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Synthesis, Chiral Separation, Absolute Configuration Assignment, and Biological Activity of Enantiomers of Retro-1 as Potent Inhibitors of Shiga Toxin

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The Shiga toxin (Stx) family is composed of related protein toxins produced by the bacteria *Shigella dysenteriae* and certain pathogenic strains of *E. coli*. No effective therapies for Stx intoxication have been developed yet. However, inhibitors that act on the intracellular trafficking of these toxins may provide new options for the development of therapeutic strategies. This study reports the synthesis, chromatographic separation, and pharmacological evaluation of the two enantiomers of Retro-1, a compound active against Stx and other such protein toxins. Retro-1 works by inhibiting retrograde transport of these toxins inside cells. In vitro experiments proved that the configuration of the stereocenter at position 5 is not crucial for the activity of this compound. X-ray diffraction data revealed (*S*)-Retro-1 to be slightly more active than (*R*)-Retro-1.

The Shiga toxin (Stx) family is composed of related protein toxins that are produced by the bacteria *Shigella dysenteriae* and certain pathogenic strains of *Escherichia coli*.[1,2] Infections with Shiga-toxin-producing *E. coli* (STEC) are responsible for acute and severe hemorrhagic diarrhea and its life-threatening complication, hemolytic uremic syndrome (HUS).[3] In 2011, a major outbreak caused by *E. coli* O104:H4 spread throughout Germany[4] and infected about 4000 people in Europe, causing more than 900 cases of HUS, resulting in 54 deaths.[5] To date, there is no effective therapy for Stx intoxication.[6] Many antibiotics used to treat bacterial infections, including quinolones, stimulate the induction of Stx-converting prophages, enhancing the severity of the disease symptoms. Hence, the use of antibiotics for the management of STEC infection is controversial[7] or not recommended.[8] Even the promising monoclonal anti-C5 antibody eculizumab, used successfully to treat three 3-year-old patients with neurological HUS complications,[9] gave mixed results during the German outbreak.[10] Shiga toxins have one moiety (B-subunit) that binds to their respective cellular receptors, the glycosphingolipid Gb3. Shiga toxins are then transported in a retrograde manner from the plasma membrane via endosomes and the trans-Golgi network (TGN) to the endoplasmic reticulum,[11] before translocation of the enzymatic moiety (A-subunit) into the cytosol. Finally, the Shiga toxin A subunit inactivates the 28S RNA of the 60S ribosomal subunit (reviewed in references [11–14]). This is an irreversible process that results in the inhibition of protein biosynthesis. Inhibitors that act on the intracellular trafficking of these toxins likely offer new options for the development of therapeutic strategies.[15]

In the course of a high-throughput screening campaign, we have identified two compounds, Retro-1 and Retro-2, which protect human cells against Stx.[16] These compounds were shown to act as inhibitors of the retrograde route used by toxins to enter into cells. Retro-1 also exhibits an enhancement of pharmacological action of antisense and splice-switching oligonucleotides in vivo, although the mode of action might be different and remains elusive.[17] Herein we report the synthesis and evaluation of the two enantiomers of Retro-1 against Shiga toxin and establish that the biological activity is almost equally dispatched between the two enantiomers with a slight preference for the *S* isomer, a less distinct behavior relative to Retro-2 analogues.[18]

The synthesis of Retro-1 (**6**) was carried out by starting from the commercially available 2-aminobenzophenone **1** (Scheme 1) in 39% yield over five steps. First, regioselective bromination was performed with NBS with complete conversion. Acetylation with bromoacetyl bromide was immediately followed by cyclization with ammonia to yield benzodiazepine **4** in 66% yield over two steps. Reduction of the imino moiety with sodium cyanoborohydride offered a racemic mixture of benzodiazepine **5**, which was treated with propionyl chloride to obtain Retro-1 as a 50:50 mixture of two enantiomers. In addition, *rac*-Retro-1 was obtained as a 1:1 mixture of conformers as detected by ^{1}H and ^{13}C NMR spectroscopy. High-temperature NMR in various solvents allowed us to obtain coalescence of the two conformers' signals (Supporting Information).

