A Targeted Therapeutic Rescues Botulinum Toxin-A Poisoned Neurons

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Author Contributions P.Z. (molecular biology & neuroscience), R.R. (chemistry & neuroscience), B.R.S. (chemistry & neuroscience) and P.R. (chemistry & neuroscience) contributed equally to this work and co-wrote the paper. All authors discussed the results and commented upon the manuscript.

The authors declare no competing financial interests.

Botulinum neurotoxin (BoNT), a Category A biothreat agent, is the most potent poison known to mankind. Currently no antidote is available to rescue poisoned synapses. BoNT acts specifically by blocking neurotransmission primarily at peripheral nerve-muscle junctions causing severe flaccid muscle paralysis, which is fatal if proper medical care is not provided. The neurotoxin acts by specifically entering the presynaptic nerve endings where it interferes with the biochemical machinery involved in the process of neurotransmitter release, i.e., neuroexocytosis. Most serotypes of BoNT are known to remain active for weeks to months after entering the nerves, but BoNT/A is the most potent and long lasting in causing muscle paralysis. An effective medical countermeasure strategy requires developing a drug that could rescue poisoned neuromuscular synapses, and would include its efficient delivery specifically to presynaptic nerve terminals. Here we report rescuing of botulinum poisoned nerve cells by Mastoparan-7 (Mas-7), a peptide constituent of bee venom, that was delivered through a drug delivery vehicle (DDV) constructed from the non-toxic fragment of botulinum neurotoxin itself. We found that Mas-7 that was delivered into BoNT/A intoxicated cultured mouse spinal cord cells restored over 40% of stimulated neurotransmitter release. The rescue of the cell poisoning did not occur from inhibition of the endopeptidase activity of BoNT/A against its well known substrate, SNAP-25 that is mechanistically involved in the exocytosis process. Rather, Mas-7 induced a physiological host response apparently unrelated to SNAP-25, but linked to the phospholipase signal transduction pathway. In addition to providing the first effective antidote against botulism, our results open new avenues to study the mechanism of exocytosis, and also to examine an

alternative cellular mechanism of botulinum neurotoxin action. An effective BoNT-based DDV can also be utilized for drug delivery against many neuronal and neuromuscular disorders.

BoNTs are produced by the anaerobic *Clostridium botulinum* species of bacteria and are the cause of botulism, a life-threatening neuroparalytic disease. They are extremely potent food poisons, with a mouse LD_{50} of 1 ng/kg or less for type A [1, 2]. Contamination of restaurant, catered or commercial foodstuffs or beverages could cause illness in a large number of consumers [1]. Aerosol exposure of BoNTs does not occur naturally, but could be attempted by bioterrorists to achieve a widespread effect. It has been estimated that a single gram of crystalline toxin, evenly dispersed and inhaled, could kill more than one million people [2].

BoNTs are large proteins with molecular weight of 150 kDa. They are produced by bacteria as a complex, containing the neurotoxin and its associated proteins [3, 4]. They are synthesized as relatively inactive single chain protoxins and are activated by protease nicking to form a dichain molecule (a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC)) linked through a disulfide bond[5]. The HC is responsible for the binding to the target nerve cells (through its C-terminus) and translocating the LC into the cell cytoplasm (through its N-terminus) [6, 7].

Inside the neuronal cytosol, the LC acts as a Zn^{2+}-endopeptidase against specific intracellular protein targets present either on the plasma membrane or on the synaptic

vesicle, and inhibits neurotransmitter release by disabling the exocytotic docking/fusion machinery [6, 7]. BoNTs catalyze proteolysis of specific proteins of the soluble NSF attachment protein receptor (SNARE) complex that have been implicated in the exocytotic machinery [6, 8]. BoNT/A, /C, and /E cleave a 25 kDa synaptosomal associated protein (SNAP-25); while synaptobrevin is the target of BoNT/B, /D, /F, /G, and tetanus neurotoxin (TeNT) [9-15].

