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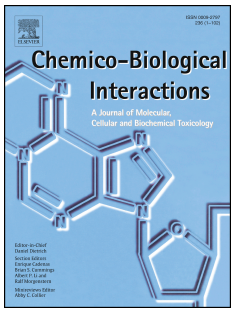

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Inhibitors of retrograde trafficking active against ricin and Shiga toxins also protect cells from several viruses, Chlamydiales and *Leishmania*

N. Gupta[1], R. Noël[1], A. Goudet[1], K. Hinsinger[1], A. Michau[1], V. Pons[1], H. Abdelkafi[1], T. Secher[2], A. Shima[2], O. Shtanko[3], Y. Sakurai[3], S. Cojean[4], S. Pomel[4], V. Liévin-Le Moal[4], V. Leignel[5], J.-A. Herweg[6], A. Fischer[6], L. Johannes[7,8,9], Kate Harrison[10], Philippa M. Beard[10,11], P. Clayette[12], R. Le Grand[13,14,15,16], J. O. Rayner[17], T. Rudel[6], J. Vacus[5], P.M. Loiseau[4], R. A. Davey[3], E. Oswald[2], J.-C. Cintrat[1], J. Barbier[1] and D. Gillet[1]*

[1] *Institute of Biology and Technology of Saclay (IBITECS), CEA, LabEx LERMIT, Université Paris-Saclay, F-91191, Gif Sur Yvette, France.*
[2] *Inserm, UMR1043, CPTP, Toulouse, France.*
[3] *Texas Biomedical Research Institute, San Antonio, Texas, USA.*
[4] *Antiparasitic Chemotherapy, UMR 8076 CNRS BioCIS, LabEx LERMIT, Université Paris-Sud, Université Paris-Saclay, F-92290, Chatenay-Malabry, France.*
[5] *DRUGABILIS (French research performer SME), F-92290, Chatenay-Malabry, France*
[6] *University of Würzburg, Biocenter, Chair of Microbiology, Am Hubland, D-97074, Würzburg, Germany.*
[7] *Institut Curie, PSL Research University, Endocytic Trafficking and Therapeutic Delivery group, 26 rue d'Ulm, F-75248, Paris Cedex 05, France.*
[8] *CNRS UMR3666, F-75005, Paris, France.*
[9] *INSERM U1143, F-75005, Paris, France.*
[10] *The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian, EH25 9RG, United Kingdom.*
[11] *The Pirbright Institute, Ash Rd, Pirbright, Surrey GH24 0NF, United Kingdom.*
[12] *ImmunoPharmacology and Biosafety Laboratory, BERTIN Pharma, CEA, F-92265, Fontenay-aux-Roses, France.*
[13] *Institute of Emerging Diseases and Innovative Therapies, CEA, U1184, Immunology of Viral Infections and Autoimmune Diseases, Infectious Disease Models and Innovative Therapies Infrastructure, F-92265, Fontenay-aux-Roses, France.*
[14] *INSERM, U1184, F-94276, Le Kremlin-Bicêtre, France.*
[15] *University of Paris South, U1184, F-92265, Fontenay-aux-Roses, France.*
[16] *Vaccine Research Institute, Henri Mondor Hospital, F-94010, Créteil, France.*
[17] *Infectious Disease Research, Southern Research, 2000 Ninth Avenue South, Birmingham, AL 35205, USA.*

Corresponding author:

Daniel Gillet

Institute of Biology and Technology of Saclay (IBITECS),

CEA,

LabEx LERMIT, Université Paris-Saclay

F-91191 Gif Sur Yvette, France

E-mail: Daniel.GILLET@cea.fr

Abstract. Medical countermeasures to treat biothreat agent infections require broad-spectrum therapeutics that do not induce agent resistance. A cell-based high-throughput screen (HTS) against ricin toxin combined with hit optimization allowed selection of a family of compounds that meet these requirements. The hit compound Retro-2 and its derivatives have been demonstrated to be safe *in vivo* in mice even at high doses. Moreover, Retro-2 is an inhibitor of retrograde transport that affects syntaxin-5-dependent toxins and pathogens. As a consequence, it has a broad-spectrum activity that has been demonstrated both *in vitro* and *in vivo* against ricin, Shiga toxin-producing O104:H4 entero-hemorrhagic *E. coli* and *Leishmania sp.* and *in vitro* against Ebola, Marburg and poxviruses and Chlamydiales. An effect is anticipated on other toxins or pathogens that use retrograde trafficking and syntaxin-5. Since Retro-2 targets cell components of the host and not directly the pathogen, no selection of resistant pathogens is expected. These lead compounds need now to be developed as drugs for human use.

Keywords: Bioterrorism; Biothreat agents; Emerging infectious diseases; Ricin toxin; Shiga-like toxins; Retrograde cell transport; High-throughput cell-based assays.

1. Bioterrorism, biothreat agents and biodefense

For several decades, the rate of health crises related to emerging infectious diseases has increased (H1N1 influenza virus, *E. coli* O104:H4, Chikungunya virus, Ebola virus, etc) (1, 2). In parallel, biological attack by disseminating a pathogen or a biotoxin has been demonstrated (e.g. anthrax letters in 2001 or ricin letters to president Obama more recently) (3). These events led to an increased awareness of health authorities for intensification of research into the development of medical countermeasures for a wide range of biothreat agents, either naturally emerging or deliberately introduced as an act of bioterrorism (4).

According to the U.S. Centers for Disease Control and Prevention (CDC), a bioterrorism attack is the deliberate release of viruses, bacteria, toxins or other harmful agents to cause illness or death in people, animals, or plants. The CDC bioterrorism agents are listed in (5). Biodefense is defined as the means or methods of preventing, detecting, or managing an attack involving biological weapons.

