

## Amiodarone and Bepridil Inhibit Anthrax Toxin Entry into Host Cells<sup>∇</sup>

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Received 21 September 2006/Returned for modification 22 October 2006/Accepted 24 April 2007

**Anthrax lethal toxin is one of the fundamental components believed to be responsible for the virulence of *Bacillus anthracis*. In order to find novel compounds with anti-lethal toxin properties, we used a cell-based assay to screen a collection of approximately 500 small molecules. Nineteen compounds that blocked lethal toxin-mediated killing of RAW 264.7 macrophages were identified, and we report here on the characterization of the two most potent antitoxic compounds, amiodarone and bepridil. These drugs are used to treat cardiac arrhythmia or angina in humans at doses similar to those that provide protection against lethal toxin in vitro. Our results support a model whereby the antitoxic properties of both drugs result from their ability to block endosomal acidification, thereby blocking toxin entry. Amiodarone was tested in vivo and found to significantly increase survival of lethal toxin-challenged Fischer rats.**

*Bacillus anthracis* is a gram-positive bacterium that is the causative agent of anthrax. Two major virulence factors that contribute to disease are lethal toxin (LT) and edema toxin (ET), and these genes are located on an extrachromosomal plasmid, pXO1 (10). Both LT and ET are bipartite toxins that rely on the same binding moiety, protective antigen (PA), to mediate the delivery of their respective enzymatic subunits into the host cytosol. LT consists of PA and lethal factor (LF), a metalloprotease that cleaves the N terminus of mitogen-activated protein kinase kinases 1 to 4, 6, and 7 (15, 49). ET, on the other hand, consists of PA and edema factor (EF), a calcium- and calmodulin-dependent adenylate cyclase that raises cyclic AMP (cAMP) levels once inside the host cell (30).

Cellular entry of either EF or LF begins with PA binding to a cell surface receptor. Receptor-bound PA undergoes furin cleavage, a step that allows it to heptamerize and form a structure known as the prepore (26, 34). The prepore then associates with up to three catalytic subunits and undergoes receptor-mediated endocytosis (38). Acidic pH of the endocytic compartment triggers conformational changes in the PA heptamer, causing it to insert into the membrane of the endosome (34). Insertion results in formation of a pore through which LF and EF translocate, thus gaining access to the cytosol of the host cell (28, 29, 52).

Importantly, both LT and ET are lethal when administered independently to laboratory animals, suggesting a role for both toxins in the pathogenic process (14, 17, 35). At the cellular level, however, the response to each toxin depends on cell type. LT treatment of macrophages derived from certain inbred mouse strains results in rapid cell lysis (6, 18), whereas a less dramatic cytotoxic effect has been seen in endothelial cells and

dendritic cells, among others, where prolonged treatment over several days results in reduced viability (reviewed in reference 3). Yet, most cell types tested so far do not die in response to LT. Rather, more subtle effects have been characterized mostly in immune cells, such as neutrophils and B and T lymphocytes whose immunological functions are impaired. The cellular response to ET is also quite cell type specific. Thus far, only macrophages have been shown to die in response to ET (46). Other, noncytotoxic effects of ET include hindering the phagocytic capacity of neutrophils, interfering with immune cell function and platelet aggregation, and induction of anthrax toxin receptor (ANTXR) expression (1, 8, 11, 33, 40, 41, 47). While we are still in the early stages of understanding the specific contributions of each toxin to the overall pathology of anthrax, it is clear that therapeutics to counteract their actions are needed.

In order to identify compounds that block LT-induced cell death, we screened a collection of biologically active small molecules with the intent of finding novel toxin inhibitors. We took advantage of the RAW 264.7 macrophage cell line, which undergoes a rapid necrotic death in response to LT, to develop a cell-based assay suitable for high-throughput screening. Using this assay we were able to identify approximately 20 compounds that improve cell viability following LT challenge. As the exact cellular response to LT and ET depends on cell type, we focused our efforts on those compounds that block steps involved in toxin entry, which are common to both toxins and to all cell types. In doing so, we aimed to identify compounds that would protect from either toxin regardless of the nature of the target cell population. Here we report on the characterization of two of these drugs, amiodarone and bepridil, which provide the strongest protection in cell-based assays and have been used in humans to treat cardiac arrhythmia or angina.

### MATERIALS AND METHODS

**Cell culture.** Cell lines were grown at 37°C under 5% CO<sub>2</sub> in medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× GlutaMAX-I supplement (Invitrogen). RAW 264.7 cells

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<sup>∇</sup> Published ahead of print on 7 May 2007.

were grown in Dulbecco modified Eagle medium supplemented with glucose, sodium pyruvate, and 25 mM HEPES (Mediatech) and CHO-K1 cells were grown in F-12 medium (Gibco).

**Reagents.** PA and EF were expressed and purified as described previously (51). LF and LF<sub>N</sub>DTA (a fusion of the amino terminus of LF with the diphtheria toxin A chain) were gifts from Jeremy Mogrige (University of Toronto). Diphtheria toxin (DT) and cholera toxin (CT) were purchased from List Biological Laboratories (Campbell, CA). Amiodarone, bepridil, GF109203X, and *N,N*-dimethylsphingosine were obtained from BioMol (Plymouth Meeting, PA). Chloroquine diphosphate, bafilomycin A1, and anti- $\alpha$ -tubulin antibody were purchased from Sigma Aldrich. Anti-PA rabbit serum was obtained from Covance (Berkeley, CA). Anti-MEK-2 N-terminal antibody was purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was obtained from Invitrogen. HRP-conjugated anti-mouse antibody was purchased from AnaSpec (San Jose, CA).

**High-throughput screen.** RAW 264.7 cells were seeded in 384-well, white, flat-bottom plates at  $4 \times 10^5$  cells/well. The next day, 0.1  $\mu$ l of each compound was added into 40  $\mu$ l medium per well by pin transfer. Premixed LT (150 ng/ml PA, 100 ng/ml LF) was then added in 40  $\mu$ l to bring the final volume to 80  $\mu$ l, and cells were incubated overnight at 37°C and 5% CO<sub>2</sub>. The final concentration of compounds in the assay wells was 125 nM, 1.25  $\mu$ M, or 12.5  $\mu$ M, depending on the compound. Each compound was tested at only one concentration in the primary screen. The following day, cell viability was measured using Cell Titer Glo according to the manufacturer's protocol (Promega). Luminescence was measured using an Analyst HT luminometer (Molecular Devices). A full list of compounds tested is available at <http://mssr.pharmacology.ucla.edu/biomol.html>.

