A screening pipeline identifies a broad-spectrum inhibitor of bacterial AB toxins with cross protection against influenza A virus H1N1 and SARS-CoV-2

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ABSTRACT

A challenge for the development of host-targeted anti-infectives against a large spectrum of AB-like toxin-producing bacteria encompasses the identification of chemical compounds corrupting toxin transport through both endolysosomal and retrograde pathways. Here, we performed a high-throughput screening of small chemical compounds blocking active Rac1 proteasomal degradation triggered by the Cytotoxic Necrotizing Factor-1 (CNF1) toxin, followed by orthogonal screens against two AB toxins hijacking defined endolysosomal (Diphtheria toxin) or retrograde (Shiga-like toxin 1) pathways to intoxicate cells. This led to the identification of the molecule N-(3,3-diphenylpropyl)-1-propyl-4-piperidinamine, referred to as C910. This compound induces the swelling of EEA1-positive early endosomes, in absence of PIKfyve kinase inhibition, and disturbs the trafficking of CNF1 and the B-subunit of Shiga toxin along the endolysosomal or retrograde pathways, respectively. Together, we show that C910 protects cells against 8 bacterial AB toxins including large clostridial glucosylating toxins from *Clostridium difficile*. Of interest, C910 also reduced viral infection *in vitro* including influenza A virus subtype H1N1 and SARS-CoV-2. Moreover, parenteral administration of C910 to the mice resulted in its accumulation in lung tissues and reduced lethal influenza infection.

Keywords

CNF1, bacterial toxins; UPEC, Rac1, GTPase; high-throughput screening; anti-infectives; endolysosomal pathway; retrograde pathway; lung infection

Running title

Broad anti-infective targeting vesicular trafficking

67 INTRODUCTION

68 Host-directed anti-infective chemical compounds that interfere with intracellular membrane
69 trafficking hold promise to alleviate pathophysiological manifestations triggered by
70 bacterial toxins, thus shifting the host-pathogen balance in favor of the host (1).
71 Furthermore, conservation of physicochemical requirements for cell invasion by pathogens
72 and toxins allows to re-direct small molecules targeting endocytic pathways against a
73 wider number of infectious agents notably viruses (2–4). Discovery of such molecules is a
74 current challenge to expand available therapeutics against highly prevalent infections,
75 multidrug-resistant pathogens and to halt pandemics (5).

76 Bacterial AB toxins are sophisticated nanomachines that rely on both specific
77 physicochemical conditions found in intracellular compartments and accessory cell factors
78 to translocate their enzymatic A-domain into the cytosol. Selective disruption of vesicular
79 trafficking can be leveraged to develop host-directed prophylactic and therapeutic
80 measures against toxin-producing bacteria with broader applications in infectiology (2, 4).
81 To enter the host cells, AB toxins exploit different endocytic pathways which converge
82 towards early endosomes from which they are dispatched to different vesicular pathways
83 (6–8). AB toxins translocate their enzymatic part along the endolysosomal pathway or after
84 retrograde transport from the endoplasmic reticulum (ER), which represents another hot
85 spot of translocation (9, 10). Early endosomes receive cargos, receptors and pathogenic
86 agents coming from the plasma membrane (11). They serve as an essential sorting station
87 to re-route receptors and/or ligands to the cell surface through recycling endosomes, to the
88 trans-Golgi network and the ER through retrograde transport, or the lysosomes for
89 degradation. Each critical step along these vesicular pathways is specified by specialized
90 membrane-associated protein complexes (12). Owing to their initial sorting functions, early
91 endosomes represent targets of interest to develop anti-infectives displaying a large
92 spectrum of action against toxin-producing bacteria and viruses.

93 The Cytotoxic Necrotizing Factor-1 (CNF1) toxin is produced by extraintestinal pathogenic
94 *Escherichia coli* responsible for urinary tract infections, neonatal meningitis and sepsis (13,
95 14). The *cnf1* gene also displays a prevalence estimated to 15% in *E. coli* sequence type
96 (ST)131 that underwent an unprecedented global expansion in the last decade and
97 represents a predominant multidrug-resistant (MDR) lineage of *E. coli* in extra-intestinal
98 infections (15). CNF1 binds to Lu/BCAM and 67-kDa laminin receptor (67LR) to enter cells
99 and reaches acidic endosomes from which it translocates into the cytosol to deamidate

100 host Rho GTPases (16–19). The activation of Rho GTPases by CNF1 confers to
101 pathogenic *E. coli* high capacities of host cell invasion (20, 21).

102

103 Chemical compounds interfering with the intracellular trafficking of bacterial toxins have
104 shown their efficacy against several infections by bacteria, viruses and parasites *in vitro*
105 and *in vivo* as summarized in Supplementary Table 1 and 2. Known inhibitors act
106 specifically on either of the two main endocytic pathways hijacked by toxins. Retro-2 acts
107 specifically on the endoplasmic reticulum (ER) exit site component Sec16A thereby
108 compromising the interaction between the retrograde trafficking chaperone GPP130 and
109 syntaxin-5 for proper transport of Shiga toxin to the ER (22, 23). Both inhibitors EGA and
110 ABMA affect exclusively the trafficking of toxins along the endolysosomal pathway by
111 unidentified mechanisms (2, 4). Currently, no single compound conferring protection to AB
112 toxins hijacking either the endolysosomal or retrograde pathways are available.

113

114 Here, we developed a high-throughput screening (HTS) pipeline allowing the identification
115 of a small chemical compound that protects host cells against eight unrelated bacterial AB
116 toxins. Moreover, C910 properties can be leveraged to confer protection of mice against
117 flu caused by influenza A virus subtype H1N1 and to reduce cell infection by SARS-CoV-2.
118 Mechanistically, we provide evidence that C910 interferes with the sorting function of
119 EEA1/Rab5-positive early endosomes.

120 RESULTS

121 Screening of compounds conferring resistance to a large spectrum of AB toxins

122 We developed a pipeline aiming at screening small chemical compounds protecting host
 123 cells against a broad spectrum of bacterial AB toxins hijacking either the endolysosomal
 124 (CNF1 and diphtheria toxin, DT) or the retrograde pathways (Shiga-like toxin 1, Stx1)
 125 (Figure 1A). We first performed HTS of 16,480 available chemical compounds from the
 126 ChemBridge DIVERSet library for their capacity to protect primary human umbilical vein
 127 endothelial cells (HUVECs) against CNF1-induced cellular depletion of Rac1 (Figure 1B).
 128 The CNF1 toxin hijacks the endolysosomal pathway to penetrate into cells and catalyzes
 129 the deamidation of a specific glutamine residue on small Rho GTPases, notably Rac1 (17,
 130 18). This post-translational modification activates Rac1 and thereby sensitizes this
 131 GTPase to ubiquitin-mediated proteasomal degradation (24). Contrary to previous screens
 132 for antitoxins based on terminal cytotoxic effects, the readout of the screen presented here
 133 relies on direct monitoring of the target of CNF1. Briefly, cells were treated with the CNF1
 134 toxin at 10 nM to achieve maximal proteasomal degradation of Rac1 in 6 h. Quantitative
 135 immunofluorescence staining of Rac1 cellular levels was applied directly to the cells. The
 136 threshold of the chemically-induced protection against Rac1 depletion was set to 30% to
 137 sort robust candidate hits (Figure 1B).

138
 139 In total, 320 hits were cherry-picked and subjected to a second round of screening at two
 140 concentrations in triplicate. 66 compounds passed the second round of screening. These
 141 hits were subsequently reordered and freshly prepared for further validation and then
 142 filtered from pan-assay interference compounds (PAINS), i.e. unstable molecules,
 143 irreversible modifiers or compounds that are frequently active in other screens (25). A total
 144 of 10 compounds were shortlisted as robust inhibitors of CNF1-mediated cellular
 145 degradation of Rac1. These compounds were subsequently analyzed for their protective
 146 action against two bacterial toxins following either endolysosomal or retrograde pathways.
 147 These orthogonal screens were set to counter-select compounds acting directly on the
 148 proteasomal degradation of Rac1, and to identify compounds endowed with a large
 149 spectrum of action against AB toxins. Here, we made use of diphtheria toxin (DT), as it
 150 shares with many enzyme-based AB toxins the need to reach early acidic compartments
 151 along the endolysosomal pathway from which the enzymatic A-domain translocates into
 152 the cytosol (26, 27). The ADP-ribosyltransferase activity of DT poisons host cells by
 153 catalyzing the post-translational modification of elongation factor-2 (EF-2), thereby

blocking protein synthesis. We also made use of Stx1 as it undergoes retrograde transport from the early endosomes to the Golgi before reaching the ER where it exits into the cytosol (10, 28). The *N*-adenine glycohydrolase activity of Stx1 inhibits protein biosynthesis. The cytotoxic effects induced by DT and Stx1 were quantified by a measure of [¹⁴C]-leucine incorporation into neosynthesized proteins. Among the 10 compounds tested, three molecules turned to be active against DT and one of these was active against Stx1 as well. Taken collectively, this screening strategy allowed us to isolate one compound out of a total of 16,480 molecules, here referred to as C910. The chemical structure of N-(3,3-diphenylpropyl)-1-propyl-4-piperidinamine can be divided into four parts (Figure 1A). The western half bears two aromatic phenyl groups while the eastern part bears a central amino group, a piperidine ring with a terminal propyl chain.

We next conducted a dose-response analysis to determine the relationship between compound concentration and C910 antitoxin activity. We determined half maximal effective concentrations (EC₅₀s) of 35.6 μM for Stx1 and 60.1 μM for DT, as well as 4.9 μM for Stx2 (Figure 1C-D and Sup. Figure 1). We also evaluated the effective concentration of C910 against Exotoxin A (PE) from *Pseudomonas aeruginosa*, that shares with DT and STx the capacity to inhibit protein biosynthesis. This toxin exploits intertwined endolysosomal and retrograde pathways (29, 30) to reach the ER and get extruded into the cytosol (7, 31). Cells were intoxicated with increasing doses of PE in the presence of C910 or DMSO vehicle establishing a value of EC₅₀ of 12.6 μM (Figure 1E and Sup. Figure 1A-D). Finally, we determined that C910 inhibited CNF1 (10 nM) with an IC₅₀ = 11.3 μM (Figure 1F). We showed that C910 exhibits no detectable toxicity on HUVECs and HeLa cells at concentrations below 75 μM (Sup. Figure 1E). Thus, we have identified a chemical entity that decreases the susceptibility of host cells to five different bacterial toxins exploiting defined intracellular trafficking pathways of intoxication.