Emerging infectious diseases are defined by the US National Institute of Allergy and Infectious Disease (NIAID) as infectious diseases that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range, or that are caused by one of the NIAID Category A, B, or C priority pathogens (6). Category A includes high-priority agents that pose a risk to national security because they can be easily transmitted and disseminated, result in high mortality, have potential major public health impact, may cause public panic, or require special action for public health preparedness. Category B includes agents that are moderately easy to disseminate and have low mortality rates. Category C agents are emerging pathogens that might be engineered for mass dissemination because of their availability, ease of production and dissemination, high mortality rate, or ability to cause a major health impact.

Although the causes of emergent diseases and bioterrorism are different, they share some characteristics: biothreat agents are highly diverse (bacteria, viruses, toxins, etc.), the agent that will provoke the next crisis is always indefinite, unknown pathogens may emerge, medical countermeasures are too long to develop in front of an immediate threat and some suspected biothreat agents may never provoke a crisis. Therefore, medical countermeasures against biothreat agents require broad-spectrum therapeutics that do not induce agent resistance (4). In this review we describe the development of new compounds that target the intracellular retrograde transport process. These compounds have been shown to be safe in animals and they demonstrated efficacy against toxins, such as ricin or Shiga toxins, in *in vitro* and *in vivo* models. Moreover, recent studies have shown that these compounds provided protection against filoviruses, poxviruses, Chlamydiales and *Leishmania*. Ricin, Shiga toxin-producing *Escherichia coli*, filoviruses, poxviruses and Chlamydiales are found both in the CDC bioterrorism biothreat agent list and the NIAID emerging disease list (5, 6). Although *Leishmania* are not, a recent outbreak of 446 cases in Madrid, Spain, between 2009 and 2012 had the characteristics of an emerging infectious disease (7).

2. Identification of ricin antidotes

Ricin is a highly toxic lectin produced in the seeds of the plant *Ricinus communis*. This toxin is used as a bio-crime and bio-suicide poison and is considered a potential bio-terrorist agent. The plant is used for ornamentation and industry. The oil (castor oil) contained in the seeds is treated for the production of ricinoleic acid, a fatty acid entering in the composition of many manufactured products such as cosmetics, soaps, shampoos, inks, paints, varnishes, brake fluids, etc. The toxin, which is hydrophilic, remains in the seed pulp after oil extraction and can be easily purified. Doses as low as a few micrograms are lethal for injected mice; in humans, 1 to 20 mg can be fatal after ingestion. There is no antidote for ricin poisoning and treatment relies on supportive care.

Ricin is a glycosylated protein which is composed of two subunits: ricin toxin A and B chains (8). The A subunit has ribosomal RNA N-glycosidase activity and thus it inhibits protein biosynthesis (adenine at position 4324 of the 28S ribosomal RNA is removed). The B subunit binds to cell surface receptors (galactose and N-acetylgalactosamine moieties of glycoproteins and glycolipids). After cell adhesion, ricin is internalized (via clathrin-dependent and independent mechanisms) and transported from early endosomes to the endoplasmic reticulum via the Golgi apparatus (8).

Different therapeutic approaches to treat ricin toxicity have been considered such as vaccination (9, 10) or production of neutralizing antibodies (11, 12). Mass immunization against ricin is however unrealistic. Neutralizing antibodies have efficacy against ricin poisoning in animal models and have their place in a therapeutic arsenal (13). However, they need to be administered within the first 10-24 h of intoxication and cannot access ricin already internalized by cells. Due to the potentially high severity of ricin intoxication, co-administration of small therapeutic molecules with anti-ricin antibodies may greatly improve disease outcome. Another approach was to target the catalytic activity of the A chain of ricin. Small-molecule compounds have been reported to inhibit enzymatic activity in *in vitro* tests, but they generally failed to protect cells or animals from ricin toxicity (14-22).

Cell-based high-throughput screens (HTS) have been used to identify small-molecule inhibitors of ricin (23). An advantage of cell-based assays in which compounds are selected for rescuing cells from intoxication is the selection of bioactive compounds that do not affect cell viability (24-26). **Figure 1** describes high-throughput cell-based phenotypic assays that measure the effects of small-molecule compounds on cell toxicity induced by ricin. Cells are exposed to ricin and protein biosynthesis that is the target of the toxin is measured in order to assess cell protection against ricin toxicity for each compound.

In 2007, Saenz *et al.* described a luciferase-based HTS with a chemical library of 14,400 small-molecule compounds. Monkey Vero cells were transfected with cDNA encoding a destabilized luciferase with short half-life; luciferase activity decreased rapidly in cells incubated in the presence of toxin. Two compounds that protected against ricin inhibition of protein synthesis were reported (24). In 2010, Stechmann *et al.* used HTS to identify small-molecule inhibitors that protected cells from ricin (25). 16,480 molecules from a commercial library of drug-like compounds (ChemBridge DIVERSet™) were tested at a concentration of 25 µM on A549 human epithelial pulmonary cells (60,000 cells per well in 96-well scintillation microplates) for their capacity to rescue cells from intoxication by ricin at a concentration of 0.1 nM. [^{14}C]-leucine incorporation in cell proteins was used as a marker of protein synthesis. Thus, ricin-intoxicated cells failed to incorporate [^{14}C]-leucine while cells protected by a given compound did. Two compounds named Retro-1 and Retro-2 were identified and studied in detail on HeLa cells.

These two inhibitors showed an unexpected degree of specificity. Indeed, these compounds blocked retrograde toxin trafficking at the early endosome/trans-Golgi network (TGN) interface and did not affect compartment morphology, endogenous retrograde cargos or other trafficking steps (**Figure 2**) (25). This transport pathway is also named the retrograde route and it is involved in many physiological and pathological situations (27).

