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 \((\pm)-2-(5\text{-methylthiophen-2-yl})-3\text{-phenyl-2,3-dihydroquinazolin-4(1H)-one}\) 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 \((\pm)-2-(5\text{-methylthiophen-2-yl})-3\text{-phenyl-2,3-dihydroquinazolin-4(1H)-one}\) as a promising RIP inhibitor and for chemical characterization of drug leads obtained from high-throughput screens.

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 \(\text{IA4CL}\) (a close analog of Retro-2; Figure 1). 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. We obtained \(\text{Retro-2}^{\text{rac}}\) in 60% yield using the same reaction conditions reported for the synthesis of \(\text{IA4CL}\), and found that these reaction conditions actually produced A4CL (a close analog of \(\text{Retro-2}^{\text{rac}}\); Figure 1). Evidence that \(\text{Retro-2}^{\text{rac}}\) 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 \(\text{Retro-2}^{\text{rac}}\) because Retro-2 and \(\text{Retro-2}^{\text{rac}}\) 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 \(\text{Retro-2}^{\text{rac}}\) 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\textsuperscript{md} 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\textsuperscript{md} 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\textsuperscript{md}. 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\textsuperscript{md} 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\textsuperscript{md}. These results indicate that Retro-2 is unstable and spontaneously converts to Retro-2\textsuperscript{md}.

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

Discussion

The conversion of imines to 2,3-dihydroquinazolin-4(1H)-ones is well established in the literature. 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. The abundant literature information on 2,3-dihydroquinazolin-4(1H)-one synthesis and our synthetic work described above show unequivocally that Retro-2 is unstable and spontaneously converts to Retro-2\textsuperscript{md}.

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

We have previously reported caveats for the use of chemical screens for potential drug leads. In a reported virtual screen for farnesyltransferase inhibitors, we found that 6 of 27 compounds purchased from chemical vendors had serious chemical identity or purity issues. In another study of RIP inhibitors, 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, 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.

Methods

General description of chemical synthesis. All commercially available reagents were used as received: H NMR (400 MHz) and 13C 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 ES. IR spectra were obtained on a Therm Nicolet Avatar 570 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-(methylthio)phen-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-carboxaldehyde (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 0.39 g of yellow powder determined to be a 1:1 mixture of Retro-2 and Retro-2\textsuperscript{md} (solid), the spectral data were collected within 30 minutes after MPLC purification. H NMR: 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); 13C NMR NMR (400 MHz, CDCl3) 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) v 3463 (w), 3346 (w), 3060 (w), 1560 (m, C=O), 1608 (s, C=N), and 1538 (m) cm\textsuperscript{-1}; LRMS-ESI m/z 320 ([M]+, 100%); HRMS-ESI m/z 321.1050 ([M+H]+, C\textsubscript{19}H\textsubscript{17}N\textsubscript{2}O\textsubscript{5}) requires 321.1062.

Figure 3 | Cell protection against ricin and Stx2 by Retro-2\textsuperscript{md} and A4CL in Vero cells.
Synthesis of 2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one (Retro-2cycl) Method 1: To a stirred solution of 2-amino-N-phenylbenzamide (0.10 g, 0.48 mmol) in acetic acid (3 mL) was added 5-methylthiophene-2-carboxaldehyde (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 (3 mL) was added 5-methylthiophene-2-carboxaldehyde (0.21 g, 1.00 mmol) in acetic acid (3 mL) at room temperature was added 5-methylthiophene-2-carboxaldehyde (107 mL, 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 as a pale yellow solid. The spectral data of Retro-2 prepared using methods 1 and 2 were identical. mp 152–154 °C; 'H NMR (400 MHz, CDCl3) δ 8.03 (d, J = 7.8 Hz, 1H), 8.02 – 7.23 (m, 7H), 6.94 (1, 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 (3H, 3H); 13C NMR (100 MHz, CDCl3) 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, 115.70, 115.58, 71.38, and 15.63; IR (KBr) v = 3289 (m, N-H), 3235–3242 (m, N-H). HRMS-ESI m/z: [M+H]+ C20H19N2O2S requires 321.1046, found 321.1046. Anal. calcd for C16H11N2O2S: 0.5 H2O C, 69.43; H, 5.23; N, 8.61. Found: C, 69.43; H, 5.23; N, 8.61.

Conversion experiment of Retro-2 to Retro-2cycl. A small amount (~2 mg) of Retro-2 was dissolved in CD2OD (1.5 mL), and 2H NMR spectra were taken every 12 hours. The half-life of Retro-2 to Retro-2cycl was ~24 hours at room temperature in CD2OD.

([15S]-Methionine incorporation assay. Vero cells were maintained in Dulbecco’s modified Eagle medium with 10% fetal calf serum and 1 mM glutamate. The cells were resuspended after trypsin treatment at 4×10⁶ 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% CO2, the medium was changed to Dulbecco’s modified Eagle medium without Met, Gln, or fetal calf serum and diluted 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. [15S]-Met was added 2 hours after ricin exposure or 3 hours after Stx2 exposure. The [15S]-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.15 Proteins were precipitated with 10% trichloroacetic acid, harvested on glass fiber filters, and counted. The control incorporation was determined after treatment with 150 μL of 0.2 M KOH to dissolve cells, as described elsewhere.15

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