C910 inhibits CNF1-mediated invasion of cells by UPEC

Invasion of epithelial and endothelial cells by UPEC leads to severe and recurrent forms of infections (32). Here, we investigated whether C910-induced resistance of cells to CNF1 cytotoxicity could decrease the susceptibility of host cells to bacterial invasion. The protective action of C910 was assessed in gentamicin-protection experiments, upon infecting cells with a *cnf1*-deleted strain of UPEC in the control condition versus complementation with exogenously added CNF1 at 1 nM. This setting allowed to measure

reproducible rates of cell invasion (33). Remarkably, cells treated with C910 at 15 μ M were protected against CNF1-mediated invasion by UPEC without affecting bacterial adhesion to the cells (Figure 2A and 2B). C910 had no impact on bacterial viability (data not shown). We also verified in parallel that C910 abolished CNF1-mediated depletion of Rac1 during cell infection (Figure 2C). In parallel, we found no protective effect of C910 on the Cdc42/Rac1-dependent invasion of cells by *Salmonella typhimurium* (Figure 2D and E). In contrast to CNF1 toxin-producing UPEC, cell-bound *S. typhimurium* directly injects Cdc42/Rac1-activating effectors through the type-3 secretion system (T3SS)-1, thereby bypassing endocytosis and intracellular trafficking steps required for AB toxins action (34). The specificity of chemically-induced cellular protection against UPEC points toward an action of C910 on the endocytic machinery that is exploited by CNF1, rather than an action on Cdc42/Rac1-driven large-scale membrane deformations that are at play for cell invasion by pathogenic bacteria.

Extended spectrum of protection against bacterial AB-like toxins

We pursued the characterization of the antitoxin spectrum of C910 by studying a paradigm of AB toxins that translocate their A-enzymatic components at acidic pH through a pore formed by B-subunit oligomers. Anthrax toxin from *Bacillus anthracis* is composed of the protective antigen (PA), lethal factor (LF) and edema factor (EF) (35). The 63-kDa matured form of PA undergoes octamerization at the cell surface in association with receptors leading to toxin-receptor endocytosis (36–38). The acidic environment of late and matured early endosomes then drives the insertion of oligomerized PA into the lipid bilayer through which LF and/or EF translocate into the cytosol either directly or via a two-step mechanism involving internal vesicles of late endosomes (39). HUVECs were intoxicated with the lethal toxin (LT: PA + LF) at two different concentrations sufficient to induce a complete cleavage of MEK2 substrate of LF, between 2 and 4 h (Figure 3A). We found that addition of C910 at 40 μ M markedly delayed the kinetics of MEK2 cleavage by protection factors comprised between 2.2 (LT) < P < 2.5 (LT_{1/10}) (Figure 3A and 3B). This demonstrates the protection conferred overtime by C910 to host cells intoxicated by LT from *B. anthracis*.

We went on to test the effect of C910 on large clostridial glucosylating toxins (LCGTs) TcdA and TcdB from *Clostridium difficile* (40). These toxins hijack different host receptors to enter into cells via endocytosis and translocate from acidified endosomes (41–43). The

internalization of LCGTs occurs via a dynamin-dependent process that largely involves clathrin. LCGTs glucosylate a threonine residue in the effectors-binding loop of Rho GTPases to short circuit the signaling cascades that control the actin cytoskeleton. Consequently, this produces a rounding of cells. Highly sensitive Vero cells were intoxicated with 8 cytotoxic units of TcdA or TcdB. These conditions were set to reach 100% of cell rounding at 8 h of intoxication. In these conditions, co-treatment of cells with C910 at 5 μ M markedly reduced the percentage of intoxicated cells (Figure 3C). The extent of protection conferred by C910 on LCGTs cytotoxicity was quantified by anti-Rac1 immunoblotting (Figure 3D). This is based on the observation that glucosylation of the threonine-35 of Rac1 blocks its recognition by the anti-Rac1 [clone 102]-monoclonal antibody (44). This allowed direct visualization of the dose-dependent inhibition conferred by C910 on LCGTs-driven post-translational modification of Rac1, which was maximal at 20 μ M (Figure 3D).

Altogether, our results show that C910 confers to host cell resistance against eight bacterial AB toxins relevant to public health or produced by a pathogen of concern of misuse.

C910 acts between CNF1 cell entry and catalytic steps

We showed that C910 protects cells against multiple AB toxins with distinctive catalytic activities and host cell receptors, thereby pointing for a direct action on host cells. Nevertheless, we verified the absence of an inhibitory effect of C910 on CNF1-mediated deamidation of RhoA measured *in vitro* (Sup. Figure 2A). We also verified that C910 did not affect the association of CNF1 to cell surface-exposed receptors. For this, CNF1 was chemically coupled to the Cy3 fluorophore (CNF1-Cy3) and incubated with cells at 4°C to allow its binding to host cells. Fluorescence-activated cell sorting (FACS) analysis showed that a concentration of C910 above its IC₅₀ against CNF1, did not significantly alter the mean fluorescence intensity (MFI) of CNF1-Cy3 bound to the cells (Figure 4A). We also recorded an absence of effect of C910 on the cellular binding of the B-subunit of Shiga toxin (STxB) (Sup. Figure 2B). Furthermore, C910 had no detectable effect on the internalization of CNF1-Cy3 and STxB (Figure 4B). Taken collectively, these data indicate that C910 operates neither at initial steps of host cell receptor binding and endocytosis of CNF1 and STxB nor at a later stage of CNF1 enzymatic activity.

These exploratory results prompted us to examine a broader impact of C910 on the intracellular transport to lysosomes, by using a protease-dependent dequenching of self-quenched red fluorescent DQTM Red BSA (DQ-BSA). At a late timing of the chase of 18 h, we observed an accumulation of cleaved DQ-BSA with no detectable effect of C910 (data not shown). When the chase was carried out during a shorter period of 4 h, we observed a strong reduction of cleaved DQ-BSA in C910-treated cells, as compared to control conditions (Figure 4C). In parallel, we recorded no significant effect of C910 on lysosomal cathepsin B activity measured *in vitro* and in cells (Sup. Figure 3A and 3B). Together, these data argue for an action of C910 on endolysosomal trafficking thereby preventing toxin actions.

C910 affects early endosome homeostasis and sorting of toxins

The above data prompted us to analyze the impact of C910 treatment on the morphology of vesicular compartments along the endolysosomal and retrograde pathways. This was conducted after a short period of time to avoid cascading effects on the endomembrane system. Immunolabeling of several protein markers failed to detect a significant effect of C910 on the distribution and morphology of endolysosomal or retrograde pathway-related compartments except for Rab5-positive early endosomes (Figure 5A). Indeed, compartments positive for Rab5 and the early endosome antigen 1 (EEA1) appeared significantly enlarged and devoid of the late endosomal marker lysobisphosphatidic acid (LBPA) (Figure 5B and Sup. Figure 3C). The extent of swelling of EEA1-positive compartments increased as a function of time (Sup. Figure 4A) and dose of C910 (Sup. Figure 4B). Transmission electron microscopy analysis of BSA-gold loaded endosomes from HUVECs, at an early time point of endocytosis, showed vacuoles containing gold-particles that had an electron-lucent lumen and few internal vesicles (Sup. Figure 4C). In parallel, we recorded an increase in the size distribution of BSA-gold positive compartments (Sup. Figure 4C).

We went on to define how C910 treatment affects the trafficking of CNF1. After 90 min of intoxication, CNF1-Cy3 accumulates in Rab7-positive late endosomes (Figure 5C). In contrast, we observed that CNF1-Cy3 accumulates in the lumen of enlarged EEA1-positive endosomes in C910-treated HUVECs (Figure 5C). This was accompanied with a significant decrease of co-localization between CNF1-Cy3 and Rab7 signals in C910-treated cells compared with control cells (Sup. Figure 5A). In parallel, we found that C910

significantly promotes the accumulation of the Alexa Fluor 488 conjugated STxB in EEA1-positive endosomes at the expense of STxB accumulation in the Golgi area, defined by Giantin immunostaining (Figure 5D).

Altogether, our results establish that C910 acts on EEA1-positive early endosomes homeostasis compromising the progression of two AB toxins along their respective endolysosomal or retrograde pathways.

C910 inhibits cell infection by influenza A virus H1N1 and SARS-CoV-2

The emergence of SARS-CoV-2, the etiologic agent of coronavirus disease (COVID) 2019, resulted in a global pandemic affecting more than one billion people. Flu pandemics include the devastating Spanish flu (> 50 million death worldwide) and the more recent 2009 H1N1 pandemic. These repeated pandemic episodes of severe pneumonia of viral origin call for developing broad anti-infective strategies against viral pathogens. Though more sophisticated than toxins, several viruses share with them physicochemical and host proteases requirements found inside endosomes to invade host cells. Well-defined influenza A virus (IAV) trafficking in host-cell compartments shows that viral particles enter cells by multiple endocytic mechanisms and traffic through EEA1-positive early endosomes prior to the release of genetic material into the cytosol from late endocytic compartments (45, 46). Infection of cells by SARS-CoV-2 involves its binding to the angiotensin converting enzyme 2 (ACE2) receptor (47) and processing of the spike glycoprotein by host proteases at the plasma membrane and in late endosomes for membrane fusion (48). Processing of the spike protein expressed at the plasma membrane of infected cells by TMPRSS2 serine protease also facilitates cell-cell fusion in syncytia formation (49).

We first examined the effect of C910 on a single IAV infectious cycle. A549 human pulmonary epithelial cells, pretreated with C910 or vehicle alone, were infected with a reporter H1N1_{WSN} virus expressing the mCitrine fluorescent protein. Expression of mCitrine from this reporter virus reflects viral genome replication (50). We monitored the efficiency of cell infection by flow cytometry at a short timing of 6 h and recorded that C910 treatment reduced the percentage of infected cells by 3.6 fold (Figure 6A, left panels). We then monitored the effect of C910 on multiple-cycle of infection. At a longer time of

infection, C910 decreased the viral titer by 25.8 fold (Figure 6A, right panel), thereby establishing the strong inhibitory action of C910 on cell infection by IAV.