3. Protection of mice challenged with ricin

The two compounds selected by Stechmann *et al.* after HTS were nontoxic for animals after intraperitoneal administration up to 400 mg/kg (25). The molecules were solubilized in pure DMSO at 30 mM and diluted to final concentration in saline. A model of ricin intoxication by nasal instillation was used in order to mimic exposure by aerosols, which is a likely modality in bioterror attacks. A dose of ricin leading to 90% deaths at day 21 (LD90) was chosen. With this dose, the first clinical signs of toxicity were observed within 24 h. A statistically

significant prophylactic protection was observed in first experiments with a single intraperitoneal dose of 2 mg/kg of Retro-2 one hour prior toxin challenge: 49% of mice survived vs. 11.5% in control group ($p = 0.001$). Complementary experiments were performed indicating a dose-response relationship (**Figure 3**). After administration of 20 mg/kg of Retro-2, the survival was 60% at 20 days. Finally, 200 mg/kg of Retro-2 fully protected mice against ricin challenge. Therefore, these results clearly demonstrated that a small molecule can protect animals exposed to a lethal dose of ricin. Retro-2 was described as a lead compound for the development of inhibitors of ricin. Moreover, the retrograde route was identified as a potential therapeutic target for other toxins that follow this route.

4. Inhibitor of retrograde trafficking protects mice infected with entero-hemorrhagic *E. coli*

Shiga toxins are members of a family that includes Shiga toxin produced by *Shigella dysenteriae* and two Shiga-like toxins (SLTs) named Stx1 and Stx2 produced by entero-hemorrhagic strains of *Escherichia coli* (28). Shiga toxin-producing *E. coli* (STEC) cause hemorrhagic colitis, hemolytic-uremic syndrome (HUS) and death (29). The most common sources for SLTs are entero-hemorrhagic *E. coli* with serotypes O157:H7 and O104:H4. There is no approved treatment of STEC-induced HUS. Despite efficacy in non-STEC-induced HUS (atypical HUS), the use of the anti-C5 complement component antibody eculizumab® in STEC-induced HUS remains inconclusive (30). In addition, antibiotics may worsen the disease by further inducing toxin release by the bacteria (31).

SLTs share structural and functional characteristics with ricin (8, 28). They are composed of an A catalytic subunit and a pentameric B subunit. The subunit A of SLTs inhibits protein biosynthesis through ribosomal RNA N-glycosidase activity and the subunit B binds to cell surface receptors (glycosphingolipid globotriaosyl ceramide; Gb3 or CD77) (28). After

internalization, these toxins are transported from early endosomes through the Golgi apparatus to the endoplasmic reticulum (28).

Since SLTs share with ricin the trafficking via the retrograde route, inhibitors that were shown to be effective on ricin intoxication via inhibition of this pathway were also tested as potential inhibitors of intoxication by SLTs. As for ricin, Retro-2 has been demonstrated to protect HeLa cells from the toxic effects of Stx1 and Stx2 (25).

Secher *et al.* have studied the effect of Retro-2 in a murine model of *E. coli* O104:H4 infection (32). As shown in **Figure 4**, O104:H4 infection led to a 70% mortality rate in untreated control mice. Systemic treatment with two injections of Retro-2 at 100 mg/kg (solubilized in pure DMSO at 30 mM and diluted to final concentration in saline) significantly reduced mortality rate to 40%. Body weight loss and clinical scores were reduced by more than half.

5. Effects of Retro-2 on viral infections

Viruses are internalized into host cells through various routes (33). In the case of enveloped viruses, direct fusion at the plasma membrane may allow deposition of the nucleocapsid directly into the cytoplasm. However, non-enveloped viruses and some enveloped viruses are unable to access the host cytoplasm directly from the cell surface. After endocytosis these viruses exploit the host vesicular trafficking that leads them to the endosomes, the Golgi apparatus or to the endoplasmic reticulum where they are released into the cytoplasm (34). The use of retrograde transport suggested that infection by some viruses could be blocked by the Retro-2 compound via similar mechanisms that blocked retrograde trafficking of ricin and SLTs.

Adeno-associated viruses. Gene therapy is a promising biomedical strategy, and adeno-associated virus (AAV) vectors are currently being evaluated for the treatment of various

diseases. AAV vectors must reach the nucleus and retrograde transport of capsids via the trans-Golgi network is necessary for gene delivery. Nonnenmacher *et al.* showed that the endosome-to-TGN/Golgi apparatus transport step of AAV is dependent on syntaxin-5 function and that this step can be inhibited by Retro-2 (35). Therefore these results support the concept that inhibition of retrograde transport could protect not only against ricin or SLTs, but also against viruses.

Polyomaviruses and papillomaviruses. Polyomaviruses are non-enveloped DNA viruses that cause severe disease in immunocompromised individuals. Thus, JC polyomavirus (JCPyV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy, and BK polyomavirus (BKPyV) is the causative agent of polyomavirus-induced nephropathy and hemorrhagic cystitis. There is no vaccine or antiviral therapy for these viruses (36). Human papillomaviruses (HPVs) are also non-enveloped DNA viruses. They are associated with the development of cancer of uterine cervix and oropharynx. Vaccination against some types of HPV has been successful, but there are no approved drugs to treat or prevent papillomavirus infections and these viruses remain a major public health concern (37).

Nelson *et al.* (38) demonstrated in tissue culture cells that Retro-2 inhibited infection by JCPyV, BKPyV and simian virus 40, which is another polyomavirus. Infectivity was reduced to 30% for simian virus 40 and 20% for BKPyV and JCPyV with 100 µM of Retro-2 as compared to control. Retro-2 inhibited retrograde transport of polyomaviruses to the endoplasmic reticulum, which is a step essential for infection (38). Carney *et al.* confirmed these results on polyomaviruses and demonstrated that Retro-2 protected also cell lines from infection by papillomaviruses (37).

