INHIBITION OF ANTHRAX PROTECTIVE ANTIGEN OUTSIDE AND INSIDE THE CELL

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Running title: Inhibition of anthrax PA

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ABSTRACT

In the course of anthrax infection, anthrax lethal factor (LF) and edema factor bind to protective antigen (PA) associated with cellular receptors ANTXR1 (TEM8) or ANTXR2 (CMG2), followed by internalization of the complex via receptor-mediated endocytosis. A new group of potential anti-anthrax drugs, β-cyclodextrins, has been recently described. A member of this group, per-6-(3-aminopropylthio)-β-cyclodextrin (AmPrβCD), was shown to inhibit LF toxicity in vitro and in vivo. In order to determine which steps in lethal factor trafficking are inhibited by AmPrβCD, we developed two targeted fluorescent tracers based on LFn, a catalytically inactive fragment of LF: 1) LFn site-specifically labeled with a fluorescent dye AlexaFluor594 (LFn-Al), and 2) LFn-decorated liposomes loaded with a fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid (LFn-Lip). Both tracers retained high affinity to PA/ANTXR complexes, and were readily internalized via receptor-mediated endocytosis. Using fluorescent microscopy, we found that AmPrβCD inhibits receptor-mediated cell uptake, but not binding of LFn-Al to PA/ANTXR complexes, suggesting that AmPrβCD works outside the cell. Moreover, AmPrβCD and LFn-Al synergistically protect RAW 264.7 cells from PA-mediated LF toxicity, confirming that AmPrβCD did not affect binding of LFn-Al to receptor-associated PA. In contrast, AmPrβCD did not inhibit PA-mediated internalization of LFn-Lip, suggesting that multiplexing of LFn on liposomal surface overcomes the inhibiting effects of AmPrβCD. Notably, internalized LFn-Al and LFn-Lip protected cells overexpressing anthrax receptor TEM8 from PA-induced/LF-independent toxicity suggesting an independent mechanism for PA inhibition inside the cell. These data suggest a potential use of β-cyclodextrins in combination with LFn-Lip loaded with anti-anthrax drugs against intracellular targets.
INTRODUCTION

*Bacillus anthracis*, Gram-positive bacteria that cause anthrax, produce two bipartite AB-type toxins comprising protective antigen (PA) and either lethal or edema factor (LF or EF). According to a current mechanistic model of anthrax toxicity (39), 83-kDa PA can bind to two different receptors on the target cells, tumor endothelial marker 8 (ANTXR1 or TEM8) that is expressed on epithelial cells of the skin, lung, and intestine (11, 12) and capillary morphogenesis protein 2 (ANTXR2 or CMG2) that is widely expressed in different tissues (38). Upon binding to cellular receptors, PA is cleaved by furin-like protease(s), and the resulting 63-kDa PA fragment forms a bagel-like heptameric prepore that can bind up to three molecules of LF, forming lethal toxin (LeTx), or EF, resulting in edema toxin. The complex is then internalized via receptor-mediated endocytosis and trafficked to the endosomal compartment, where, under mild acidic conditions, PA undergoes a conformational transition from a prepore to a pore, leading to formation of a 14-stranded, membrane-spanning β-barrel through which LF and EF are released into cytosol (reviewed in 16).

