(S)-N-Methyldihydroquinazolinones are the Active Enantiomers of Retro-2 Derived Compounds against Toxins

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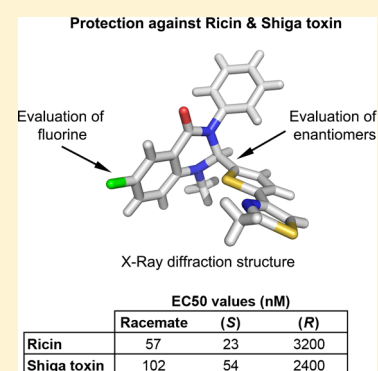[†]CEA, iBiTec-S/SIMOPRO, CEA-Saclay, LabEx LERMIT, F-91191 Gif-sur-Yvette, France
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S Supporting Information

ABSTRACT: This study reports the synthesis, chromatographic separation, and pharmacological evaluation of the two enantiomers of a new compound, named Retro-2.1, active against toxins by inhibiting intracellular trafficking via the retrograde route. The absolute configuration of the bioactive enantiomer has been assigned from X-ray diffraction to the (S)-enantiomer. To date, (S)-Retro-2.1 is the most potent molecule to counteract the cytotoxic potential of ricin and Shiga toxin, with EC50 values of 23 and 54 nM, respectively.

Protection against Ricin & Shiga toxin

Evaluation of fluorine

Evaluation of enantiomers

X-Ray diffraction structure

	EC50 values (nM)		
	Racemate	(S)	(R)
Ricin	57	23	3200
Shiga toxin	102	54	2400

KEYWORDS: Retro-2.1, Shiga toxin, ricin, dihydroquinazolinones

Ricin is a highly toxic protein abundant in castor beans and classified as a potential bioweapon. After ingestion or inhalation, ricin causes major organ damages that can ultimately lead to the death of the exposed organism. To date no approved treatments or prophylaxis for human use is available. The Shiga toxin (Stx) family consists of related protein toxins that are produced by the bacteria *Shigella dysenteriae* and certain pathogenic strains of *Escherichia coli*.[1,2] Bacteria producing these toxins are responsible for a number of diseases such as the life-threatening complication hemolytic uremic syndrome (HUS).[3] In 2011, a major outbreak caused by *E. coli* O104:H4 infected about 4000 people in Europe, causing more than 900 cases of HUS resulting in 54 deaths.[4] So far, no effective therapy exists for Stx intoxication.[5,6] Ricin and Stx share several characteristics. They have one moiety (B-subunit) that binds to their respective cellular receptors (glycoproteins and glycolipids for ricin; the glycosphingolipid Gb3 for Stx), while another moiety (A-subunit) enters the cytosol and inactivates protein biosynthesis. Both toxins are transported in a retrograde manner from the plasma membrane via endosomes and the TGN to the endoplasmic reticulum (ER),[7] before translocation to the cytosol, where they enzymatically inactivate the 28S RNA of the 60S ribosomal subunit (reviewed in refs 7−10). Inhibitors acting on the intracellular routing of these toxins likely offer novel options for the development of therapeutic strategies.[11]

We have recently identified a compound named Retro-2 that protects human cells against ricin and Stx by blocking retrograde toxin transport at the early endosomes-TGN interface.[12] Retro-2 also protects mice against lethal ricin challenge.[12] A structure−activity relationship (SAR) study of Retro-2 led to an improved activity against Stx up to 100-fold for compounds sharing a dihydroquinazolinone scaffold (cyclized Retro-2).[13,14] We also demonstrated that only one enantiomer (compound **62**,[13] Scheme 1) of dihydroquinazolinones was bioactive, but its absolute configuration was unsolved.

Here, we report the synthesis and evaluation of a new enantiopure dihydroquinazolinone compound, named Retro-2.1, acting against Stx cytotoxicity with improved potency (∼500-fold compared to Retro-2) and demonstrate that Retro-2.1 is also effective against ricin *in vitro* (∼1000-fold increased activity compared to Retro-2). We establish from crystal *X-ray diffraction* data that the antitoxin activity resides mainly in the *S*-enantiomer.