Ebola and Marburg filoviruses. Ebola and Marburg viruses are filamentous enveloped viruses that are members of the family *Filoviridae*. Both Ebola virus and Marburg virus cause

severe diseases in humans in the form of viral hemorrhagic fevers, which are associated with a high mortality rate of up to 90% (39, 40).

There is no approved vaccine, and only a few experimental drugs have been tested in animals or humans with no proven efficacy for patient treatment (favipiravir) (41). As a consequence, filoviruses are considered biosafety level-4 pathogens. Moreover, due to the emergence of a new variant of Ebola virus in West Africa, there is an urgent need for efficient therapeutics. The only small molecule drug with potential efficacy in mice is favipiravir, with an IC_{50} of 67 µM (42). Shtanko *et al.* have demonstrated that Retro-2 blocked infection by Ebola virus and Marburg virus *in vitro* in a dose-dependent manner with IC_{50} of 8.4 µM (article under revision). Fifty µM of Retro-2 reduced cell infection bellow 10% as compared to control. Retro-2 appeared to act on a late step of virus entry at the level of intracellular endocytic compartments.

In summary, Retro-2 is a potential antiviral therapy that broadly inhibits viruses that use retrograde trafficking (adeno-associated viruses, polyomaviruses and papillomaviruses), although other mechanisms of viral inhibition appear to be possible as suggested for filoviruses.

Poxviruses. Endosome to Golgi retrograde transport pathway proteins have been identified as pro-viral host factors in two independent high throughput siRNA screens of Vaccinia virus (VACV) (43, 44). Two recent studies investigated the role of this pathway in poxvirus replication in detail and showed that Retro-2 potently prevented spread of Vaccinia and Monkeypox viruses in cell cultures (45, 46). Most interestingly, this protective effect was not linked with virus entry but rather a membrane wrapping process which occurs at late stages of virion maturation. Two viral proteins, F13 and B5 are required for the wrapping of a small percentage of the single membrane intracellular mature virions into triple membrane intracellular enveloped virions. These are then processed further to become responsible for

long range spread of the virus *in vivo* and *in vitro* (47). F13 uses the endosome to trans-Golgi retrograde trafficking pathway to travel from early endosomes to the trans-Golgi where it co-localizes with B5. Retro-2 treatment causes mis-localization of B5 and F13, blocks the wrapping process and thereby inhibits the formation of the triple-wrapped intracellular enveloped virions and reduces viral spread.

6. Effects of Retro-2 on intracellular parasites

Leishmania is a parasite responsible for leishmaniasis that affects twelve million people worldwide, with two million new cases each year. Although *Leishmania* is not considered a bioterrorism agent, a recent outbreak of *Leishmania infantum* of 446 cases in Madrid, Spain, between 2009 and 2012 had the characteristics of an emerging disease (7). Current treatments are either toxic or induce the development of drug resistant strains of the parasite and there is need for new anti-leishmanial drugs (48-50). *Leishmania* species are unicellular eukaryotes with well-defined nucleus and cell organelles. These parasites are internalized by macrophages into membrane-bound compartments called *Leishmania* parasitophorous vacuoles (LPVs) that share many characteristics with phagosomes. Previous observations established a role for syntaxin-5 in the development of LPVs. Since pathways involving syntaxin-5 had been shown to be inhibited by Retro-2 (25), Canton and Kima studied the effect of this compound on LPVs development (51). Retro-2 blocked LPV development within 2 h in cells infected with *Leishmania amazonensis*. In infected cells incubated for 48 h with Retro-2, LPV development was significantly limited and infected cells harbored four to five times fewer parasites than controls. *In vivo* experiments in mice showed that Retro-2 limited experimental *L. amazonensis* infections: a 20 mg/kg intraperitoneal dose had no effect on the course of infection, but a 100 mg/kg dose of Retro-2 resulted in approximately a log less parasites compared to control (**Figure 5**). No toxicity was evidenced in experimental animals.

The efficacy of Retro-2 was recently demonstrated on *L. donovani infantum*. Retro-2 was active *in vitro* both on axenic and intramacrophage amastigotes of *L. donovani infantum* in a range from 3 to 20 µM. In addition, Retro-2 exhibited a significant reduction of parasite burden after a treatment at 100 mg/kg/day × 5 days on the *L. donovani infantum* Balb/c mouse model (**Table 1**). Therefore, this compound is able to act *in vitro* and *in vivo* on parasites exhibiting two different systems of housing conditions. The amastigote forms of the *L. amazonensis* complex reside in large, communal LPVs housing many parasites, whereas those of *L. donovani infantum* are located in individual LPVs. Such observations encourage to study the effects of Retro-2 and analogues on common pathways used by both New-World (*L. amazonensis*) and Old-World (*L. donovani infantum)* parasites to set up their LPVs.

7. Effects of Retro-2 on intracellular bacteria

Simkania negevensis is an obligate intracellular Gram-negative bacterium of the order Chlamydiales. Its natural host is not known and it is widespread among humans. *S. negevensis* has been associated with transmissible infections of the upper respiratory tract. Infections with the two closely related human pathogenic bacteria *Chlamydia pneumoniae* and *Chlamydia psittaci* can cause pneumonia, chronic bronchitis and chronic asthma. *Chlamydia trachomatis* is responsible for ocular and sexually transmitted infections. *S. negevensis* grows in host cells within a membrane-bound vacuole forming endoplasmic reticulum contact sites. Herweg *et al.* recently demonstrated that 75 µM of Retro-2 decreased bacterial replication both during primary infection down to 50% and progeny infection down to less than 40%-20% (52). Most interestingly, *S. negevensis* progeny from cells cultured in the presence of Retro-2 were markedly less efficient in infecting cells cultured in the absence of Retro-2. The compound seemed to alter the morphology of *S. negevensis*–containing vacuoles and replication of the bacteria (52). Similar results were obtained against *C. trachomatis*.