Current therapy for botulism involves respiratory supportive care and the administration of antitoxin. The only antitoxins available are equine antitoxin (neutralizing antibodies against BoNT/A, /B, and /E, an investigational heptavalent (against ABCDEFG) antitoxin BabyBIG®, derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine, is only available for infant botulism [16]. An antitoxin has to be administered before toxins reach the nerve cells. The therapeutic window for using an antitoxin is short. Once the syndrome is developed, the antitoxin is less effective since the antitoxin can not get into the nerve cell to neutralize the toxin. The flaccid muscle paralysis caused by BoNT/A lasts for several months [17]. Therefore patients who have already developed the syndrome have to be put under respiratory intensive care for this long duration of paralysis [1, 2, 18]. The estimated cost for each botulism patient under respiratory supportive care could be as high as US $350,000 [19]. This puts a large burden on hospitals, both financially, and in resource management. Should a bioterror attack occur, there will be a public health crisis due to the lack of effective antidotes against botulism, especially in the absence of reliable presymptomatic diagnostics.

Mass immunization is not feasible or desirable, primarily because BoNT is an effective therapeutic agent against numerous neuromuscular disorders, and also has a wide range of cosmetic applications [20].

For relief from BoNT-mediated paralysis, it is important that the poisoned nerve cells are rescued through restoration of the neurotransmitter release process. While drugs have been designed to block the endopeptidase activity which is believed to be responsible for the inhibition of neurotransmitter release, delivery of the drugs specifically to the poisoned nerve terminals has remained a major hurdle.

We designed a drug delivery vehicle (DDV) utilizing recombinant BoNT/A heavy chain (rHC), which is known to specifically bind to the presynaptic nerve terminals and be internalized via endocytosis. The DDV construct was a modification of that developed by Goodnough et al.[21] consisting of a targeting molecule, Cy3 labeled rHC, linked by a disulfide bond to a drug stimulant, Oregon green 488 (OG488) labeled 10 kDa dextran. We attached Mas-7 with the DDV (DDV-Mas-7) (See supplementary figure S1 for details on DDV and DDV-Mas-7 structures) and tested its efficacy in rescuing cultured mouse spinal cord neuronal cells poisoned with BoNT/A (Fig. 1). Mas-7 is a wasp venom derived peptide known to be a phospholipase A_2 (PLA_2) activator, which effectively stimulated acetylcholine exocytosis in PC12 cells in a SNAP-25 independent manner [22]. [^{3}H]glycine release assay was used to assess the recovery from BoNT/A poisoning. The experimental design was to mimic a therapeutic application of the DDV strategy to treat

individuals poisoned with BoNT/A and exhibiting clinical symptoms of botulism. Three-week old mouse spinal cord neuronal cultures were treated with 1 pM BoNT/A at 37^{o}C for 8 hours. After washing to remove excess toxin, cells were treated with DDV-Mas-7 at a concentration of 100 nM for 16 hours at 37^{o}C. Vesicular neurotransmitter release, measured by the 80 mM K^{+}-evoked [^{3}H]glycine release assay, was substantially restored (~40%; Fig. 1A).

Interestingly, there was only a partial cleavage of SNAP-25 (28% of total SNAP-25 protein) in neurons treated with 1 pM BoNT/A, a concentration that almost completely blocked the [^{3}H]glycine release due to 80 mM K^{+} stimulation (Fig. 1B). This suggested that SNAP-25 cleavage may not be the only factor responsible for the inhibition of neurotransmitter release due to BoNT/A. Keller and Neale also reported that a dose-response data with BoNT/A produced non-overlapping curves for SNAP-25 proteolysis and blockade of neurotransmitter release [23]. It is noteworthy that in BoNT/A poisoned neurons, DDV-Mas-7 treatment had no effect on SNAP-25 cleavage, suggesting that Mas-7 in DDV was effective in rescuing neurons from BoNT/A toxicity via a pathway independent of SNAP-25. To our knowledge, this is the first experimental demonstration of a prospective therapeutic approach to treat botulism in a relevant peripheral neuronal model combined with a feasible targeted drug delivery technology.