Since targets on the cell surface are more accessible to drugs than intracellular targets, several strategies have been proposed for inhibition of PA receptor binding and proteolytic cleavage, PA oligomerization, and binding of LF and/or EF to PA prepore (34, 36, 40). Recently, it has been reported that a β-cyclodextrin derivative, per-6-(3-aminopropylthio)-β-cyclodextrin (AmPrβCD), inhibits conductance of PA pore reconstituted in a bilayer lipid membrane and inhibits LF toxicity *in vitro* and *in vivo*, suggesting that AmPrβCD might inhibit translocation inside the cell (24, 25). However, AmPrβCD activity might be due to inhibition of earlier steps that take place on the cell surface, such as binding of LF to PA prepore or internalization of the ligand-receptor complex.
To establish whether PA function is inhibited inside or outside the cell, we developed two targeted fluorescent tracers based on LFn, a catalytically inactive fragment of LF capable of PA-dependent binding and internalization into cells (2). LFn expressed with C-terminal Cys-tag for site-specific modification (4) was labeled either with a fluorescent dye AlexaFluor594, yielding LFn-Al tracer, or with PEGylated phospholipid for decorating liposomes loaded with membrane-impermeable fluorescent dye 8-hydroxy pyrene-1,3,6-trisulfonic acid (HPTS), yielding LFn-Lip tracer. We found that both fluorescent tracers retain high affinity to PA/receptor complexes and undergo TEM8- and CMG2-mediated endocytosis, providing new opportunities for “tagging” cells expressing these receptors. Although LFn was used previously for intracellular delivery of fused protein fragments (31, 32), we were surprised to find that LFn can also provide for intracellular delivery of significantly larger liposomes.

We report here that AmPrβCD inhibits receptor-mediated cell uptake of PA-bound LFn-Al by both receptors, suggesting that AmPrβCD acts via extracellular mechanism(s). However, AmPrβCD does not inhibit receptor-mediated endocytosis of LFn-Lip, suggesting that multiplexing of LFn on liposome surface overcomes the effects of AmPrβCD on PA/receptor complexes. Since internalized LFn-Lip protects cells overexpressing anthrax receptor TEM8 from PA-induced/LF-independent toxicity, we hypothesize that LFn-Lip inhibits PA intracellularly. These data suggest a potential use of β-cyclodextrins in combination with LFn-Lip loaded with anti-anthrax drugs against intracellular targets.

**MATERIAL AND METHODS**

**Materials** - PA and LF (List Biological, Campbell, CA) were reconstituted in 5 mM HEPES pH 7.5, 50 mM NaCl, 0.1% BSA to final concentrations of 1 mg/ml, and stored at -70 °C in small aliquots. Per-6-(3-aminopropylthio)-β-cyclodextrin (AmPrβCD) was custom synthesized by
Pinacle Pharmaceuticals (Charlottesville, VA). AlexaFluor-594 maleimide was from Molecular Probes (Eugene, OR). Poly(ethylenglycol)-α-Distearoyl Phosphatidylethanolamine-ω-maleimide (DSPE-mPEG-maleimide, FW 3,400) was from Nectar Therapeutics. A fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) was from Sigma. APO-screen kit and caspase-2 fluorogenic substrate were from CalBiochem. Cell Probe HT Caspase3/7 Whole Cell Assay kit was from Beckman Coulter.

Synthesis of LFn-based fluorescent tracers - Construction of LFn fused to C-terminal Cys-tag was previously described (4). An additional His-tag was introduced by cloning Cys-tagged LFn ORF into NcoI-SalI sites of the pET/28b(+) vector (Novagen). The resulting protein, LFn-Cys, was expressed in BL21(DE3) E. coli and purified as described (4), with an additional metal affinity chromatography step on His-resin (Novagen) that was done according to the manufacturer’s instructions. The yield of LFn-Cys was ~20 mg/L of more than 98% pure protein, as judged by RT-HPLC analysis as described (5). For site-specific conjugation of AlexaFluor-594, LFn-Cys (25 nmoles) was mixed with AlexaFluor-594 maleimide to a final protein-to-dye molar ratio of 1:2, in a volume of 0.1 ml of 50 mM NaPi pH 7.2. The reaction mixture was incubated for 30 min, and purified by desalting (PD-10 column, GE Healthcare). The extent of AlexaFluor-594 modification was analyzed by RT-HPLC on a C4 column and calculated as a ratio of integral peak intensities at 216 nm (for protein) and at 598 nm (for AlexaFluor-594).