8. Optimization of Retro-2

In order to identify more potent compounds, a structure-activity relationship (SAR) study was performed by Noel *et al.* (53). During the course of the SAR process, it appeared that the bioactive compound was not Retro-2 but the cyclized analog Retro-2^{cycl}. The *N*-methyldihydroquinazolinone derivatives of Retro-2 were tested *in vitro* for their protective effect against Stx2 on cellular protein synthesis. After cyclization and optimization of the three main moieties of Retro-2, a dihydroquinazolinone compound was identified with approximately 100-fold improvement of the EC_{50} against Stx2 cytotoxicity. Only the (*S*)-enantiomer was active (**Figure 6**). The mode of action of this compound was similar to that of Retro-2, namely a selective inhibition of the retrograde transport.

In a next step, Gupta *et al.* have reported the synthesis and the evaluation of a new enantiopure dihydroquinazolinone compound, named Retro-2.1, with improved *in vitro* protection against Stx2 (approximately 500-fold compared to Retro-2) and ricin (approximately 1,000-fold increased activity) (54). (*S*)-Retro-2.1 is currently the most potent molecule to counteract the cytotoxic potential of ricin and SLT with EC_{50} values of 23 and 54 nM, respectively. By comparison, the (*R*)-enantiomer shows EC_{50} values of 3200 and 2400 nM against ricin and Stx2, respectively.

Carney *et al.* also described dihydroquinazolinone analogs of Retro-2^{cycl} with improved potency as suppressors of human polyoma- and papillomavirus infection *in vitro* (37).

Therefore, as previously demonstrated *in vitro* and *in vivo* for Retro-2, optimized derivatives have the potential to be developed as broad-spectrum antidotes to a wide array of pathogens, including toxins, viruses, intracellular bacteria and parasites that exploit retrograde trafficking to enter and infect the cell.

9. Toxins and pathogens not affected by Retro-2

We have found bacterial toxins and viruses against which Retro-2 had no effect, neither on cell intoxication nor on cell infection. These include diphtheria toxin (DT), *Clostridium botulinum* neurotoxin A (BoNT/A), dengue virus serotype 4 (DENV-4), chikungunya virus (CHIKV) and Venezuelan equine encephalitis virus (VEEV). **Table 2** indicates the maximum concentration at which Retro-2 was tested against each mentioned pathogen, the target cells and the type of assay used.

In the case of DT and BoNT/A, the lack of action of Retro-2 is easily understood because the retrograde transport from the early endosomes to the trans Golgi network is not involved in their mechanism of action. During cell intoxication, DT is internalized into early endosomes. Following acidification of these compartments its catalytic domain is translocated into the cytosol during trafficking through the endocytic carrier vesicles of the endosome to lysosome degradation pathway (55). In the case of BoNT/A, the toxin is directly endocytosed in recycling synaptic vesicles or clathrin-coated vesicles of the nerve terminus. These vesicles are acidified, which triggers the translocation of the catalytic chain of the toxin into the cytosol where it finds its target, the SNARE protein SNAP-25 (56).

In the case of the viruses, the absence of effect of Retro-2 on cell infection suggests that enveloped, positive-sense, single-stranded RNA viruses such as DENV-4, CHIKV and VEEV do not involve the endosome to Golgi retrograde transport machinery for entry or other steps of their cycle. It has been suggested that DENV-4 enters the cytoplasm after trafficking along the Rab5-positive early endosomes through the Rab7-positive late endosomes to the lysosomes or further compartments (57, 58). The alphaviruses such as CHIKV and VEEV are believed to enter the cell via receptor-mediated endocytosis followed by membrane fusion in the acidified endosomes (59, 60); however, more recent studies suggest an alternate mode of entry directly at the plasma membrane (61).

10. Conclusions

Medical countermeasures against biothreat agents require therapeutics that are safe and have a large spectrum of activity without inducing possible mechanisms of resistance in case of living pathogens. Retro-2 and its derivatives meet these requirements. Indeed, the use of HTS coupled to cell-based assays allowed selecting bioactive compounds that did not affect cell viability (25, 32, 51). *In vivo*, Retro-2 and its derivative have been demonstrated to be safe, even at high doses (25, 32, 51). Moreover, Retro-2 is an inhibitor of retrograde transport and it affects syntaxin-5-dependent pathogens. As a consequence, it has a broad-spectrum activity that has been demonstrated both *in vitro* and *in vivo* in mice against ricin, SLT-producing O104:H4 *E. coli* and *Leishmania* and *in vitro* against AAV, polyoma-, papilloma-, Ebola, Marburg and poxviruses and Chlamydiales. An effect is anticipated against other toxins or pathogens that use retrograde trafficking. Since Retro-2 targets cell components of the host and not directly the pathogen, no selection of resistant pathogens is expected (4). These lead compounds need now to be developed as drugs for human use. This implies solving drugability issues (solubility, pharmacology, efficacy post disease onset) in order to obtain a true drug candidate.

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Table 1. Comparison of the effects of Retro-2 and the anti-leishmanial reference molecule Miltefosine on the growth of *L. donovani infantum in vitro* on RAW macrophages and *in vivo* in Balb/c mice.

