Common Pharmacophore of Structurally Distinct Small-Molecule Inhibitors of Intracellular Retrograde Trafficking of Ribosome Inactivating Proteins

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We reported previously (±)-2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one [(±)-Retro-2cycl] as the chemical structure of Retro-2 that showed mouse protection against ricin, a notorious ribosome inactivating protein (RIP). Herein we report our chemical resolution of (±)-Retro-2cycl, analog synthesis, and cell-based evaluation showing that the two optically pure enantiomers and their achiral analog have nearly the same degree of cell protection against ricin as (±)-Retro-2cycl. We also report our computational studies explaining the lack of stereo preference and revealing a common pharmacophore of structurally distinct inhibitors of intracellular retrograde trafficking of RIPs. This pharmacophore comprises a central aromatic ring o-substituted by an aromatic ring and a moiety bearing an O or S atom attached to sp² C atom(s). These results offer new insights into lead identification and optimization for RIP antidote development to minimize the global health threat caused by ribosome-inactivating proteins.

Results

Chemical resolution of (±)-Retro-2cycl. Although asymmetric syntheses have been reported for substituted 2,3-dihydroquinazolin-4(1H)-ones that are the scaffold of Retro-2cycl, we found that enantioselective reaction of 5-methylthiophen-2-carboxaldehyde with 2-aminobenzanilide using the readily available Sc(III)-inda-pybox as a catalyst gave the desired product with <10% yield and ~20% enantiomeric excess. Recognizing the

Ricin produced by the castor bean plant enzymatically depurinates the 28S rRNA and thereby functions as a ribosome-inactivating protein (RIP). This notorious RIP has been used repeatedly for bioterrorism as in letters sent to U.S. government officials in 2013. Escherichia coli produces Shiga-like toxins (Stx1 and Stx2; the latter being the most toxic), Stx1 and Stx2 also function as RIPs and are responsible for food-borne outbreaks, including the deadliest outbreak in 2011, with significant morbidity and mortality. Presently there are no U.S. Food and Drug Administration approved vaccines or therapeutics to protect against ricin or other RIPs.

In an effort to minimize the global health threat caused by RIPs, we have been working on developing small-molecule RIP inhibitors that target the RIP catalytic domain using our doorstop approach. In this endeavor we attempted to synthesize Retro-2 (Figure 1) as a benchmark for our in vivo studies, because it was reported that 200 mg/kg Retro-2 protected all inhibitor-treated mice against a dose of ricin that killed 90% of an unprotected control mouse population. Serendipitously we discovered that the chemical structure of Retro-2 is (±)-2-(5-methylthiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one rather than (E)-2-((5-methylthiophen-2-yl)methyleneamino)-N-phenylbenzamidine. The former is a racemic, cyclic compound abbreviated as (±)-Retro-2cycl (Figure 1), and the latter is an achiral, acyclic chemical (Figure 1). Because (±)-Retro-2cycl is a mixture of two stereoisomers, we set out to separate the mixture, determine the cell protection activities of the two enantiomers, and develop analogs of the active enantiomer(s) as a better benchmark for our in vivo studies.