Preparation of HPTS-loaded liposomes and modification of LFn-Cys with DSPE-mPEG-maleimide were performed as described (4, 6). Briefly, a two-fold molar excess of mPEG-DSPE maleimide was added to LFn, incubated for 10 min at RT, and then mixed with equal volume of pre-formed HPTS-loaded liposomes. After a 16-h incubation at 37 °C, LFn-Lip was purified by gel-filtration on Sepharose 4B and characterized by SDS-PAGE and RP-HPLC. An average liposome
preparation contained 1-2 µM LFn, which corresponded to 50-100 LFn molecules per liposome. LFn-Lip was stored at 4 °C for several weeks without any loss of LFn functional activity.

*Cells* - CHO-K1 ovary hamster cells (CCL-61) and RAW 264.7 mouse monocytes (TIB-71) were from ATCC (Rockville, MD). TEM8 overexpressing cells were made by stable transfection of CHO-K1 cells with the pEF6/V5-His-TOPO/TEM8 plasmid (kindly provided by Dr. B. Terman, Albert Einstein School of Medicine, Bronx, NY, USA) encoding the full-length human TEM8 splice variant 1 fused to c-myc, V5 and 6xHis epitopes (22). Lipofectamine 2000 reagent (Gibco Life Technologies) was used for transfection. Selected blasticidine-resistant clones were screened for TEM8 expression by Western blotting using c-myc monoclonal antibody (Invitrogen). A clone with the highest TEM8 expression was designated CHO-TEM8 and used for this work. CHO-K1 and CHO-TEM8 cells were grown in F-12 medium, RAW 264.7 cells were grown in DMEM. Both media included 10 % FBS (HyClone), 2 mM L-glutamine and antibiotics. Cells were maintained at 37 °C, 5% CO₂.

*Microscopy* - Cells were incubated in complete culture medium containing 8 nM PA, with or without AmPrβCD (10 or 100 µM), for 15 min at 37 °C. Cells were either supplemented with an LFn-tracer followed by incubation at 37 °C for 1 h, or shifted to 4 °C for 15 min first, and then supplemented with LFn-tracer for a 1-h incubation at 4 °C. After incubations with LFn-tracers, cells were washed twice with PBS, fixed in 4% formaldehyde (Polysciences, Warrington, PA) for 5 min at RT, and mounted in mounting medium for fluorescence with DAPI or propidium iodide (Vector Labs) for nuclear counterstaining. Digitized images were captured on Zeiss Axiovert 2000 fluorescent microscope and Zeiss LSM 510 confocal microscope.

*Caspase activation assay* - CHO-TEM8 cells were plated in 75-cm² flasks, 1.5 mln cells/flask. Twenty hrs later, PA was added to a final concentration of 30 nM, either alone or in a combination...
with 10 μM AmPrβCD. Cells were incubated for 24 hrs under normal culture conditions, then harvested in 2 mM EDTA/PBS, spun down and lysed at 4 °C for 5 min in a lysis buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 1% NP-40, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 25 μg/ml antitripsin, 25 μg/ml aprotinin). Cell lysates were clarified by centrifugation (10,000 x g for 2 min) and supplemented with 2 mM DTT to preserve caspase enzymatic activity. Total protein in cytosolic fractions was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Aliquots containing 40 μg of cytosolic protein were analyzed in a final volume of 100 μl of caspase activity buffer (0.1 M HEPES pH 7.4, 2 mM DTT, 0.1% NP-40, 1% sucrose) containing 50 μM of caspase substrate. Reaction mixtures were incubated for 1 hour at 37 °C, and fluorescence was measured at λ<sub>ex</sub> = 400 nm and λ<sub>em</sub> = 505 nm.