Compound	***In vitro***	***In vivo***	
	Intra-macrophage amastigotes	Treatment regimen : mg/kg/day × 5 days by i.p. route	Reduction of parasite burden in the liver (%)
Retro-2	20.25 ± 5.91	100	77*
Miltefosine	0.92 ± 0.07	10	82*

In vitro activity: IC_{50} is the drug concentration inhibiting the intramacrophage parasite growth by 50% after a 72 h incubation time. Molecules were solubilized in pure DMSO at 30 mM and diluted in culture medium. *In vivo* activity: the experiment was performed on female Balb/c mice 18-20 g according to the protocol described in (62). Retro-2 was solubilized in pure DMSO at 30 mM and diluted in saline The non-parametric Mann-Whitney U-test was performed to compare liver parasitic load; p value <0.05 was considered as significant versus untreated control mice. *p<0.01.

Table 2. Toxins and viruses against which no protective activity was found for Retro-2. Cell types, maximum concentrations of Retro-2 and assay type are given. Retro-2 was added at least 1 h before and maintained during intoxication/infection.

Toxins	**Cell line**	**Retro-2 maximum concentration tested**	**Assay type**
DT	HeLa cells	30 µM	Protein synthesis
BoNT/A	Newborn rat cerebellum neuron primary culture	20 µM	0.5 nM BoNT/A, SNAP25 cleavage monitoring by Western blot
Viruses			
DENV-4	HEK293	500 µM (no toxicity)	Viral cytopathic effect
CHIKV	HEK293	500 µM (no toxicity)	Viral cytopathic effect
VEEV	VeroE6	100 µM	Viral cytopathic effect

Figure 1. High-throughput cell-based assays. These phenotypic assays measure the effects of small-molecule compounds on cellular cytotoxicity induced by ricin or other toxins (23). Chemical compounds from stock plates (1) are added to microplates seeded with cells (2); toxin is then added. After incubation, different methods (3) are used to assess the effects of each compound on toxicity induced by ricin or other toxins: a) the CellTiter-Gloluminescent cell-viability assay quantifies ATP that assessed metabolically active cells. b) In luciferase reporter-gene assays, the enzyme activity is a measure of ongoing protein biosynthesis. c) In the third method, the inhibitory effect of ricin on protein biosynthesis in intact cells is measured through the incorporation of radioactive amino acids into neosynthesized polypeptides.

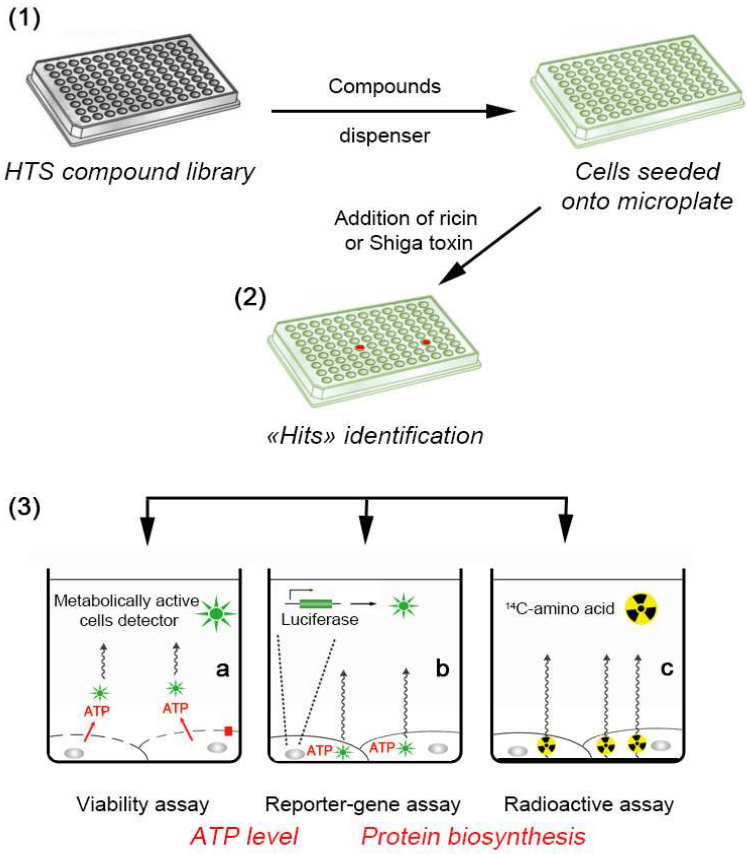


Figure 2. Cellular target of Retro-2. Toxins such as ricin and SLTs penetrate cells through the retrograde transport route from the plasma membrane to the endoplasmic reticulum, via endosomes and the Golgi apparatus (27). Retro-2 blocks toxin transport between early endosomes and the Golgi apparatus (25).