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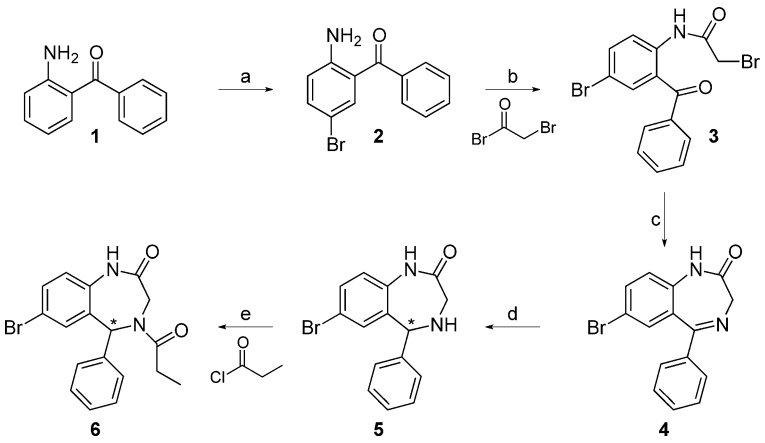

Scheme 1. Synthesis of Retro-1. *Reagents and conditions*: a) NBS, CH_2Cl_2, 0 °C, 1 h, then RT, 2 h, 99%; b) $NaHCO_3$ (aq. 2 M), CH_2Cl_2, 0 °C, 2 h; c) NH_3 (7 M in MeOH), 0 °C→RT, overnight, 66% (two steps); d) $NaBH_3CN$, AcOH, MeOH, RT, 6 h, 74%; e) Et_3N, CH_2Cl_2, RT, overnight, 79%.

To assign biological activity to one enantiomer and/or one conformer, a chiral-phase HPLC separation was performed with a ChiralPak IA column (see Figure 1 and Supporting Information page 4). Among the four possible isomers, only two peaks were detected that correspond to the two enantiomers (no separation of the conformers). No contamination of Retro-1-E1 by Retro-1-E2 or vice versa could be identified on the chromatograms (Figure 1 A). ^{1}H NMR analysis was undertaken on each enantiomer, confirming the presence of the two conformers.

To unequivocally assign the absolute configuration of each enantiomer, X-ray crystal structure determination was obtained for separate enantiomers. Amongst the various crystallization conditions that we screened, a mixture of dichloromethane and methanol (1:1) allowed us to obtain monocrystals suitable for X-ray diffraction (slow evaporation method). These studies revealed that Retro-1-E1 has the *S* configuration at the C5 position of the benzodiazepine core (Figure 1), whereas Retro-1-E2 is the *R* isomer.

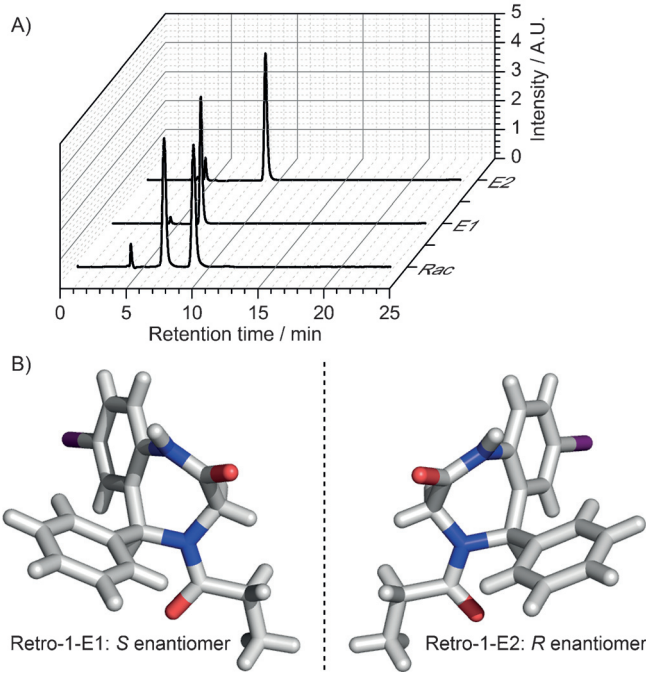

Figure 1. A) Chiral HPLC separation of enantiomers of Retro-1. B) Structural determination of the Retro-1 enantiomers by X-ray crystallography.