RESULTS

To develop LFn-based fluorescent tracers, we fused LFn to a 15-aa long cysteine-containing Cys-tag for site-specific modification (4) and His-tag for purification (Fig. 1A, inset). Given the crucial importance of the N-terminal portion of LFn for binding to PA (45), we have chosen C-terminus for tag fusion. Wild type LFn does not contain cysteine residues, and the only reactive thiol group in LFn-Cys fusion protein is provided by a cysteine in position 4 of C-terminal Cys-tag. To obtain LFn-Al, LFn-Cys was modified with a thiol-reactive fluorescent dye AlexaFluor-594 maleimide and purified by gel-filtration. RP-HPLC analysis confirmed a 1:1 ratio of protein to dye in the conjugate, LFn-Al (Fig. 1A). To obtain LFn-Lip, LFn-Cys was lipidated with a thiol-reactive PEGylated phospholipid, mPEG-DSPE maleimide, yielding ~50% of modified protein within minutes (Fig. 1B). For insertion of lipidated LFn-Cys into liposomal membrane, the lipidation reaction mixture was added to pre-formed liposomes loaded with HPTS, a
membrane-impenetrable fluorescent dye, and incubated for 16 hrs at 37 °C followed by purification of the resulting LFn-Lip from free LFn-Cys by gel-filtration on Sepharose 4B (Fig. 1B).

The specificity of binding to PA was established for both LFn-based tracers by competition with full-length LF for binding to cellular PA/ANTXR complexes (assay described in 4). In this assay, varying amounts of either tracer or unmodified LFn-Cys are added to RAW 264.7 cells exposed to LeTx formed by combination of LF and PA. LeTx is highly toxic for RAW 264.7 cells, and therefore the ability of non-toxic LFn to compete with full-length LF for binding to cell-associated PA is readily detected by the number of viable RAW 264.7 cells after a 3-h exposure to LeTx. We found that under selected conditions, unmodified LFn-Cys rescued RAW 264.7 cells with IC\textsubscript{50} of 3.5±0.4 nM, which is close to activity of LFn (IC\textsubscript{50} ~ 3 nM) reported earlier (4). Notably, both LFn-Al and LFn-Lip tracers displayed similar activity in this assay with IC\textsubscript{50} of 4.3±0.3 nM (Fig. 1C), indicating that site-specific C-terminal attachment of either a highly charged fluorescent dye or a bulky liposome did not dramatically affect binding of LFn-Cys to cell-associated PA.

To monitor PA-mediated LFn-Al internalization, we initially tested two ANTXR positive cell lines: RAW 264.7 mouse monocytes expressing CMG-2 (8), and CHO-K1 hamster ovary fibroblasts expressing an unidentified ANTXR (12, 30). Receptor-mediated endocytosis was observed by fluorescent microscopy of cells incubated with LFn-Al in the presence of PA, while cells incubated with LFn-Al alone served as control for non-specific uptake. We found that punctate Alexa-594 fluorescence was readily detectable in RAW cells in the presence, but not in the absence of PA, indicating PA/CMG2 mediated endocytosis of the tracer (Fig. 2A). However, CHO-K1 cells did not accumulate detectable amounts of Alexa-594 (data not shown), suggesting
that these cells do not express sufficient levels of PA receptors. Indeed, according to a recent report (21), wild type CHO cells can internalize only ~800 LF molecules per cell. Therefore, for this study we developed CHO-K1 cells overexpressing TEM8. A selected clone with the highest expression of TEM8, named CHO-TEM8, demonstrated PA-dependent uptake of LFn-Al (Fig. 2A), and was used for further studies. It should be noted that a prolonged incubation (1 h) with LFn-Al at 37 °C was necessary to reach detectable levels of the fluorescent probe in both cell types, while no cell-associated fluorescence was detected after a shorter (15-min) incubation (data not shown).

