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Article

In Vivo Activity of Repurposed Amodiaquine as a Host-Targeting Therapy for the Treatment of Anthrax

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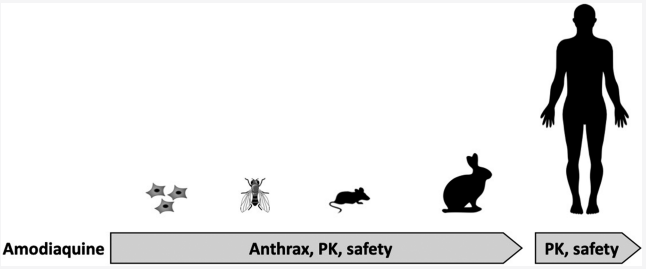
ABSTRACT: Anthrax is caused by *Bacillus anthracis* and can result in nearly 100% mortality due in part to anthrax toxin. Antimalarial amodiaquine (AQ) acts as a host-oriented inhibitor of anthrax toxin endocytosis. Here, we determined the pharmacokinetics and safety of AQ in mice, rabbits, and humans as well as the efficacy in the fly, mouse, and rabbit models of anthrax infection. In the therapeutic-intervention studies, AQ nearly doubled the survival of mice infected subcutaneously with a *B. anthracis* dose lethal to 60% of the animals (LD_{60}). In rabbits challenged with 200 LD_{50} of aerosolized *B. anthracis*, AQ as a monotherapy delayed death, doubled the survival rate of infected animals that received a suboptimal amount of antibacterial levofloxacin, and reduced bacteremia and toxemia in tissues. Surprisingly, the anthrax efficacy of AQ relies on an additional host macrophage-directed antibacterial mechanism, which was validated in the toxin-independent *Drosophila* model of *Bacillus* infection. Lastly, a systematic literature review of the safety and pharmacokinetics of AQ in humans from over 2 000 published articles revealed that AQ is likely safe when taken as prescribed, and its pharmacokinetics predicts anthrax efficacy in humans. Our results support the future examination of AQ as adjunctive therapy for the prophylactic anthrax treatment.



KEYWORDS: *Bacillus anthracis, anthrax toxin, amodiaquine, pharmacokinetics, safety, efficacy*

Bacillus anthracis is a toxin-producing bacteria that causes anthrax, which can result in a nearly 100% mortality rate.[1] The 2001 United States anthrax mail attacks illustrated the use of *B. anthracis* spores as a biological weapon of mass destruction, where despite aggressive treatment with antibiotics, the fatality rate was 45% for anthrax victims.[2] This incident demonstrated that, unfortunately, antibiotics are ineffective against the toxins, which remain in circulation at lethal levels despite the antibiotic treatment and emphasize the need to develop antitoxins.

B. anthracis secretes protective antigen (PA), lethal factor (LF), and edema factor (EF), three toxin components encoded on the pXO1 plasmid that lead to hemorrhages, edema, and necrosis of mammalian host cells.[3] Monomeric 83 kDa PA (PA_{83}) binds to the mammalian cell-surface receptors, where furin cleaves PA_{83} into a free 20 kDa (PA_{20}) and a host receptor-bound 63 kDa (PA_{63}) subunits.[3] Seven or eight receptor-bound PA_{63} multimerize to form a prepore, which binds EF and LF before clathrin-mediated endocytosis.[4] The decrease in the endosomal pH leads to the formation of an endosomal membrane PA_{63} channel. The endosomal fusion with lysosomes induces the translocation of LF and EF into the cellular cytoplasm.[5] Cathepsin B (Ctsb) is a lysosomal protein necessary to deliver LF and EF from the intraluminal vesicles of the late endosomes into the cytoplasm through a back fusion process.[6] Once in the cytosol, EF and LF exert their lethal effects on mammalian host cells.

Several approved therapies exist to treat anthrax-associated bacteremia and toxemia. Both antibacterials and antitoxins are recommended for postexposure treatment.[7] Ciprofloxacin,

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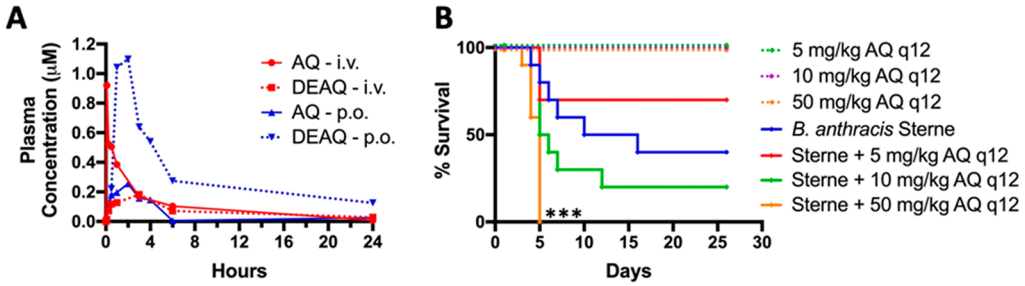

Figure 1. Pharmacokinetics and anthrax-efficacy of AQ in mice. (A) Measurement of plasma concentrations of AQ and metabolite DEAQ in mice. Plasma concentrations of AQ and DEAQ were measured in A/J female mice given a single dose AQ, either 1 mg/kg intravenously (i.v.) or 20 mg/kg orally (p.o.). Blood samples were collected before AQ administration and 0.083, 0.25, 0.5, 1, 3, 6, 8, and 24 h postadministration for both administration routes. (B) Effects of varying doses of AQ on the survival of Sterne-infected mice. Ten A/J female mice per group were treated with 5, 10, or 50 mg/kg of AQ by oral gavage every 12 h for 5 days in the presence or absence of *B. anthracis* exposure. The survival of mice was observed for 26 days. *P* values based on Log-rank (Mantel−Cox) test indicate statistical significance compared to the *B. anthracis* Sterne conditions (***, $p = 0.0008$).

Table 1. Pharmacokinetics of AQ and DEAQ in Mice and NZW Rabbits[a]

animal	drug (route)	M/F ($n = 3$)	analyte	T_{max} (h)	C_{max} (μM)	$t_{1/2}$ (h)	AUC_{last} (h μM)	AUC_{inf} (h μM)	Cl (mL/h/kg)	V_z (mL/kg)
A/J mice	AQ (i.v.)	F	AQ	0.08	0.92	0.5	2.484		1 131	816
			DEAQ	3.0	0.18	2.0	1.678		1 818	3 636
	AQ (p.o.)	F	AQ	2.0	0.26	2.0	1.154			
			DEAQ	2.0	1.1	2.0	7.313			
	AQ (i.v.)	M	AQ	0.083 ± 0.00	11.47 ± 1.92	9.0 ± 0.4	8.02 ± 0.63	8.13 ± 0.65	3 169 ± 153	41 136 ± 3 667
			DEAQ	5.3 ± 1.2	0.333 ± 0.06	20.4 ± 2.5	8.15 ± 0.96	10.50 ± 1.81		
		F	AQ	0.083 ± 0.00	14.06 ± 4.95	9.3[b]	15.37[b]	15.58[b]	1 668[b]	22 366[b]
			DEAQ	4.0[b]	0.506[b]	18.5[b]	11.24[b]	13.89[b]		
NZW rabbits	DEAQ (i.v.)	M	DEAQ	0.083 ± 0.00	7.53 ± 0.31	12.7 ± 1.8	22.53 ± 1.96	23.92 ± 2.65	1 266 ± 133	22 798 ± 1 444
		F	DEAQ	0.083 ± 0.00	6.86 ± 1.72	12.6 ± 0.6	18.93 ± 1.91	20.03 ± 2.03	1 508 ± 160	27 487 ± 2 692
	AQ (p.o.)	M	AQ	1 ± 0.6	0.801 ± 0.22	3.1 ± 1.0	3.52 ± 1.56	3.88 ± 1.39		
			DEAQ	2 ± 0.6	2.05 ± 0.77	11.3 ± 1.5	26.07 ± 8.26	27.84 ± 9.19		
		F	AQ	2 ± 0.6	1.07 ± 0.16	3.7 ± 1.5	4.68 ± 1.42	4.84 ± 1.31		
			DEAQ	2 ± 0.6	3.04 ± 0.44	10.5 ± 1.0	33.23 ± 5.54	34.88 ± 5.33		

[a]The pharmacokinetics of AQ and DEAQ were determined in A/J mice given a single dose AQ, either 1 mg/kg intravenously (i.v.) or 20 mg/kg orally (p.o.). Moreover, the pharmacokinetics of AQ and DEAQ were determined in NZW rabbits given a single dose of 10 mg/kg of AQ or DEAQ i.v. or 20 mg/kg of AQ p.o. [b]Animals ($n = 2$) exhibited severe reactions postdose and were euthanized. Therefore, a complete drug concentration profile is not available.

levofloxacin, and doxycycline are approved antibiotics as a treatment for inhalational anthrax.[7] To combat toxemia, three anti-PA_{83} antibodies exist: raxibacumab, anthrasil, and obiltoxaximab.[8] However, there are limitations to the existing antitoxins. The currently approved antitoxins require more expensive manufacturing, special storage, and intravenous (IV) administration. Additionally, because multidrug resistance has been observed in naturally occurring *B. anthracis* infections, there is a possibility of drug resistance against anthrax therapies.[9] Moreover, the limitations of antibody-based antitoxins include their inability to act intracellularly and reduced ability to cross the blood-brain barrier.[8] A wide-area aerosol of *B. anthracis* could cause a large number of casualties.[10] In such a mass-exposure event, the standard of care is to send asymptomatic patients home with a self-administered oral postexposure prophylactic.[10] However, because no oral antitoxin is available, such prophylaxis would be limited to an antibiotic only, which reduces survival rates for high bacteremia cases. Therefore, identifying and developing orally administered antitoxins with an alternative mechanism of action is urgently needed.