**Secondary assays.** RAW 264.7 cells were seeded in white, flat-bottom, 96-well plates at  $2 \times 10^4$  cells/well and allowed to grow overnight. On day two, the medium was replaced with 100  $\mu$ l of fresh medium, followed by the addition of 100  $\mu$ l of a 2 $\times$  compound/toxin mix with either LT (150 ng/ml PA or 100 ng/ml LF) or PA (150 ng/ml) plus LF<sub>N</sub>DTA (40 ng/ml). Final compound concentrations were either 1.25  $\mu$ M or 12.5  $\mu$ M, depending on the agent. Samples were incubated overnight, and viability was measured using Cell Titer Glo as described above. For dose-response curves, cells were seeded as described above and challenged with toxin at 50 to 300 ng/ml per subunit (PA and LF or LF<sub>N</sub>DTA) at different concentrations of compound.

**IC<sub>50</sub> determination.** A total of  $2 \times 10^4$  RAW 264.7 cells/well were seeded in clear, flat-bottom, 96-well plates and allowed to grow overnight. On day 2, the medium was replaced with 100  $\mu$ l of fresh medium. Threefold serial dilutions were made for amiodarone, bepridil, GF-109203X, and *N,N*-dimethylsphingosine to give 4 $\times$  working stock solutions. Fifty microliters of each 4 $\times$  compound dilution was added to each well followed by the addition of 50  $\mu$ l LT (200 ng/ml of each PA and LF) or PA plus LF<sub>N</sub>DTA (200 ng/ml each). Samples were incubated for 24 h, and relative viability was measured using WST-1 viability reagent (Roche). Fifty percent inhibitory concentration (IC<sub>50</sub>) curves were graphed using XL-fit4 software (IDBS). One hundred percent inhibition was defined as the compound concentration providing maximal viability. This viability value was then used to calculate the percent inhibition for the remaining compound concentrations.

**DT assay.** A total of  $5 \times 10^3$  CHO-K1 cells were seeded per well in 96-well plates. On day 2, the medium was replaced twice with 100  $\mu$ l of 50  $\mu$ M, 25  $\mu$ M, or 12.5  $\mu$ M compound, followed by treatment with either PA plus LF<sub>N</sub>DTA (100 ng/ml each) or DT (5 ng/ml) in a 100- $\mu$ l volume. Samples were incubated for 48 h at which time viability was measured by WST-1 assay.

**CT assay.** White, flat-bottom, 384-well plates were seeded with  $8 \times 10^2$  CHO-K1 cells/well and allowed to grow overnight. Samples were then treated with ET or CT in the presence of either amiodarone or bepridil at 25  $\mu$ M, 12.5  $\mu$ M, or 6.25  $\mu$ M. Control samples for each condition were treated with the compound in the absence of toxin. Samples were incubated for 4 h at 37°C. Relative cAMP levels were measured using HTRF cAMP dynamic reagent (CISBIO). Time-resolved fluorescence was measured on a Perkin-Elmer Victor3 V multilabel counter.

**PA binding.** A total of  $1 \times 10^6$  RAW 264.7 cells/sample were stained with Alexa Fluor 647-labeled PA(E733C) as described previously (33). Samples were incubated with 200 ng/ml labeled PA in the presence of amiodarone (12.5  $\mu$ M), bepridil (12.5  $\mu$ M), GF-109203X (3.125  $\mu$ M), or *N,N*-dimethylsphingosine (5  $\mu$ M) for 2 h on ice. Samples were washed with cold phosphate-buffered saline (PBS) prior to fixing in a 3% formaldehyde-PBS solution. Samples were analyzed with a BD FACSCalibur at the Jonsson Cancer Center Flow Cytometry Facility.

**MEK2 cleavage assay.** Six-well plates were seeded with  $1 \times 10^6$  RAW 264.7 cells/well and allowed to grow overnight. Sample cells were then treated with 100 ng/ml each PA and LF in the presence or absence of 12.5  $\mu$ M compound. Samples were incubated at 37°C for 4 h, washed with cold PBS, and lysed with

NP-40 lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40) supplemented with complete mini protease inhibitor tablet (Roche). Ten micrograms of each protein sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-N-terminal MEK-2 antibody, HRP-conjugated anti-mouse horseradish peroxidase secondary antibody, and detected by Immuno-Star HRP detection reagent (Bio-Rad).

**Pore assay.** Six-well plates were seeded with  $5 \times 10^6$  RAW 264.7 cells/well and allowed to grow overnight. Plates were chilled on ice for 10 min prior to PA binding (200 ng/ml) in the presence of amiodarone (12.5  $\mu$ M) or bepridil (12.5  $\mu$ M) at 4°C for 1 h. Binding was followed by 1 to 4 of h incubation at 37°C, at which time samples were washed with cold PBS and harvested as described previously. Seventy micrograms of each protein sample was subjected to SDS-PAGE and immunoblotting with anti-PA serum and HRP-conjugated anti-rabbit secondary antibody.

**Medium pH-pore experiment.** A total of  $5 \times 10^6$  RAW 264.7 cells/well were seeded in six-well plates and allowed to grow overnight. Samples were then pretreated with amiodarone (12.5  $\mu$ M), bepridil (12.5  $\mu$ M), bafilomycin (25 nM), or chloroquine (CQ) (200  $\mu$ M) for 1 h at 37°C. Samples were chilled for 10 min on ice, treated with 200 ng/ml PA in the presence or absence of drug, and incubated on ice for 1 h. Four different medium pH conditions were tested for each drug: 6.25 mM HEPES, pH 7.4; 12.5 mM HEPES, pH 6.8; 50 mM morpholineethanesulfonic acid (MES), pH 6.5; and 50 mM MES, pH 6.0. Samples were transferred to 37°C for 1 h and subsequently washed and harvested. Seventy-five micrograms of protein from total cell lysate was analyzed by immunoblotting as described above.