For BoNT/A, the widely accepted molecular explanation of its mechanism of action is that toxicity is due to its zinc-dependent endopeptidase activity via proteolysis of SNAP-25, an essential component of the exocytotic SNARE complex. An alternative

mechanism, although not mutually exclusive, is that the BoNT/A affects phospholipase A_2 (PLA_2) [22, 24-25] and Rho-GTPase (RhoB) [26] in stimulus-induced neurotransmitter release. According to this mechanism, stimulus-induced increase in intraterminal free Ca^{2+} concentration ($[Ca^{2+}]_i$) activates PLA_2, which causes arachidonic acid (AA) release from membrane phospholipids. AA has been proposed to act as a fusogen in the vesicle fusion process. Lysophosphatidic acid (LPA), the other product of PLA_2 action, acts via LPA receptors to stimulate Rho-GTPase (Rho-B), which regulates actin cytoskeletal organization. Actin disorganization has been proposed to be a prerequisite for intraterminal vesicle migration and exocytosis. Our results have shown that BoNT/A inhibits neuroexocytosis by interfering with both PLA_2-mediated AA release in PC12 cells [22, 24-25] and RhoB-mediated actin disorganization[26]. Our observation of Mas-7 based rescue of BoNT/A-poisoned neuronal cells is likely to engage this pathway of neurotransmitter release.

A series of control and background experiments were carried out to ensure understanding and utility of DDV-Mas-7 as a viable therapeutic approach against botulism. First, we observed that in 3-week old cultured mouse embryonic spinal cord neurons poisoned with 1 pM BoNT/A, Mas-7 alone or Mas-7 plus high (80 mM) K^+ was able to induce neurotransmitter ([^{3}H]glycine) release; high K^+ alone was ineffective (data not shown). Second, since the targeted DDV approach is based on the premise of a selective entry of DDV into presynaptic nerve terminals via BoNT/A receptor mediated endocytosis, we demonstrated by competition experiments that the uptake of the DDV-Mas-7 was via BoNT/A receptors. In these experiments (Fig. 2), 3-week old spinal cord cultures were

exposed for 16 hours to DDV (200 nM) alone or to DDV plus a 1-, 3-, or 10-fold excess of unlabeled rHC or BoNT/A holotoxin added to cultures simultaneously. As seen in Fig. 2, in the absence of rHC or BoNT/A, DDV uptake and dextran separation were as expected; however, a 10-fold excess of rHC or BoNT/A holotoxin totally blocked the uptake of DDV. These results suggested that DDV entry into neurons occurred by the same route as that used by BoNT/A. Third, we considered the possibility that in neurons, the processes of exocytosis and endocytosis might be tightly coupled, i.e., interruption of exocytosis, as in BoNT/A poisoning, might halt endocytosis as well. To discount this possibility, we demonstrated uptake of labeled DDV-Mas-7 (Cy3 labeled rHC, red fluorescence) in spinal cord neurons previously exposed to a high concentration (1 nM) of Alexa 488-labeled BoNT/A (green florescence) which had completely blocked K^+-stimulated [^{3}H]glycine release. To examine DDV-Mas-7 uptake in these cells, the cells were washed once using warm culture medium and reincubated at 37^{o}C with 100 nM DDV-Mas-7 for 16 hours. Confocal microscopy results indicated that both BoNT/A and DDV-Mas-7 were taken up in the same cell pool, but localized in separate population of endosomes (Fig. 3), demonstrating internalization of DDV-mas-7 via endocytosis into BoNT/A poisoned neurons. Neale et al. also reported that BoNT/A blocked synaptic vesicle exocytosis, but not endocytosis at nerve terminal [27]. It is suggested that the exocytosis and endocytosis are not tightly coupled in BoNT/A poisoned neurons.