Alternative strategies to reduce drug-resistance in *B. anthracis* could include host-targeting approaches and drug repurposing of small molecule drugs. Host-oriented therapies blocking the host proteins exploited by pathogens are less likely to be circumvented by microbial resistance, since these drugs do not directly target pathogens. Drug repurposing can be used to foresee the side effects caused by such host-directed drugs. Approved drugs already have well-established safety and pharmacokinetic (PK) profiles in humans and animals as well as existing manufacturing and distribution networks, which would potentially accelerate their approval.

It has been previously discovered that a small molecule antimalarial amodiaquine (AQ) effectively inhibited LF-PA cytotoxicity by targeting host Ctsb.[11] AQ, also known as Camoquin, Basoquin, and Flavoquine, is a synthetic 4-aminoquinoline patented in 1949 by Parke-Davis (today a Pfizer subsidiary) used as an alternative to treat chloroquine-resistant malaria-causing *Plasmodium falciparum* at 10 mg/kg/day for 3 days. Both AQ and its primary active metabolite, desethyl-amodiaquine (DEAQ), are known to be efficacious against malaria.[12] While the WHO Model List of Essential

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Medicines currently recommends AQ for malaria treatment, AQ is efficacious against other deadly and aggressive infectious diseases such as Ebola and other Ctsb-depended viruses and toxins.[11,13] *In vitro* studies have shown that AQ and DEAQ inhibit the cytosolic entry of LF into mouse macrophage RAW264.7 cells and protect the cells from apoptosis.[11] An *in vivo* study revealed that AQ inhibits LF-PA-induced death in the Sprague−Dawley rat model.[11]

In this study, we test the safety and efficacy of AQ for the treatment of inhalational anthrax. We show toxicity and PK profiles in mice and rabbits and demonstrate appreciable increases in survival during anthrax in both models. Additionally, we demonstrate *in vitro* and *in vivo* data suggesting an additional host-oriented antibacterial mechanism of AQ. Finally, we provide a comprehensive analysis of all currently published articles on AQ, revealing any known occurrence of adverse drug reactions (ADRs) and PK in humans. Collectively, these results support the future exploration of AQ in nonhuman primates and its potential application as a human adjunct therapy in the treatment of inhalational anthrax.

RESULTS

Pharmacokinetics of Amodiaquine and Desethyl-Amodiaquine in Mice. A single-dose toxicity assay in female A/J mice (3 mice per time point) was performed, where AQ was administered intravenously at doses ranging from 1 to 200 mg/kg. We observed that doses of AQ higher than 5 mg/kg were toxic to mice, resulting in increased clinical signs and, in some cases, death. Therefore, we determined the maximal plasma concentration (C_{max}), a time when C_{max} is achieved (T_{max}), clearance (Cl), volume of distribution (V_d), and plasma half-life ($t_{1/2}$) of AQ and DEAQ by administering a single dose of 1 mg/kg AQ intravenously or 20 mg/kg orally and collecting blood samples at predose and throughout the 24 h (Figure 1A and Table 1). Oral administration of 20 mg/kg AQ was chosen because it was used to determine PK profiles of AQ and DEAQ in the rhesus macaque model of the Ebola virus.[14]

The oral administration of AQ resulted in plasma DEAQ with $t_{1/2}$ and C_{max} of 2 h and 1.1 μM, which is within the range of effective antitoxin and antiviral concentrations of AQ and DEAQ.[11] Therefore, oral administration is an effective route of delivery of AQ into plasma. Moreover, since both AQ and DEAQ are short-lived in the plasma of smaller mammals, multiple drug administrations per day are required in mice during subsequent animal efficacy tests.

Amodiaquine Protects Mice Challenged with *B. Anthracis* Spores. We tested the ability of orally administered AQ to protect anthrax model A/J mice[15] infected with *B. anthracis* toxigenic Sterne strain. AQ was administered every 12 h for 5 days at 5, 10, and 50 mg/kg. The infection with 1.1×10^4 spores resulted in the survival of 40% of mice (Figure 1B). When provided with 5 mg/kg of AQ, the survival rate improved from 40% to 70%, although statistical significance was not reached. Interestingly, infected mice that received AQ at either 10 or 50 mg/kg demonstrated a dose-dependent, statistically significant decrease in survival to 20% and 0%, respectively. None of the AQ doses were lethal to uninfected mice. These results reveal 5 mg/kg as a therapeutic dosage of AQ in Sterne-infected mice while demonstrating decreased survival times at increased AQ doses.

Pharmacokinetics of Amodiaquine and Desethyl-Amodiaquine in Rabbits. Three male and female NZW rabbits were each administered a single intravenous 10 mg/kg dose or a single dose of 20 mg/kg via oral gavage of AQ or DEAQ. While male rabbits of either group exhibited no ADRs, female rabbits were more sensitive to AQ and DEAQ (Tables S1 and S2). In intravenous studies, the toxicity was evident in all AQ-treated female rabbits, with ADRs occurring immediately after dosage. DEAQ had only a minor effect on females, with one female experiencing minor hypoactivity. In contrast to intravenous studies, all rabbits experienced no severe ADRs postorally administered AQ. Only 2 rabbits (one male and one female) exhibited reduced appetite and/or reduced fecal output.

In rabbits administered 10 mg/kg of AQ or DEAQ intravenously, mean plasma concentrations of both AQ and metabolite, DEAQ, were measured throughout the 48 h postadministration to determine the PK profile (Figure 2A, Table 1, and Table S3). AQ's oral administration resulted in C_{max} values of DEAQ of 2.05 μM and 3.04 μM for males and females, respectively (Figure 2B, Table 1, and Table S4). The $t_{1/2}$ for AQ's oral administration was 3.1 and 3.7 h for males and females, respectively. The $t_{1/2}$ of DEAQ was longer in both genders: 11.3 h for males and 10.5 h for females. Overall, the toxicity and PK profiles of AQ in rabbits show oral administration results in little to no ADRs as well as favorable plasma concentrations of AQ and DEAQ.

Amodiaquine Delays the Death of Rabbits Challenged with Aerosolized *B. anthracis* Spores. We evaluated the efficacy of AQ given orally against toxigenic, encapsulated, and aerosolized *B. anthracis* Ames in NZW rabbits, and we tested the effect of the drug on bacteremia and antigenemia (measured by plasma PA) (Table S5). All rabbits were challenged by aerosol with 200 LD_{50} of spores in the presence or the absence of AQ. The animals' respiration was monitored and recorded to ensure aerosol deposition of 2.0×10^7 spores. Immediately postchallenge, the animals either received 0, 5, or 20 mg/kg of AQ via oral gavage twice daily for 3 consecutive days. The weights of animals were recorded throughout the study, showing an expected weight loss postinfection (Figure S1).

As seen in Sterne-infected mice, higher doses of AQ significantly decrease survival time in rabbits: 20 mg/kg of AQ decreased the median survival time by a statistically significant 21.5 h (Figure 2C). The stomachs of rabbits that received 20 mg/kg revealed visible signs of tissue injury to the gastric lining. In contrast, the animals receiving a lower AQ dose of 5 mg/kg yielded a statistically significant 12 h longer median survival time, although all of the animals succumbed to anthrax.

Since 5 mg/kg given to rabbit twice daily corresponded to the human equivalency dose of 3.2 mg/kg/day and is lower than the approved dose of 10 mg/kg/day of AQ in humans, we tested the efficacy of 5 mg/kg AQ when given twice daily for 5 days to *B. anthracis* infected rabbits. We also tested AQ's anthrax efficacy at a lower dose of 1.25 mg/kg. While the median survival times of treated animals yielded no significant difference compared to untreated rabbits (70−75 h), 40% of the rabbits given 5 mg/kg exhibited 40−50 h longer survival times than the control animals (Figure 2D). Although AQ did not protect any rabbits from inhalational anthrax, 5 mg/kg consistently showed the trend of delaying infected animals' death. These experiments determined the efficacious dose, frequency, and AQ administration duration as 5 mg/kg given twice a day orally for 5 days.

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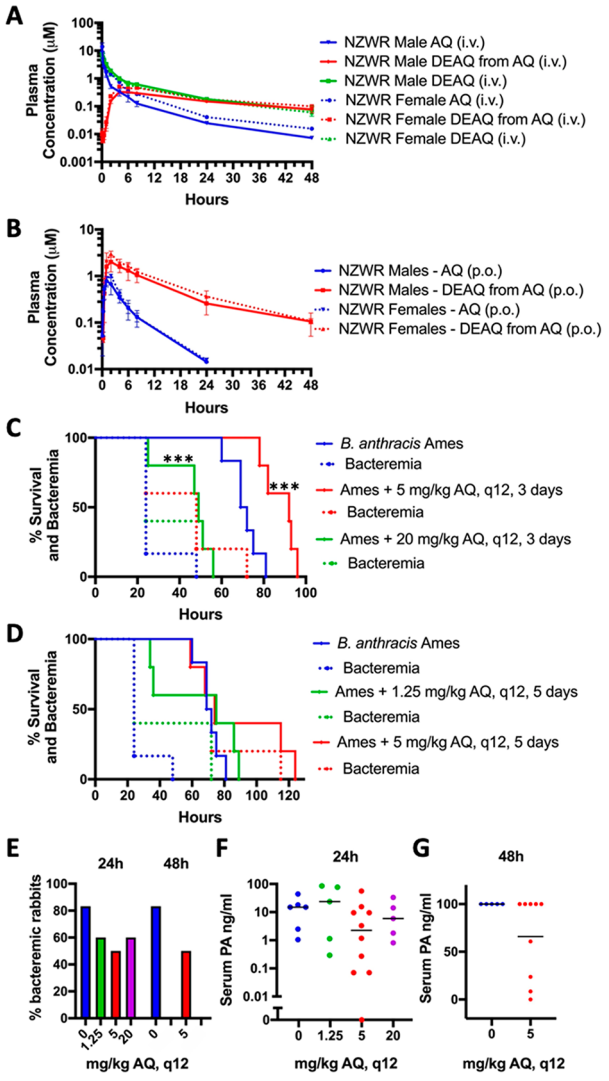