**Animal studies.** The animal LT challenge was performed according to protocols approved by the Scripps Institutional Animal Care and Use Committee. The in vivo study was performed in Fischer 344 male rats (180 to 200 g; Harlan, Indianapolis, IN). Amiodarone hydrochloride (BioMol International, Plymouth Meeting, PA) in 20% dimethyl sulfoxide (DMSO) in PBS was prepared with either 10 mg/ml (10 $\times$ ; high dose) or 1 mg/ml (1 $\times$ ; low dose) in a final volume of 1 ml per rat. LT was prepared with 10 times the minimal lethal dose of PA and LF (40  $\mu$ g and 8  $\mu$ g, respectively, List Biological Laboratories, Campbell, CA) in PBS in a final volume of 500  $\mu$ l per rat. Protein concentrations were quantified for PA and LF by Bradford assay (Bio-Rad protein assay reagent; Bio-Rad, Hercules, CA). Rats were anesthetized with isoflurane and then inoculated with amiodarone followed by LT through a jugular vein cannula. Control groups included rats that received only drug or carrier. Rats recovered from anesthesia within 5 minutes of administration of the LT and were monitored for symptoms of intoxication and death. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey posttest (Prism).

## RESULTS

**High-throughput screen to identify compounds that block LT cytotoxicity.** In order to identify small molecules that block anthrax toxin-mediated cytotoxicity, we established a high-throughput cell-based screen using LT-sensitive RAW 264.7 cells. Cells were incubated with LT in the presence of compounds from a library of approximately 500 biologically active small molecules (BioMol). Specifically, the collection consisted of a variety of compounds described as ion channel inhibitors, enzyme inhibitors, bioactive lipids, kinase inhibitors, endocannabinoids, and orphan ligands. Control wells received carrier (DMSO) in place of compound in the presence or absence of LT. Twenty-three compounds that gave some degree of protection against LT were identified. Table 1 shows the hits resulting from the initial screen where compounds were tested as a singlet. Their protective ability is expressed as the change in viability relative to the toxin-treated control.

A secondary assay was performed on hits from the initial screen in which cells were challenged with either LT or PA plus LF<sub>N</sub>DTA. LF<sub>N</sub>DTA is a chimeric protein consisting of the fusion of the N terminus of LF to the DT catalytic domain. LF<sub>N</sub>DTA binds to PA and is translocated into the host cell cytosol in the same manner as LF is. However, once

TABLE 1. High-throughput screen for anthrax antitoxic compounds<sup>a</sup>

Sample	Activity	Fold protection	Toxin specificity
Toxin-DMSO	Negative control	1.0	
DMSO (no toxin)	Positive control	13.7 <sup>b</sup>	
Soluble ANTXR1-DMSO	Positive control	7.5	
Bepridil	Calcium channels	8.8	LT, PA + LFnDTA
Amiodarone	Calcium channels	7.9	LT, PA + LFnDTA
GF109203X	PKC inhibitor	6.7	LT, PA + LFnDTA
<i>N,N</i> -Dimethylsphingosine	Bioactive lipid	6.4	LT, PA + LFnDTA
KN-93	CaM <sup>c</sup> kinase II inhibitor	6.3	— <sup>c</sup>
Nicardipine	Calcium channels	5.6	LT, PA + LFnDTA
U-50488	Calcium channels	5.2	LT, PA + LFnDTA
SB-203580	p38 MAP <sup>f</sup> kinase inhibitor	4.3	LT, PA + LFnDTA
SB-202190	p38 MAP kinase inhibitor	4.2	LT, PA + LFnDTA
AM-580	Bioactive lipid	3.9	LT
U-37883A	Potassium channels	3.9	LT
Enantio-PAF <sup>d</sup> C-16	Bioactive lipid	3.2	LT
13- <i>cis</i> -Retinoic acid	Bioactive lipid	3.2	LT
Propafenone	Potassium channels	3.1	—
Retinoic acid, all <i>trans</i>	Bioactive lipid	3.0	LT
PCO-400	Potassium channels	3.0	—
H-89	PKA inhibitor	2.9	—
SDZ-202791 [ <i>R</i> -(—)]	Calcium channels	2.8	LT, PA + LFnDTA
<i>O</i> -Phospho-L-serine	Possible endogenous glutamate ligand	2.8	LT
Phenamil	Sodium channels	2.8	LT
ML-9	MLC <sup>g</sup> kinase inhibitor	2.8	LT, PA + LFnDTA
NS-1619	Potassium channels	2.8	LT
9- <i>cis</i> -Retinoic acid	Bioactive lipid	2.6	LT

<sup>a</sup> RAW 264.7 cells were seeded in 384-well plates, exposed to compounds at a single concentration (between 0.125 and 12.5  $\mu$ M), and then challenged with LT (75 ng/mL PA plus 50 ng/mL LF). Each compound was tested as a singlet. The following day, cell viability was measured using Cell Titer Glo (Promega). Results for the 23 compounds that showed protection are listed. Positive-control wells received no toxin or LT plus a soluble version of the receptor ectodomain (ANTXR), a known inhibitor of toxin entry. Negative-control wells received toxin and DMSO only. Hits from the primary screen were cherry picked and rescreened against LT in triplicate or against PA (75 ng/ml) plus LFnDTA (40 ng/ml) in duplicate. Compounds were then classified as blocking both toxins (likely entry inhibitors), blocking LT specifically (likely inhibitors of events downstream of entry), or false positives (did not repeat in secondary assay). Our classification is shown in the Toxin specificity column.

<sup>b</sup> Fold increase in viability signal over toxin-DMSO sample.

<sup>c</sup> —, compound was not protective during secondary assay.

<sup>d</sup> PAF, platelet-activating factor.

<sup>e</sup> CaM, calmodulin.

<sup>f</sup> MAP, mitogen-activated protein.