Therapeutic targeting is important for two main reasons: (a) delivering an effective high concentration of the therapeutic compound to the site of toxicity, e.g., nerve terminals for botulism, and (b) minimizing systemic toxicity, if any, due to treatment compounds. To

demonstrate the feasibility of delivering a therapeutic compound via the DDV, we examined the separation of the prototype drug carrier OG488-dextran from DDV. Spinal cord neurons were treated with 200 nM labeled DDV for 16 hours at 37^{o}C. Confocal microscopic images were analyzed by utilizing the Bio-Rad AutoDeblur and AutoVisualize software (see supplementary information Fig. S2 for detail). The results indicated that about 40% of the drug carrier components were separated from DDV and diffused into cytosol from endosome in 3 week-old cultures (Fig. 4). Results also revealed that both the separation of the drug carrier from DDV and the neuronal function of glycine release was cell maturation dependent (supplementary information Fig. S2, table ST1). To test the toxicity of rHC in our experimental model, we compared the inhibition of 80 mM K^+ stimulated [^{3}H]glycine release due to increasing concentrations of rHC or native BoNT/A holotoxin by the assay described in Methods (supplementary information). Results obtained with a given batch of toxin (Figure S3) showed that BoNT/A was effective as expected with an IC_{50} of <1 pM and a total inhibition at ~0.1 nM. However, the rHC was relatively nontoxic at 10 nM or higher (supplementary information Fig. S3).

In conclusion, this report provides new knowledge of endocytosis and exocytosis as well as of BoNT trafficking and action. In particular, the results showing the efficacy of DDV-Mas-7 in rescuing neurons from botulism is consistent with our previous reports on a PLA_2- and RhoB-mediated mechanism of BoNT/A toxicity. In addition, these results suggest an alternative approach towards botulism intervention other than the one commonly emphasized, i.e., protection of vesicle fusion proteins, e.g., SNAP-25 for

BoNT/A. Notably, application of this DDV approach to antagonize botulism is not necessarily limited to the neuronal targeting of a PLA_2 activator as shown here, but also should be useful for delivery of other prospective antidotes such as protease inhibitors to protect the vesicle fusion proteins as applicable. Finally, the success in the DDV strategy against botulism shown here may open new avenues in developing technologies to treat other neurological disorders that require a targeted delivery of therapeutics to affected neurons or tissues.

METHODS SUMMARY

See Supplementary Information for detailed methods.

^{3}[H]glycine release assay. 3[H]glycine release was determined by a modification of the method described by Williamson et al.[28] Spinal cord cells were incubated at 37^{o}C for 30 min in HEPES-buffered saline (HBS) containing 2 mCi/ml ^{3}[H]glycine. The cells were washed briefly with Ca^{2+}-free HBS and incubated sequentially for 7 min in each of the following modified HBS solutions: 5 mM KCl/0 mM Ca^{2+}, 80 mM KCl/2 mM Ca^{2+} and 5 mM KCl/0 mM Ca^{2+}. Each incubation solution was collected, and the radioactivity was determined by scintillation counting.

Fluorescent images. Uptake of DDV by spinal cord neurons, release of dextran from DDV and rHC localization were determined by confocal microscopy. Cells were exposed to DDV, Cy3-labeled rHC, or OG488-labeled dextran in growth medium for 16 h at a concentration of 200 nM at 37^{o}C. Cells were subsequently washed and fixed overnight with 2% paraformaldehyde. The anti-endosome antibody was then used to determine rHC localization. The coverslips containing fixed cells were mounted between a glass slide and glass coverslip and fluorescent images were viewed on a Bio-Rad 2000 laser confocal microscope.

SNAP-25 Immunoblotting. Immunoblotting was performed using an anti-SNAP-25 antibody. After electrophoresis in 4-12% gradient polyacrylamide gels, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The anti-SNAP-25 antibody

was used to determine the full-length and truncated SNAP-25 protein. Scanned images of Western blots were produced and analyzed utilizing Bio-Rad quantity-one software.

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FIG LEGENDS

Fig.1. Restoration of stimulated [^{3}H]glycine release function in BoNT/A poisoned neurons by treatment with DDV-Mas-7.