During the course of our previous SAR study starting from Retro-2, we obtained *N*-methylated cyclic analogues leading to
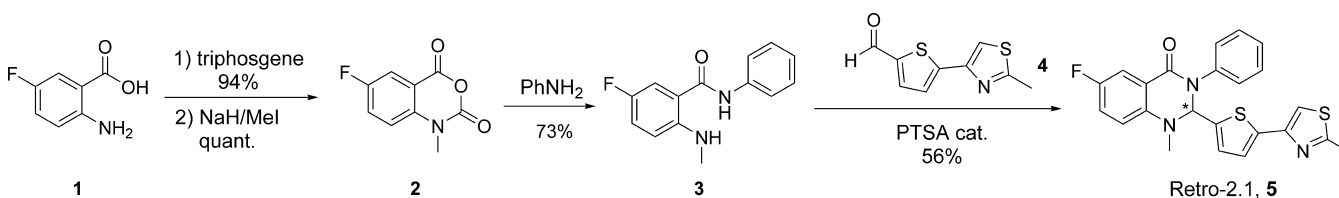
Scheme 1. Previous Studies on Retro-2 Optimization and Objectives of This Work (with EC$_{50}$ against Shiga Toxin)
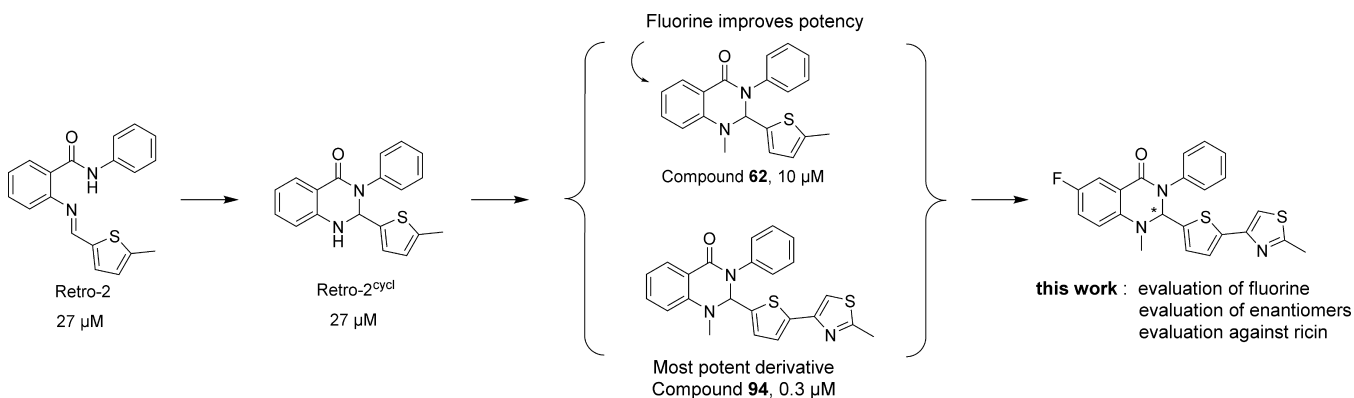
Scheme 2. Synthesis of 6-Fluoro-1-methyl-2-(5-(2-methylthiazol-4-yl)thiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one, Retro-2.1


a potent inhibitor of Stx action in cells (compound **94**, EC$_{50}$ = 0.3 μM,[13] Scheme 1). In parallel, substitution of the phenyl ring of the dihydroquinazolinone scaffold of **62** was evaluated. Among all the substituents, only the 6-fluoro derivative showed a better activity compared to the parent Retro-2^{cycl} (EC$_{50}$ = 11 μM vs 27 μM, respectively)[13] (Scheme 1). We describe here the synthesis and evaluation of a compound combining the best pharmacophores.

The synthesis of compound **5** was achieved according to our previously published procedure starting from the commercially available 4-fluoro anthranilic acid **1** (Scheme 2) in 39% yield over 4 steps.