The biological activity of Retro-1 and each enantiomer was then evaluated against Stx (Figure 2). *rac*-Retro-1 inhibited Stx action on HeLa cells with an EC_{50} value of 6.2 ± 0.7 μM (Figure 2 A top and 2 B) with an *R*-value of 62.4 ± 9.6 (Figure 2 C; see Supporting Information for *R*-value calculation). We were also interested to determine whether compounds **4** and **5** are active against Stx; both were completely inactive at 30 μM (data not shown). These results confirm the importance of the propionyl group for bioactivity, but do not allow us to clearly assign the impact of the conformation of the seven-membered ring (or the rotamers around the amide bond) on biological activity.

Both (*S*)- and (*R*)-Retro-1 were evaluated for inhibition of Stx cytotoxicity. (*S*)-Retro-1 inhibited Stx cellular action with an EC_{50} value of 3.6 ± 0.3 μM (Figure 2 A middle and 2 B), whereas (*R*)-Retro-1 exhibited an EC_{50} value of 9.4 ± 1.4 μM against Stx (Figure 2 A bottom and 2 B). In addition, (*S*)-Retro-1 offered an *R*-value of 144 ± 24.5 (Figure 2 C), and (*R*)-Retro-1 an *R*-value of 51.6 ± 14.8 (Figure 2 C). A eudismic ratio of 2.6 was calculated from the EC_{50} values of cytotoxicity assays reported herein, reflecting low enantioselectivity. The same ratio was obtained in comparing the *R*-values of each enantiomer.

Numerous proteins have been shown to regulate retrograde transport at the early endosome–TGN interface. However, it was previously demonstrated that only the SNARE protein syntaxin-5 is strongly relocalized in Retro-1-treated cells, whereas the subcellular distribution of the Golgi was not affected.[16] We further investigated whether Retro-1 enantiomers can also affect the subcellular distribution of syntaxin-5. Syntaxin-5 (Figure 3, green) was strongly relocalized in Retro-1-treated cells after 1 h (Figure 3 B), in comparison with control (Figure 3 A). The same phenotype was observed with either (*S*)- or (*R*)-Retro-1 used at their EC_{50} values (Figure 3 C and D, respectively). These data suggest that both enantiomers might have the same cellular target.

The small difference in biological activities between the two enantiomers cannot be readily assigned to a three-point contact model.[19] Nevertheless, a complex behavior based on the influence of stereochemistry at C5 combined with the M and P conformations of the seven-membered ring[20] (and also possibly rotamers around the amide bond) could justify the results obtained in evaluating the enantiomers of Retro-1 against Stx, showing similar activities between the two. Similar results were previously demonstrated for the binding of benzodiazepines to the GABA receptor.[21]

In summary, we describe the synthesis and chromatographic separation of Retro-1 enantiomers. In vitro experiments proved that the configuration of the stereocenter at position 5 is not