Figure 2. Pharmacokinetics and anthrax-efficacy of AQ in rabbits. (A) Plasma concentrations of AQ and DEAQ in male and female NZWR were measured after a single intravenous (i.v.) dose of 10 mg/kg of AQ or DEAQ. (B) Plasma concentrations of AQ and DEAQ of male and female rabbits given 20 mg/kg of AQ orally (p.o.). (A,B) Blood samples were collected before and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24- and 48 h postadministration of AQ. (C−G) Effects of varying doses of AQ on anthrax-related events in infected rabbits. (C,D) Survival (solid) and the first detection of bacteremia, defined as the presence of any *B. anthracis* in the blood (dashed) of infected rabbits, were measured in the absence or presence of 1.25, 5, or 20 mg/kg of AQ. Beginning at the time of aerosol exposure to 200 LD_{50} of *B. anthracis* Ames spores, animals were given AQ treatment for either 3 days (C) or 5 days (D) via oral gavage every 12 h. Treatment groups consisted of 5 rabbits each, while the control group consisted of 6 rabbits. *P* values based on Log-rank (Mantel−Cox) test indicate statistical significance of survival curves compared to *B. anthracis* Ames conditions (***, $p < 0.001$). (E) Percentage of bacteremic rabbits, whose blood contained any *B. anthracis*, as measured by the presence of *B. anthracis* in serum, in each infected group at 24- and 48-post infection. (F,G) Serum anthrax toxin component, Protective Antigen (PA), was measured from animals in each anthrax-infected group at 24- and 48 h postchallenge. The assay's upper limit of quantitation of PA was 100 ng/mL. Animals given 5 mg/kg of AQ were grouped, regardless of whether given for 3 or 5 days (E−G). Bars indicate median values.

In addition to examining the protective effects of AQ, we sought to determine if it affected the serum levels of PA and *B. anthracis*. Bacteremia and antigenemia developed in most animals 24 h postchallenge (Figure 2C,D and Table S6). Our data showed a correlation between PA levels and bacteremia, as noted in previous studies in the rabbit model of inhalation anthrax.[16] Interestingly, at 24 and 48 h postinfection, we observed that 5 mg/kg of AQ results in a decrease in bacteremia and antigenemia, which correlates with the corresponding delay in mortality, although statistical significance is not achieved (Figure 2E−G). Ultimately, however, all animals become bacteremic and/or toxigenic before death, indicating that AQ affects bacteremia and PA levels transiently (Table S6).

Amodiaquine Adds Therapeutic Benefit to Antibacterial Levofloxacin. During the 2001 attack, despite anthrax victims receiving antibiotics, the fatality rate was 45%.[2] In order to simulate this fatality rate in the laboratory setting with rabbits, we determined the "suboptimal" treatment with levofloxacin that results in a partial survival rate. We determined that 1.6 mg/kg levofloxacin administered intravenously for 5 consecutive days beginning 24 h postchallenge was sufficient to protect 25−50% of rabbits challenged with aerosolized spores. This partial protection allows us to test whether AQ provides an "added benefit" to the suboptimal amount of levofloxacin.

Rabbits were challenged with 200 LD_{50} Ames spores by aerosol exposure. One group ($n = 12$) received 5 mg/kg of AQ twice a day for 5 days orally within 30 min postchallenge. In addition to AQ, the same animals were given suboptimal levofloxacin. The second group of animals ($n = 12$) received only suboptimal doses of levofloxacin, while the last group ($n = 4$) remained untreated after infection (Tables S7 and S8). The animals' weights were monitored, showing weight loss after the challenge and at the peak of infection when animals succumbed (2−4 days postchallenge) (Figure S2).

Treatment with a suboptimal levofloxacin dose alone resulted in a 33% survival of animals 21 days postinfection (Figure 3A). In contrast, concomitant administration of AQ with levofloxacin increased survival to 67%. This difference in survival between the treated groups did not reach a statistical significance ($p = 0.11$).

Since the levofloxacin regimen begins 24 h postinfection, treated animals received only AQ during the first 24 h postinfection. Bacteremia was detected in all groups by 24 h postchallenge. After 24 h of infection, AQ treatment reduced bacteremia and antigenemia: bacteremia was detected in 83% (10/12) of rabbits given levofloxacin alone, while only 33% (4/12) of rabbits were bacteremic from the AQ + levofloxacin group (Figure 3B and Table S8).

By 48 h postchallenge, 75% of rabbits in each of the two treatment groups and all untreated animals were bacteremic (Figure 3B). Slightly fewer animals were bacteremic in the AQ + levofloxacin group than in the levofloxacin-alone group by 72 h postchallenge, but the difference was not statistically significant (Table S8).

The animals treated with AQ + levofloxacin had a 2-day (24- and 48 h postchallenge) average serum PA concentrations that were lower than that of the levofloxacin only group ($p = 0.1$) (Figure 3C,D). Unfortunately, due to the high variability in serum PA concentrations and small group sizes, significant differences between these two groups could not be detected at any single time point.

Timing of the Amodiaquine Dose Postchallenge Improves Overall Survival. As seen in the 2001 U.S. mail attacks, anthrax patients may not receive treatment immediately. To determine the efficacy of delayed treatment, 5 mg/kg

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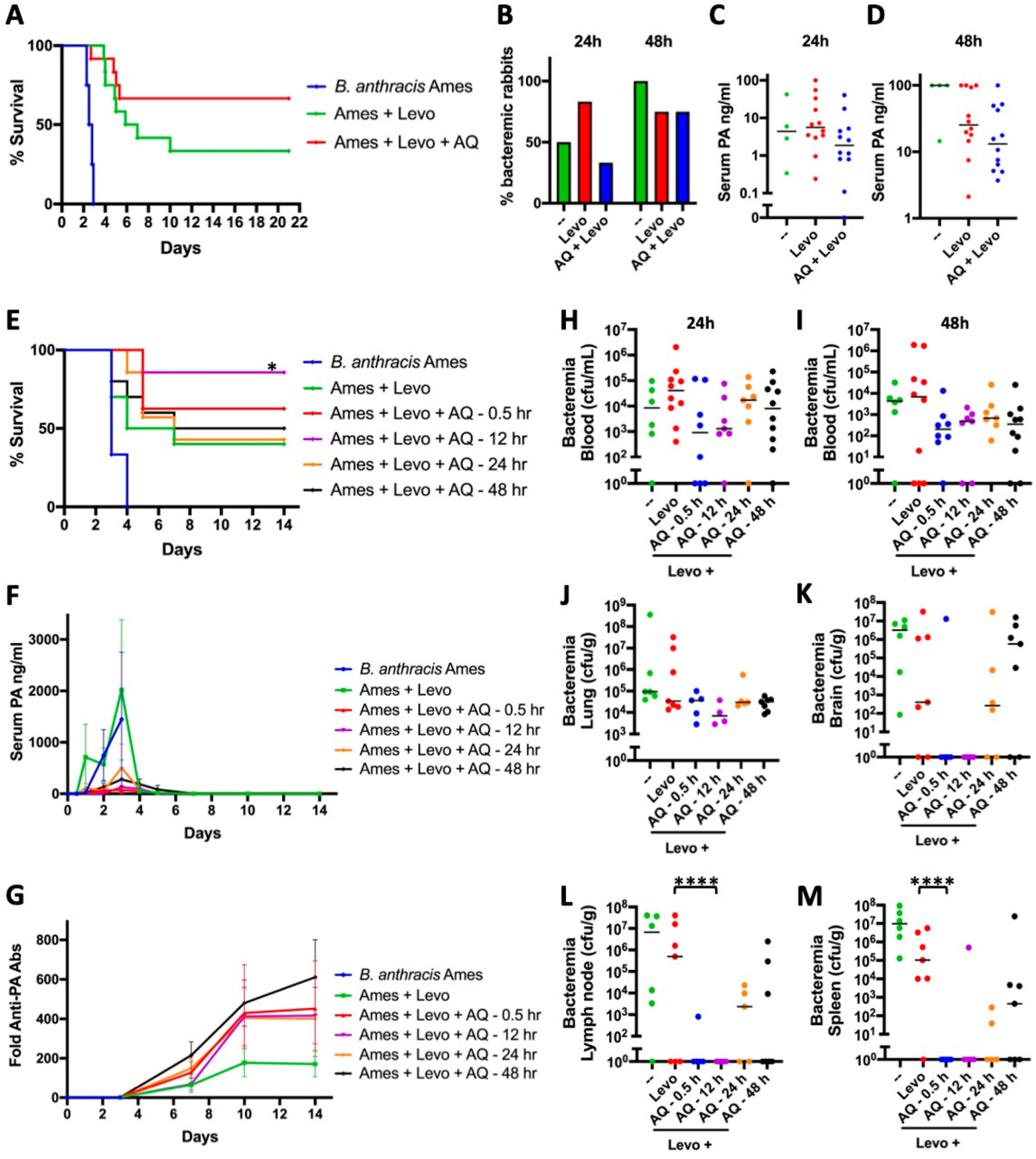