As for many viruses, SARS-CoV-2 relies on components of endocytic pathways to infect cells (51, 52). This encompasses the activity of PIKfyve kinase that resides predominantly on early endosomes to regulate endomembrane homeostasis, also being the specific target of Apilimod, a compound that blocks SARS-CoV-2 replication (51). We tested the potential protective effect of C910 on U2OS cells expressing human ACE2 (U2OS-ACE2), which are highly sensitive to SARS-CoV-2 (49). These cells express functional GFP upon productive infection and fusion with neighboring cells, providing a convenient readout of infection. U2OS-ACE2 were preincubated with C910 before viral exposure, and viral infection was revealed after a long time of infection. The compound C910 inhibited SARS-CoV-2 infection with an IC_{50} of $0.74 \pm 0.07 \mu M$ (Figure 6B). Collectively, these results widen the protective spectrum of C910 against two RNA viruses that proceed through the endolysosomal pathway.

Both C910 and the PIKfyve inhibitor Apilimod induced swelling of EEA1-positive early endosomes (Sup. Figure 6A) (53). This suggested a possible PIKfyve inhibition in C910 action. Nevertheless, we found that Apilimod had no protective effect on CNF1-mediated degradation of Rac1 (Sup. Figure 6B) and conversely C910 did not inhibit PIKfyve kinase activity in vitro (Sup. Figure 6C). In conclusion, C910 bears broad anti-infective properties through a molecular mechanism different from that of Apilimod.

C910 accumulates in lung tissues and protects mice against lethal Influenza

The pharmacokinetic (PK) parameters and pulmonary biodistribution of C910 following intraperitoneal administration was first defined. Supplementary Figure 7A shows that with a dose of 10 mg/kg, the plasmatic concentration of C910 reached a maximum (C_{max}) of 164 ± 33 nM at 15 min, followed by a sharp drop to a prolonged plateau of about 25 nM for at least 24 h. C910 displays a half-life ($T_{1/2}$) of 28.5 ± 4.3 h in the plasma, indicating a long exposure and slow elimination from the organism. Following a single intraperitoneal injection of C910 at 20 mg/kg/mouse, we estimated its concentration in lung tissues to 100-116 μM at 1 h and 61-110 μM at 4 h. We pursued by investigating the effect of intraperitoneal injection of 20 mg/kg of C910 on major blood chemistry parameters, e.g. markers of hepatic, pancreatic, renal and cardio-muscular functions using an approved

357 field portable clinical device. No toxicity and no statistically significant differences were
 358 found between treated and vehicle control animals 24 h after administration (Sup. Figure
 359 7B). Thus, despite low plasmatic concentrations, C910 accumulates in lung tissues with no
 360 recorded toxic effects and reaches for several hours effective inhibitory concentrations
 361 against IAV H1N1.

362

363 We then proceeded to proof of concept experiments starting with a prophylactic setting.
 364 C57BL/6 mice were intraperitoneally treated with C910 and then challenged with an
 365 infectious IAV (H1N1 PR/8 strain) (Figure 7A). At a low dose of virus known to induce
 366 weight loss and subsequent mortality, we recorded a complete protection of C910-treated
 367 mice, while the vehicle control-treated animals had to be euthanized during the course of
 368 infection due to excessive weight loss or serious symptoms (Sup. Figure 7C and 7D).
 369 When animals were challenged with a higher dose of virus in the same experimental
 370 setting, we recorded that C910 treatment improved mice survival (Figure 7B and 7C).
 371 Indeed, half of C910 treated-mice survived while all of the vehicle-treated mice had to be
 372 euthanized, starting at day 5. In a therapeutic setting (Figure 7D), repeated injections of
 373 C910 starting 24 hours after viral infection significantly increased mice survival and
 374 attenuated weight loss (Figure 7E and 7F). Taken collectively, these findings document a
 375 beneficial effect of C910 on mice infected with the influenza A virus, which can be
 376 attributed to the accumulation of C910 in lung tissues at an effective dose defined *in vitro*.

377 We conclude that C910 displays an extended protective spectrum against eight bacterial
 378 AB toxins and two viruses via an original molecular mechanism of action on early
 379 endosome sorting functions.

DISCUSSION

We have isolated by HTS a piperidinamine-derived chemical compound that protects host cells against a wide spectrum of structurally unrelated AB toxins hijacking endolysosomal or retrograde membrane trafficking pathways. Mechanistically, we establish that C910 affects the morphology of Rab5/EEA1-positive endosomes and the routing of CNF1 and STxB to late endosomes and to the trans-Golgi network (TGN), respectively. We leveraged C910 properties to protect cells against infection by UPEC, IAV and SARS-CoV-2, and to confer protection of mice against IAV subtype H1N1 as well.

We show that direct monitoring of Rac1 cellular levels in response to CNF1 intoxication coupled to orthogonal screens allow the identification of a chemical agent acting on the two major endosomal pathways hijacked by AB toxins and several viruses. Direct measurement of the protective effect of chemical compounds on the target of toxins likely avoid identification of inhibitors acting indirectly via rescuing the downstream cytotoxic effects of specific toxin's catalytic action. For example, the autophagy inhibitor 3-MA reverted autophagy-mediated cell death triggered by Stx2 via ER-stress pathway (54). Interestingly, we show that including orthogonal screens with DT and Stx1 is a stringent procedure that nonetheless allows the identification of a compound targeting the two major vesicular pathways hijacked by AB-like toxins, as compared to other anti-toxin chemicals identified to date (Sup. Table 2). These compounds comprise EGA and ABMA that were selected from HTS against the lethal toxin from *Bacillus anthracis* and the plant toxin ricin, respectively (2, 4). Retro-2 was selected according to the same procedure than ABMA, showing protection against ricin and Stx toxins (22). As summarized in the supplementary Table 2, these inhibitors display a specificity of action on a large panel of toxins trafficking through one of the two major pathways, as opposed to the broader spectrum of action of our newly discovered C910 compound. For example, C910 is able to inhibit Stx1/2, which are excluded from the anti-toxin spectra of ABMA and EGA. Moreover, Retro-2 is devoid of a protective effect against endolysosomal based-toxins, e.g. DT and CNF1 (unpublished data).

We attribute a vesicular traffic disruptor function to C910 on EEA1-positive early endosomes. Indeed, C910 does not affect the binding and endocytosis of CNF1 and Stx1 while it induces the swelling of Rab5/EEA1-positive early endosomes and alters their

sorting functions. C910 affects the morphology of early endosome, in this window of treatment without inducing detectable changes on the subcellular distribution and morphology of other organelles studied. Diphtheria toxin translocation occurs primarily at the level of matured early endosomes (27). Our findings suggesting that C910 corrupts early endosome maturation are consistent with the recorded protection against DT that exits from matured early endosomes. Alteration of early endosome morphology by C910 resembles the phenotype induced by inhibition of the phosphoinositide 5-kinase PIKfyve that synthesizes phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂) from PtdIns(3)P (55). Of note, the knock-down of PIKfyve also affects the retrograde trafficking from early endosome to the TGN, and PIKfyve inhibitor shows cell protection against SARS-CoV-2 (51, 56, 57). Nevertheless, PIKfyve inhibition by Apilimod did not protect cells against CNF1, while it triggered early endosome enlargement at nanomolar range. Moreover, C910 has no inhibitory effect on recombinant PIKfyve kinase activity *in vitro*. Together, these data indicate that C910 displays an original molecular mechanism of action, different from that of Apilimod, although both molecules affect EEA1-positive early endosomes and display anti-infective properties. Characterization of the host target of C910 will thus contribute to define key steps in the vesicular trafficking exploited by numerous bacterial toxins and viruses.

We have previously screened 1,120 off-patent drugs from the Prestwick Chemical Library for their capacity to protect cells against CNF1-induced Rac1 depletion for drug repurposing. Nevertheless, the identified compounds did not allow further development in the clinic. Indeed, their antitoxin activities required concentrations higher than their safety range, and in some instances, they had effects on multiple cellular organelles (58, 59). The extended spectrum of protection conferred by C910 here points to its valuable pharmacological properties as much as it displays animal protection against IAV H1N1 with no recorded toxicity. As found for UPEC, C910 might protect cells from the pathogenicity triggered by bacterial pathogens responsible for severe or highly prevalent infections with limited susceptibility to antibiotics. This also relates to recurrent infections caused by *Clostridium difficile*. Development of host-targeted therapy is of particular interest against infections caused by Shiga toxin (Stx)-producing *E. coli* (STEC) as adverse effects cannot be fought by antibiotics (60) or CNF1-producing UPEC for which anti-microbial resistance is a growing concern (5). Interestingly, we show that C910 displays a higher protection against Stx2 compared with Stx1. Each toxin hijack different

early endosome-Golgi transport pathways but Stx2 is responsible for triggering the hemolytic and uremic syndrome as a consequence of STEC infection in humans (8). Owing to the synthesis of LCGTs, *Clostridium difficile* induces antibiotic and healthcare-associated diarrhea with roughly 10% mortality rate and a high recurrence rate of approximately 20%. There is an urgent need in identifying novel treatments targeting LCGTs pointing to the importance of further characterizing C910 mechanism of action (61). The broad-spectrum of protection conferred by C910 also endows this molecule with promise against toxins from *Bacillus anthracis*, a pathogen rarely responsible for human infections and which is not the object of large vaccination campaigns but of concern of misuse. Therefore, host-targeted therapy targeting host vesicular trafficking represents an alternative strategy in the event of diversion of such a deadly pathogen.

Previous studies have shown that host-targeted prophylactic strategy endows the anti-toxin inhibitors an expanded efficacy against viruses (2, 4, 62, 63). Nevertheless, this is the first time to our knowledge that an anti-toxin inhibitor exhibits prophylactic potential against influenza A virus H1N1. In pilot experiments, we recorded a protective response induced by C910 in the group of male mice contrary to females (data not shown). This bias likely relates to the reported exacerbation of IAV-associated pathogenesis in female mice (64). Moreover, differences of C910 efficacy between male and female mice may reflect differences of pharmacokinetics.

