of the chemical shifts for the imine proton (8.71 ppm) and the methyl proton (2.59 ppm) of **Retro-2** and the gradual and simultaneous appearance of the chemical shifts for the proton at the chiral center (6.26 ppm) and the methyl proton (2.34 ppm) of **Retro-2^{cycl}**. The half-life of **Retro-2** is ~24 hours in neat deuterated methanol (Figure 2). In the presence of a catalytic amount of acid, however, the conversion of **Retro-2** to **Retro-2^{cycl}** was completed within ~30 minutes.

More conclusively, we purchased **Retro-2**—listed as 2-{[(5-methyl-2-thienyl)methylene]amino}-*N*-phenylbenzamide with ID 5374762—from ChemBridge and found that the proton and carbon NMR spectra of the product we received were identical to those of **Retro-2^{cycl}**. These results indicate that **Retro-2** is unstable and spontaneously converts to **Retro-2^{cycl}**.

Cell-based assays of retro-2^{cycl} and A4CL. To investigate whether **Retro-2^{cycl}** is the actual chemical structure associated with cell protection against RIPs, we tested the cell-protection activities of **Retro-2^{cycl}** and **A4CL** using a [^{35}S]-Met-incorporation–based protein synthesis assay in Vero cells. As shown in Figure 3, the presence of 20 μM **Retro-2^{cycl}** moved the dose-response curve of protein synthesis in the presence of ricin or Stx2 to increased Met incorporation, indicating cell protection by **Retro-2^{cycl}** against ricin and Stx2. **A4CL** also showed cell protection against both toxins but was slightly less effective than **Retro-2^{cycl}**.

Discussion

The conversion of imines to 2,3-dihydroquinazolin-4(1*H*)-ones is well established in the literature[7-11]. The erroneous characterization of **IA4CL** rather than **A4CL** as the product of the reaction of 2-amino-*N*-phenylbenzamide with 4-chlorobenzaldehyde in ethanol with a catalytic amount of *p*-toluenesulfonic acid was, in our view, probably due to the omission of the crucial carbon NMR spectrum[6]. The abundant literature information on 2,3-dihydroquinazolin-4(1*H*)-one synthesis and our synthetic work described above show unequivocally that **Retro-2** is unstable and spontaneously converts to **Retro-2^{cycl}**.

Given that the NMR spectra of **Retro-2** from ChemBridge, which provided **Retro-2** for the reported biological studies[4], are identical to those of **Retro-2^{cycl}** and that **Retro-2^{cycl}** and its structurally similar analog **A4CL** protect cells against ricin and Stx2, it is conceivable that **Retro-2^{cycl}**—which is a racemate—is the compound responsible for the reported biological activities[4]. In this context, we measured the optical rotations of **Retro-2** from ChemBridge and **Retro-2^{cycl}** and found both to be zero. These results further support our assertion that a racemic mixture of **Retro-2^{cycl}** produced the reported biological data[4].

We have previously reported caveats for the use of chemical screens for potential drug leads[5,12]. In a reported virtual screen for farnesyltransferase inhibitors[12], we found that 6 of 27 compounds purchased from chemical vendors had serious chemical identity or purity issues. In another study of RIP inhibitors[5], spectroscopic analyses required to confirm the stereochemistry of two chemicals revealed that the stereochemistry of one had been assigned incorrectly by the vendor. In the **Retro-2** report[4], of two promising chemical structures discovered with high-throughput screening, one appears to have been incompletely characterized by the chemical vendor. These repeated problems raise concerns and call for chemical characterization of leads identified from high-throughput screens.