Herein we report our chemical resolution of (±)-Retro-2cycl, synthesis of an achiral analog of (±)-Retro-2cycl, computational studies of Retro-2cycl isomers and other known inhibitors of intracellular transport of RIPs, and cell-based studies of Retro-2cycl isomers and their achiral analog. The results led to the development of a common pharmacophore of structurally distinct RIP transport inhibitors and offer important insight into RIP antidote development.
presence of the aniline moiety in Retro-2\textsuperscript{cycl}, we wanted to perform fractional crystallization of (±)-Retro-2\textsuperscript{cycl} salts to be prepared from chiral acids. Unexpectedly, mixing (±)-Retro-2\textsuperscript{cycl} with acids such as (1S)-(−)-10-camphorsulfonic acid caused decomposition of (±)-Retro-2\textsuperscript{cycl} to 5-methylthiophene-2-carboxaldehyde and 2-aminobenzanilide, while weak acids such as (R)-(−)-mandelic acid, (−)-di-p-toluoyl-L-tartaric acid, (−)-dibenzoyl-L-tartaric acid, and (D)-(−)-tartaric acid did not form salts with (±)-Retro-2\textsuperscript{cycl}. We eventually resorted to high-performance liquid chromatography (HPLC) separation using a chiral column. Although diastereomeric chiral stationary phases\textsuperscript{13} was reported for separation of thiadizones that possess an aminal stereo center analogous to the one in (±)-Retro-2\textsuperscript{cycl}, we used the Lux\textsuperscript{TM} Amylose-2 chiral column from Phenomenex (Torrance, California) that has the 5-chloro-2-methylphenylcarbamate derivative of amylose as a chiral stationary phase\textsuperscript{14}. Our rationale for using this type of stationary was that the phenyl rings of the column could form pi-pi interactions with the three aromatic rings of (±)-Retro-2\textsuperscript{cycl} and that the carbamate NH group of the column would permit hydrogen bonds with the amide oxygen atom of (±)-Retro-2\textsuperscript{cycl}. Indeed, using the Lux\textsuperscript{TM} Amylose-2 chiral column, we readily separated (±)-Retro-2\textsuperscript{cycl} as evidenced by the HPLC chromatogram showing two well-separated peaks with retention times of 9.350 and 17.517 minutes and areas under the peaks of 49.769% and 50.231%, respectively, under conditions described in Method (Figure 2). The [α]\textsubscript{RT} D values of the two enantiomers are +118.7° and −126.5°, respectively. As described below, the cell protection studies showed that the two optically pure isomers demonstrated nearly the same
degree of cell protection against ricin as the racemate. Therefore, we did not carry out the X-ray crystallography study to determine whether (+)-Retro-2⁴⁻⁷ corresponds to (R)-Retro-2⁴⁻⁷ or (S)-Retro-2⁴⁻⁷, but rather synthesized and tested an achiral analog of (±)-Retro-2⁴⁻⁷.

**Design and synthesis of an achiral analog of Retro-2⁴⁻⁷.** Energy minimizations at the HF/6-31G(d) level indicate that both enantiomers can adopt conformations at local minima of their potential energy surfaces (local minimum conformations). In those conformations the thiophene group is at the equatorial position of the heterocyclic ring in Retro-2⁴⁻⁷. The superimposition of the local minimum conformations of the two isomers shows nearly identical molecular shapes in terms of both steric and electrostatic properties (Figure 3), which explains the nearly equal potency of the two isomers for cell protection against ricin. This overlay also prompted us to synthesize and test 2-(5-methylthiophen-2-yl)-3-phenylquinazolin-4(3H)-one (DA2MT, Figure 1) as an achiral analog of (±)-Retro-2⁴⁻⁷. As apparent from Figure 3, the local minimum conformation of DA2MT derived at the HF/6-31G(d) level is nearly identical to those of the two isomers. Furthermore, DA2MT does not carry an aminal carbon, so it is chemically more stable than (±)-Retro-2⁴⁻⁷. The latter decomposes in the presence of (1s)-(−)-10-camphorsulfonic acid as described above. Accordingly, we synthesized DA2MT in quantitative yield through oxidation of (±)-Retro-2⁴⁻⁷ using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone rather than following a published procedure that uses potassium permanganate in acetone and has a solubility issue for (±)-Retro-2⁴⁻⁷.

**Cell-based assays of Retro-2⁴⁻⁷ Enantiomers and DA2MT.** To determine the relative potencies of (±)-Retro-2⁴⁻⁷, (±)-Retro-2⁴⁻⁷, (−)-Retro-2⁴⁻⁷, and DA2MT in protecting cells against ricin, we examined protein synthesis inhibition by ricin in Vero cells by measuring [35S]-Met-incorporation. The concentrations used were 12% of protein synthesis in the presence of 50 ng/mL ricin. Interestingly, all four inhibitors showed nearly equal potency of cell protection against ricin. This observation offers important insight into generalizing the structurally distinct chemicals that inhibit the intracellular transport of RIPs to be discussed below.