To establish the effects of AmPrβCD on uptake of LFn-Al, RAW 264.7 and CHO-TEM8 cells were pre-incubated with PA alone or PA and AmPrβCD at 37 °C, and then incubated with LFn-Al for 1 hour at 37 °C. A dramatic decrease of intracellular fluorescence was observed in both cell types in the presence of 10 µM and 100 µM AmPrβCD (Fig 2A for 100 µM AmPrβCD), indicating that either binding or uptake was inhibited by the drug. Given relatively low affinity of LFn to PA even at room temperature (Kd~1 nM) (19), the lack of membrane-bound LFn-Al at 37 °C in the presence of the drug might be temperature- rather than drug-dependent. We therefore tested how AmPrβCD affects binding of LFn-Al at 4 °C, when LFn/PA complexes are 3-7 fold more stable than at room temperature (19). CHO-TEM8 cells were pre-incubated at 37 °C with PA, alone or with 10 µM AmPrβCD, shifted to 4 °C for 15 min, then supplemented with LFn-Al and incubated for 1 hour at 4 °C. In both systems, membrane binding of LFn-Al was readily detectable as a distinctive band around significantly smaller DAPI-stained nuclei (Fig. 2B). Judging by fluorescence intensity, not only did AmPrβCD fail to inhibit but, in fact, it stimulated LFn-Al binding to PA/TEM8 (Fig. 2B).
In similar experiments performed on RAW 264.7 cells, binding of LFn-Al to RAW 264.7 cells at 4 °C was not detectable (data not shown). Although it was, most likely, due to the low level of the endogenous CMG2, we could not rule out a possibility that AmPrβCD might directly inhibit binding of this tracer to PA/CMG2. We therefore used the protection assay described above (Fig. 1C) to compare the effects of AmPrβCD alone, or in combination with LFn-Al, on CMG2-mediated toxicity of LeTx. We reasoned that if AmPrβCD does not affect PA-mediated LFn-Al binding, a combination of these two compounds might be more effective than each one alone. In our standard LeTx protection assay (0.2 nM LF, 2 nM PA, 3-h exposure), AmPrβCD alone protected RAW 264.7 cells at micromolar concentrations with IC50 of ~7 µM (Fig. 3A), while LFn-Al was active in this assay, with IC50 of 4 nM (Fig. 1C). To evaluate the relationship between LFn-Al and AmPrβCD effects, if any, we used an approach pioneered by Bernbaum to analyze whether drugs given simultaneously are additive, synergistic, or antagonistic (9). AmPrβCD was mixed with LFn-Al at the corresponding working concentrations resulting in a molar ratio of 2,000:1, serially diluted in complete culture medium and added to cells exposed to LeTx. We found that the combination of two compounds was significantly more effective in this assay than each compound alone, with IC50 values of ~1.2 µM for AmPrβCD and ~0.6 nM for LFn-Al. Fractional IC50 values (ratio of IC50 for a drug in combination to IC50 for a drug alone) were determined as 0.17, for each compound (Fig. 3A, compare to Fig. 1C). Since the sum of fractional IC50 values was significantly less than 2, our results, according to Bernbaum (9), indicated synergism between LFn-A and AmPrβCD, excluding the possibility that AmPrβCD inhibited binding of LFn-Al to PA/CMG2.

Since our data indicated that AmPrβCD acted outside of the cell, it was conceivable that, due to its high positive charge, AmPrβCD could bind to the cell surface and non-specifically affect
endocytosis of many different cellular receptors. We therefore tested if AmPrβCD could inhibit
endosytosis of an anthrax-unrelated ligand/receptor complex. For these experiments we used
SLT-VEGF, a chimeric toxin comprising the full-length Shiga-like toxin (SLT) subunit A fused
to vascular endothelial growth factor (VEGF) described in (3). SLT-VEGF is internalized via
VEGF receptor-mediated endocytosis and is highly toxic for 293/KDR cells expressing VEGF
receptor VEGFR-2 at nanomolar levels. AmPrβCD was serially diluted in complete culture
medium containing 1 nM SLT-VEGF and added to 293/KDR cells at the same concentration
range that was used for protection of RAW 264.7 cells from LeTx (Fig 3A). As a positive control
for cell rescue we used VEGF that protects 293/KDR cells from toxicity of SLT-VEGF by
competing for VEGFR-2 binding (4, 5, 7). We found that AmPrβCD did not protect 293/KDR
cells from SLT-VEGF-induced toxicity (Fig. 3B), indicating that its effects were limited to
PA/ANTXR complexes.