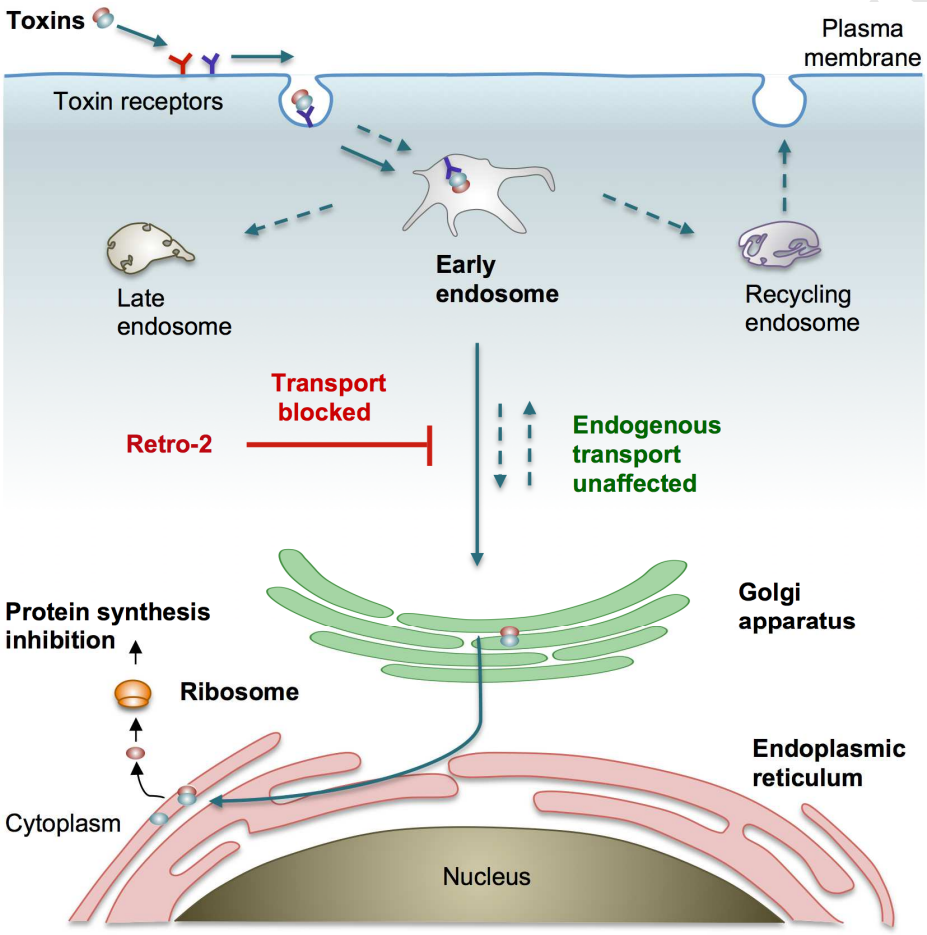


Figure 3. Protection of ricin challenge by Retro-2 (25).

Mice were treated with the indicated doses of Retro-2 and then exposed to ricin via the nasal route. In each experiment, treated mice received a single intra-peritoneal dose of Retro-2 (solubilized in pure DMSO at 30 mM and diluted in saline) 1 h prior to toxin exposure (2 µg/kg by nasal instillation); control animals received vehicle prior to ricin administration. The survival curves for treated animals were statistically different from control animals (log rank test; $p < 0.0001$ for 2 mg/kg of Retro-2, $p = 0.031$ for 20 mg/kg; $p = 0.0007$ for 200 mg/kg). The data are reproduced from (25).

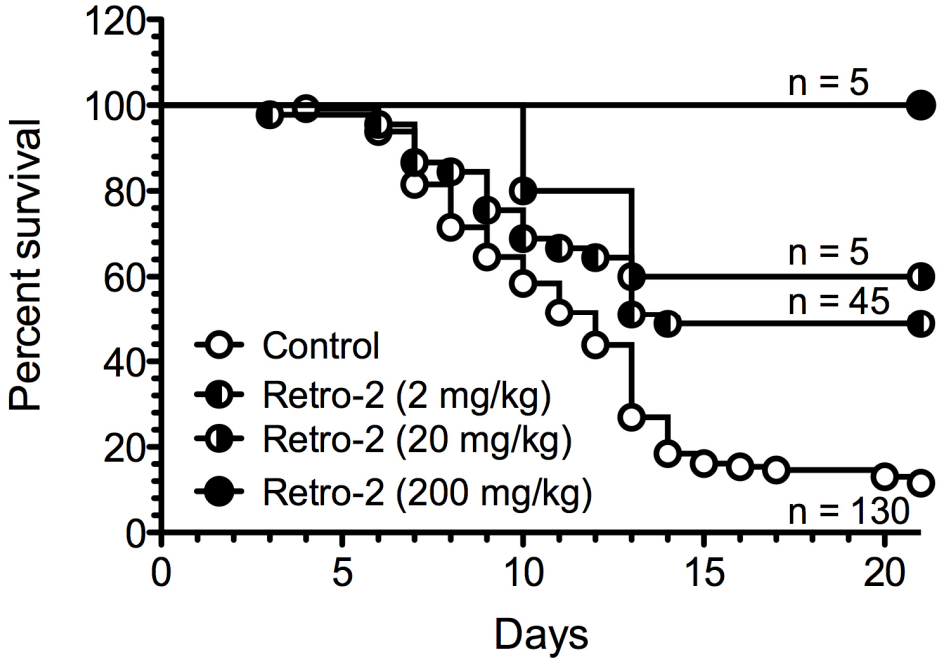


Figure 4. Mice protected from *E. coli* O104:H4 infection with Retro-2 (32).

BALB/c mice received the O104:H4 strain by oral gavage. Intraperitoneal administration of mitomycin C (MMC) induced toxin release at 18, 21, and 24 h after infection. Mice received intraperitoneal administration of Retro-2 (100 mg/kg) at 16 and 26 h after infection. Retro-2 was solubilized in pure DMSO and diluted in saline at 10% DMSO final concentration. Body weight loss (A), clinical scores (B), and survival rates (C) were monitored for 10 days after infection. N=20 for uninfected controls, n=40 for O104:H4-infected mice treated or not treated with Retro-2. * P<0.05; ** P <0.01; ***, P < 0.001. The data are reproduced from (32).