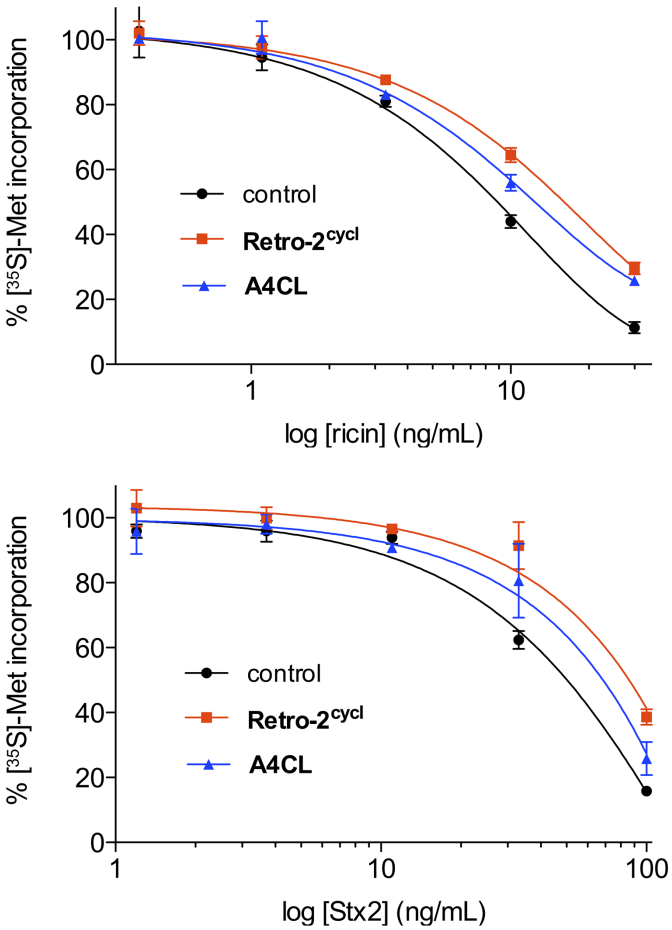

Figure 3 | Cell protection against ricin and Stx2 by Retro-2^{cycl} and A4CL in Vero cells.

Methods

General description of chemical synthesis. All commercially available reagents were used as received. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Mercury 400 spectrometer from Varian (Palo Alto, CA). Chemical shifts are reported in ppm using the solvent peak as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, and m = multiplet), coupling constant, and integration. Low-resolution mass spectra (LRMS) were recorded using either a Hewlett Packard 5973 Mass Spectrometer with SIS Direct Insertion Probe (Palo Alto, CA) or a Waters ZQ/EMD 1000 Mass Spectrometer (Milford, MA). High-resolution mass spectra (HRMS) were obtained on a Bruker BioTOF II ESI. IR spectra were obtained on a ThermoNicolet Avatar 370 FT-IR (Waltham, MA) using a KBr pellet. A Biotage SP-1 (Charlotte, NC) was used for medium pressure liquid chromatography (MPLC) purification using silica gel as the packing material.

Synthesis of (*E*)-2-(((5-methylthiophen-2-yl)methylene)amino)-*N*-phenylbenzamide (Retro-2). To a stirred solution of 2-amino-*N*-phenylbenzamide (0.42 g, 2.00 mmol) in methanol (6 mL) at room temperature was added 5-methylthiophene-2-cabaldehyde (214 μL, 2.00 mmol). After 1.5 hours of stirring, the reaction mixture was chilled to −20°C for 1 hour. The resulting yellow short needles were collected via filtration, washed with methanol, and dried under high vacuum to give 0.35 g of yellow powder determined to be a 1 : 1 mixture of **Retro-2** and **Retro-2^{cycl}** using ^{1}H NMR. The filtrate and washings were combined, concentrated in vacuo, purified with MPLC (silica gel, 100% hexanes to 30% EtOAc-hexanes) to give **Retro-2** (0.16 g) as yellow viscous syrup and 0.11 g of a 1 : 1 mixture of **Retro-2** and **Retro-2^{cycl}**. The estimated yields of **Retro-2** and **Retro-2^{cycl}** were 0.39 g (61%) and 0.23 g (36%), respectively. Because **Retro-2** (oil) was cyclized spontaneously to **Retro-2^{cycl}** (solid), the spectral data were collected within 30 minutes after MPLC purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.34 (s, 1H), 8.47 (s, 1H), 8.40 (dd, *J* = 1.4, 6.6 Hz, 1H), 7.86 (d, *J* = 8.0 Hz, 2H), 7.58 (d, *J* = 7.8 Hz, 1H), 7.51−7.47 (m 1H), 7.40−7.33 (m, 3H), 7.15−7.06 (m, 2H), 6.85 (d, *J* = 3.3 Hz, 1H), and 2.59 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.24 (C=O), 154.42 (C=N), 148.95, 148.52, 139.64, 138.95, 135.67, 132.90, 131.84, 129.25, 129.11, 127.57, 126.97, 124.10, 120.82, 118.98, and 16.49; IR (KBr) ν 3463 (w), 3346 (w), 3060 (w), 1659 (m, C=O), 1608 (s, C=N), and 1538 (m) cm^{-1}; LRMS-EI *m/z* 320 ([M$^+$], 100%); HRMS-ESI *m/z* 321.1050 ([M+H]$^+$, $C_{19}H_{17}N_2OS^+$ requires 321.1062).