Figure 3. Added therapeutic benefit of AQ with levofloxacin on anthrax-related events. (A) Survival of anthrax-infected NZWR was measured in the absence or presence of treatment. Animals were treated with a suboptimal dose of levofloxacin (1.6 mg/kg/day) beginning 24 h postchallenge or with 5 mg/kg of AQ 30 min postchallenge of aerosol exposure to 200 LD_{50} of *B. anthracis* Ames spores. Levofloxacin was administered intravenously once daily and AQ every 12 h via oral gavage for 5 consecutive days. Treatment groups consisted of 12 rabbits each, while the control group consisted of 4 rabbits (equal ratio of males and females in each group). (B) Percentage of bacteremic rabbits, as measured by *B. anthracis* colony-forming units in serum, in each infected group at 24- and 48-post infection with inhaled *B. anthracis* spores. Bacteremia is defined as the presence of any *B. anthracis* in the blood. At 24 h postchallenge, blood samples of animals in groups given levofloxacin (Levo) were collected and tested for bacteremia before the initial levofloxacin dose, and they lack any drugs. (C,D) Serum PA concentrations were measured from animals in each anthrax-infected group at 24- and 48 h postchallenge. At 24 h postchallenge, blood samples of animals in groups given levofloxacin were collected and quantified for serum PA levels before the initial levofloxacin dose. The assay's upper limit of quantitation of PA was 100 ng/mL. Bars indicate median values. (E) Survival of anthrax-infected rabbits was measured in the absence or presence of Levo without or with AQ. Animals were treated with a suboptimal dose (1.6 mg/kg/day) of levofloxacin beginning 24 h postchallenge or 5 mg/kg of AQ 0.5, 12, 24, or 48 h postchallenge of aerosol exposure to 200 LD_{50} of *B. anthracis* Ames spores. Levofloxacin was administered intravenously once daily and AQ orally every 12 h for 5 consecutive days (*, $p = 0.0326$). (F,G) Serum PA (F) and anti-PA IgG (G) concentrations were measured from animals in each anthrax-infected group for 14 days postchallenge. IgG are plotted as fold increase compared to unchallenged animals. (H–M) Bacterial counts in blood (H,I, 24 and 48 h postinfection), lungs (J), brain (K), lymph nodes (L), and spleen (M) were measured from infected animals. Animals given 5 mg/kg of AQ beginning 0.5, 12, 24, or 48 h postinfection. Bars indicate median values (****, $p < 0.0001$).

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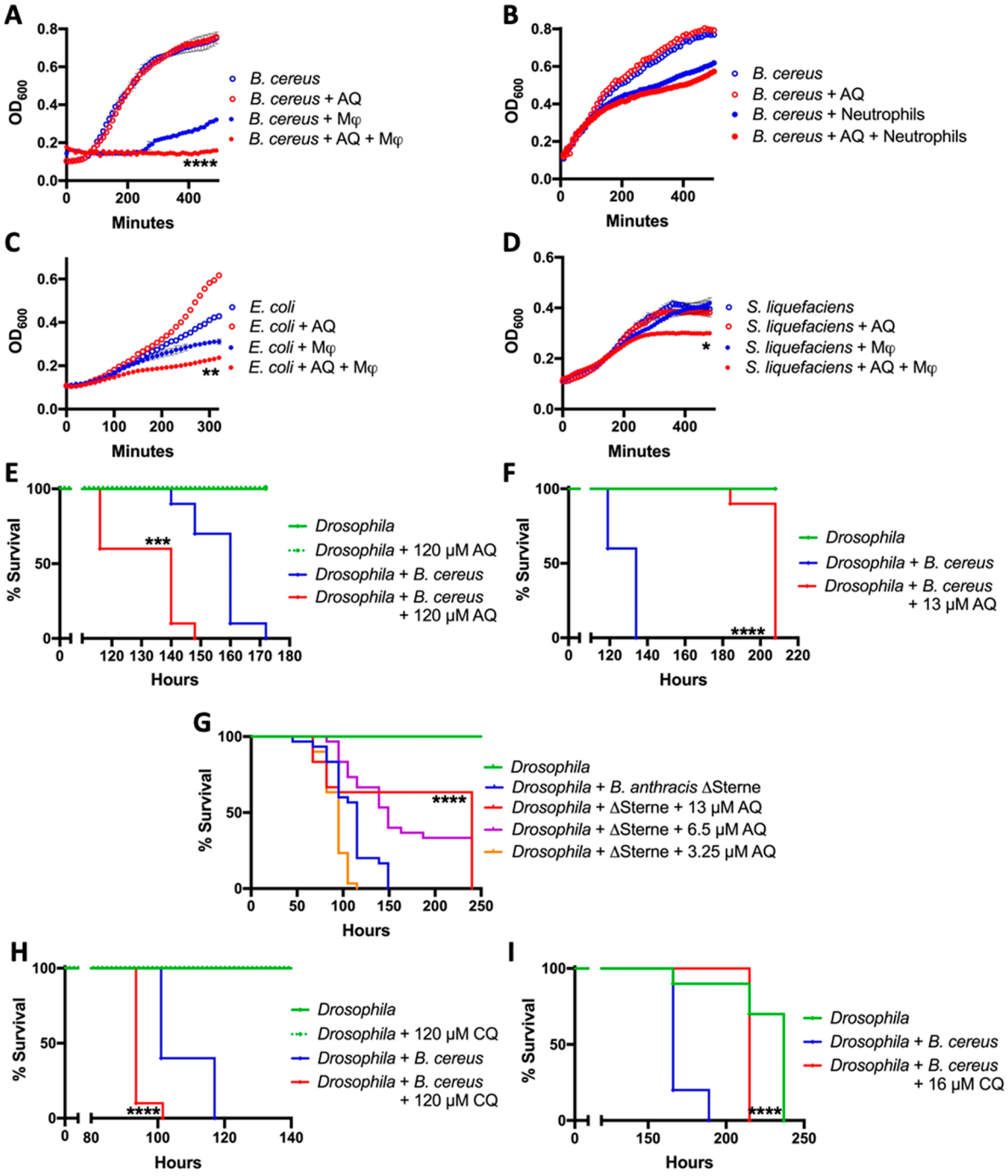

Figure 4. Host-directed antibacterial activity of AQ. (A−D) Effect of AQ on the ability of macrophages or neutrophils to reduce the bacterial growth rate was measured. Murine macrophage (Mφ) cells were treated with and without 660 nM of AQ during *B. cereus* (A), *E. coli* (C), or *S. liquefaciens* (D) exposures. Neutrophil-like differentiated HL-60 cells were treated with and without AQ during *B. cereus* exposure (B). AQ's effect on the bacterial growth rate was also measured in the absence of mammalian cells. Bacterial growth kinetics were measured every 10 min for 320−500 min. Each data point shown indicates the mean ± SD value obtained in triplicate assays done in a representative experiment (****, $p < 0.0001$; **, $p = 0.0026$; *, $p = 0.0384$). (E−I) Survival of *Drosophila melanogaster* orally exposed to varying amounts of AQ (E−G) or chloroquine (CQ) (H,I) during *B. cereus* (E, F, H, I) or *B. anthracis* (G) infections. The effect of each compound on the survival of uninfected flies was also measured. Flies were continuously exposed to each compound at the start of the bacterial challenge. Each group contains 10 male flies. *P* values based on the Log-rank (Mantel−Cox) test indicate statistical significance of survival curves compared to *Bacillus* conditions (***, $p < 0.001$; ****, $p < 0.0001$).

of AQ was administered in combination with levofloxacin at various 12-h time intervals postinfection. After aerosol exposure to 200 LD_{50} Ames spores, rabbits were separated into six groups: saline control ($n = 6$), 1.6 mg/kg/day of levofloxacin for 5 days starting at 24 h postchallenge ($n = 10$), and four groups of levofloxacin with 5 mg/kg of AQ twice daily for 5 days starting 30 min ($n = 8$), 12 h ($n = 7$), 24 h ($n = 7$), and 48 h ($n = 10$) postchallenge (Table S9). The weights and core body temperature of the animals were measured, showing the weight loss and temperature increase postinfection (Figures S3 and S4).

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All animals in the saline control group succumbed to *B. anthracis* infection within 4 days (Figure 3E). Treatment with AQ + levofloxacin 12 h postinfection yielded the highest protection: 86% of animals survived compared to 40% survival of those receiving levofloxacin alone ($p = 0.0326$). As previously observed, the administration of AQ 30 min postchallenge yields 63% overall survival of anthrax-infected animals (Figure 3A,E). Animals who received AQ 24- or 48 h postchallenge exhibited similar overall survival to levofloxacin alone, suggesting AQ provides little to no benefit for rabbits when administered in combination with levofloxacin a day or two after the infection.

PA concentration in plasma of infected rabbits was measured throughout the experiment (Figure 3F). The animals treated with AQ + levofloxacin had average serum PA concentrations lower than those of the levofloxacin only group. Unfortunately, due to the high variability in serum PA concentrations and small group sizes, the statistical significance in differences between these two groups could not be achieved ($p = 0.1$). Concurrently, the levels of plasma anti-PA IgG antibodies were measured in infected rabbits throughout the experiment. In animals treated with levofloxacin alone, the PA-specific antibodies increased by the seventh day after the infection and further increased by 174-fold on the 10th day of anthrax and remained at that level by the 14th day (Figure 3G). In contrast, animals in all groups treated with AQ + levofloxacin had a higher antibody-response to PA, resulting in a 401−611-fold average increase in the antibody response.

At 24 h postinfection, the number of bacteria in blood was lower in animals that had received AQ 30 min or 12 h postinfection (Figure 3H). By 48 h postchallenge, bacterial loads in blood samples of all AQ + levofloxacin treated animals were lower than levofloxacin-treated and untreated animals (Figure 3I).