In addition, since only one enantiomer was active on previous dihydroquinazolinones synthesized as racemates,[13] a chiral phase HPLC separation of Retro-2.1 was performed on a ChiralPak IB column (see Experimental Section and Figure 1). No contamination of Retro-2.1a by Retro-2.1b or *vice versa* could be identified on the chromatograms (Figure 1).

To unequivocally assign the absolute configuration of each enantiomer, crystal structure determinations were obtained on separate enantiomers. Among the different crystallization

conditions that we screened, a mixture of CH$_2$Cl$_2$ and methanol (1:1) proved efficient to obtain monocrystals suitable for *X-ray diffraction* (slow evaporation method). These studies revealed that Retro-2.1a had a *S*-stereochemistry at the C-2 position of the dihydroquinazolinone core (Figure 2), while Retro-2.1b was the *R*-isomer.
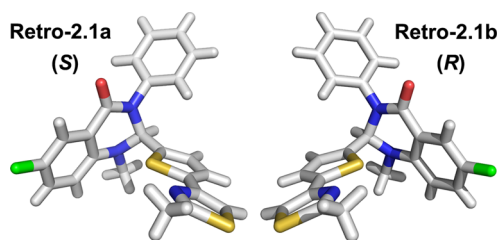

Figure 2. Structure determination of the Retro-2.1 enantiomers by X-ray crystallography.

The biological activity of Retro-2.1 and each enantiomer was then evaluated against Stx and ricin (Figure 3). Retro-2.1 racemate inhibited Stx action on HeLa cells with an EC$_{50}$ of 102 $\pm$ 15.0 nM (Figure 3, upper left panel; n = 9) more potently than the previously described best compound (compound **94**,[13] EC$_{50}$ = 0.3 μM). It also inhibited ricin induced-toxicity on human pulmonary A549 cells with an EC$_{50}$ of 57 $\pm$ 2.4 nM (Figure 3, upper right panel; n = 3).