<sup>g</sup> MLC, myosin light chain.

in the cytosol, LFnDTA interferes with protein synthesis by modifying elongation factor 2, ultimately leading to cellular apoptosis. Thus, we reasoned that compounds that protected against both LT and PA plus LFnDTA were likely to block entry processes, as these steps are common to both toxins. Of 23 hits identified in the primary screen, 19 were confirmed in the secondary assay with respect to their ability to block LT-mediated cytotoxicity (Table 1). Ten of the 19 confirmed hits protected equally well against both catalytic moieties, suggesting that they likely block steps mediated by PA, rather than steps related to the catalytic activities of either A moiety (Table 1).

IC<sub>50</sub> values for the four compounds with the greatest LT inhibitory activity in the secondary screen were determined (Fig. 1a). IC<sub>50</sub> values were in the low micromolar range, with values from 1.5  $\mu$ M to 4.8  $\mu$ M. The values obtained for amiodarone and bepridil are within the range of serum blood levels found in patients undergoing treatment with these agents (13, 31, 42). This was encouraging, as protection may be possible at dosages tolerated by humans. We therefore decided to focus our future efforts on these two compounds. Dose-response curves for amiodarone and bepridil were performed to determine the ability of each drug to protect cells challenged with LT or PA plus LFnDTA (Fig. 1b). In each case, similar

IC<sub>50</sub> values were obtained for both toxins, further supporting the hypothesis that these drugs block PA-mediated events.

**Amiodarone and bepridil protect against DT but not CT.** To further determine the mechanism of action of amiodarone and bepridil, we tested the abilities of these drugs to protect cells from two additional toxins that enter via endocytosis but differ in their requirements for acidic pH, CT and DT. Like LT, CT and DT enter the cell via receptor-mediated endocytosis (16). Once endocytosed, DT, like LT, translocates out of the endosome in a manner that requires low pH. On the other hand, entry of CT is acid independent, and the catalytic chain translocates into the cytosol from the endoplasmic reticulum after undergoing vesicular retrograde transport. Based on these differences, we reasoned that the effects of amiodarone or bepridil on the activity of either toxin could provide clues as to the drugs' mechanism of action.

We first tested whether amiodarone and bepridil increase cellular viability in response to the DT. CHO-K1 cells were used for this experiment because, unlike RAW 264.7 cells, they have a functional DT receptor (39). For a control, we used PA plus LFnDTA which contains the same catalytic domain but enters via a PA-dependent mechanism. CHO-K1 cells were treated with DT or PA plus LFnDTA in the presence or absence of various drug concentrations for 48 h prior to mea-