Frequently affected organs in anthrax patients are the lungs, brain, lymph nodes, and spleen.[17] We observed that AQ treatment starting 12 h postinfected reduced the average of the bacterial counts in the lungs (Figure 3J). While levofloxacin alone reduced bacterial burden in the brain, lymph nodes, and spleens of infected rabbits (Figure 3K−M), AQ + levofloxacin treatments starting 0.5- and 12 h postinfection resulted in undetectable levels of bacteria in these organs (except for one animal) ($p < 0.0001$). The treatment with AQ + levofloxacin 24- and 48 h postinfection reduced bacterial counts in the lymph nodes and spleen (Figure 3L,M). These data collectively show that AQ adds a benefit to suboptimal amounts of antibacterial levofloxacin even when given within 12 h postinfection.

Discovery of the Additional Host Macrophage-Oriented Antibacterial Mechanism of Amodiaquine. Previously, AQ was discovered as a host-oriented antitoxin for anthrax.[11] However, to our surprise, the data from the current study shows that AQ also reduces the bacterial burden in *B. anthracis* infected rabbits. We investigated the mechanism of this antibacterial action of AQ.

We tested whether AQ directly affects the in vitro growth rate of the *B. anthracis* Ames strain in liquid media. The antibacterial activity of AQ against Ames cells was tested for an AQ concentration range of 0.13−275 μM, which spans the C_{max} of AQ in rabbits. Interestingly, none of the tested AQ concentrations decreased the growth rate of *B. anthracis*, demonstrating that AQ does not have pathogen-directed efficacy, thus reducing bacterial growth *in vivo* likely by targeting host antibacterial processes. Doxycycline was included as a control, and the minimal concentration that inhibited the growth of Ames was 60.4 nM.

Mammalian macrophages and neutrophils phagocytose the invading *B. anthracis*.[18] We investigated whether AQ affects the antibacterial activity of macrophages and neutrophils *in vitro*. To avoid anthrax toxin-mediated killing of phagocytes,[11] we cocultured host cells with a related Gram-positive *B. cereus* (ATCC 10987) and Gram-negative *Escherichia coli* (MM294) and *Serratia liquefaciens* (ATCC 27592). Murine macrophage (RAW264.7) cells were treated with 660 nM of AQ during exposure to vegetative *B. cereus*. When exposed to AQ, macrophages completely reduced the *Bacillus* growth rate in comparison to untreated macrophages (Figure 4A). This phagocyte-directed antibacterial activity of AQ is not as pronounced in neutrophil-like (HL-60) cells (Figure 4B). Lastly, we demonstrated that the effect of AQ on macrophages extends to Gram-negative bacteria, as the treatment of macrophages with AQ led to a significant reduction of growth rates of *E. coli* and *S. liquefaciens* (Figure 4C,D). AQ did not affect the growth rate of any bacteria in the absence of macrophages. These data show that AQ suppresses the growth rate of bacteria through a host macrophage-directed mechanism.

We tested whether AQ affects *Drosophila melanogaster*'s sensitivity to *B. cereus* and a pXO1-cured derivative of the Sterne strain of *B. anthracis*, ΔSterne. Fruit flies were chosen because they defend against bacteria with macrophage-like plasmatocytes.[19] When flies are orally exposed to 120 μM of AQ, it reduced the survival of flies infected with *B. cereus* by significantly decreasing the median survival from 160 to 140 h (Figure 4E). Interestingly, while uninfected flies feeding solely on sucrose can survive for 240−336 h, lower concentrations of 13 μM of AQ significantly enhanced the survival of flies to *B. cereus* and the toxin-negative ΔSterne, adding 74 and 125 h to median survival to yield 208 and 240 h, respectively (Figure 4F,G). Just as in mice and rabbits, high AQ increased the sensitivity of infected flies and low AQ protected them from bacteria.

Historically, AQ is an improved structural analogue of the antimalarial chloroquine (CQ). CQ also reduces the endocytosis of anthrax toxin by reducing host Ctsb activity, although it is less potent than AQ.[11] Similar to AQ, a high concentration of CQ (120 μM) reduced the survival of infected flies, and a lower 16 μM of CQ reduced the sensitivity of flies to *B. cereus* (Figure 4H,I). Thus, the host-directed antibacterial activity of AQ extends to other structurally related 4-aminoquinolines. Our data collectively suggest the anthrax efficacy of AQ relies on two host-directed mechanisms: a previously known antitoxin and a newly discovered macrophage-mediated antibacterial mechanisms.

Systematic Review to Establish AQ Pharmacokinetics and Safety Profiles in Humans. Previously a monotherapy, AQ is currently used in combination with other antimalarials. Unfortunately, previously published systematic literature reviews assessing AQ's PK and safety in humans include data from malarial patients and treatments combining AQ with other drugs, which may affect AQ's PK and safety profiles. We conducted a systematic review to establish AQ PK and safety profiles in the absence of malaria and other drugs. Following PRISMA guidelines,[20] we searched multiple databases on September 10, 2020, with the term "amodiaquine," and identified 2,005 citations published from 1948 to 2020. Of those, 1,807 were written in English. We further excluded 447

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in vitro studies, 158 animal trials, 154 reviews/commentaries, 295 papers that mention AQ in passing, 120 drug combinations articles, and 601 malaria articles (Figure 5). The remaining 32 articles included 25 papers on AQ safety[21−45] and 7 articles on the PK of AQ and DEAQ.[46−52]

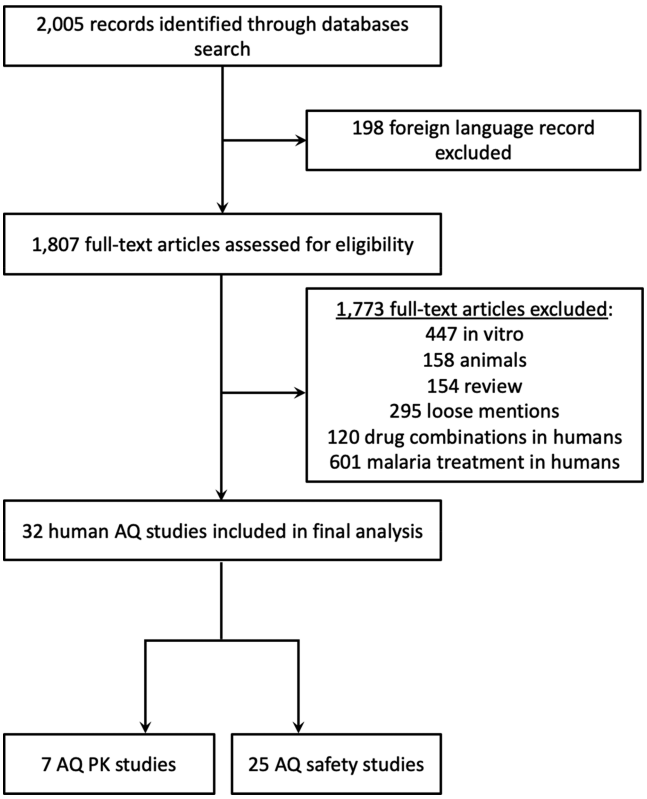

Figure 5. PRISMA flow-chart. Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) was used to analyze 2,005 AQ articles. A systematic review was done to identify studies that report on AQ PK and safety.

A total of 25 papers reporting on AQ's safety were identified from various countries (Table 2). The total number of subjects reported in these papers is 664, of which 240 are adults and 424 are children (2−18 years old). To achieve the recommended 10 mg/kg/day, AQ is sold in pills of 200, 400, or 600 mg to be taken over 3 days (a total of 600−1,800 mg). However, in all 25 studies, the total dose and the treatment length exceeded the approved dose and the treatment length. Specifically, the treatment's total length ranged from 8 days to over 6 years, with the total number of doses ranging from 5 to 1,564. The mild and severe side effects were reported in 86 (36%) adults and 102 (24%) children. Severe side effects include agranulocytosis/leukopenia/neutropenia (10 studies), hepatotoxicity (5 studies), eye disorders (5 studies), neuromyopathy (2 studies), and reduction in spleen size (1 study). Milder side effects included abdominal discomfort (6 studies), fever (3 studies), and oral/skin pigmentation changes (10 studies).

To validate our manual systematic literature review, Python Parser Library (Jupiter notebook in Anaconda environment) and Matlab Text Analytics Toolbox were independently used to identify, compare, and count words/phrases in the main body of each article (excluding references). Both programs were written to sift through many articles and identify side effects using a comprehensive list of 7,058 side effects,[53] which excluded "malaria". As a result, 161 side effects were found by both programs in 25 preselected articles, confirming agranulocytosis, jaundice, hepatitis, nausea, vomiting, and headache as the most frequently reported side effects (Tables S10 and S11).

After excluding 13 side effects related to malaria, arthritis, and lupus, the list of 161 side effects was reduced to 148, which was used to search all 1,807 AQ papers. The database of all AQ articles was analyzed by Python code. Consistent with the previous analysis, the same AQ side effects were found by Python code among those frequently mentioned in the large database (Table S12).

Concurrently, the database of all AQ articles was analyzed by Matlab. Filters were introduced to eliminate articles describing animal trials, drug combinations, or *in vitro* experiments. This brought the number of analyzed articles to 411, which included all 25 manually selected studies. Matlab confirmed that the same side effects as in the set of preselected 25 articles were frequently mentioned (Table S13). Overall, this human safety review analysis suggests that AQ is likely safe when taken as prescribed, since all subjects in all of the analyzed papers took more than the approved amount of AQ.