To establish if AmPrβCD inhibition of PA-mediated LF uptake depends on the presence of
LF in the complex, we used a novel PA cytotoxicity assay. While characterizing CHO-TEM8
cells, we found that PA alone was cytotoxic to these cells in a dose-dependent manner (Fig. 4A).
Parental CHO-K1 cells were not sensitive to PA in the same concentration range (Fig. 4A),
indicating a causative role of high-level TEM8 expression for PA-induced toxicity. A similar
PA-induced/LF-independent toxicity has been recently reported for RAW 264.7 cells engineered
to overexpress TEM8 (37). We found that PA-induced toxicity was completely blocked by 5 μM
AmPrβCD (Fig. 4A). Using fluorogenic assays with pan- and specific caspase substrates, we also
found that AmPrβCD inhibited PA-induced apoptosis in CHO-TEM8 cells by blocking the
overall caspase activity, and more specifically initiator caspase-2 and executioner caspases -3
and -7 (Fig. 4B).
PA-induced toxicity observed on TEM8 overexpressing RAW 264.7 cells was tentatively attributed to a large number of pores formed by internalized PA in endosomal compartments (37). If this suggested mechanism is correct, LF and its derivatives should also rescue CHO-TEM8 cells from PA-induced toxicity by blocking PA membrane channel, as it was recently described for PA channels reconstituted in artificial lipid bilayer membranes (35). Indeed, we found that LFn and both LFn-targeted fluorescent tracers successfully rescued CHO-TEM8 cells in a similar dose-dependent manner (Fig. 4C, for LFn-Lip). As expected, untargeted HPTS-loaded liposomes did not prevent cell death in this assay (Fig. 4C).

Judging by the ability to protect RAW 264.7 cells from LeTx (Fig. 1C) and CHO-TEM8 cells from PA-induced toxicity (Fig. 4C), LFn-Lip tracer was capable of specific binding to PA/ANTXR complex. Since LFn has been used for intracellular delivery of fused proteins fragments (31, 32), we could not exclude the possibility of intracellular delivery of LFn-targeted liposomes. Indeed, confocal microscopy of CHO-TEM8 cells incubated with LFn-Lip and PA revealed intracellular localization of fluorescent signals (Fig. 5A for reconstructed image, supplemental Fig. 1s for individual Z-sections). Similar results were obtained with RAW 264.7 cells, but with a smaller number and weaker punctuate signals (data not shown). For both types of cells, intracellular fluorescent signals were negligible in the absence of PA, or when using untargeted HPTS-loaded liposomes (data not shown). Surprisingly, PA-dependent uptake of LFn-Lip was readily detected in both cell lines regardless of the presence of AmPrβCD, clearly indicating that AmPrβCD did not inhibit uptake of LFn-Lip (Fig. 5B). This unexpected result suggested that multiplexing of LFn might overcome AmPrβCD-induced inhibition of PA-mediated endocytosis.
DISCUSSION

Blocking heptameric PA pore with heptameric β-cyclodextrins appears to be a rational starting point for the development of effective anti-anthrax treatments. Recent data from Karginov et al. (25) indicate that β-cyclodextrins derivatized on a primary face with amino groups inhibited the toxicity of LeTx \textit{in vitro} and provide protection against anthrax infection in highly susceptible Fischer F344 rats. Comparison of \textit{in vitro} activity of several amino-substituted β-cyclodextrins demonstrated structure-activity relationships within this class of compounds (24), supporting the hypothesis of specific interactions between β-cyclodextrins and PA. Based on the ability of AmPrβCD to inhibit ion currents through PA pore reconstituted into a lipid bilayer, it was suggested that this compound might inhibit translocation of LF or EF inside the cell through PA pore formed in endosomes at mildly acidic conditions (24, 25). However, we report here that AmPrβCD selectively inhibits receptor-mediated endocytosis of PA/LFn-Al complex by either of two anthrax receptors, TEM8 and CMG2. Furthermore, using a new assay for TEM8-mediated LF-independent PA cytotoxicity, we found that AmPrβCD action does not even require binding of LF to PA/TEM8 complex. Taken together, these data indicate that AmPrβCD inhibits LF toxicity by acting in a PA-specific manner on the cell surface rather than inside the cell. On the other hand, judging by inhibition of PA-induced/LF-independent toxicity by LFn-Al and LFn-Lip, PA can be also inhibited inside the cells.