A. Potassium (80 mM)-evoked glycine release was measured in 3 week-old mouse spinal cord neuronal cultures. B. Western blots showing cleavage of SNAP-25 due to 1 pM BoNT/A. Spinal cord cells were solubilized in sample buffer and 5 mg of total cellular protein was used for Western blotting with SNAP-25 antibody (SMI-81, Sternberger Monoclonals, Inc. Lutherville, MD). SNAP-25 cleavage was determined by densitometric analyses of Western blots. SNAP-25, a full-length 25 KDa SNAP-25 protein; SNAP-25A, a truncated from of SNAP-25 protein cleaved with a 9 c-terminal residues by BoNT/A. (1) control spinal cord cultures not exposed to BoNT/A, (2) Spinal cord cultures incubated with 1 pM toxin for 8 h at 37°C, (3) spinal cord cultures incubated with 1 pM toxin for 8 h at 37°C washed to remove toxin, and then incubated with 100 nM of toxin-free DDV-Mas-7 for 16 h at 37°C,

Fig.2. Fluorescently labeled DDV uptake in neurons is via BoNT/A receptors. Fluorescent images of mouse spinal cord neurons demonstrated that both unlabeled BoNT/A holotoxin and rHC compete with fluorescent DDV molecules for entry into cells. The DDV consisted of Cy3 (red fluorescence) labeled rHC conjugated to OG488 (green fluorescence) labeled 10 kDa dextran. Images in upper panel were obtained from cultures exposed for 16 h under the following conditions: (A1) DDV (200 nM in all experiments) in the absence of rHC; (B1) DDV and rHC (200 nM); (C1) DDV and rHC (600 nM); and (D1) DDV and rHC (2 mM). Images in lower panel were obtained from

cultures exposed for 16 h under the following conditions: (A2) DDV in the absence of BoNT/A; (B2) DDV and BoNT/A (200 nM); (C2) DDV and BoNT/A (600 nM); and (D2) DDV and BoNT/A (2 mM). DDV and rHC or BoNT/A were added simultaneously. Fluorescent images were analyzed by Bio-Rad 2000 laser confocol microscope. Alexa 488 was excited at 488 nm line of an argon laser and detected with a 530-nm cutoff filter; Cy3 was excited at 543 nm line of an argon laser and detected with a 565-nm cutoff filter.

Fig.3. Exocytosis and endocytosis are not tightly coupled in BoNT/A poisoned neurons.

BoNT/A was labeled with Alexa 488 (green fluorescence). Three week-old cultured mouse spinal cord neurons were incubated with a culture medium containing 1 nM of Alexa 488-BoNT/A for 8 hours at 37^{o}C. Excess Alexa 488-BoNT/A was removed by washings 3 times with fresh culture medium. The cells were incubated with fresh medium for 1 hour and then incubated in medium containing 100 nM Cy3 (red fluorescence) labeled DDV-Mas-7 (Fig. S1B) for 16 hours. Fluorescent images were analyzed by Bio-Rad 2000 laser confocol microscope. Alexa 488 was excited at 488 nm line of an argon laser and detected with a 530-nm cutoff filter; Cy3 was excited at 543 nm line of an argon laser and detected with a 565-nm cutoff filter.

Fig.4. The drug carrier molecule in DDV separates and translocates into neuronal cytosol.

Three-week old spinal cord cultures were incubated for 16 h with 200 nM solutions of fluorescently labeled DDV and then labeled with anti-endosome antibody as described

under methods. Confocal images shown are as follows: A, red-rHC: fluorescence elicited at an excitation wavelength of 543 nm; B, green-OG488-dextran: fluorescence elicited at an excitation wavelength of 488 nm; C, bright blue-Alexa 633-endosomes: fluorescence elicited at an excitation wavelength of 632 nm; D, overlay of red and green showing either co-localization (orange) or separation of rHC and dextran; E, overlay of red and blue showing either the localization (magenta) of rHC in the endosomes as believed or its release into the cytosol; F, overlay of green and blue showing either localization (light blue or greenish blue) of dextran in the endosomes or its release into the cytosol.