Owing to its capacity of targeting two major vesicular trafficking pathways exploited by bacterial AB toxins and several viruses, C910 is a valuable tool to study membrane trafficking and help in the development of anti-infective compounds. The rapid evolution of the influenza virus complicates effective vaccine design therefore small molecule inhibitors for the treatment of viral infections are urgently needed. There is a number of antivirals in the clinic or in development, many of them targeting viral proteins. However, resistance against these antivirals rapidly emerges due to mutations in viral proteins (51, 65). Thus, the principle of new host-directed antitoxin and antiviral molecules such as C910 deserves further studies.

478 MATERIALS AND METHODS

479 Cell culture and bacterial toxins

480 Human umbilical vein endothelial cells (HUVECs) (PromoCell, Heidelberg, Germany) were
 481 cultured as described (24). HeLa, L929, Vero, A549, Vero E6 and U2OS-ACE2 cell lines
 482 were cultured at 37°C in 5% CO₂ in DMEM/GlutaMax (Invitrogen) supplemented with 10%
 483 heat-inactivated FBS (F9665, Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen).
 484 DT(D0564) was purchased from Sigma-Aldrich, Stx1 (#161) and Stx2 (#162) from List
 485 Biological, CNF1 was purified as described previously (58); TcdA and TcdB were purified
 486 from *C. difficile* VPI10463 as described (66); protective antigen and lethal factor from
 487 *Bacillus anthracis* were purified as described (67); PE toxin was kindly provided by Bruno
 488 Beaumelle (UMR5236 CNRS, University of Montpellier, France); STxB was purified and
 489 labeled with Alexa Fluor 488, as described (68, 69).

490 Antibodies and reagents

491 C910 was purchased from Chembridge (ID: 5454910, San Diego, CA, USA) or
 492 Synthenova (ID: SN0218L3, Hérouville Saint-Clair, France). The following products were
 493 purchased from the indicated commercial sources: L-[¹⁴C(U)]-leucine was from Perkin-
 494 Elmer; DMSO (D4540), Hoechst 33342 (B2261), Gelatin (G9361), Saponin (S-7900),
 495 Bafilomycin A1 (B1793), mouse anti-actin (ac-74), HIS-tagged B subunit of Shiga toxin
 496 1(SML0655) and Apilimod (A149227) from Sigma; rabbit anti-EEA1 (#3288), rabbit anti-
 497 Rab7 (#9367), rabbit-anti-his tag (#12698) from Cell Signaling; rabbit anti-Rab7
 498 (ab137029), mouse anti-Giantin (ab37266) and Cathepsin B Activity Assay Kit (ab65300)
 499 from Abcam; mouse-anti-6x-his (R930-25 [clone 3D5]), DQ™ Red BSA (D-112051) from
 500 ThermoFisher Scientific; mouse-anti-EEA1 (BD610457), mouse-anti-Rab5 (610724),
 501 mouse anti-Rac1 (610651 [clone 102]), mouse anti-Rab4 (610888), mouse anti-Lamp1
 502 (611043) was from BD Bioscience; mouse anti-TGN38 (sc-101273), rabbit anti-MEK2 (SC-
 503 524) from Santa Cruz; rabbit anti-Calnexin (ADI-SPA-860-D) from Enzo; mouse-anti-LBPA
 504 (Z-PLBPA) was from Echelon Biosciences; Paraformaldehyde (PFA) (15710) from
 505 Electron Microscopy science. Fluorescence-tagged secondary antibodies were purchased
 506 from Thermofisher and used at 1:500 dilutions: Alexa Fluor 594 donkey anti-mouse (A-
 507 21203), Alexa Fluor 594 donkey anti-rabbit (A-21207), Alexa Fluor 488 donkey anti-mouse
 508 (A-21202), Alexa Fluor 488 donkey anti-rabbit (A-20206), Alexa Fluor 647 donkey anti-
 509 mouse (A-31571). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (P0399) or

swine anti-rabbit secondary antibodies (P0447) were from Dako.

Orthogonal screening pipeline

All steps of compound screening were performed in gelatin-coated Nunc MicroWell 96 Well opaque plates (Thermo Scientific) seeded with 20,000 HUVECs per well. During the first round of screen, we tested 16,840 compounds from the ChemBridge DIVERSet library at a working concentration of 50 μ M in the presence of CNF1 10 nM during 6 h (Z' values > 0.5).

$$Z' = 1 - \frac{(3SDx + 3SDy)}{\text{mean}(x) \text{mean}(y)}$$

Rac1 cellular levels were determined by direct immunolabeling of cells, as followed. Fixation of HUVECs was performed in 4% PFA, 15 min. Permeabilization and saturation were performed concomitantly in D-PBS supplemented with 1% donkey serum, 0.1% Triton X-100 and 0.05% Tween-20 during 1 h. Cells were next labelled with an anti-Rac1 mouse antibody [clone 102] for 1 h and revealed with Alexa Fluor 488-coupled anti-mouse secondary antibody. Rac1 immunostaining was quantified using a Cytation 5 reader (BioTek) in a fluorescence intensity mode (λ_{Ex} = 485 nm; λ_{Em} = 520 nm) with a 3 x 3 area scan mode per well. Hit compounds were cherry-picked and reevaluated following the same procedure with another secondary antibody coupled with Alexa Fluor 594 (λ_{Ex} = 590 nm; λ_{Em} = 620 nm) to exclude auto fluorescent molecules. Selected hits were then reordered and freshly prepared for a third round of screen. Defined hit compounds were further filtered from pan-assay interference compounds (PAINS), i.e. unstable molecules, irreversible modifiers or compounds that are frequently active in other screens (25). Eleven compounds were shortlisted as robust inhibitors of CNF1-mediated degradation of Rac1 and went through a series of two orthogonal screens to define their protective effects first against DT and then Stx1. Compound N-(3,3-diphenylpropyl)-1-propyl-4-piperidinamine, here referred as C910, passed all the screens. Of note, C910 and the unrelated (S)-2-amino-N-(1-((5-chloro-1*H*-indol-3-yl)methyl)piperidin-4-yl)-3-(1*H*-indol-3-yl)propanamide (referred to as API or SN0209) have been isolated for their capacity to alter the interaction of SOS with p21-Ras GTPase *in vitro* (70). Co-treatment of cells with API ranging from 5 to 10 μ M did not protect cells against CNF1 ruling out a possible protective action of C910 through the SOS/Ras signaling (not shown).

Evaluating efficacy of C910 against intoxication

Protein synthesis inhibition was measured as described (22). Briefly, cells were plated overnight in 96-well Cytostar-T microplates with scintillator incorporated into the polystyrene. Cells were then challenged with increasing doses of toxins for the indicated periods of time (DT: 6 h; Stx1, Stx2, PE: 18 h) in presence of vehicle or various concentrations of C910. The medium was replaced with DMEM supplemented with L-[¹⁴C(U)]-leucine at 0.5 µCi/ml for an additional 3 h (Stx1, Stx2, PE) or 15 h (DT). Protein synthesis was determined using a Wallac 1450 MicroBeta scintillation counter (PerkinElmer). All values are expressed as means of duplicates ± s.e.m., data were fitted using the nonlinear regression dose-response and the goodness-of-fit for toxin alone (carrier) or with drugs was assessed by r^2 values and confidence intervals with Prism ver. 8 software (GraphPad, San Diego, CA).

Cytotoxic effects of the LCGTs TcdA and TcdB on Vero cells (Sigma-Aldrich) were evaluated by measure of cell rounding. Vero cells seeded in 96-well plates at a density of 10,000 cells per well one day before, were intoxicated with 8 cytotoxic units (CU) of TcdA or TcdB for 8 h in the absence or presence of C910 at 0, 5, 10 and 20 µM. Cell rounding was visualized by phase-contrast microscopy. In parallel, cell lysates from intoxicated cells were examined for glucosylated-Rac1 that no longer react to anti-Rac1 monoclonal antibody [clone 102].

The activity of LT from *B. anthracis* was assessed by determination of MEK2 cleavage. Cell lysates from HUVECs intoxicated with two concentration of LT (0.3 µg/ml protective antigen (PA) + 0.1 µg/ml Lethal factor (LF) or 0.03 µg/ml PA + 0.1 µg/ml LF) in the absence or presence of C910 at 40 µM for 1, 2, 4, 8 h were collected and examined for MEK2 protein levels by immunoblot. GAPDH was used as the loading control.

Western blot analysis

Samples were size-separated via SDS-PAGE, transferred to Immobilon-P PVDF membranes (Millipore) and labelled by primary antibodies. Signals were revealed using horseradish peroxidase-conjugated goat anti-mouse or swine anti-rabbit secondary antibodies (DAKO) followed by chemiluminescence using Immobilon® Western (Millipore). The chemiluminescent signals were recorded on a Syngene PXi imager (OZYME), and the data were quantified using Fiji software (NIH).

Binding of CNF1 to cells analyzed by FACS

573 Recombinant CNF1 toxin was fluorescently labeled with Cy3 (Cy®3 Mono 5-pack, GE
574 healthcare, PA23001) according to the vendor's recommendations. Briefly, purified toxin
575 preparations (1 mg/ml) were incubated with the Cy3 dye in 100 mM bicarbonate buffer for
576 18 h at 4°C and applied to dialysis to separate the toxin from the free dye. CNF1-Cy3 (16
577 nM) was absorbed to the cells at 4°C for 30 min and cells processed for FACS analysis
578 using a BD LSRFortessa™ flow cytometer. Data were analyzed with FlowJo (BD
579 Bioscience).

580 Fluid-phase transport to lysosomes

581 HUVECs were seeded in fibronectin pretreated-μ-Slide 8 Well Glass Bottom (80827, ibidi)
582 one day before. Cells were pulsed with DQ-BSA (DQ™ Red BSA) at a concentration of 10
583 μg/ml for 30 min (37°C, 5% CO₂). The cells were then washed three times with PBS before
584 being treated with compounds in the complete medium at 37°C for the indicated time,
585 nuclei were labeled with Hoechst 33342. Fluorescence of live cells were acquired with a
586 Perkin Elmer Ultraview spinning disk confocal microscope using 60X/1.2 NA water
587 objective.