An additional 7 PK papers reported the C_{max}, T_{max}, $t_{1/2}$, AUC, and Cl of AQ and/or DEAQ in adults (Table 3). Apart from one study where AQ was administered intravenously at 3 mg/kg, AQ was given orally in all remaining 6 studies as 200, 400, and/or 600 mg doses. In 5 studies, only 1 dose of AQ was given, while in 1 other study, a total of 3 AQ doses were given either every week or every 6 weeks. Most notably, the C_{max} and $t_{1/2}$ of DEAQ ranges are 155−3,055 nM and 19−436.8 h, respectively. Given that in humans the C_{max} of DEAQ can approach the low micromolar range, and its plasma half-life is much longer in than in small animals, we predict efficacy for AQ against anthrax in humans.

DISCUSSION

We demonstrate the efficacy of AQ *in vivo*, where it adds benefit to levofloxacin, reduces bacteremia in plasma and lungs, and prevents bacteremia in the brain, lymph nodes, and spleen. We demonstrate that AQ protects hosts from anthrax by both host-directed antitoxin and antibacterial mechanisms, making AQ host-oriented and broad-spectrum against other pathogens.[11] AQ reduces the activities of pathogens that enter into the host cytoplasm from endosomes,[11] but it does not inhibit the entry of toxins and viruses that enter through a retrograde route to the endoplasmic reticulum, such as ricin: no difference in weight loss and survival of ricin-intoxicated mice were observed in the absence or presence of AQ. In the future, AQ should be tested *in vivo* against other Ctsb-dependent pathogens.

Our tests revealed a narrow therapeutic range of AQ dose for the treatment of anthrax. While 10 mg/kg/day was effective, 2.5 mg/kg/day was not effective, and 20 mg/kg/day reduced the survival of infected animals. Many antimicrobial drugs have been known to have narrow therapeutic indexes. For example, it has been shown that antibiotics used to treat anthrax, such as ciprofloxacin and tetracycline, are immunosuppressive at high concentrations.[54] At lower doses, AQ activates the ability of macrophages to kill bacteria (Figure 4A−D). At a higher concentration it reduces the survival *in vivo* (Figures 1B, 2C, and 4E) potentially due to the tissue injury to the gastric lining or by the inhibition of macrophages, which would be consistent with agranulocytosis (lowering of monocytes) in humans (Table 2 and Tables S10−S13).

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Table 2. Systematic Review to Establish AQ Safety Profiles in Humans

report[b]	*n* (M/F)	age	location	dose frequency	oral dose (mg)	treatment length	no. of side effects (%)	side effects
Massaga, 2008	10 (10/0)	16−33	Tanzania	1/day	25/kg	8 days	1 (10%)	headache, abdominal discomfort, fever, body malaise
Markham, 2007	1 (0/1)	39	Algeria	1/week	200	3 months	1 (100%)	fulminant hepatitis
Vrbova, 1992	127 (79/48)	8−10	Papua New Guinea	1/week	10/kg	13 weeks	84 (66%)	reduction in spleen size, fall in hemoglobin levels
Clarke, 1991	7 (1/6)[c]	29−71	U.K.	n.a.	2400−7400	n.a.	7 (100%)	agranulocytosis and hepatotoxicity
Rouveix, 1989	1 (0/1)	47	France	1/week	400	7+ weeks	1 (100%)	agranulocytosis
Bernuau, 1988	3 (2/1)[c]	12−51	France	1/week	200−700	22−59 weeks	3 (100%)	fulminant hepatitis, jaundice, liver cell necrosis, encephalopathy, coma, death
Wittes, 1987	1 (0/1)	42	Niger	1/week	780	10 months	1 (100%)	neuromyopathy
Larrey, 1986	5 (5/0)	36−64	France	1/week	200−800	4−15 weeks	5 (100%)	hepatitis, asthenia, vomiting, fever, chills, abdominal pain
Hatton, 1986	1 (0/1)	49	Zaire	1/week	400	3 months	1 (100%)	agranulocytosis, neutropenia
McAllan, 1986	4 (0/4)[c]	2−13	Australia	daily for 5 days/week or 1/week	75 or 50−150	2−6 years	4 (100%)	gray-brown skin tone and palatal pigmentation
Hirst, 1982	1 (1/0)[c]	34	U.S.	4/day	200	14 months	1 (100%)	diffuse conjunctivitis, corneal, and skin changes and also abnormal results from retinal function tests
Gillespie, 1977	1 (0/1)[c]	26	New Guinea	1/week	3800	10 weeks	1 (100%)	agranulocytosis, sore throat, fever, neutropenia
Shee, 1963	1 (1/0)[d]	43	Mozambique	1/week	200	3 years	1 (100%)	blueish discoloration of body and nail beds, beginning on his legs, slowly increased in intensity over time (patient has a history of malaria)
Maguire, 1962	17 (8/9)	38−62	U.K.	daily until controlled; 3−4×/week after	200−300	3 weeks−9 months	6 (35%)	abdominal pain, nausea, vomiting, lethargy, blue-gray pigmentation near nail beds and hard palate, corneal deposits, leukopenia
Schloeder, 1961	1 (1/0)	33	Panama	2/day	200	4 months	1 (100%)	hip pain, leg weakness, dyspnea, poor vision, lethargy, anorexia, yellow hands/feet
Kersley, 1959	25 (n.a.)	n.a.	U.K.	1/day	200	2+ years	20 (80%)	AQ 400 mg: digestive (12/25), giddiness (7/25), headache (2/25), fatigue (2/25), rash (1/25), visual symptoms (8/25), 1 fatality possible due to agranulocytosis due to AQ 400 mg
	21 (n.a.)				400		10 (52%)	AQ 200 mg: digestive (7/21), giddiness (3/21), headache (1/21), fatigue (1/21), rash (1/21), visual (1/21)
Glick, 1957	1 (0/1)	53	U.K.	1/day	200	8 weeks	1 (100%)	fatal agranulocytosis
Raymond, 1989	1 (0/1)[e]	38	Gabon	1/week	600	5 weeks	1 (100%)	fatal acute hepatitis, preceded by asthenia, pruritus, vomiting, dark urine 10 days later disappeared
Doull, 1959	55 (n.a.)	n.a.	Philippines	1/day	200	10 weeks	30 (55%)	blueish-green skin discoloration
Maguire, 1964	5 (2/3)	25−60	U.K.	1/day	200	3 months	2 (40%)	diarrhea in one patient, and mild leucopenia in another
Campbell, 1960	391 (*f*)[c]	3−adult	Territory of Papua and New Guinea	1/week	400−600	5 weeks−6+ years	40 (40%) adults 12 (4%) children	pigmentation of (i) the nail-beds of the toes and fingers; (ii) the mucous membrane of the hard palate; (iii) the skin of the face and neck.
Young, 1958	3 (2/1)	43−47	The Netherlands	1/day	300−400	2−4 months	3 (100%)	pale yellow to dark gray discoloration of the skin
Bleil, 1958	1 (0/1)	42	U.S.	n.a.	n.a.	18 days	1 (100%)	amenorrhea, partial blindness, and yellow pigmentation
Kennedy, 1955	1 (0/1)	2	Southern Rhodesia	2/day	100	1 week	1 (100%)	absolute neutrophil leucopenia
Watson, 1974	1 (0/1)[c]	34	U.K.	n.a.	n.a.	8 years	1 (100%)	oral pigmentation

[a]Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) was used to analyze 2,005 AQ articles. The systematic review was done to identify 25 studies that report on safety in humans. n.a. not available. [b]All reports are case studies except Massaga, 2008 (open-label randomized clinical trial), Vrbova, 1992 (placebo-controlled chemoprophylaxis trial), Doull, 1959 (double-blinded clinical study), and Campbell, 1960 (field study). [c]Ethnicity is Caucasian. [d]Ethnicity is Chinese. [e]Ethnicity is French. [f]Adults: 100 (65/35). Children: 291 (n.a.).

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Table 3. Systematic Review to Establish Pharmacokinetics of AQ and DEAQ in Humans[a]

report/location	*n* (M/F)	age	weight (kg)	no. of doses × dosage (mg)	dose frequency; administration	analyte	C_{max} (nM)	T_{max} (h)	$t_{1/2}$ (h)	Cl (units)	AUC (nM[b]h)
Minzi, 2003/Tanzania	1 (1/0)	n.a.	n.a.	1 × 600	single; p.o.	AQ	31.4	1.0	14.5	n.a.	585
						DEAQ	2507	2.0	149	n.a.	116759
Laurent, 1993/France	8 (8/0)	22−29	62−73	1 × 400	single; p.o.	AQ	21.9 ± 12.4	1.5 ± 0.5	n.a.	n.a.	121 ± 149
						DEAQ	155 ± 39	2.7 ± 0.5	97.5 ± 77.7	n.a.	6406 ± 4881
White, 1987/Liverpool	7 (7/0)[b]	21−35	41−59	1 × 3/kg	10 min infusion; i.v.	AQ	179−5398 (average: 1166)	n.a.	2.1	4.7−56.6 (L/kg[b]h)[d]	n.a.
						DEAQ	n.a.	n.a.	n.a.	n.a.	n.a.
Winstanley, 1987/Liverpool	7 (7/0)	22−44	n.a.	1 × 600	single; p.o.	AQ	89.9 ± 8.4	0.5 ± 0.3	5.2 ± 1.7	n.a.	433 ± 107
						DEAQ	552 ± 79	3.4 ± 0.8	n.a.	n.a.	24517 ± 4219
Winstanley, 1987/Liverpool	6 (6/0)	22−46	n.a.	1 × 200	1 dose every 6 weeks; p.o.	AQ	45.0−73.1	0.6−1.3	5.7−7.9	102−267 (L/min)[e]	84−208
				1 × 400		DEAQ	156−622	3.0−5.5	n.a.	1070−1700 (mL/h)[f]	10067 ± 28065
				1 × 600							
Pussard, 1987/France	4 (3/1)	27−50	46−80	1 × 10/kg	single; p.o.	AQ	n.a.	1.0	n.a.	n.a.	n.a.
						DEAQ	281−699	1.0−7.0	216−436.8	n.a.	n.a.
Ntale, 2007/Uganda	3 (2/1)[c]	n.a.	n.a.	1 × 600	single; p.o.	AQ	632	1.0	18.65	n.a.	n.a.
						DEAQ	3055 ± 1080	2.0 ± 1.0	19 ± 5.0	n.a.	n.a.