Given known activities of cyclodextrins, AmPrβCD might inhibit receptor-mediated endocytosis of PA or LeTx through two different mechanisms. In a “protein”-centered model, binding of AmPrβCD to protein component(s) of the complex could stabilize protein conformations that are poorly suitable for endocytosis (13, 41, 43, reviewed in 18). Alternatively, in a “membrane”-centered model, reversible binding of cyclodextrin to PA/receptor complex...
might lead to depletion of cholesterol from underlying lipid raft rendering the whole membrane region poorly suitable for endocytosis. Indeed, at millimolar concentrations, β-cyclodextrin derivatives are known to affect many membrane processes, including receptor-mediated endocytosis of viral and bacterial proteins, by sequestering cholesterol from lipid rafts (10, 14, 15, 17, 20, 26-29, 33, 44, reviewed in 18). In fact, Abrami et al. convincingly demonstrated that β-methyl cyclodextrin inhibits lipid raft mediated endocytosis of LF/PA (1). Judging by a relatively high IC₅₀ of ~7 µM, binding of AmPrβCD is, most likely, reversible, and therefore it might continuously deplete cholesterol from the lipid raft in the area of PA/receptor complex. This putative mechanism, however, still requires PA/AmPrβCD interaction, since lipid-raft mediated internalization of VEGFR-2 (23) was not affected in the presence of AmPrβCD. Further experiments are in progress to discriminate between “protein” and “membrane” centered mechanisms of AmPrβCD action and to accommodate a recent discovery that low-density lipoprotein receptor-related protein 6 (LRP6) acts as an accessory protein in toxin internalization (42).

Regardless of the mechanism, it is particularly interesting that cells uptake liposomes targeted by LFn via a PA-mediated mechanism despite the presence of AmPrβCD. It suggests that properly designed LFn-Lip loaded with specific inhibitors of LF or EF might be used together with cyclodextrins to target the same cells. Indeed, cyclodextrin would inhibit PA-mediated internalization of LF and EF but not LFn-Lip, while internalized LFn-Lip might release drugs that inhibit LF and EF inside the same cell. It should be noted, however, that it would require a clear demonstration that the entrapped drugs are released into the cytosol, which might depend on the selective structure of drug and composition of liposomes. We hypothesize that
such combination of a targeted drug and targeted delivery of a different drug might provide significant therapeutic benefits for anthrax post-exposure treatment.

REFERENCES


The abbreviations used are: AmPrβCD, per-6-(3-aminopropylthio)-β-cyclodextrin; LFn-Al, LFn-AlexaFluor594 conjugate; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; LFn-Lip, LFn-decorated liposomes loaded with HPTS.