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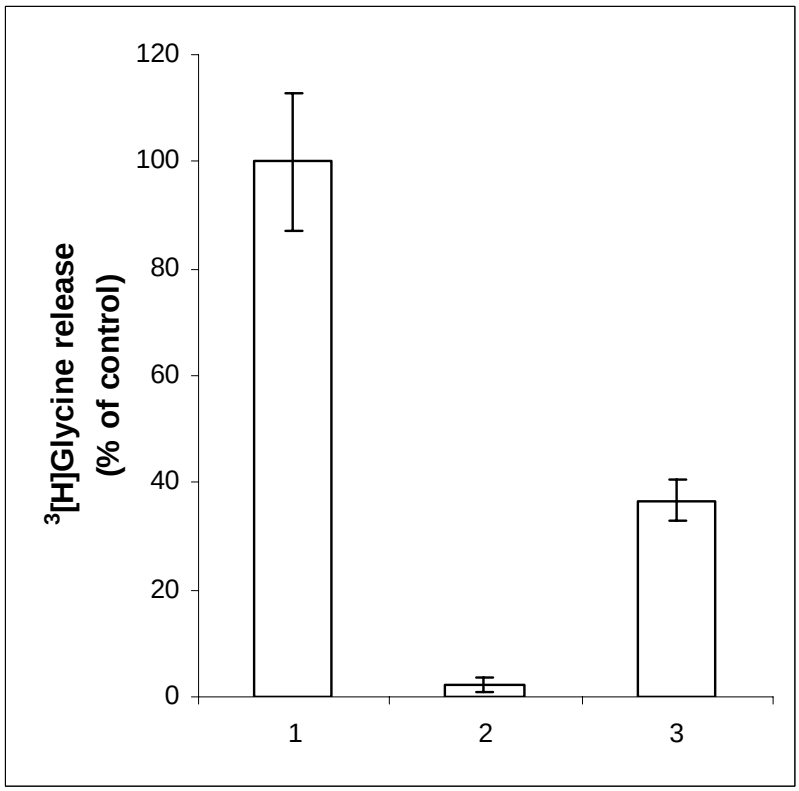
A



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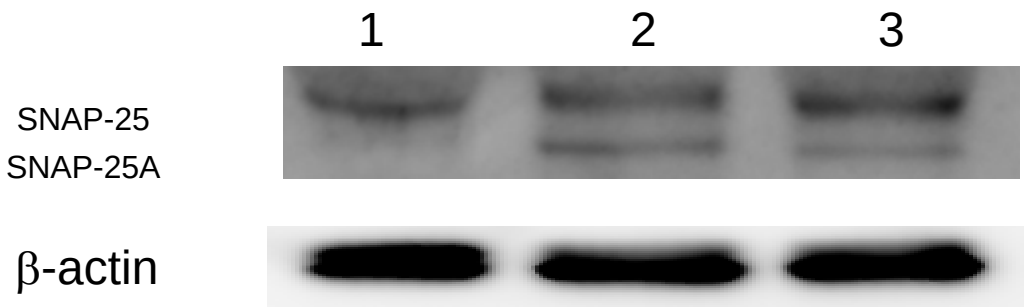