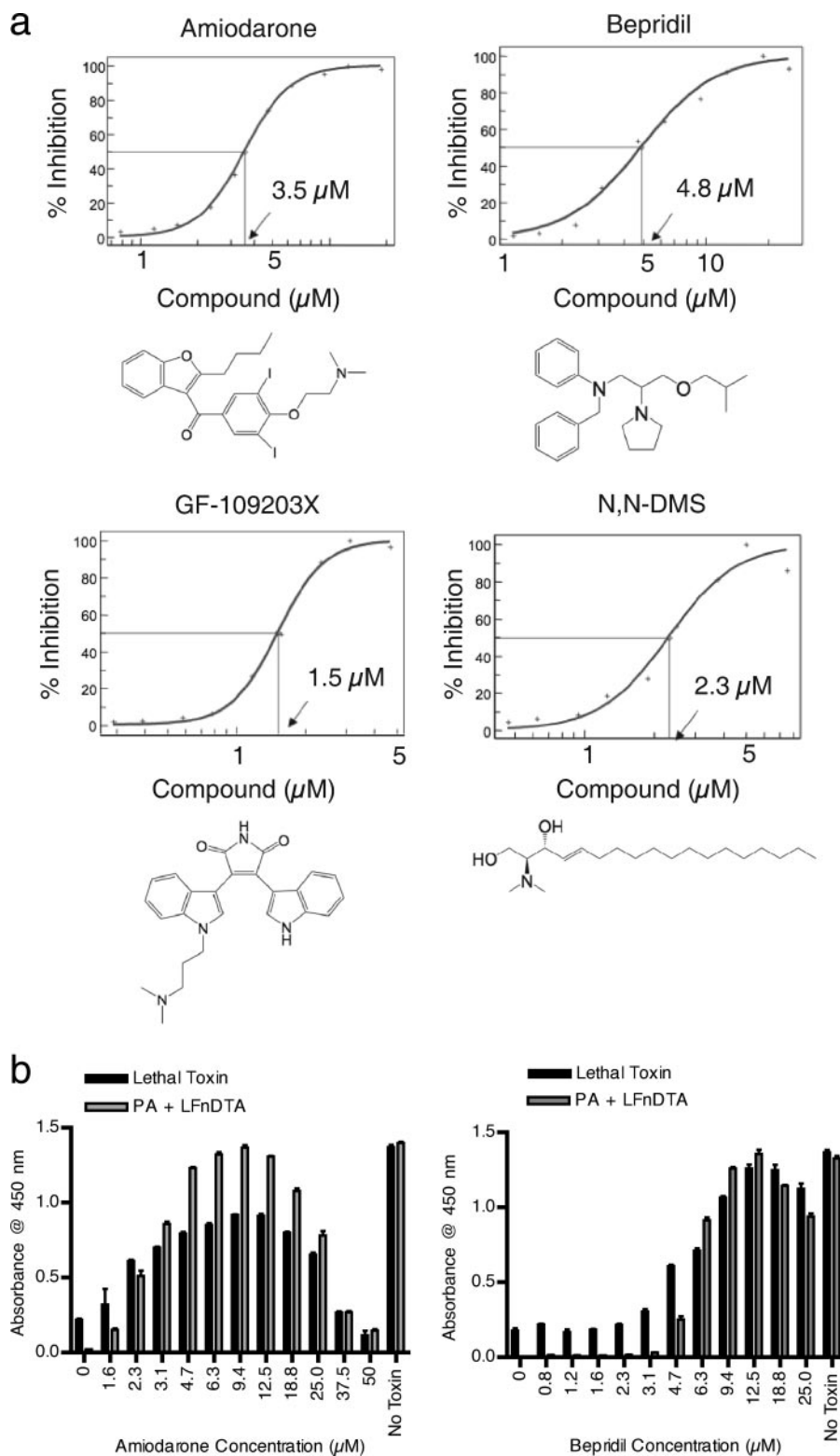


FIG. 1. Characterization of small molecules with antitoxic activity. Following initial high-throughput screening, four compounds were identified that protect RAW 264.7 cells from LF- and LF<sub>N</sub>DTA-mediated killing. (a) IC<sub>50</sub> values were determined for each compound using 50 ng/ml PA plus 50 ng/ml LF. N,N-DMS, *N,N*-dimethylsphingosine. (b) Amiodarone and bepridil show dose-dependent protection against both LT and PA plus LF<sub>N</sub>DTA. RAW 264.7 cells were incubated with 50 ng/ml PA and either 50 ng/ml LF or 50 ng/ml LF<sub>N</sub>DTA. Twenty-four hours later, viability was determined using WST-1 reagent (Roche) and measuring absorbance at 450 nm. Average absorbance values of triplicate samples are graphed with standard deviations (error bars) shown. Cytotoxicity is observed at high doses of either drug. Experiments were performed a minimum of three times with similar results. Results from a single representative experiment are presented.

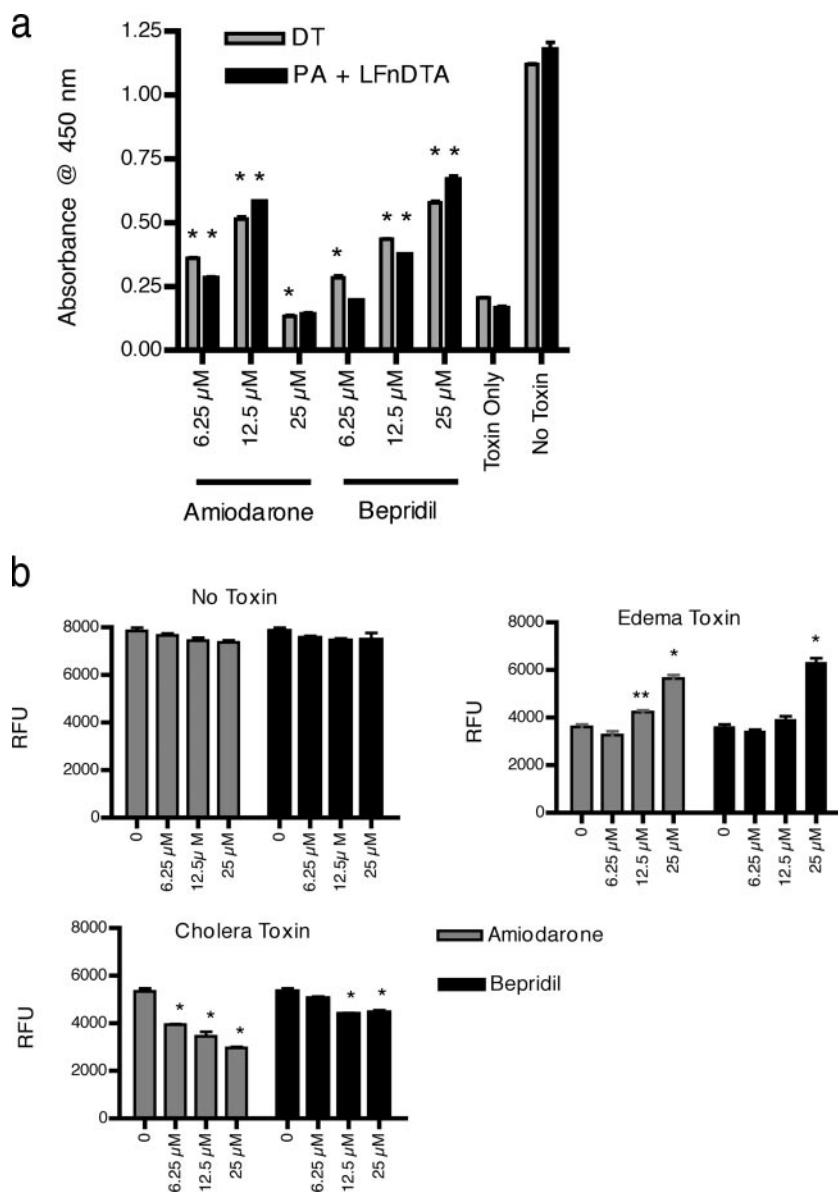


FIG. 2. Amiodarone and bepridil inhibit DT but not CT. (a) Amiodarone and bepridil both block intoxication of CHO-K1 cells by PA plus LFnDTA as well as DT in a dose-dependent manner. Cells were incubated with 50 ng/ml of PA plus 50 ng/ml LFnDTA or 2.5 ng/ml DT in the presence of the indicated concentration of either drug. Forty-eight hours later, cell viability was measured using WST-1 reagent (Roche) and measuring absorbance at 450 nm. Average absorbance values of triplicate samples are graphed with standard deviations (error bars) shown. (b) Amiodarone and bepridil do not block intoxication of CHO-K1 cells by CT. Cells were incubated with either ET (25 ng/ml each PA and EF) or CT (31.25 ng/ml) in the presence of the indicated concentration of either drug. Four hours later, cellular cAMP was measured using HTRF cAMP dynamic reagent (CISBIO). Using this assay, cAMP levels are inversely proportional to relative fluorescence units (RFU). Average fluorescence values of triplicate samples are graphed with standard deviations (error bars) shown. Values that are significantly different from the mean values compared to the toxin-only control calculated by one-way ANOVA with Dunnett's posttest are indicated as follows: \*,  $P < 0.01$ ; \*\*,  $P < 0.05$ .

suring viability. Both amiodarone and bepridil improved viability significantly compared to toxin-only controls (Fig. 2a).