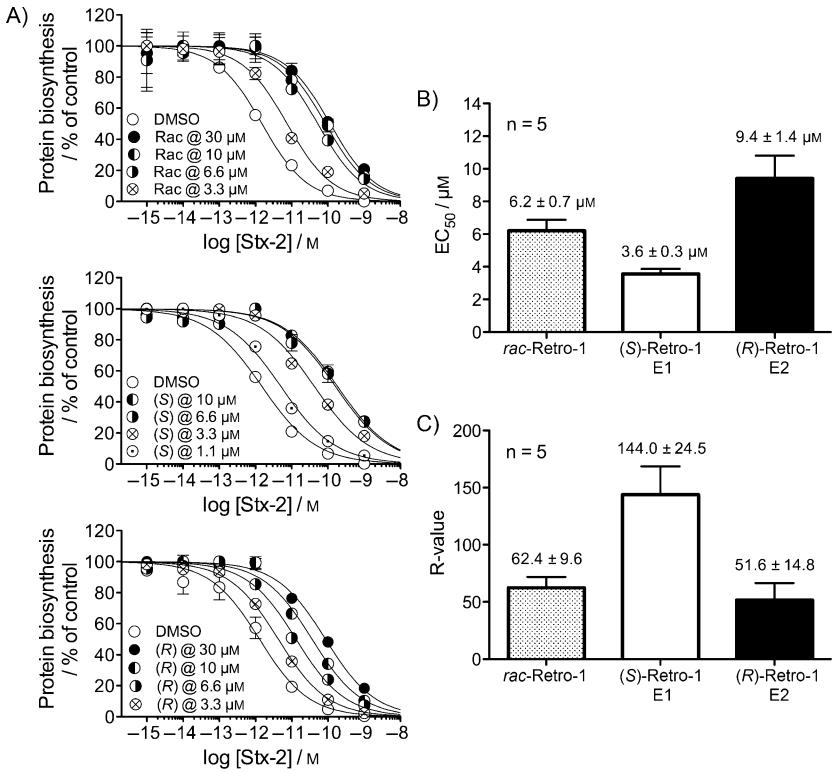

Figure 2. Biological activity of Retro-1 and its enantiomers toward Stx. A) HeLa cells were incubated for 4 h with *rac*-Retro-1 (top), (*S*)-Retro-1 (middle), (*R*)-Retro-1 (bottom), or carrier only (DMSO, open circles) before the addition of Stx at the indicated concentrations for 20 h. Media was removed and replaced with DMEM containing [^{14}C]leucine at 0.5 μCi mL^{-1} for 7 h before quantifying radioactivity. Each data point represents the mean ± SEM of duplicates of a representative experiment. B) EC_{50} values ± SEM of *rac*-Retro-1, (*S*)-Retro-1, and (*R*)-Retro-1 determined from five independent experiments. C) R-values ± SEM of *rac*-Retro-1, (*S*)-Retro-1, and (*R*)-Retro-1 determined from five independent experiments.

crucial for the activity of this compound, as both enantiomers are active at protecting cells against Stx and relocalizing syntaxin-5. Nevertheless, the absolute stereochemistry of the eutomer has been assigned by X-ray diffraction experiments. The *S* isomer proved to be slightly more active than the *R* isomer. Because no 1,3,4,5-tetrahydro-2*H*-benzo[*e*][1,4]diazepin-2-ones possess bioactive properties, only little information is available regarding the influence of chirality at C5, and we are currently working on a few hypotheses to decipher the impact of M or P helicity on the bioactive properties of Retro-1 against Stx. We have also started a structure–activity relationship study to optimize the activity of the hit compound in order to assess the impact of the enantioselectivity of analogues both in vitro and in vivo; results from these studies will be reported in due course.

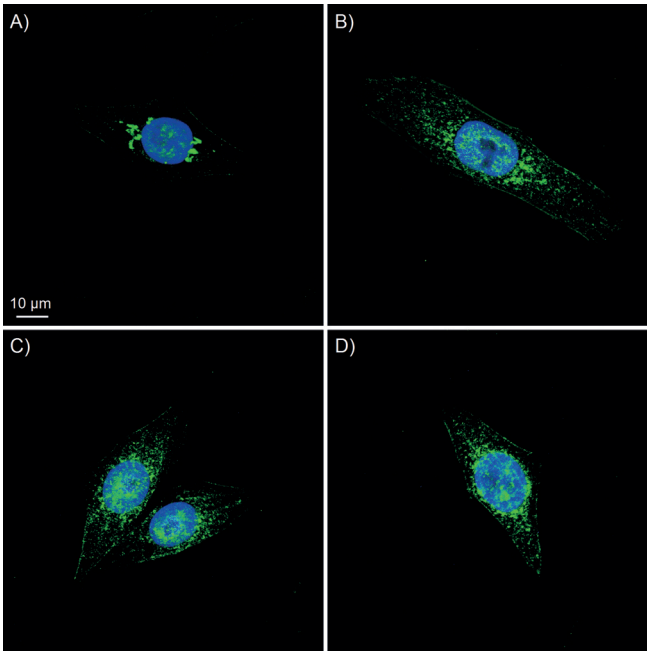

Figure 3. Retro-1 and its enantiomers relocalize syntaxin-5. Cells were treated for 1 h with A) carrier (0.05% DMSO), B) *rac*-Retro-1 (6 μM), C) (*S*)-Retro-1 (3.5 μM), or D) (*R*)-Retro-1 (9.4 μM), then fixed and labeled for syntaxin-5 protein (green) and nuclei (blue). Relocalization of syntaxin-5 is observed after treatment with *rac*-Retro-1, (*S*)-Retro-1, and (*R*)-Retro-1.

Abbreviations

Stx: Shiga toxin, HUS: hemolytic uremic syndrome, Gb3: globotriaosylceramide, EC_{50}: 50% effective concentration, HPLC: high-performance liquid chromatography, TGN: trans-Golgi network, DMSO: dimethyl sulfoxide, SEM: standard error of the mean, NBS: *N*-bromosuccinimide.

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Keywords: benzodiazepines · Retro-1 · retrograde transport · Shiga toxin

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