**Discussion**
Substituted 2,3-dihydroquinazolin-4(1H)-ones have been well studied for various biological properties. Racemization of the aminal stereo center in the substituted 2,3-dihydroquinazolin-4(1H)-ones has been reported. One would suspect that the aminal stereo center in Retro-2⁴⁻⁷ could be sensitive to racemization and possibly cause the lack of the stereo preference for cell protection that we observed. However, the Retro-2⁴⁻⁷ structure has the substitution by a phenyl ring at its amide nitrogen atom. Theoretically this substitution lowers the propensity of the aminal stereo center for racemization. Indeed, through HPLC separation using a chiral column, we were able to separate (±)-Retro-2⁴⁻⁷. The optical activities and purities of the two enantiomers are demonstrated by the specific optical rotations and the HPLC chromatogram shown in Figure 2. In addition, we measured the rotations of the two enantiomers 14 months after the HPLC separation and found no racemization of the two isomers over the 14-month period. Furthermore, DA2MT, an achiral analog of (±)-Retro-2⁴⁻⁷, showed similar cell protection against ricin as the two isomers. These results preclude the possibility that racemization of the enantiomers led to the paucity of stereo preference for the cell protection against ricin.

DA2MT has been briefly reported to show no protection of HeLa cells against Shiga-like toxin 1, while a procedure for preparing DA2MT and the standard structure characterization have not been reported. In this study, we found that DA2MT is as active as Retro-2⁴⁻⁷ in protecting Vero cells against ricin. In view of its chemical stability and the paucity of chirality, DA2MT is a chemically improved analog of Retro-2⁴⁻⁷ for cell protection against ricin and a useful lead for RIP antidote development as exemplified below.

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**Figure 3 | Local minimum conformations of structurally distinct RIP inhibitors derived at the HF/6-31G(d) level and their superimpositions.** A (upper left): (S)-Retro-2⁴⁻⁷ and (R)-Retro-2⁴⁻⁷; B (upper middle): (S)-Retro-2⁴⁻⁷, (R)-Retro-2⁴⁻⁷, and DA2MT; C (upper right): DA2MT, (S)-Retro-1, and Cpd 75; D (lower panel): local minimum conformations of (S)-Retro-2⁴⁻⁷, (R)-Retro-2⁴⁻⁷, DA2MT, (S)-Retro-1, and Cpd 75 in sphere model. For clarity, all H atoms except for those at the chiral centers are undisplayed in the superimposed structures. The Br, S, O, N, and H atoms are colored in dark red, dark yellow, red, blue, and white, respectively. The C atoms in (S)-Retro-2⁴⁻⁷, (R)-Retro-2⁴⁻⁷, DA2MT, Cpd 75, and (S)-Retro-1 are in green, magenta, cyan, orange, and yellow, respectively.
transport inhibitor leads. Of the lack of structural information about the targets of current RIP optimization of RIP transport inhibitors, which may address the pharmacophore described above may result in new leads for RIP Alternatively, in silico database screening for chemicals with the promise in identifying improved RIP transport inhibitors. It offers new insights into lead identification and optimization for RIP antitoxin development to minimize the global health threat caused by RPs. For example, focused combinatorial library synthesis to derivate DA2MT with the guidance of the pharmacophore holds promise in identifying improved RIP transport inhibitors. Alternatively, in silico database screening for chemicals with the pharmacophore described above may result in new leads for RIP transport inhibitors. Reverse engineering with 20 essential amino acids for proteins with cavities that can accommodate the pharmacophore is now technically feasible with today's computing capability. Such reverse engineered proteins can thus offer guidance in lead optimization of RIP transport inhibitors, which may address the concern that the RIP transport inhibitors are undesirable because of the lack of structural information about the targets of current RIP transport inhibitor leads.