We next tested the abilities of amiodarone and bepridil to block CT, which enters the cytosol via an acid-independent route. CT ADP ribosylates the host  $G_s$  protein, resulting in constitutive activation of cellular adenylate cyclase activity and thus an increase in cAMP levels. In addition, we assayed the effects of bepridil and amiodarone on the activity of anthrax ET, which is itself an adenylate cyclase. For this experiment,

we used CHO-K1 cells, as they have a strong cAMP response to both CT and ET (30). Cells were exposed to either CT or ET in the presence or absence of various concentrations of amiodarone and bepridil for 4 h, at which point cellular cAMP levels were measured. As expected, amiodarone and bepridil interfered with the cAMP response to ET (Fig. 2b). However, neither drug affected the cellular response to CT, indicating that CT entry is not blocked by these drugs. Combined with the DT results, these data are consistent with amiodarone and

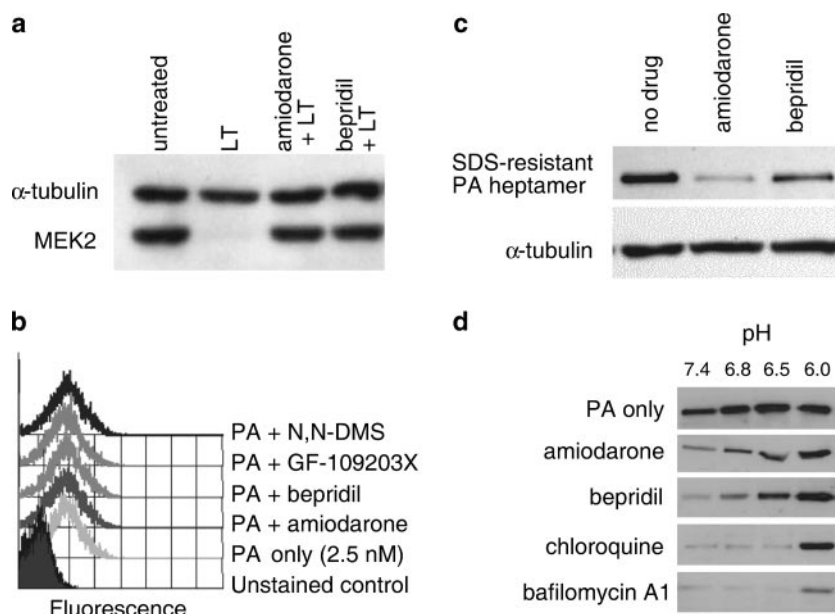


FIG. 3. Amiodarone and bepridil block toxin entry in a pH-dependent manner. To determine at which step intoxication is blocked, cells were incubated with drugs in the presence of PA and/or LT and analyzed as follows. (a) Compounds block MEK cleavage. RAW 264.7 cells were incubated with LT (100 ng/ml each PA and LF) in the presence of 12.5  $\mu$ M amiodarone or bepridil for 4 h, and then cells were lysed. Ten micrograms of protein from total lysates was analyzed by SDS-PAGE and Western blotting using an antibody that recognizes the N terminus of MEK2. (b) Compounds do not block PA binding. RAW 264.7 cells were incubated with Alexa Fluor 647-labeled PA (2.5 nM) in the presence of the indicated amount of each compound for 2 h on ice, then washed, and analyzed by flow cytometry. N,N-DMS, *N,N*-dimethylsphingosine. (c) Compounds block PA pore formation. RAW 264.7 cells were incubated with 200 ng/ml PA in the presence of 12.5  $\mu$ M compounds for 1 h on ice and then shifted to 37°C for 1 h to allow for PA uptake and pore formation. Cells were then lysed and analyzed by Western blotting for the presence of the SDS-stable pore form of the PA heptamer. (d) Block to PA pore formation is dependent on medium pH. RAW 264.7 cells were pretreated with drugs for 1 h at 37°C and pH 7.4. Cells were then incubated with drug plus 200 ng/ml PA for 1 h on ice at pH 7.4. Cells were then shifted to buffered medium containing drug and 200 ng/ml PA at the indicated pH and incubated at 37°C for 1 h to allow for internalization and pore formation.

bepridil blocking intoxication by inhibiting a pH-dependent process in the host cell.

**Amiodarone and bepridil block PA pore formation.** To gain further insight into the mechanism of action of these antitoxic compounds, we treated cells with LT in the presence or absence of each drug and analyzed effects on substrate processing by LF. Consistent with a block to toxin entry processes, much of the cellular MEK2 remained intact when either drug was present (Fig. 3a). The initial step of entry is receptor binding. However, neither drug affected cell surface binding of AF-647-labeled PA (Fig. 3b). Taken together, our results thus far suggested that amiodarone and bepridil act on toxin entry steps that occur subsequent to receptor binding but prior to toxin translocation into the cytosol.

Receptor-bound PA heptamerizes on the cell surface forming a structure known as the prepore, which after relocating to lipid rafts, undergoes endocytosis and is trafficked to a low-pH endosome. Exposure to the low pH causes PA to insert into the membrane of the endosome and form a pore that translocates the catalytic moieties into the cytosol. Unlike the prepore, the pore structure is highly stable to denaturation and remains oligomerized even when exposed to detergents, such as SDS (34). The possibility that amiodarone and bepridil affect pore formation seemed plausible, since both have tertiary amines, characteristic of agents that raise endosomal pH (Fig. 1a). Such pH neutralization would prevent membrane insertion of the PA heptamer. Consistent with this hypothesis, PA pore

formation was reduced in the presence of these compounds (Fig. 3c).

Lysosomotropic agents, such as ammonium chloride and CQ, protect cells from LT by raising the pH of the endosome (18). This, in turn, interferes with the insertion of the PA heptamer into the membrane of the endosome and thereby prevents the translocation of LF into the cytosol of the host. The effects of amiodarone and bepridil on anthrax toxin entry are consistent with endosomal pH neutralization. However, it is possible to block toxin entry by inhibiting the ability of PA to heptamerize, as was shown recently to be the mechanism of action for cisplatin (37). Thus, we wished to determine whether the block to intoxication induced by amiodarone and bepridil correlated with pH-induced changes in PA. Medium pH has been shown to alter the ability of lysosomotropic agents to protect cells from anthrax LT (18). Using a similar approach, we compared the effect that medium pH has on the abilities of amiodarone and bepridil to block pore formation. Indeed, acidic media were able to restore pore formation activity in the presence of either drug (Fig. 3c), indicating that the most likely mechanism of antitoxic activity for amiodarone and bepridil is through endosome pH neutralization.