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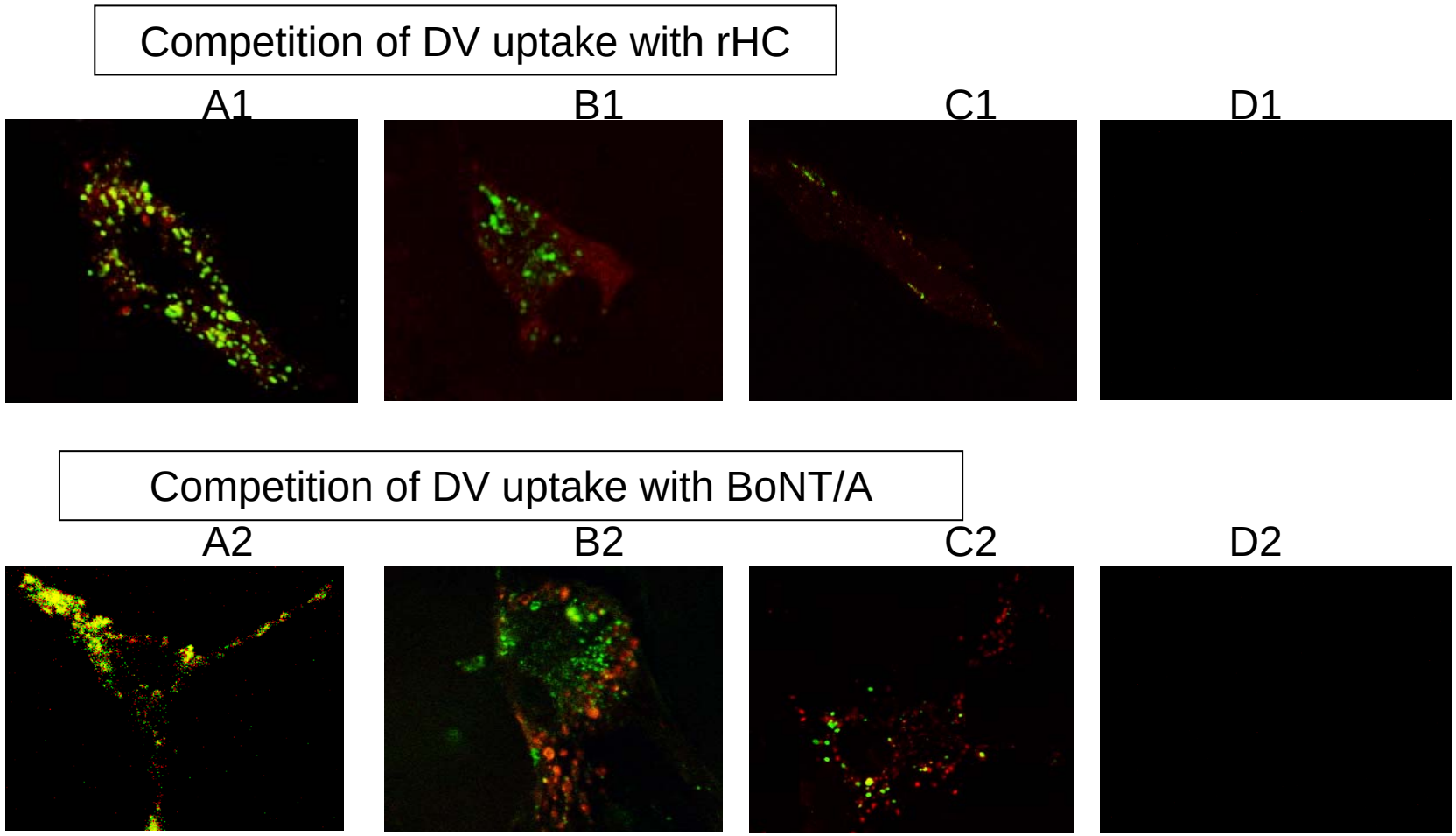

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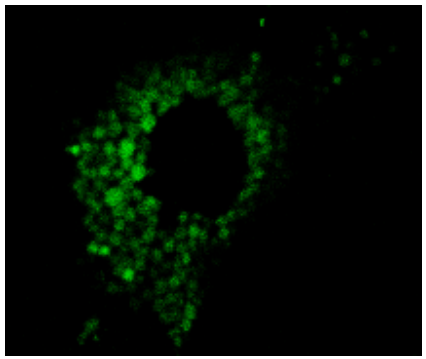

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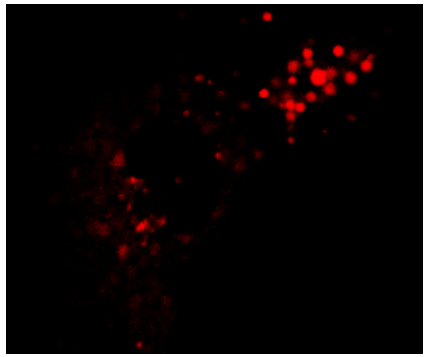

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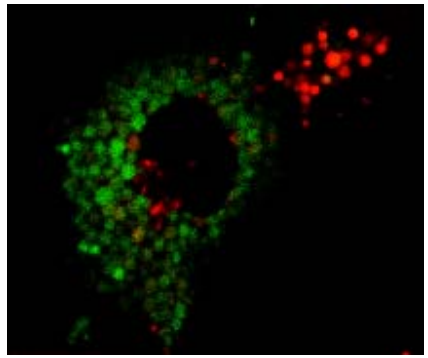