[a]PRISMA was used to analyze 2,005 AQ articles. The systematic review identified 7 studies that report on pharmacokinetic parameters of AQ and DEAQ in humans. Administration: i.v. intravenous; p.o. oral. [b]Ethnicity is Thai. [c]Ethnicity is Ugandan. [d]Systemic clearance [e]Oral clearance. [f]Renal clearance.

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Our data also revealed a narrow efficacious timing of AQ when given to animals postexposure. AQ doubled the survival of animals when given at the time of and 12 h (Figure 3) after infection, but it offered limited to no added benefit when given 24−48 h postchallenge. It has been previously shown that anthrax symptoms appear in rabbits 20 h postinfection, and they include bacteremia, toxemia, and an increase in body temperature.[55] Consistent with our AQ data, all other approved anthrax antitoxins have been shown to have limited therapeutic timing. Raxibacumab protected 100% of NZWR when given at 0 and 12 h after infection, but the survival of rabbits decreased to 50% and 42% when the drug was given 24 and 36 h postexposure, respectively.[56] Similar results have been seen in anthrax-infected NZWR treated with anthrasil and obiltoxaximab.[57,58] The limited therapeutic timing of these antitoxins has also been observed in the cynomolgus macaque, the model that is believed to most closely resemble anthrax in humans.[59]

Our literature review analysis revealed that after oral administration of AQ by healthy subjects, the plasma $t_{1/2}$ of DEAQ is 1−18 days, and the C_{max} is in the low micromolar range (Table 3). These values are comparable to those determined in macaques,[14] where $t_{1/2}$ of DEAQ is 13−51 h, and the C_{max} is 1.17−2.01 μM. Importantly, C_{max} is comparable to antitoxin and antiviral EC_{50}s.[11] Since macaques and humans reach higher AQ and DEAQ in plasma and longer half-lives than mice or rabbits, we predict AQ will be more effective in larger mammals.

Our study showed that AQ adds benefit to suboptimal antibacterial drugs in anthrax-infected animals by doubling survival rates. The previously approved antitoxins showed a modest added benefit to antibiotics, and no added benefit reached statistical significance.[60−62] Since AQ has a different mechanism of action against anthrax toxin from the approved antitoxins, we propose AQ could be used in combination with the approved antitoxins to provide a synergistic added benefit to antibiotics. Based on the AQ efficacy limited to presymptomatic anthrax, it could potentially be used prophylactically.

Our initial drug screen identified AQ as an anthrax antitoxin, and our research efforts were primarily focused on AQ. Because DEAQ is the active metabolite of AQ, it was also included in our previous[11] and current tests. AQ and DEAQ are structurally very similar compounds, but there are some key differences between them that may also prompt us to proceed with the development of DEAQ to treat anthrax. For example, DEAQ displays a superior PK profile than AQ (Tables 1 and 3). Additionally, AQ has shown some toxicity issues associated with its long-term use as a malaria prophylactic (Table 2) and in our mice and NZWR tests. Because DEAQ does not need to be metabolized by the liver to exert its effect, it might not cause hepatotoxicity. However, the mechanism by which AQ causes liver injury is unknown, and it has been postulated that AQ metabolites might cause similar complications.[63,64] DEAQ could be developed as orally or intravenously administered anthrax therapy for adult and pediatric use. The dosing of DEAQ for either route of administration would need to be determined to achieve effective and safe concentrations in plasma necessary for antianthrax efficacy.

There could never be ethical human efficacy Phase 2 and 3 clinical trials for anthrax, although the FDA's Animal Rule does not obviate the need for Phase 1 human trials. Accordingly, the FDA approves anthrax countermeasures based on the Animal Rule, stipulating that the anthrax therapy would be approved for humans if it shows antianthrax efficacy in two different animals, rabbits and macaques, as well as the safety in Phase 1 human trials. Based on our review of more than seven decades of clinical usage, we believe that AQ, but not DEAQ, could also skip Phase 1 safety trials. Because AQ is already approved and manufactured for malaria treatment, AQ's approval as a new antitoxin may be accelerated, and manufacturing and scale-up of this molecule will be significantly simplified compared to the previously approved biologics.

METHODS

Murine Pharmacokinetics. A/J (complement deficient) mice (7-to-10 weeks old) were obtained from Jackson Laboratories. Three female mice were given a single intravenous dose of 1 mg/kg of AQ (Sigma-Aldrich, A2799, purity ≥99%). Three additional female mice were given a single oral dose of 20 mg/kg of AQ via oral gavage. Blood was collected via cardiac sampling from mice under deep anesthesia and placed in tubes containing K_2 EDTA (Becton Dickinson, 365974) and processed to plasma. Calibration standards and quality control samples were prepared in blank mouse plasma. Following protein precipitation of the plasma samples with an equal volume of ice-cold acetonitrile (Fisher Scientific, A955-500), the samples were centrifuged and the supernatant was filtered through a 0.2 μm filter and transferred to glass autosampler vials. Samples were injected into the Waters Acquity UPLC-I mass spectrometer for analysis.

***B. anthracis* Sterne Mouse Infections.** Ten female A/J mice per group were used. Each mouse received approximately 1.1×10^4 *B. anthracis* Sterne (strain 7702) spores subcutaneously. AQ was administered via oral gavage every 12 h for 5 days. The mice were observed for 26 days. Before injection, spores were heat-shocked at 65 °C for 30 min, serially diluted, and grown on LB plates to determine cfu/mL. Uninfected mice were injected with water. Mice were observed for morbidity/mortality once daily until symptoms developed, and then thrice daily. Mortality rates were analyzed by a Fisher's exact test and by Log-rank (Mantel−Cox) test. Survival was analyzed by a Kaplan−Meier log-rank test.

Ricin Intoxication Mouse Models. *Oral Challenge.* Two CD-1 mice were dosed with a 200 μL sublethal dose of ricin of 875 μg ricin/mouse (ricin stock solution at 5 mg/mL in phosphate-buffered saline) with or without AQ via gavage. In the absence of AQ, mice were treated with 200 μL of ricin (175 μL of stock ricin with 25 μL of PBS) and in the presence of 10 mg/kg of AQ, the same concentration of toxin was mixed with 0.1 mg per mouse AQ (25 μL of 10 mg/mL AQ in water).

Intravenous Challenge. Two CD-1 mice each were dosed intravenously with 100 μL of ricin (1 μg per mouse) equivalent to 6 LD_{50} in the presence or absence of 0.1 mg/mouse (∼4 mg/kg) of AQ by tail vein injection. Animal survival was measured over time.

NZWR Pharmacokinetics. Nine male and nine female NZW rabbits (5−7 months old) were obtained from Covance Research Products. Clinical observations, including morbidity/mortality, were recorded immediately postdose the day of treatment, minimum once daily, and before the last blood collection. Every effort was made to minimize, if not eliminate, pain and suffering in all animals in this study. Euthanasia, if necessary, occurred as follows: subcutaneous administration of a sedative cocktail (as recommended by a veterinarian), followed by intravenous administration of an overdose of sodium pentobarbital.

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Three males and three females received either 10 mg/kg AQ (Fitzgerald 51R-U031004, USP reference standard, purity 99.9%) or DEAQ (ALFA Chemistry ACM79352786, purity ≥98%) intravenously or 20 mg/kg AQ orally. For intravenous administrations, a slow bolus via an ear vessel was given over an ∼2−6 min, followed by a 0.2 mL flush of saline to ensure complete dose administration. Oral administration of AQ occurred via a gavage tube followed by an air flush to ensure the entire dose administration. Fresh AQ and DEAQ doses were corrected for salt content and administered as a single dose.

Blood samples from all animals were collected from an ear vessel (not where the test article was administered). Samples were collected in tubes containing K_3 EDTA (BioIVT) and processed into plasma. Drug concentrations were determined by analyzing blood samples via LC−MS/MS. Amodiaquine-d10 (Medical Isotopes, Inc.) and *N*-desethylamodiaquine-d_5 dihydrochloride were stable label internal standards for AQ and DEAQ, respectively (Cerilliant). Plasma drug level data were analyzed using Phoenix WinNonlin (version 6.3) software to perform noncompartmental modeling.

***B. anthracis* Ames Aerosol Challenge in NZWR.** Rabbits were challenged by aerosol with 200 LD_{50} of *B. anthracis* Ames spores using a qualified Biaera aerosol control platform fitted with a muzzle-only aerosol chamber using computer control of humidity, pressure, and airflow. Each rabbit was anesthetized with an intramuscular injection of ketamine-HCl (35 μg/kg) and xylazine-HCl (5 μg/kg) before immediate transfer into the ABSL-3 class III biosafety glove cabinet. Real-time plethysmography was performed on each rabbit using a pair of DSI elastic band sensors placed around each animal's thorax and abdomen, which were then calibrated with a pneumotach fitted to the face of each animal. After removing the mask, the rabbit's muzzle was inserted into a Biaera aerosol chamber fitted with a latex diaphragm. The target dose of spores for aerosol deposition in the lungs was 2.0×10^7 spores. A six-jet collison nebulizer generated the aerosol, yielding a spray factor of $1.0-2.0 \times 10^{-6}$. The nebulizer concentration to deliver this dose was calculated and combined with a standard volume of air (7,000 mL) to deliver the target challenge dose of spores. Aerosol samples were collected continuously to confirm the challenge dose of spores for each animal by serial dilution and plating on blood agar plates. The duration of aerosol delivery was based on each animal's respiration intensity and controlled by the Biaera aerosol system computer.