**Amiodarone increases survival in Fischer 344 rat intoxication model.** Finally, we wished to determine whether amiodarone could protect animals from a lethal challenge of LT. Bepridil was not tested, as it is no longer available for human use in the United States due to adverse side effects. Cannu-

TABLE 2. Amiodarone increases survival in Fischer rats challenged with LT<sup>a</sup>

Treatment	Avg time to death (min) (SD)	P value (Tukey test)
LT only	61.2 (2.28)	
Amiodarone (5.4 mg/kg) + LT	64.8 (7.57)	>0.05
Amiodarone (54 mg/kg) + LT	82.6 (2.28)	<0.001

<sup>a</sup> Cannulated Fischer rats (five rats per condition) were used to test toxin inhibitory efficacy of amiodarone. Amiodarone was injected via the cannula at the indicated doses (in milligrams per kilogram of body weight) followed immediately by injection of LT (40  $\mu$ g PA, 8  $\mu$ g LF). Animals were constantly monitored to determine time to death. One-way ANOVA analysis indicated that differences in time to death were significant ( $P < 0.0001$ ). Further statistical analysis was performed using the Tukey test to determine whether individual treatment groups were significantly different from the control group (shown in table).

lated Fischer 344 rats were injected with either a high or low dose of amiodarone, followed immediately by injection of LT (40  $\mu$ g PA and 8  $\mu$ g LF). High-dose amiodarone treatment gave statistically significant increases in the time to death compared to animals receiving toxin only (Table 2). Animals receiving only drug or carrier displayed no adverse side effects (data not shown). Amiodarone clearance in vivo is biphasic and is initially cleared rapidly from the serum following single bolus injection likely due to lipid partitioning (45). However, animals and humans can be given loading doses to increase serum concentrations of amiodarone. Thus, future experiments should include multiple dosing regimens to increase serum concentrations of the drug.

**Amiodarone and CQ provide additive protection from LT.** Amiodarone-based protection from LT in rats was encouraging yet incomplete, consistent with high doses of this drug (10  $\mu$ M) required for maximal protection in cell culture experiments. In humans, amiodarone serum concentrations are limited by toxicity to 2.5 to 5  $\mu$ M. Several lysosmotropic drugs are known to block LT activity, including CQ. Indeed, CQ protects cells in culture (18) and can protect mice from LT challenge (2). In previous reports, the dose of CQ required to protect cultured cells is  $\sim$ 100  $\mu$ M, which is much higher than the serum concentration safely achievable in humans ( $\sim$ 10  $\mu$ M) (50). Indeed, Comer and colleagues were unable to achieve therapeutic doses of the related drug quinacrine in animal models due to drug toxicity (11). Therefore, we next wished to determine whether protection from LT could be achieved at lower concentrations of amiodarone and CQ by using a combination of both drugs. CQ was first tested in isolation and found to protect RAW 264.7 cells at 33  $\mu$ M (Fig. 4a), a dose slightly lower than previously reported, yet higher than achievable serum concentrations or the protective dose of amiodarone. A mixture of both drugs showed increased protection at lower concentrations of each, indicating an additive protective effect (Fig. 4b).

## DISCUSSION

Here we present evidence that two drugs previously used to treat cardiac arrhythmia or angina can also function as inhibitors of anthrax toxin. Amiodarone is a commonly prescribed drug, recently accounting for nearly one-third of all antiarrhythmic prescriptions in North America (13). In contrast to their

predicted actions as cardiac drugs, the most likely mechanism of toxin inhibition for both amiodarone and bepridil appears to be via neutralization of endosomal pH, thereby blocking toxin entry. These two drugs join the growing list of compounds that have been reported to block anthrax toxin activity in animal models via endosomal pH neutralization (2, 36). We report that protection from LT in cell culture models correlates with IC<sub>50</sub> values of 3.5  $\mu$ M for amiodarone and 4.8  $\mu$ M for bepridil. These in vitro concentrations compare favorably with reported concentrations in serum from human patients, which reach 3.75  $\mu$ M amiodarone and 6.8  $\mu$ M bepridil (31, 42). Interestingly, amiodarone and bepridil were recently reported to also function as antimalarial drugs with IC<sub>50</sub> values of approximately 2.5  $\mu$ M each (32).

Amiodarone has multiple activities on host cells and has

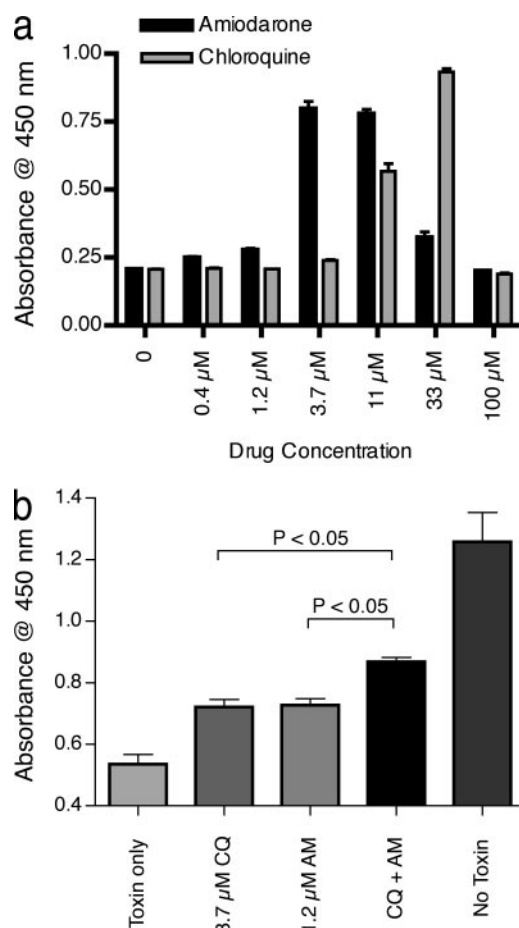


FIG. 4. Amiodarone and CQ provide combined protection from LT. RAW 264.7 cells were challenged with 300 ng/ml LT in 96-well plates. Cell viability was measured 24 h later using WST-1 as described in Materials and Methods. Column values represent average absorbance values of triplicate samples and error bars represent standard deviations. (a) Intoxication was performed in the presence of increasing concentrations of CQ or amiodarone. (b) Intoxication was performed in the presence of 3.7  $\mu$ M CQ, 1.2  $\mu$ M amiodarone (AM), or a combination of both drugs. Statistical analysis for data in panel b was performed using one-way ANOVA ( $P < 0.001$ ). Tukey's multiple comparison test showed that all column values differed significantly from each other ( $P < 0.05$ ) with the exception of 3.7  $\mu$ M CQ versus 0.4  $\mu$ M amiodarone ( $P > 0.05$ ).