588 Immunocytochemistry, confocal imaging and image quantification

589 Immunocytochemistry was performed on cells fixed with 4% PFA in PBS for 15 min at
590 room temperature. Next, cells were permeabilized with 0.1% Saponin in PBS for 10 min
591 and blocked with 0.2% gelatin in PBS. Immunolabeling with indicated antibodies was
592 performed in 0.05% Saponin and 0.1% gelatin in PBS Buffer. Images were acquired using
593 a Perkin Elmer Ultraview spinning disk confocal microscope using 60X/1.2 NA oil objective.
594 Fiji ImageJ software (National Institutes of Health) was used for image processing and
595 quantification.

596

597 Measures of co-localization between two fluorescence signals of interest are expressed as
598 Pearson's coefficient correlation or Mander's overlap coefficient. Cells in each condition
599 were randomly selected and analyzed under the same threshold. Briefly, each individual
600 cell was selected from a single mid-z stack of the confocal image by hand-drawing a
601 "region of interest" (ROI) from Fiji software. The two channels of fluorescence were next
602 separated and analyzed with JACop plug-in.

603

604 Measures of the size of EEA1-positive vesicles were performed from the single mid-z

stack of confocal images that were next quantified with the Analyze particles tool from Fiji Software. Threshold values were maintained throughout each experimental set.

Bacterial infection experiments

The UTI89 uropathogenic *E. coli* (UPEC) mutant strain was described previously (71). *Salmonella enterica* serovar Thyphimurium strain SL1344 was kindly provided by Jost Enninga (Institut Pasteur, France). Bacteria internalization into HUVECs was assessed using the gentamycin protection assay. Briefly, HUVECs were seeded on 12-well plates at the density of 250.000 cells/well overnight (triplicate wells). When indicated, 1 nM CNF1 toxin was added after 30 min pre-treatment with 15 μ M C910. Exponentially growing UPEC ($OD_{600} = 0.6$ in LB) or *Salmonella* ($OD_{600} = 0.6$ in LB with 0.3 M NaCl) were added onto cells at MOI 100, followed by 20 min centrifugation at 1,000 x g. Cell infection was performed for 10 min at 37°C, 5% CO₂. Infected cells were washed three times with PBS and either lysed for cell-bound bacteria measurements or incubated another 30 min in the presence of 50 μ g/ml gentamicin before lysis for internalized bacteria measurements. Cells were lysed in PBS + 0.1% Triton X-100 and serial-diluted bacteria plated on LB-agar supplemented with 200 μ g/ml streptomycin or ML-agar for CFU counting.

IAV infection

For single-cycle IAV infection assays, A549 cells (70,000/ well) were infected with a reporter H1N1 A/WSN/33 (H1N1_{WSN} PB2-2A-mCitrine), at a MOI of 5 PFU/cell for 6 h. Cells were fixed in 4% PFA and analyzed by flow cytometry (Attune NxT; Thermo Fisher Scientific) as described in (50). For multicycle growth assays, A549 cells were infected with a seasonal A/Bretagne/7608/2009 (H1N1_{pdm09}) strain adapted to human cell lines (50), at the MOI of 10⁻³ infective particles/cell and the production of infectious particles in the culture supernatant was determined at 24 h using a standard plaque assay on the highly sensitive canine MDCK-SIAT cells, as described previously(72).

SARS-CoV-2 infection

U2OS-ACE2 GFP1-10 and GFP 11 cells, also termed S-Fuse cells, become GFP+ when they are productively infected by SARS-CoV-2 (49). Cells were mixed (ratio 1:1) and plated at 4x10³ per well in a μ Clear 96-well plate (Greiner Bio-One). The following day, cells were incubated with the indicated concentrations of C910 for 2 h at 37°C. SARS-

CoV-2 (BetaCoV/France/IDF0372/2020) (MOI 0.1) was then added. 20 h later, cells were fixed with 2% PFA, washed and stained with Hoechst 33342 (dilution 1: 1,000, Invitrogen). Images were acquired with an Opera Phenix high content confocal microscope (PerkinElmer). The GFP area and the number of nuclei were quantified using the Harmony software (PerkinElmer). The percentage of inhibition was calculated using the GFP area value with the following formula: $100 \times (1 - (\text{value with IgA/IgG} - \text{value in "non-infected"}) / (\text{value in "no IgA/IgG"} - \text{value in "non-infected"}))$.

In vivo IAV infection model

All mice were housed under specific-pathogen-free conditions at Seattle Children's Research Institute and all animal experiments performed at Seattle Children's Research Institute were approved by the Institutional Animal Care and Use Committee (IACUC00580). A pilot experiment showed a protective effect of C910 in male animals therefore we used male mice for further experiments (data not shown). Viral challenges were carried out on groups of 9~10 week-old C57BL/6 male mice (Charles River Laboratories). Animals were treated by intraperitoneal injection with either 20 mg/kg of C910 or vehicle control DMSO as described in the figures. Anesthetized mice were infected intranasally with the IAV H1N1 PR/8 strain (Charles River Laboratories) diluted in 25 μ L PBS, at a high dose (1.5×10^5 PFU/mouse, Figure 7A-C) and at a low dose (5×10^3 PFU/mouse, Sup. Figure 7D-E) in prophylactic setting as well as a dose of 9.5×10^3 PFU/mouse (Figure 7D-F) in treatment setting. Mice were monitored daily for weight loss and any other signs of disease.

Statistics

All data are presented as mean $\pm$ s.d. or mean $\pm$ s.e.m., unless specified in the figure legends. Student's *t*-tests were applied to determine the statistical significance between two datasets and one-way ANOVA was used to perform analyses between more than two datasets, unless specified in the figure legends. A value of $p \leq 0.05$ was considered significant (denoted * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Graph Pad Prism Software 8 was used to produce the graphs and to perform statistical analyses.

ACKNOWLEDGEMENT

We thank Cezarela Hoxha, Laurent Audry, Yuen-Yan Chang, Sylvain Meunier (Institut

Pasteur, Paris, France) and Imène Belhaouane (Institut Pasteur, Lille) for technical involvement as well as Caroline Stefani (Benaroya Research Institute, USA), Daniel Ladant (Inst. Pasteur) and Jean-Nicolas Tournier (IRBA) for fruitful discussions. This work was funded by the joint ministerial program of Research and Development against Chemical, Biological, Radiological, Nuclear and Explosive risks (CBRNE), a grant from the Agence Nationale de la Recherche (ANR-19-ASTR-0001), Labex IBEID (ANR-10-LABX-62-IBEID), the French Alternative Energies and Atomic Energy Commission (CEA). N.M., L.C., F.V. and E.P. were supported by DGA-MRIS/AID scholarships with CEA and Pasteur, respectively. SIMoS and SCBM are members of the Laboratory of Excellence LERMIT, supported by a grant from the Agence Nationale de la Recherche (ANR-10-LABX-33).

COMPETING INTERESTS

All authors do not have any financial or other interests related to the submitted work.

AUTHOR CONTRIBUTIONS

E. L. and D.G. coordinated the research; J.B. directed HTS; N.M., J-C.C. and J.B. operated the HTS and analyzed results; Y.W. and E.P. performed cell biology experiment; L.S., Y.W., N.M., E.P., and V.K. performed measurements of C910 efficacy on toxins; IAV H1N1 experiments were performed by C.D and S.V. (*in vitro*) and S.G and M.A (*in vivo*); SARS-CoV-2 experiments was performed by F.G. and O.S.; S.P. and A. Mettouchi. conceived and operated bacterial invasion experiments; M.S. and Y.W. performed electron microscopy experiments; L.C., P.C., R.T., P. Barbe., M.K., P. Bordin., A. Machelart., V.S. and F.T. contributed to pharmacokinetics and biodistribution studies, M.K. and R.T. established C910 concentration in biological samples; G.C. and B.P. performed *in vitro* PIKfyve activity measurements; M-R.P. and L.J. supplied LCGTs and STxB respectively; J-C.C. and F.V. supplied chemical compounds; Y.W., D.G. and E.L. drafted the manuscript. All authors read and approved the manuscript.

FIGURE LEGENDS

Figure 1. High-throughput screen of broad-spectrum anti-toxin inhibitors.

A: General scheme and results of the screening pipeline developed to isolate small chemical compounds inhibiting CNF1 and bacterial AB toxins hijacking the endolysosomal (DT for diphtheria toxin) or retrograde (Stx 1 for Shiga like toxin) pathways, respectively. Chemical structure of N-(3,3-diphenylpropyl)-1-propyl-4-piperidinamine, referred to as C910. B: ChemBridge library compounds were screened on HUVECs. Upper black points represent positive controls (100% Rac1 signal in control HUVECs), lower black points are negative controls corresponding to Rac1 level set to 0% in HUVECs exposed to 10 nM CNF1, 6 h, while red points correspond to HUVECs incubated with library compounds in the presence of 10 nM CNF1, 6 h. Cutoff was set for 30% of Rac1 signal. C-E: Graphs show protein synthesis inhibition induced by Stx1, DT and PE. (C and D) HeLa cells were pretreated 1 h in DMEM with C910 at indicated concentrations (color filled circles), or DMSO vehicle (black circles) prior to addition of increasing concentrations of Stx1 (C) or DT (D) for 18 h and 6 h, respectively. Each point represents the mean of duplicates $\pm$ s.e.m. of one representative experiment ($n = 2$). E: L929 cells were incubated for 1 h with C910 (color filled circles), or with DMSO vehicle (black circles) before addition of PE for 18 h at indicated concentrations. Protein synthesis was measured by scintillation counting. Each point represents the mean of duplicates $\pm$ s.e.m. of one representative experiment ($n = 2$). F: Graph shows Rac1 cellular level in the presence of increasing concentrations of C910. HUVECs were intoxicated with 10 nM CNF1 for 6 h in the presence or absence of C910 at the indicated concentrations. Each point represents the mean of duplicates $\pm$ s.e.m. of one representative experiment ($n = 2$).

Figure 2. C910 protects host cells against UPEC invasion mediated by CNF1.