When tested separately, Retro-2.1a ((*S*)-Retro-2.1) potently inhibited toxin cytotoxicity (eutomer), while the second eluted compound, Retro-2.1b ((*R*)-Retro-2.1), only showed moderate activity at micromolar concentrations (distomer). Indeed, (*S*)-Retro-2.1 inhibited both Stx cellular action (EC$_{50}$ of 54 $\pm$ 16 nM; Figure 3, middle left panel; n = 7) and ricin (EC$_{50}$ of 23 $\pm$ 4 nM; Figure 3, middle right panel; n = 4), whereas (*R*)-Retro-2.1 exhibited only an EC$_{50}$ of 2.4 $\pm$ 1 μM against Stx (Figure 3, lower left panel; n = 4) and an EC$_{50}$ of 3.2 $\pm$ 0.1 μM against ricin (Figure 3, lower right panel; n = 3). These results
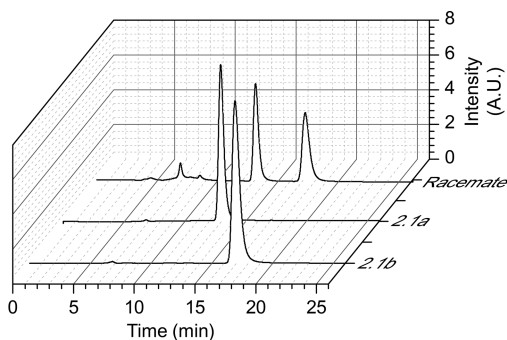
Figure 1. Chiral HPLC chromatograms of rac-Retro-2.1 and purified enantiomers, Retro-2.1a and Retro-2.1b.
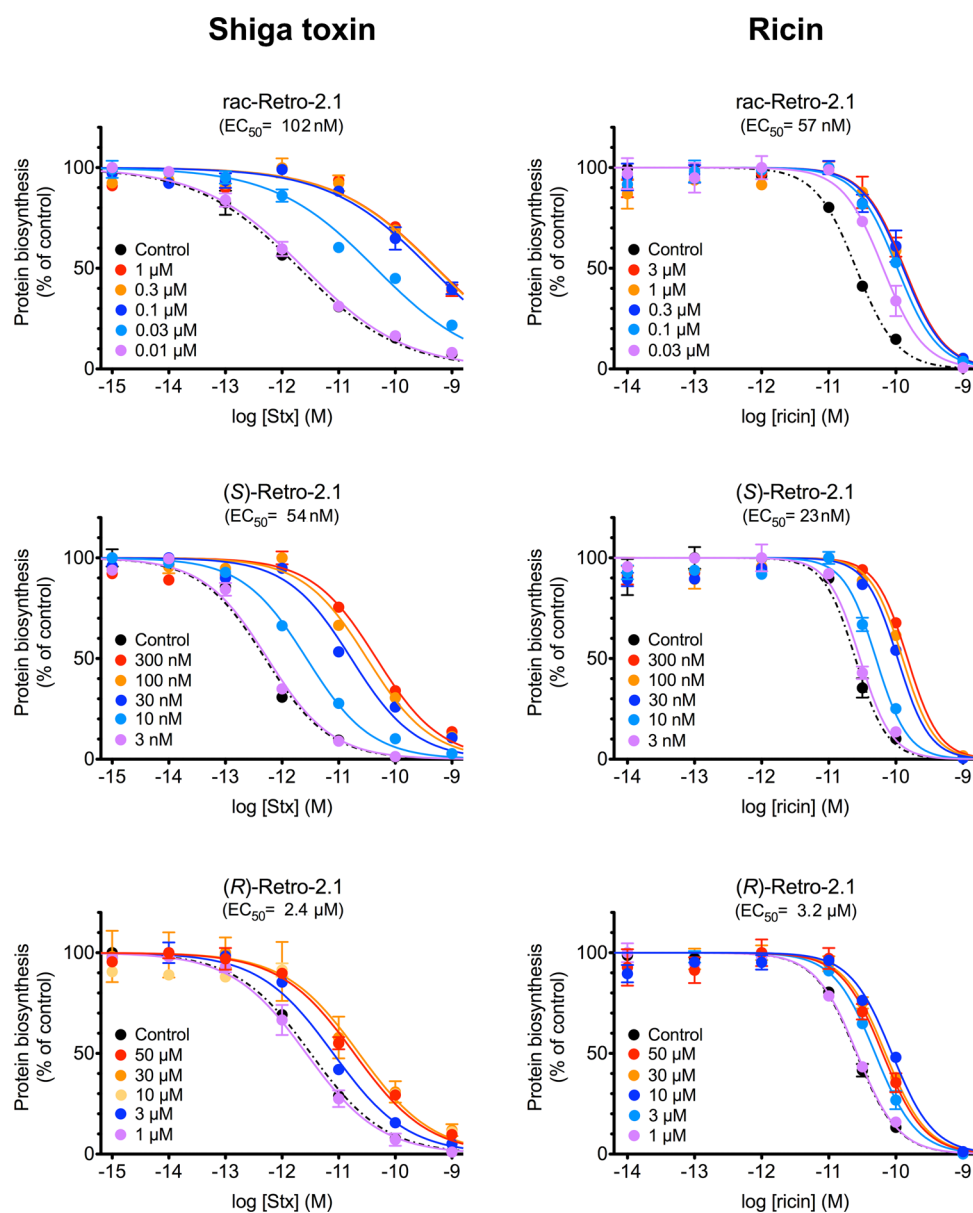
Figure 3. Retro-2.1 protects cells against Stx and ricin. Intoxication of HeLa cells by Stx (left) and intoxication of A549 cells by ricin (right) in the presence of inhibitors. Cells were incubated 1 h at 37 °C with DMSO (0.04%, black data points in all parts of the figure), rac-Retro-2.1 (various concentrations; upper panel), (S)-Retro-2.1 enantiomer (various concentrations; middle panel), or (R)-Retro-2.1 enantiomer (various concentrations; lower panel) before addition of toxins at the indicated concentrations. Each data point represents the mean of duplicate ± SEM of a representative experiment. EC$_{50}$ values are the mean of n independent experiments (see text for more details). Note that the concentrations used for racemate and enantiomers are different.