been reported to block calcium, sodium, and potassium channels and also interferes with phospholipases and protein kinase C (PKC) activity (48). Additionally, bepridil and several other initial hits from our primary screen had reported inhibitory effects on PKC. PKC inhibitors were previously reported to block cellular intoxication by anthrax LT (5). Thus, it was important to determine the mechanism of action for amiodarone and bepridil. A separate hit, GF-109203X, also displayed PKC-inhibitory and lysosomotropic activities. This was useful, as there are several bisindolylmaleimide compounds related to GF-109203X that target different isoforms of PKC or have no PKC inhibitory activity but retain tertiary amines and thus have the potential to neutralize endosomal pH. Using a subset of these compounds as well as a dominant-negative form of PKC- $\beta$ II, we found that toxin inhibitory activity correlated strongly with the presence of tertiary amines, but not PKC inhibition (A. M. Sanchez and K. A. Bradley, data not shown). While we cannot exclude a role for PKC in anthrax toxin sensitivity, our data support only a role for neutralization of endosomal pH.

Conflicting data exist as to whether amiodarone is lysosomotropic or whether the tertiary amine present in amiodarone neutralizes lysosomal pH (4, 9, 19, 20, 23, 25, 43). Interestingly, our findings differ from those of Baritussio et al. who reported that 10  $\mu$ M amiodarone did not block DT activity in rabbit alveolar macrophages (4). In contrast, Futamura reported that amiodarone inhibits endosomal acidification and cellular pH using fluorescein isothiocyanate-dextran or acridine orange-based fluorescence assays (19, 20). These effects were seen as early as 30 min postexposure, consistent with our data in which drugs are added immediately before toxin. Furthermore, both Futamura and Quaglino et al. reported differential cellular effects of amiodarone based on its cationic, amphiphilic, or unique properties (19, 43). Thus, it may be possible to identify analogs that retain antitoxic activity while removing antiarrhythmic and/or toxic properties.

Lysosomotropic agents have previously been shown to block the acid-dependent entry of anthrax toxin (12, 18, 22, 24, 27, 36). Recently, CQ was shown to be effective in reducing LT-induced mortality in animal models (2). Furthermore, oxidized ATP and other nucleotides were shown to both neutralize endosomal pH and protect animals from LT challenge (36). In addition, many steps of anthrax toxin entry and catalytic activity have been targeted for inhibitor development (44). The importance of identifying additional drugs that block anthrax toxins may be in their future combinatorial uses. Several drugs identified as anthrax toxin inhibitors, including amiodarone, bepridil, and CQ, have known side effects that may limit their use. However, it may be possible to use these drugs in combination, each at a lower concentration, to achieve efficient toxin-blocking activity. Here we demonstrate that amiodarone and CQ can be used in combination *in vitro* to enhance protection from LT. However, this specific combination of drugs is not likely to represent a viable therapy *in vivo*, as the use of CQ with antiarrhythmic drugs is contraindicated. Rather, our results reaffirm the strategy of using multiple toxin inhibitors simultaneously to achieve enhanced protection or to achieve therapeutic effects at subtoxic drug doses. Indeed, the combination of CQ with furin inhibitors, which block processing of PA, was shown to protect murine macrophages from LT more efficiently than protection conferred by either compound alone

(27). This synergistic protection was observed at CQ concentrations of 10  $\mu$ M, much lower than the concentration required to achieve protection in the absence of furin inhibitors.

Futamura reported cell type specificity in the response to amiodarone (20). However, we have observed that the antitoxic activities of amiodarone and bepridil are maintained in multiple cell types, including monocytes or macrophage-like cells (THP-1 [data not shown] and RAW 264.7), Raji B cells (data not shown), and epithelial cells (CHO-K1). In addition to protecting against LT, these drugs also protected cells from ET and PA plus LF<sub>N</sub>DTA, suggesting that they block steps mediated by PA and thus would function to block lethal effects of both anthrax toxins *in vivo*.

Using the well-established Fischer 344 rat model for LT challenge, we were able to demonstrate protective effects of amiodarone *in vivo*. That complete protection was not observed in the Fischer rats is not surprising, as a single dose of amiodarone is predicted to clear rapidly from circulation (13). Indeed, oral administration of amiodarone in humans involves loading doses of 800 to 1,600 mg/day followed by maintenance doses of 200 mg/day (13, 42). Following this dosing regimen, serum concentrations in humans can exceed 3.75  $\mu$ M and remain in the bloodstream with a serum half-life of up to 60 days (13, 42). Therefore, future studies should be designed to address the antitoxic efficacy of amiodarone in animals preloaded with drug. While preloading may not be feasible in anthrax patients following spore exposure, the large number of individuals that are effectively preloaded as a result of using amiodarone to treat arrhythmia may represent a subpopulation with altered susceptibility to anthrax toxin. Other factors will likely come into play when considering aerosolized spore challenges rather than intoxication models. Indeed, amiodarone has been reported to alter phagocytic activity of alveolar macrophages, which are believed to play a role in initiation of inhalation anthrax (20, 21, 43). How amiodarone influences the outcome of inhalation anthrax is an important question for future studies.

#### ACKNOWLEDGMENTS

This research was funded by National Institutes of Health (NIH) awards AI-057870 (K.A.B.) and P01AI056013 (M.M.). A.M.S. was supported by National Research Service award 1-F31-AI-061847. We also acknowledge support of the UCLA Jonsson Comprehensive Cancer Center (JCCC) and the UCLA AIDS Institute and the flow cytometry, virology, and mucosal immunology cores which are supported by NIH awards CA-16042 and AI-28697 (UCLA CFAR grant) and by the Jonsson Comprehensive Cancer Center (JCCC), the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA.

We thank David J. Banks and Steven LeVine for helpful discussions.

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