A-E: Measure of cell invasion by uropathogenic *E. coli* UTI89 Δ hlyA Δ cnf1 (UPEC) and *Salmonella enterica* serovar Typhimurium SL1344 (Salmonella). HUVECs were pre-incubated 30 min with 15 μ M C910 or vehicle prior to infection during 2.5 h by UPEC in the absence or presence of 1 nM CNF1. HUVECs were incubated 3 h with 15 μ M C910 or vehicle prior to infection with *Salmonella* in the presence or absence of C910 during 1 h. Graphs show number of viable bacteria associated to the cells (A and D) or intracellular bacteria resistant to gentamicin treatment (B and E). Values correspond to colony forming units (CFU)/ml, presented as mean of $\pm$ s.e.m. from three independent experiments. One-

way ANOVA was performed overall ($p < 0.0001$) with Tukey's multiple comparison test to compare the treated conditions in (A) and (B), **** $p < 0.0001$ or ns: not significant. Data in (D) and (E) were analyzed with unpaired two-tailed t -test, ns: not significant. (C) Immunoblot anti-Rac1 shows that C910 blocked CNF1-mediated Rac1 depletion during cell infection by UPEC, as described in (B) and that C910 and/or *Salmonella* infection, as described in (E), did not affect Rac1 cellular levels. Anti-GAPDH immunoblot was used as a loading control.

Figure 3. C910 protects host cells against lethal toxin from *B. anthracis* and large glucosylating toxins from *C. difficile*.

A: Immunoblots show kinetics of MEK2 cleavage at two concentrations of lethal toxin, PA 0.3 $\mu\text{g/ml}$ / LF 0.1 $\mu\text{g/ml}$ (LT) or diluted 1/10 (LT_{1/10}) together with the protection conferred by C910 at 40 μM . Anti-GAPDH immunoblots show loading control. The blots are representative of four independent experiments. B: Graph shows nonlinear regression curves of normalized MEK2 level as a function of time (h) ($R^2 > 0.9$), in conditions described in (A). Percentage of MEK2 signal in HUVECs intoxicated for the indicated periods of time with LT (red circles) and LT_{1/10} (orange circles) in the presence of DMSO vehicle (plain circles) or C910 at 40 μM (open circles). Each point represents mean $\pm$ s.d. from four independent experiments. Data were fitted to obtain the mean time ($t_{1/2}$) of MEK2 cleavage and the protection factors (i.e., $P = t_{1/2, \text{LT+C910}} / t_{1/2, \text{LT}}$). C: Vero cells intoxicated with 8 cytotoxic units (cu) of TcdA or TcdB for 8 hours in the presence of DMSO vehicle or C910 at indicated concentrations. Scale bar, 10 μm . D: Anti-Rac1 immunoblots show the non-glucosylated form of Rac1. Vero cells were treated as described in (C). Lower graph corresponds to Rac1 signals normalized to GAPDH and set to 100% for control condition, data are expressed as mean $\pm$ s.d. from three independent experiments. Two-way ANOVA with Tukey's multiple comparison test was used to compare toxin-treated cells with non-intoxicated controls in the presence of C910 at different concentrations, **** $p < 0.0001$ or ns: not significant.

Figure 4. Absence of impact of C910 on CNF1 and STxB entry into cells.

A: HUVECs were pre-treated with 40 μM C910 or DMSO for 2 h, detached and incubated with labeled CNF1-Cy3 (1/500) on ice for 30 min in the presence or absence of C910 prior to flow cytometry analysis. Signal of non-treated cells was used as background. MFI (Mean Fluorescence Intensity) of C910-treated cells was normalized to that of DMSO-

treated cells. Histogram represents mean $\pm$ s.e.m. of three independent experiments. ns: not significant, paired two-tailed *t*-test. B: Representative images show the internalization of CNF1-Cy3 (red) in HUVECs and STxB-his (green) in HeLa cells, nuclei were stained with DAPI (blue). Cells were pre-treated with C910 or DMSO for 30 min at 37°C, then incubated with CNF1-Cy3 (red) or STxB-his (green) on ice for 30 min and followed by incubation for 15 min at 37°C in the continues presence of C910 or DMSO. Scale bar, 20 μ m. C: Representative images show the level of DQ-BSA cleavage (red) in live cells treated 4 h with DMSO, 20 μ M C910 or 100 nM Bafilomycin A1 (Baf A1). Scale bar, 20 μ m.

Figure 5. C910 alters EEA1-positive early endosomes morphology and function.

A-D: immunofluorescence analyses of C910 specific effect on EEA1/Rab5 compartments and CNF1 or STxB progression along the endolysosomal and retrograde pathways, respectively. Nuclei were labeled with DAPI (blue). Scale bar, 10 μ M. A: HUVECs were treated with 40 μ M C910 or DMSO for 45 min and labeled for the indicated markers of intracellular compartments. B: Images of EEA1- and Rab5-positive compartments in HUVECs treated as in (A). The size of EEA1-positive particles was quantified and normalized to control, and displayed as mean $\pm$ s.e.m., *n* > 20 cells in one representative experiment (*n* = 2). ** *p* < 0.01, unpaired two-tailed *t*-test. C: Images of EEA1- and Rab7-positive compartments in HUVECs pre-treated with 40 μ M C910 or DMSO vehicle for 30 min followed by incubation with CNF1-Cy3 for 90 min in the presence of C910 or DMSO. C: Line plots show the intensity of fluorescence along the line. D: HeLa cells were pre-treated for 5 min with 40 μ M C910 or DMSO before addition of Alexa Fluor 488-STxB (STxB, 1/5000, 4°C for 30 min; green signal), followed by incubation for 55 min at 37°C in the presence of C910 or DMSO. Cells were then labeled for EEA1 (red) and Giantin (pink). Right graphs show Mander's overlap coefficients between STxB and EEA1 as well as STxB and Giantin, expressed as arbitrary unit (a.u.). Data show mean $\pm$ s.e.m. for 44 (DMSO) and 53 (C910) cells from three independent experiments. **** *p* < 0.0001, unpaired two-tailed *t*-test.

Figure 6. C910 inhibits cellular infection by IAV H1N1 and SARS-CoV-2.

A: Left, Effect of C910 on IAV H1N1 single cycle. A549 were pre-treated with C910 (30 μ M) or DMSO alone for 1 h at 37°C prior to infection by H1N1_{WSN} mCitrin reporter at a MOI of 5 in the presence of C910 30 μ M or DMSO. At 6 h post-infection, cells were analyzed by flow cytometry. Mean Fluorescence Intensity (MFI) of non-infected or infected cells are

shown. Histogram represents mean $\pm$ s.e.m. of 6 replicates in one representative experiment. **** $p < 0.0001$, unpaired two-tailed t -test. Right, Inhibitory effect of C910 on H1N1 viral production. A549 were infected with H1N1_{pdm09} MOI at 0.001 in the presence of 30 μ M C910 or DMSO alone. Viral production was titrated 24 h later on cell supernatant by plaque forming assay. Histogram represents mean $\pm$ s.e.m of 3 replicates in one representative experiment. ** $p = 0.005$, unpaired two-tailed t -test. B: Schematic protocol of SARS-CoV-2 infection. U2OS-ACE2 GFP1-10 and GFP 11 cells were pre-incubated with C910 for 2 h and then SARS-CoV-2 (MOI = 0.1) was added for 20 h. The relative infection efficiency was calculated as the ratio between the GFP-area and the total amount of cells stained with DAPI. C: Data are shown as mean $\pm$ s.d. from duplicate wells, one representative experiment ($n = 3$).

Figure 7. C910 protects mice against IAV H1N1 infection.

A and D: time-line representation of the procedures. B-C: Survival curve for mortality (B) or weight loss (C) following intranasal infection with PR/8, as described in (A) are shown for mice treated with C910 ($n = 8$) or vehicle control DMSO ($n = 8$). E-F: Survival curve for mortality (E) or weight loss (F) following intranasal infection with PR/8 as described in (D) are shown for mice treated with C910 ($n = 11$) or vehicle control DMSO ($n = 10$). Mantel-Cox test was performed to compare the survival curve between groups injected with DMSO and C910. Weight loss data are shown as mean $\pm$ s.e.m, * $p < 0.05$, multiple t -test.

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Supplementary information

A screening pipeline identifies a broad-spectrum inhibitor of bacterial AB toxins with cross protection against influenza A virus H1N1 and SARS-CoV-2

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1123 **Materials and Methods**

1124

1125 Determination of EC₅₀s of C910 for DT, Stx1, Stx2 and PE

1126 The mean percentage of protein biosynthesis was determined and normalized from
 1127 duplicate wells. All values are expressed as means $\pm$ s.e.m. Data were fitted with Prism v8
 1128 software (Graphpad Inc., San Diego, CA) to obtain the toxin concentrations giving 50%
 1129 protein synthesis inhibition in absence or presence of compound (IC₅₀ drug vs IC₅₀ DMSO). The
 1130 protection factors R ($R = IC_{50 \text{ drug}} / IC_{50 \text{ DMSO}}$) were determined by the software's nonlinear
 1131 regression "dose-response EC₅₀ shift equation". For each concentration of C910, a
 1132 percentage of protection was determined from R values ($\% \text{ protection} = (R-1) / (R_{\text{max}} - 1)$
 1133 $\times 100$, with R_{max} corresponding to the highest value of R in the series). The concentration
 1134 of C910 was plotted against the corresponding percentage of protection of cells and the
 1135 50% efficacy concentration (EC₅₀) was calculated by non-linear regression.

1136

1137 Determination of CC₅₀s of C910 on HUVECs and HeLa cells

1138 Cells seeded in Corning™96 well Flat Clear Bottom Black Microplates (#3603) were
 1139 incubated with increasing doses of C910 for 6 h, medium was replaced with fresh medium
 1140 including 10% of Alamar Blue (final concentration 1:10). Then the fluorescence (540/590
 1141 nm) was measured 1h later by Cytation 5 cell imaging multi-mode reader (BioTek). The
 1142 mean percentage of toxicity was determined from duplicate wells. All values are expressed
 1143 as means $\pm$ s.e.m from one representative experiment. Data were fitted with Prism v8
 1144 software (Graphpad Inc., San Diego, CA) to obtain the concentrations giving 50% toxicity.