Synthesis of 2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1*H*)-one (Retro-2^{cyd}). Method 1: To a stirred solution of 2-amino-*N*-phenylbenzamide (0.10 g, 0.48 mmol) and a few crystals of *p*-toluenesulfonic acid hydrate in ethanol (3 mL) was added 5-methylthiophene-2-cabaldehyde (56.5 μL, 0.52 mmol) at room temperature. Yellow precipitates appeared in 10 minutes; the color disappeared in 30 minutes. The precipitates were collected via filtration, and the filter cake was washed with ethanol and dried under high vacuum to give 0.093 g (60%) of **Retro-2^{cyd}** as a grey powder. **Method 2:** To a stirred solution of 2-amino-*N*-phenylbenzamide (0.21 g, 1.00 mmol) in acetic acid (3 mL) at room temperature was added 5-methylthiophene-2-cabaldehyde (107 μL, 1.00 mmol). Thin-layer chromatography showed completion of the reaction in 20 minutes. The solvent was removed in vacuo, and the crude product was purified with MPLC (silica gel, 100% hexanes to 30% EtOAc-hexanes) to give 0.28 g (88%) of **Retro-2^{cyd}** as a pale yellow solid. The spectral data of **Retro-2^{cyd}** prepared using methods 1 and 2 were identical. mp 152–154°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, J = 7.8 Hz, 1H), 8.02−7.23 (m, 7H), 6.94 (t, J = 7.5 Hz, 1H), 6.70 (d, J = 8.2 Hz, 1H), 6.68 (d, J = 3.5 Hz, 1H), 6.47−6.46 (m, 1H), 6.20 (d, J = 2.3 Hz, 1H), 4.77 (s, 1H), and 2.36 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.56 (C=O), 145.14, 141.24, 140.93, 140.71, 134.07, 129.28, 129.25, 127.21, 127.09, 126.82, 124.58, 120.33, 117.50, 115.58, 71.38, and 15.63; IR (KBr) ν 3289 (m, N-H), 1637 (s, C=O), 1611 (w), 1505 (m), 1486 (m), and 1387 (m) cm^{-1}; LRMS-EI *m/z* 320 ([M$^+$], 100%); HRMS-ESI *m/z* 321.1046 ([M+H]$^+$, $C_{19}H_{17}N_2OS^+$ requires 321.1062). Anal. cald for $C_{19}H_{16}N_2OS$ 0.5 H_2O: C, 69.28; H, 5.20; N, 8.50. Found: C, 69.43; H, 5.23; N, 8.61.

Conversion experiment of Retro-2 to Retro-2^{cyd}. A small amount (~2 mg) of **Retro-2** was dissolved in CD_3OD (1.5 mL), and ^{1}H NMR spectra were taken every 12 hours. The half-life of **Retro-2** to **Retro-2^{cyd}** was ~24 hours at room temperature in CD_3OD.

[^{35}S]-Methionine incorporation assay. Vero cells were maintained in Dulbecco's modified Eagle medium with 10% fetal calf serum and 1 mM glutamine. The cells were resuspended after trypsin treatment at 4×10^4 cells/mL in the same medium, and 0.5 mL of the medium was dispensed into 24-well plates. After 24 hours at 37°C and 5% CO_2, the medium was changed to Dulbecco's modified Eagle medium without Met, Gln, or fetal calf serum and equilibrated for 1 hour. An inhibitor solution with a final dimethyl sulfoxide concentration of 0.5% was added to the medium at 25 hours. Ricin or Stx2 was added after 26 hours at varied concentrations. [^{35}S]-Met was added 2 hours after ricin exposure or 3 hours after Stx2 exposure. The [^{35}S]-Met incorporation was terminated 30 minutes after the Met addition via medium removal and addition of 150 μL of 0.2 M KOH to dissolve cells, as described elsewhere[13]. Proteins were precipitated with 10% trichloroacetic acid, harvested on glass fiber filters, and counted. The control incorporation was determined after treatment with 0.5% dimethyl sulfoxide alone. Ricin was purchased from Vector Laboratories (Burlingame, CA). Stx2 was provided by the Phoenix Laboratory (Tufts-NEMC Microbial Products & Services Facility).

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Author contributions

J.G.P. discovered the conversion of **Retro-2** to **Retro-2^{cyd}**; Y.-P.P., J.G.P., and N.E.T. designed the experiments; J.G.P. and J.N.K. performed the experiments; all authors analyzed the data; Y.-P.P. wrote the paper; all authors contributed with revisions.

Additional information

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