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FIGURE LEGENDS

Figure 1. Site-specific modification does not affect the ability of LFn to bind to cell-associated PA. A, Purified LFn-Al was analyzed by RP-HPLC with detection at 280 nm for protein (solid line) and 598 nm for Alexa-594 (dotted line). Inset: LFn-Al with a single cysteine available for SH-detected modification. B, LFn-Lip made by LFn-Cys lipidation followed by insertion into pre-formed liposomes. Samples were analyzed by reducing SDS-PAGE on 17.5% gel followed by SafeBlue staining (BioRad). Purification on Sepharose 4B column: FT, liposome-containing flow-through fraction; E, elution fraction containing free LFn-Cys. C, competition of LFn-based tracers with LF to PA associated with RAW 264.7 cells. Cells were plated on 96-well plates 15x10^3 cells/well, 20 hrs before the experiment. Varying amounts of an LFn-tracer were mixed with LF and PA in complete culture medium, and added to cells in triplicate wells to final concentrations of 2 nM PA and 0.2 nM LF. After 3 hrs of incubation at 37 °C in 5 % CO₂, viable cell numbers were determined by CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega).
Figure 2. AmPrβCD inhibits internalization, rather than binding of LFn-Al to PA/ATR complex. Cells were plated on glass cover slips, 10^5 cells/slip, and 20 hrs later shifted to fresh culture medium (no PA), or medium supplemented with 8 nM PA alone (+PA), or in combination with 100 µM AmPrβCD (PA + Drug) and pre-incubated for 15 min at 37 °C. LFn-Al was added to a final concentration of 2 nM, and cells were incubated for 1 hr at 37 °C (A) or 4°C (B). Blue, DAPI nuclear counterstaining. Red, Alexa-594. Scale bars, 20 µm.

Figure 3. AmPrβCD-mediated cell rescue is LFn-synergistic and PA-specific. A, Competition with full-length LF was done as described in legend to figure 1C. AmPrβCD alone or in mixture with LFn-Al at a molar ratio of 1000:1, was mixed with LF and PA, serially diluted and added to cells. B, VEGFR-2 expressing 293/KDR cells were plated on 96-well plates, 1,000 cells/well. Twenty hrs later, VEGF or AmPrβCD were serially diluted in complete culture medium containing SLT-VEGF and added to cells in triplicate wells to a final SLT-VEGF concentration of 1 nM. After 96 hrs of incubation at 37 °C in 5 % CO₂, viable cell numbers were determined by CellTiter 96® kit. Am-CD, AmPrβCD.

Figure 4. Rescue CHO-TEM8 cells from PA-induced death. A, CHO-K1 and CHO-TEM8 cells were plated on 96-well plates, 2,000 cells/well, and exposed to either PA alone, or PA in the presence of 5 µM AmPrβCD, 20 hrs later. After 48 hrs of incubation under normal culture conditions, viable cell numbers were determined by CellTiter 96® kit. B, Near-confluent CHO-TEM8 cells were exposed to 30 nM PA alone, or in the presence of 5 µM AmPrβCD, incubated for 24 hrs under normal culture conditions, then analyzed for caspase activation as described in
Methods. C, CHO-TEM8 cells were plated on 96-well plates, 2,000 cells/well. Twenty hrs later, LFnLFn-Lip or Lip control (untargeted HPTS-loaded liposomes adjusted to the same HPTS concentration) were serially diluted in complete culture medium containing 12 nM PA and added to cells in triplicate wells. After 48 hrs of incubation at 37 °C in 5 % CO₂, viable cell numbers were determined by CellTiter 96® kit.

Figure 5. LFn-Lip is internalized by ATR expressing cells regardless of the presence of AmPrβCD. Cells for microscopy were plated and pre-incubated with 8 nM PA with or without 100 µM AmPrβCD as described in legend to figure 2. A, LFn-Lip was added to CHO-TEM8 cells to a final concentration of 2 nM LFn, for 1 h at 37 °C, then PBS washed cells were fixed and observed. Green, HPTS fluorescence. Scale bar, 20 µm. B, incubation with 2 nM LFn-Lip with or without 8 nM PA and 100 µM AmPrβCD, for 1 h at 37 °C. Green, HPTS, blue, DAPI nuclear counterstaining. Scale bar, 20 µm.
Figure 1

A

B

C

LFn - Cys-tag - 6xHis
KESCAKKFQHMDS
Alexa-594

Lipidation, min
Seph 4B
LFn-Cys 5 10 30 60 FT E M, kDa

Cell viability (% control)

LFn, nM
Figure 3

A

Cell viability (% control) vs. LFn-Al, nM for RAW cells
- Am-CD+LFn-Al
- Am-CD

B

Cell viability (% control) vs. AmRβCD, μM for 293/KDR cells
- VEGF
- Am-CD