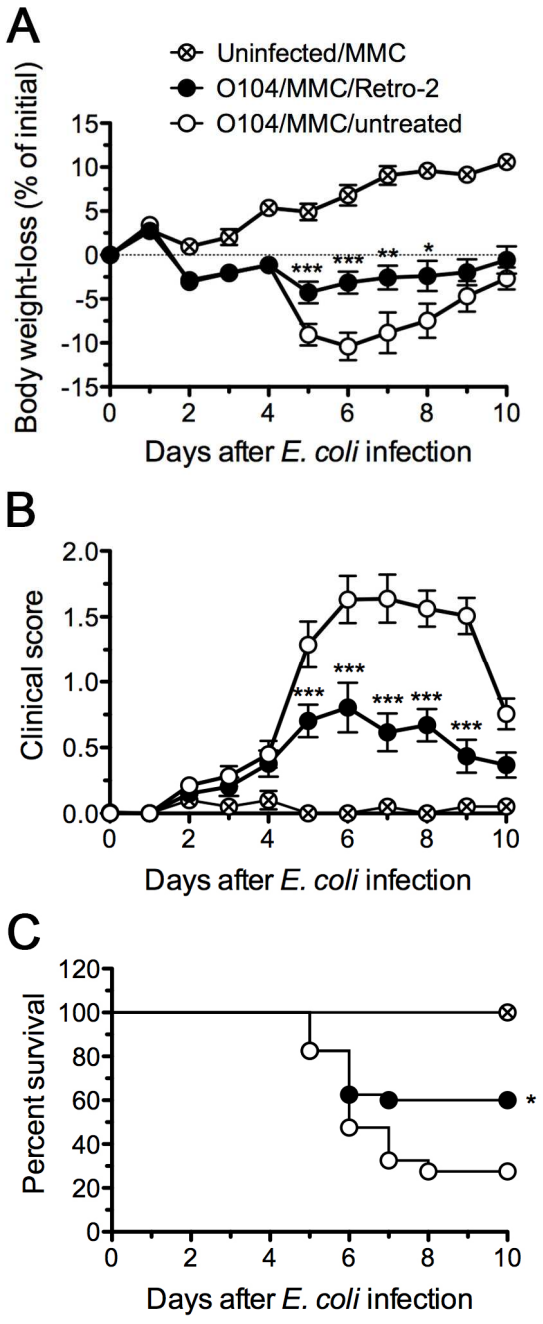


Figure 5. Retro-2 can control the course of an experimental infection with *L. amazonensis* (51).

BALB/c mice were infected in their hind feet with 2×10^6 stationary stage cultured *L. amazonensis* promastigotes. Mice received an intra-peritoneal administration of 20 or 100 mg/kg Retro-2, 24 h after infection. A group of mice was administered the 100 mg/kg dose 3 weeks after the infection was initiated. The parasite titer per foot was determined after 9 weeks of infection. The data are reproduced from (51).

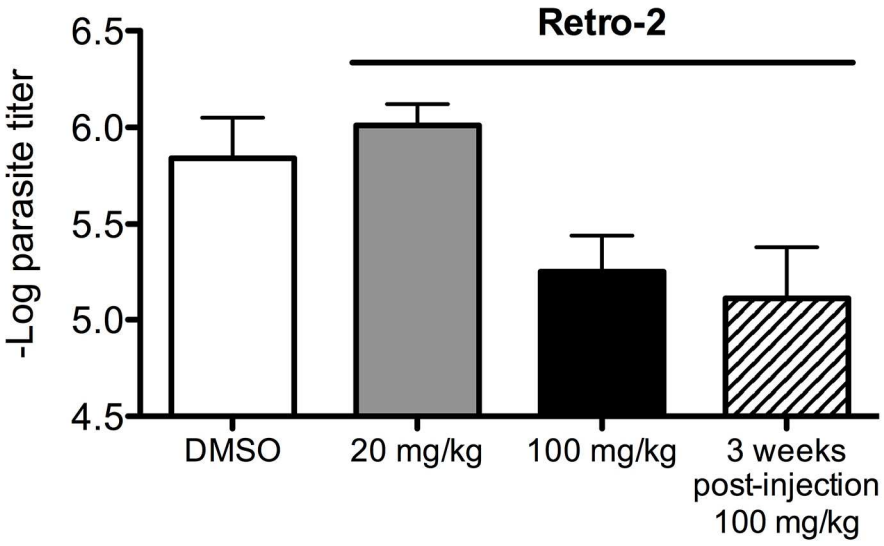
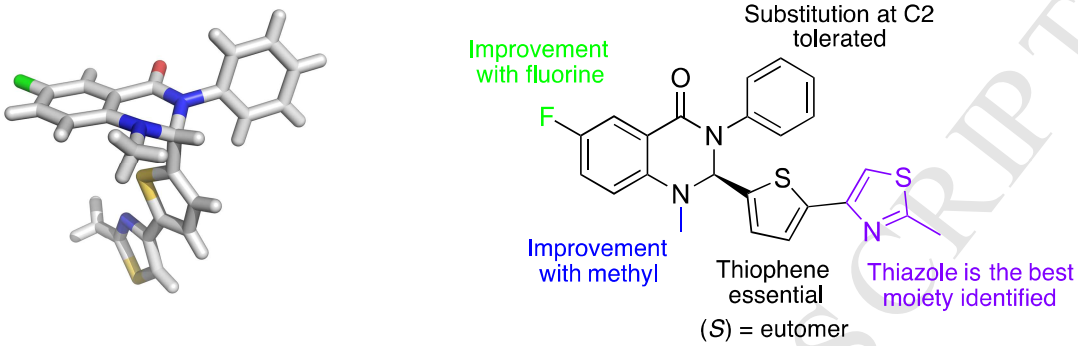


Figure 6. Left, structure determination of the (*S*)-Retro-2.1 enantiomer by X-ray crystallography, the only enantiomer bioactive against toxins in the nanomolar range (54). Right, highlight of the preferential substitutions that increase Retro-2 activity.



Highlights

The molecule Retro-2 identified by HTS protects cells from ricin and Shiga toxins

Retro-2 acts by blocking toxin trafficking from early endosomes to the Golgi

Acting on a cellular target, Retro-2 protects cells from many intracellular pathogens

Retro-2 protected mice from ricin, Shiga toxin-producing *E. coli* and *Leishmania*

The optimized analogue Retro-2.1 is 1000 fold more active than Retro-2 against ricin