are in accordance with the EC$_{50}$ values obtained from the racemate form with a 2-fold increase of potency of the eutomer. Eudismic ratios of 45 and 135, respectively for Stx and ricin, were calculated from the EC$_{50}$ values of cytotoxicity assays reported in this paper, reflecting a high enantioselectivity. This eudismic ratio may also explain the absence of activity of the distomer of compound **62** reported previously[13] on Stx, since the highest concentration tested for the distomer was 30 μM (due to low solubility) while the EC$_{50}$ of the racemate was 10 μM.

It is worth noting that besides a lower potency, determined by a higher EC$_{50}$, (R)-Retro-2.1 also displayed a reduced efficacy (lower maximal R-value) in comparison to (S)-Retro-2.1 against both Stx and ricin (for Stx, $R_{(S)}$ = 357 vs $R_{(R)}$ = 68, n = 6; for ricin, $R_{(S)}$ = 5 vs $R_{(R)}$ = 2.9, n = 3; R values are from

experiments performed in parallel). These differential pharmacological activities proved that only the (S)-enantiomer fully retained the desired antitoxin activity.

Retro-2.1 has a stronger efficacy (R-values) against Stx than against ricin, as observed for Retro-2.[12] Most likely, ricin can use several mechanisms to traffic into cells by binding to a variety of glycoreceptors. In contrast, Stx only binds to Gb3 and may be facing limited molecular redundancy for reaching TGN membranes. Ricin has also been proposed to cross endosomal membranes to reach the cytosol, as an alternative mechanism to retrotranslocation from the endoplasmic reticulum.[15] This type of endosomal escape would allow bypassing a Retro-2.1-induced retrograde transport block.

We describe herein the synthesis and chromatographic separation of Retro-2.1 enantiomers. In vitro experiments

proved the configuration of the stereocenter in position 2 is crucial for activity, since one enantiomer is active to protect cells against Stx and ricin. The absolute stereochemistry of the eutomer has been assigned by X-ray diffraction. The (S)-isomer proved to be the bioactive enantiomer, and (S)-Retro-2.1 is the most active compound against these toxins reported so far.

Since N-methyldihydroquinazolinones derived from Retro-2 block the retrograde pathway within cells, optimized (S)-Retro-2.1 has the potential to be developed as a broad-spectrum antidote to a wide array of pathogens, including toxins and parasites, as demonstrated previously *in vitro* and *in vivo* for Retro-2.[12,16]

ASSOCIATED CONTENT

Ⓢ Supporting Information

General procedures, characterization of new compounds, crystal structure determination of compound **5**, chemicals for *in vitro* experiments, intoxication assays, and determination of EC_{50} values. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Stx, Shiga toxin; *E. coli*, *Escherichia coli*; HUS, hemolytic uremic syndrome; Gb3, globotriaosylceramide; ER, endoplasmic reticulum; RNA, ribonucleic acid; SAR, structure–activity relationship; EC_{50}, 50% effective concentration; HPLC, high performance liquid chromatography; TGN, trans-Golgi network; DMSO, dimethyl sulfoxide; SEM, standard error of the mean; RP-HPLC, reversed phase–high performance liquid chromatography; THF, tetrahydrofuran; DMF, dimethyl formamide; PTSA, para-toluene sulfonic acid; NMR, nuclear magnetic resonance; MS, mass spectrometry; HRMS, high resolution mass spectrometry; LC/MS, liquid chromatography coupled to mass spectrometry; IR, infrared; UV, ultraviolet; DAD, diode array detector; DMEM, Dulbecco's modified Eagle's medium; IC_{50}, 50% inhibition concentration; TLC, thin-layer chromatography

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