AQ was dissolved fresh daily in sterile, pyrogen-free water to 4 mg/mL and given by orogastric gavage. Levofloxacin (Akorn, Inc., NDC 17478-107-20, sold as 25 mg/mL intravenous infusion injection) intravenous treatment began 24 h postchallenge. The animals' clinical condition was recorded at least four times daily for 14 days postchallenge and then twice daily for the remainder of the study, provided that the animals showed no signs of clinical illness. During the critical phase of the study (24−96 h) postchallenge, the frequency of observations increased due to the anticipated rapid disease course and to facilitate identification of moribund animals that met the criteria for euthanasia (e.g., unresponsive to touch, labored breathing, lack of motion, and flared nostrils).

NZWR Blood Collection and Processing. Blood specimens were collected via the VAP. To avoid VAP and catheter contaminations, blood specimens used to assess bacteremia after 7 days postchallenge were collected from a peripheral blood vessel. Blood specimens were collected for quantitative bacterial plate counts daily until the animal either succumbed to infection or was no longer bacteremic. Approximately, 2.0 mL of whole blood was also collected in serum separator microtubes pre- and postchallenge, and the serum was used for PA-ECL and anti-PA IgG titration.

Assessment of Bacteremia and Bacterial Load. Bacterial concentration in the blood was determined using an automatic serial diluter and plater (easySpiral Dilute; Interscience). Whole blood, diluted in sterile water, was plated onto trypticase soy agar plates containing 5% sterile sheep blood (TSAB) and incubated at 37 °C for 16−24 h. Colonies were enumerated using an automatic colony counter (Scan 500; Interscience). Bacterial colonies having morphology typical of *B. anthracis* were subcultured and confirmed as *B. anthracis* with bacteriophage γ.

Bacterial/spore load was also determined in the lung, lymph node (mediastinal), brain, and spleen. Tissues were homogenized in sterile water using a Stomacher 80 MicroBiomaster tissue homogenizer (Seward Ltd.). The homogenate was serially diluted in water and plated onto TSAB plates using the automatic diluter/plater and incubated at 37 °C for 16−24 h. Colonies were counted.

Detection of PA and Anti-PA IgG in Serum. PA was measured in serum using PA-ECL screening assay kit MesoScale Discovery (L15CA-1), which utilizes the anti-PA antibody. To quantitate the levels of PA in each serum sample, a standard curve (0−100 ng/mL) was analyzed in parallel on each assay day by using recombinant PA (List Biological Laboratories, Inc., 171E). Test samples were assayed in duplicate.

Anti-PA IgGs were measured in serum via ECL similar to the PA-ECL screening assay. Biotinylated recombinant PA_{63} were bound to streptavidin-coated plates (MSD) and used as the capture antigen. Detection was accomplished using SULFO-TAG labeled antirabbit antibody and read buffer (MSD).

Macrophage and Neutrophil Coculture with Bacteria. All mammalian cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. Before the experiment, RAW264.7 mouse macrophage cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin and streptomycin (P/S). Neutrophil-like cells were generated by inducing the differentiation of human myeloblastic leukemia cells (HL-60 cells) using all-trans retinoic acid (ATRA).[65] HL-60 cells were grown in RPMI 1640 (Gibco) supplemented with 5% FBS and P/S. ATRA (Sigma-Aldrich) was dissolved in 100% ethanol with a stock concentration of 5 mM and used at a final concentration of 1 μM. HL-60 cells were treated with 1 μM ATRA for 72 h.

Macrophages and differentiated neutrophils were seeded in 96-well plates at 1.5×10^5 cells in 100 μL per well 24 h before the assay in media lacking P/S and ATRA. On the day of the experiment, bacterial overnights were resuspended in respective mammalian cell culture media and added to 96-well plates containing macrophages or neutrophils at the final OD_{600} of 0.1, in the absence or presence of 660 nM of AQ. Control wells contained either macrophages or neutrophils without bacteria or bacteria without mammalian cells with or without AQ.

Bacterial growth kinetics were measured for up to 500 min, by incubating the plate at 37 °C, and taking absorbance measurements at 600 nm every 10 min. Plates were shaken for 10 s before every measurement by a SpectraMax Plus microplate reader (Molecular Devices).

Drosophila Oral Feeding Survival Assay. Experiments with flies were conducted with male Oregon-R *Drosophila*

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melanogaster (Bloomington Drosophila Stock Center stock no. 5) aged 4−5 days. Flies were maintained at 25 °C with 12-h light/dark cycles and fed standard cornmeal-molasses-agar fly medium with yeast flakes. Flies were infected according to the bacterial intestinal infection method.[66] Extra-thick Whatman blotting paper (Bio-Rad Laboratories, 1703965) was cut into 25 mm diameter discs, and three stacked discs were placed into the bottom of 25 mm diameter polystyrene *Drosophila* vials, following by capping with a foam plug. All overnight bacterial cultures were centrifuged, and the bacterial pellets were resuspended in a 50 mM sucrose solution to a final OD_{600} of 3.3. AQ or CQ were dissolved in the bacterial sucrose solutions before adding to vials. Finally, 2.5 mL of the respective solution was pipetted onto the Whatman paper in each vial containing *Drosophila*. Each insect experiment shown is representative of at least three independent experiments.

Systematic Review of Amodiaquine in Humans. A systematic literature search was done according to PRISMA guidelines[20] on September 10, 2020, using the search term "amodiaquine". Searches using PubMed, Science Direct, Google, and Google Scholar were conducted to identify 2,005 publications describing AQ between 1948 and 2020. The search was not restricted to the English language, but only articles written in English were analyzed. The pdf files of all 2,005 articles were acquired and analyzed in two ways: reviewers-based analysis and parallel text-mining analysis. Ten reviewers read full texts and agreed on the final study eligibility. Reviewers independently extracted data using a standardized form and database.

Statistics. Data analysis was conducted using GraphPad Prism software. All *P*-values reported are products of the respective positive control to a single experimental condition using two statistical analyses: the Log-rank (Mantel−Cox) and the Gehan−Breslow−Wilcoxon tests. An alpha of 0.05 was deemed the threshold for significance. We report *P* values adjusted by the Bonferroni correction. A delay in median survival was reported. Since the chance of dying in a small-time interval was not the same early in the study and late in the study, the values for the 95% CI of the ratio of median survivals were not meaningful and were not reported.

Study Approval. Mice euthanasia protocols follow recommendations established by the American Medical Veterinary Association Guideline to minimize animal pain and suffering. Animal research at the USAMRIID was conducted under an animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the AAALAC International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

All rabbit-related procedures for this study were conducted in accordance with an animal use protocol approved by the UTMB IACUC. General procedures for animal care and housing were in accordance with the current Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) recommendations, current requirements stated in the Guide for the Care and Use of Laboratory Animals (National Research Council), and current requirements as stated by the U.S. Department of Agriculture through the Animal Welfare Act and Animal Welfare Regulations (November 2013).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00190.

Figure S1, weights of anthrax-infected rabbits receiving varying doses of AQ; Figure S2, weights of anthrax-infected rabbits receiving levofloxacin and AQ; Figure S3, weights of anthrax-infected rabbits receiving levofloxacin and delayed AQ treatment; Table S1, clinical observations of individual rabbits after intravenous administration of AQ and DEAQ; Table S2, clinical observations of individual rabbits after oral administration of AQ; Table S3, pharmacokinetics of individual rabbits after intravenous administration of AQ and DEAQ; Table S4, pharmacokinetics of individual rabbits after oral administration of AQ; Table S5, therapeutic dosing of AQ and blood collection schedule of anthrax-infected rabbits; Table S6, anthrax-related events of individual rabbits after oral administration of AQ; Table S7, therapeutic dosing and blood collection schedule of anthrax-infected rabbits receiving levofloxacin and AQ; Table S8, anthrax-related events of individual rabbits after oral administration of AQ; Table S9, therapeutic dosing and blood collection schedule of anthrax-infected rabbits receiving levofloxacin and delayed AQ treatment; Table S10, identification of side effects in AQ safety articles via Python; Table S11, identification of side effects in AQ safety articles via Matlab; Table S12, identification of side effects in AQ articles via Python; and Table S13, identification of side effects in AQ articles via Matlab (PDF)

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Author Contributions

M.M.S. designed the research; all authors performed the research and analyzed the data; and M.M.S., G.B., and S.A. wrote the paper, which was edited by all of the authors.

Notes

The authors declare the following competing financial interest(s): M.M.S. and L.Z. are co-inventors on the AQ patent for anthrax.

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■ ABBREVIATIONS USED

PA, protective antigen; LF, lethal factor; PA_{83}, 83 kDa PA subunit; PA_{63}, 63 kDa PA subunit; Ctsb, cathepsin B; AQ, amodiaquine; DEAQ, desethyl-amodiaquine; WHO, World Health Organization; PK, pharmacokinetics; C_{max}, maximal plasma concentration; T_{max}, a time when C_{max} is achieved; Cl, clearance; V_d, volume of distribution; $t_{1/2}$, plasma half-life; ADR, adverse drug reactions; i.v., intravenously; p.o., orally; LD, lethal dose; Levo, levofloxacin; NZWR, New Zealand White Rabbit

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