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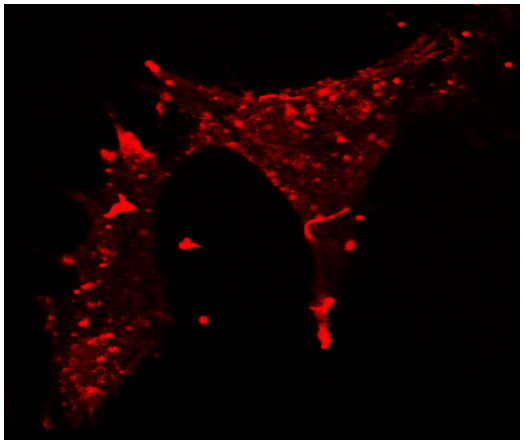
A



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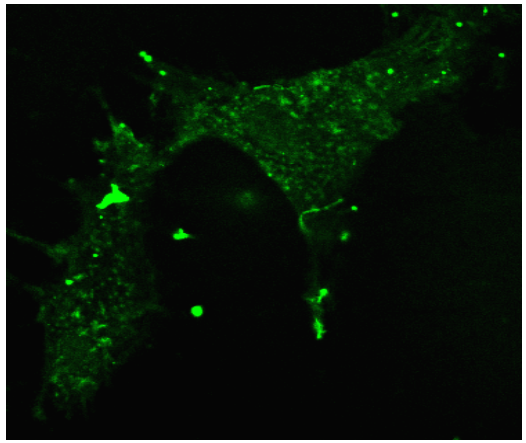
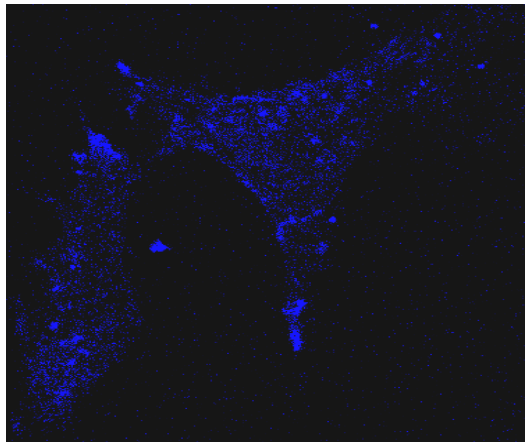


C



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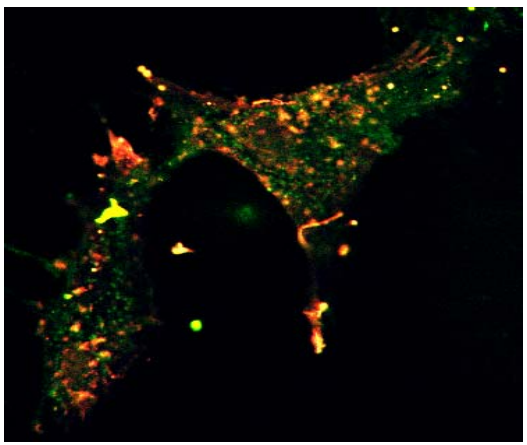

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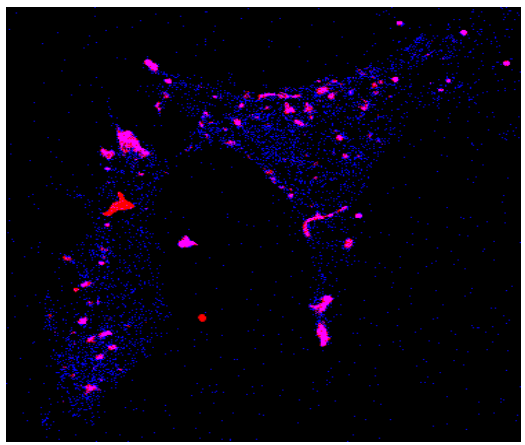

F