Methods

Separation of (±)-Retro-2
\textsuperscript{sm}.
 About 10 mg of (±)-Retro-2
\textsuperscript{sm} prepared according to our published procedure was separated using the Lux\textsuperscript{TM} Amylose-2 chiral column on a Beckman Coulter HPLC System Gold 125P eluting with n-hexane/2-propanol (60:40) at a flow rate of 1.0 mL/min. The retention times of the plus and minus enantiomers are 9.350 and 17.517 minutes, respectively. Two fractions were collected and the eluent was removed in vacuo. The chemical identities of both isomers were confirmed by proton NMR analysis. The specific optical rotations of the two enantiomers are 118.7° (c = 0.21 in CHCl₃) and −126.5° (c = 0.29 in CHCl₃), respectively, which were measured using a Jasco DIP-370 Digital Polarimeter at 598 nm at room temperature with L = 0.5 dm.

General description of chemical synthesis. All commercially available reagents were used as received. 1H 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 SIR 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 BiatoP II ESL IR spectra were obtained on a Thermo Nicolet Avatar 370 FT-IR (Waltham, MA) using a KBr pellet. Melting points were determined by using Mel-Temp II (Holliston, MA). Elemental Analysis was performed at NuMega (San Diego, CA).

Synthesis of DA2MT (2-(5-methylthiophen-2-yl)-3-phenylquinazolin-4(3H)-one). To a stirred solution of Retro-2
\textsuperscript{sm} (64.0 mg, 0.20 mmol) in chloroform (5 mL) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and 4,5-dichloro-3,6-dihydroxyphthalonitrile as the by-product. The layers were separated, and the organic layer was dried over MgSO₄, filtered, and concentrated to give 63.1 mg of pure product as a white solid in quantitative yield. Melting point 172–173.5°C. [M] (HRMS−ESI m/z) 319.0898 ([M + H]⁺, c, H\textsubscript{13}H\textsubscript{19}N\textsubscript{2}O\textsubscript{5} requires 319.0905). Anal. calcd for C\textsubscript{19}H\textsubscript{15}N\textsubscript{2}O\textsubscript{5}: C, 66.19; H, 4.19; N, 10.79. Found: C, 66.13; H, 4.23; N, 10.82.

Computational studies. Different conformations of each inhibitor shown in Figure 3 were systematically generated by alternating the bulky groups at axial and equatorial positions and subsequently energy minimized with the MMX force field using the PCModel 91 program (Serena Software). These resulting conformations were subjected to energy minimization at the HF/6-31G(d) level using the Gaussian 98 program. All the energy-minimized conformations at the HF/6-31G(d) level were checked for possible imaginary frequencies by subsequent frequency calculations using the same theory and basis set. The energy-minimized conformations with no imaginary frequencies were then manually superimposed using the Pair Fitting tool of the MacPyMOL V1.5.0 (Schrodinger LLC, Portland, OR), which led to the superimposed inhibitor structures shown in Figure 3.

[\textsuperscript{35S}]-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 × 10⁵ 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, Glu, 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 was added after 24 hours at varied concentrations. [35S]-Met incorporation was added 2 hours after ricin exposure. The [35S]-Met incorporation was terminated 30 minutes after the dissolve cells, as described elsewhere. 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).

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Acknowledgments
This work was supported by the U.S. National Institute of Allergy and Infectious Diseases (5U01 AI082120-04).

Author contributions
Y.-P.P. and N.E.T. conceived and supervised the project; S.Y. designed and performed the computational studies; all authors analyzed the data; Y.-P.P. wrote the paper; all authors contributed with revisions.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yu, S.C., Park, J.G., Kahn, J.N., Tumer, N.E. & Pang, Y.-P. Common Pharmacophore of Structurally Distinct Small-Molecule Inhibitors of Intracellular Retrograde Trafficking of Ribosome Inactivating Proteins. Sci. Rep. 3, 3397; DOI:10.1038/srep03397 (2013).

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