1145

1146 Electron microscopy

1147 After the uptake of BSA-gold (Cell Microscopy Core, Universitair Medisch Centrum Utrecht,
 1148 Netherland), HUVECs were fixed at room temperature for 2 h in 2.5% glutaraldehyde in
 1149 PHEM buffer, pH 7.2 (60 mM 1,4 piperazine diethylsulfonic acid (PIPES), 25 mM N-2-
 1150 hydroxyethylpiperazine NI-2-ethane sulfonic acid (HEPES), 10 mM EGTA, 2 mM MgCl₂,
 1151 pH 7.2). After washes with PHEM, cells were post-fixed with 1% osmium and 1.5%
 1152 potassium ferricyanide (Merck, Darmstadt, Germany) in PHEM before dehydration in a
 1153 graded series of ethanol and infiltration with epoxy resin. Resin was polymerized at 60°C
 1154 for 48 h. Sections with a nominal thickness of 70 nm were obtained with a UC7 (Leica
 1155 Microsystems, Vienna, Austria) and collected on formvar, carbon coated 200 mesh copper
 1156 grids (Electron Microscopy Sciences, Hatfield, PA, USA). Sections were contrasted with

1157 4% uranyl acetate and Reynold's lead citrate and observed with a Tecnai spirit
1158 (Thermofisher, Eindhoven, Netherlands) operated at 120 kV. Images were acquired using
1159 a Gatan Ultra Scan™ 4000 digital camera (Gatan, Pleasanton, USA).

1160

1161 *In vitro* PIKfyve activity

1162 Recombinant PIKfyve production and activity measurements were performed as described
1163 ¹. Briefly, PIKfyve was incubated with DMSO or compounds in the presence of
1164 PtdIns3P/PE lipid vesicles for 15 min at room temperature, prior to addition of [γ -³²P]-ATP
1165 and incubation at 37°C for 30 min. After lipid extraction and thin layer chromatography, the
1166 [³²P]-PtdIns(3,5)P₂ produced by PIKfyve was visualized by autoradiography.

1167

1168 C910 dosage and quantification by LC-MS/MS for pharmacokinetics and biodistribution 1169 studies

1170 Two stock solutions of C910 standard and another compound used as internal MS
1171 standard (IS) were prepared at concentrations of 10 mM in DMSO. For establishing
1172 calibration plots, known amounts of C910 were spiked into control mouse plasma or lung
1173 homogenates. To eliminate plasmatic proteins and other endogenous compounds, C910
1174 was extracted from plasma samples by two extractions with acetone. This involves
1175 addition of 4 volumes of ice-cold acetone and incubation for 1 h at -20°C. After
1176 centrifugation at 15,000 x g for 10 min, the supernatants containing C910 were collected.
1177 For each sample, both acetone supernatants were combined and dried at 40 °C for 3 h.
1178 Dried extracts were finally re-suspended into 25% acetonitrile (ACN) in water containing
1179 0.1% formic acid (FA) for analysis by LC-MS/MS Multiple Reaction Monitoring (MRM)
1180 mode.

1181

1182 Mouse lungs were homogenized using a Precellys/Cryolys Evolution homogenizer from
1183 Bertin, France, according to the manufacturer's recommendations using the CK28 ceramic
1184 beads 2 mL lysing kit. One hundred mg of organ in 400 μ L of cold PBS were grinded at
1185 4 °C by 3 to 6 cycles of 30 sec at 5,000 rpm with 15 s pause between each cycle until
1186 obtaining a smooth homogenate (3 animals per time point, two independent experiments).

1187

1188 To extract C910 from lung homogenates, samples were washed twice with ice-cold PBS
1189 (vortex, centrifugation at 15,000 x g for 10 min, elimination of the supernatant). Then, the
1190 pellets were resuspended, vortexed and centrifuged twice in DMSO to extract C910 and

both supernatants were combined. A fraction of this DMSO supernatant extract was diluted with 25% ACN in water containing 0.1% FA and analyzed by LC-MS/MS (MRM mode). The LC-MS/MS system consisted of an Agilent 1100 HPLC system on-line coupled to an Esquire-HCT ion Trap mass spectrometer (Bruker-Daltonics) equipped with an electrospray ionization (ESI) source. LC separation was carried out at a flow rate of 200 μ L/min on a ThermoScientific reverse phase Accucore-150 C18 column (2.1 x 50 mm; 2.6 μ m) with a linear gradient 0-100% B for 7 min with a mobile phase composed of 0.1% FA in water for A and 0.1% FA in ACN for B. ESI conditions and detection of C910 and products were optimized as well as for the internal MS standard IS spiked in samples for quantification. Fragmentation energy amplitudes were also optimized (SmartFrag off). MS detection was operated in MRM mode. The fragmentation transition followed for dosage of C910 was m/z 337.2 $\rightarrow$ 125.9. A second fragmentation transition was used to confirm the identity of C910 (m/z 337.2 $\rightarrow$ 238.0). The fragmentation transition followed for internal standard was m/z 436.0 $\rightarrow$ 284.9 (dosage) and m/z 436.0 $\rightarrow$ 254.9 (identity confirmation). Data Analysis software (Bruker Daltonics) was used for qualitative and quantitative processing of raw MS and MS/MS data. The ion chromatograms of MS/MS transitions of C910 as well as of the internal MS standard were extracted (EIC). These EIC peaks were integrated and the ratio C910/IS of the corresponding MS transitions enabled to plot calibration curves of C910 in plasma or lung homogenates and further quantify C910 in any of these biological matrices from mice treated with C910.

1211

1212 *In vivo* pharmacokinetics (PK), biodistribution and plasmatic diagnostic

Animal care and surgical procedures were performed according to the Directive 2010/63/EU of the European Parliament, which had been approved by the Ministry of Agriculture, France. The project was submitted to the French Ethics Committee CEEA (Comité d'Ethique en Expérimentation Animale) and obtained the authorization APAFIS#10108-2017060209348158 v3. Animal protocols in CIIL (Center for Infection and Immunity of Lille) were approved by the Minister of Higher Education and Research after favorable opinion of the Ethics Committee (CEEA Nord-Pas de Calais n°75/APAFIS#10232-2017061411305485 v6). All experiments were performed in accordance with relevant named guidelines and regulations. Experiments were conducted on adult male C57BL/6J mice (23.5 $\pm$ 0.3 g) purchased from Janvier Labs (Le Genest St Isle, France). Mice were injected intraperitoneally with 10 mg/kg, i.e. 29,716 nmol/kg, of C910. Blood samples were collected at 5 min, 15 min, 30 min, 45 min, 1 hr, 6 h and 24 h

1225 after injection, on three distinct mice at each time. Quantification of C910 concentration
 1226 contained in plasma at each time was performed by LC-MS/MS. PK parameters were
 1227 calculated from these data with the non-compartmental analysis Linear up/Log down
 1228 method, using the PKSolver add-in program. Plasmatic diagnostic was performed at 24
 1229 hrs after injection using the Piccolo® AmLyte 13 and the Piccolo Xpress® chemistry
 1230 analyzer (Abaxis, USA). Levels of glucose, blood urea nitrogen, creatinine, total bilirubin,
 1231 albumin, alanine aminotransferase, aspartate aminotransferase, creatine kinase, amylase,
 1232 sodium, potassium, and calcium were determined in heparinized plasma of five mice
 1233 injected with vehicle or C910. Quantitative data are shown as means, with error bars
 1234 indicating the standard error of the mean (SEM).

1235

Figure legends

Supplementary Figure S1. EC₅₀s of C910 for DT, Stx1 and Stx2 and its CC₅₀, corresponding to Fig 1C and E, F. Curves show different concentrations of C910 and the corresponding percentages of cell protection against Stx1(A), Stx2(B), DT(C) and PE(D). (E) Curves show different concentrations of C910 and the corresponding viabilities of HUVECs and HeLa from one representative experiment (n = 2).

Supplementary Figure S2. C910 does not affect CNF1 catalytic activity and STxB binding with membrane receptors.

A: Recombinant RhoA protein (1 µg) was incubated with CNF1 (0.1µg) in the presence or absence of C910 (1 µM) for the indicated time. The time-dependent upper-shift of RhoA shows the absence of effect of C910 on CNF1 deamidase activity. B: HeLa cells were pre-treated with 40 µM C910 or DMSO for 1 h at 37°C. Next, 3 mM EDTA-dissociated cells were incubated with STxB-his on ice for 30 min in the presence of 40 µM C910 or DMSO vehicle and subsequently fixed with 4% PFA, labelled by anti-his antibodies and Alexa Fluor 488-conjugated secondary antibodies and then analyzed by Flow Cytometry. Signal of cells without incubation with STxB-his was used as background. MFI (Mean Fluorescence Intensity) of C910-treated cells was normalized to DMSO-treated cells values. The histogram represents mean ± s.e.m. of four independent experiments, ns: not significant, paired two-tailed *t*-test.

Supplementary Figure S3. C910 does not inhibit cathepsin B activity.

A: Cell-free assay. Cell lysates mixed with cathepsin B substrate labelled with fluorescent probe, and incubated 2 h with DMSO vehicle, 20 µM C910 or a control inhibitor, provided in the kit. B: Cell-dependent assay. Lysates from HUVECs treated with DMSO vehicle or 20 µM C910 for 2 h were examined. Cell lysate supplemented with Bafilomycin A1 (Baf A1) 200 nM was used as control of inhibition of acid-dependent cathepsin B. Fluorescent intensity ($\lambda_{Ex}/\lambda_{Em}$ = 400/505 nm) from cleaved fluorescent probe was determined on FluoStar Omega (BMG Labtech) and normalized to DMSO vehicle, as a control. Data represent the mean ± s.e.m. of three independent experiments. ns: not significant, paired two-tailed *t*-test.

Supplementary Figure S4. Dose and time-dependent enlargement of EEA1-positive compartments in C910 treated cells.

1270 A: HUVECs were incubated with C910 or DMSO for the indicated periods of time, fixed,
1271 permeabilized and labeled for EEA1. Representative images are shown from two replicate
1272 experiments. Scale bar, 10 μ m. B: HUVECs were incubated with different doses of C910
1273 or DMSO for 20 h and then processed as in (A). The size of EEA1-positive vesicles from at
1274 least 20 cells in each condition are quantified by Image J and shown as mean $\pm$ s.e.m.
1275 One-way ANOVA was performed overall ($p < 0.0001$) with Dunnett's multiple comparison
1276 test to compare control and treated conditions, *** $p < 0.001$, ns: not significant. C:
1277 HUVECs were pre-treated with 20 μ M C910 or DMSO vehicle for 2 h and then pulsed with
1278 BSA-gold for 15 min in the presence of C910 or DMSO vehicle. Cells were processed for
1279 electron microscopy. Representative electron micrographs are shown. White arrows
1280 indicate BSA-gold. Right, the size of BSA-gold positive compartments was quantified and
1281 data presented as mean $\pm$ s.e.m. for 87 (DMSO) and 77 (C910) compartments. Each point
1282 represents an individual compartment, **** $p < 0.0001$, unpaired two-tailed t -test. Scale bar,
1283 100 nm.

1284

1285 **Supplementary Figure S5. C910 affects the trafficking of CNF1, corresponding to**
1286 **Figure 5C.**

1287 HUVECs were treated with 20 μ M C910 or DMSO as Figure 5C. Right, Pearson's
1288 coefficient of two fluorescence (CNF1-Cy3 and Rab7) was analyzed with Fiji software and
1289 JACoP plug-in. Data are presented as mean $\pm$ s.e.m. for 60 (DMSO) and 51 (C910) cells
1290 from three independent experiments. Each point represents an individual cell, **** $p <$
1291 0.0001, unpaired two-tailed t -tests. Scale bar, 10 μ m.

1292

1293 **Supplementary Figure S6. Distinctive mechanisms of action between Apilimod and**
1294 **C910.**

1295 A: Representative images show EEA1 staining of HUVECs incubated with DMSO or
1296 Apilimod at the indicated concentration for 2 h. Scale bar, 10 μ m. B: Immunoblots anti-
1297 Rac1 show levels of CNF1-mediated Rac1 degradation and protective effect of C910
1298 treatment on Rac1 degradation. In contrast, Apilimod has no significant protective effect at
1299 a broad range of concentrations. One representative experiment, $n = 2$. HUVECs were
1300 pre-incubated for 30 min with indicated concentrations of compounds prior to addition of
1301 CNF for 6 h. Anti-GAPDH was used as loading control. C: Representative images show
1302 radioactive labelled $PI_{(3,5)}P_2$ produced by recombinant PIKfyve in the presence of DMSO or
1303 compounds. In contrast to Apilimod, increasing doses of C910 had no effect on

1304 recombinant PIKfyve lipid kinase activity. One representative experiment, $n = 2$.

1305

1306 **Supplementary Figure S7. C910 PK, blood diagnostic and inhibition of IAV infection**
1307 ***in vivo*.**

1308 A: PK parameters calculated for C910 following an intraperitoneal administration at 10
1309 mg/kg in mice. Data are presented as mean $\pm$ s.e.m. of data from three experiments. B:
1310 Acute *in vivo* toxicology test was carried out on Balb/c mice. Five control animals received
1311 an i.p. injection of saline solution (0.9% NaCl) containing 5% DMSO and five animals
1312 received 20 mg/kg of C910 diluted in saline solution with 5% DMSO by the same route.
1313 The blood samples were collected after 24 h and then 100 μ L of plasma were analyzed by
1314 automated biochemical Piccolo Xpress® analyzer (Abaxis). Alanine aminotransferase
1315 (ALT), aspartate aminotransferase (AST), amylase (AMY), total bilirubin (TBIL), albumin
1316 (ALB), blood urea (BUN), creatinine (CRE), creatine kinase (CK). C-D: Survival (C) and
1317 weight (D) of mice following intranasal infection as in Figure 7A with a low dose (5×10^3
1318 PFU/mouse) of the IAV H1N1 PR/8 strain, and treatment with C910 ($n = 4$) or vehicle
1319 control DMSO ($n = 4$). Mantel-Cox test was performed to compare the survival curves
1320 between DMSO and C910 groups. Weight loss data are shown as mean $\pm$ s.e.m, * $p <$
1321 0.05, multiple *t*-test.

Supplementary table 1 : Mode of action of broad-spectrum inhibitors and their *in vivo* investigation

Inhibitors	Retro-2 ²⁻⁹	EGA ¹⁰⁻¹²	Amodiaquine ¹³	ABMA ¹⁴⁻¹⁷
Structure
Cellular targets and pathways	Sec16a (ER exit site component), affecting anterograde trafficking of syntaxin-5	Early endosome sorting (no effect on STx retrograde transport)	Inhibiting lysosomal cathepsin B	Early and late endosomes (no effect on STx retrograde transport)
Altered phenotype of cellular organelles or probes	Relocalization: Syntaxin 5, Syntaxin 6, Syntaxin 16	EGF is trapped in the EEA1 ⁺ compartment	Decreased lysosomal fluorescence	Rab7 ⁺ compartment enlargement; enhanced lysotracker fluorescence ; enhanced co-localization of Rab7/Rab9 with p62
<i>In vivo</i> investigations	<p>Ricin: i.p. 2/10/20/200 mg/kg 1h before intoxication; 6-week-old female Balb/c mice.</p> <p>Escherichia coli O104:H4: i.p. 100 mg/kg at 16 h and 26 h after infection, 5-week-old Balb/c mice.</p> <p>EV71: daily i.p. 2/10 mg/kg for 7 days after infection; newborn Balb/c mice.</p> <p>L. amazonensis: i.p. 100 or 20mg/kg 24h or 3 weeks after infection, 18g Balb/c mice.</p> <p>Vaccinia virus: i.p. 20 µg/mouse twice (-1d, +1d or +1, +2d after infection), 6-8-week-old female Balb/c mice.</p>	<p>BoNT/A, B, D: i.p. 12.5 mg/kg every 12 h for 3 days before infection; Swiss-Webster adult male CD1 mice</p>	<p>Anthrax toxin: i.v. 1.5/3/6 mg/kg with toxin together; Sprague-Dawley adult male rats</p>	<p>Ricin: i.p. 2/20/200 mg/kg 1h before intoxication, 6-week-old female Balb/c</p> <p>HSV-2: daily i.p 5 mg/kg for 7 days since 1h after inoculation; 6-8 week-old female Balb/c</p>

Supplementary table 2: protective spectrum of inhibitors

Effect		Retro-2 ²⁻⁹	EGA ¹⁰⁻¹²	Amodiaquine ¹³	ABMA ¹⁴⁻¹⁷	C910
Toxins	Active	Ricin toxin Shiga-like toxin 1/2 (Stx1/2) from <i>Escherichia. Coli</i>	Lethal toxin from <i>Bacillus anthracis</i> (LT) Diphtheria toxin (DT) Exotoxin A (PE) BoNT/A, B and D from <i>Clostridium botulinum</i> Iota toxin from <i>Clostridium perfringens</i> C2 toxin from <i>Clostridium botulinum</i> Cytolethal distending toxins from <i>Haemophilus ducreyi</i> (Hd-CDT) and <i>Clostridium difficile</i> (CDT)	LT and DT Lethal toxin from <i>Bacillus anthracis</i> (LT) Toxin B from <i>Clostridium difficile</i> (TcdB)	Ricin toxin Diphtheria toxin (DT) Exotoxin A (PE) Lethal toxin from <i>Bacillus anthracis</i> (LT) Toxin B (TcdB) Lethal toxin from <i>Clostridium sordellii</i> (TcsL)	CNF1 Diphtheria toxin (DT) Exotoxin A (PE) TcdA, TcdB Lethal toxin from <i>Bacillus anthracis</i> (LT) Stx1, Stx2
	Inactive	Diphtheria toxin (DT) BoNT/A	Ricin toxin Cytolethal distending toxins from <i>Escherichia. coli</i> TcdA and TcdB	Cholera Toxin Exotoxin A (PE)	STx 2 BoNT/A	Ricin
Viruses	Active	Adeno-associated virus (AAV) Polyomaviruses , Poxviruses Papillomaviruses Ebola and Marburg filoviruses Herpes simplex virus-2 (HSV-2) EV71	Influenza A virus (A/WSN/33 strain, H ₁ N ₁) Vesicular stomatitis virus (VSV) Lymphocytic choriomeningitis virus (LCMV)	Ebola virus SARS coronavirus Rabies Junin Chikungunya virus	Ebola virus Rabies virus Dengue 4 virus Herpes simplex virus-2 (HSV-2) EV71	Influenza A virus (H ₁ N ₁) SARS-CoV-2
	Inactive	Chikungunya virus Dengue 4 virus Venezuelan equine encephalitis virus (VEEV)	Amphotropic murine leukemia virus (A-MLV) Nipah virus (NiV)	Polivirus 3 Herpes simplex virus-1 Human Cytomegalovirus Respiratory syncytial virus	Chikungunya virus	
Bacteria	Active	<i>Escherichia coli</i> O104:H4 <i>Simkania negevensis</i> <i>Chlamydia trachomatis</i>			<i>Simkania negevensis</i> <i>Chlamydia trachomatis</i>	Uropathogenic <i>E. coli</i> (UPEC)

Parasites	Active	<i>Leishmania</i>			<i>Leishmania</i>	
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Figure 1

A

B

C

D

E

F

Figure 2

Figure 3

Figure 4

A

B

C

DQ-BSA, 4h

Figure 5

A **B** **C** **D**

Figure 6

Figure 7

A

D

B

E

C

F

A

B

C

D

E

A

B

A

B

C

A

B

C

A

Elimination rate (1/h)	0.025 ± 0.004
$T_{1/2}$ (h)	28.5 ± 4.3
$T_{\max}$ (min)	8.4 ± 3.4
$C_{\max}$ (nM)	164 ± 33
Area Under the Curve (AUC_{0-t}) (nM*h)	623 ± 140
Mean Residence Time (MRT_{0-t}) (h)	12.3 ± 0.6
Volume of distribution/F (L/kg)	751 ± 140
Clearance/F (L/kg/h)	19.7 ± 6.2

B **C** **D**