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RESEARCH**

APPLICATION NUMBER:

**020634Orig1s061, 020635Orig1s067,
021721Orig1s028**

PHARMACOLOGY REVIEW(S)

**DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH**

PHARMACOLOGY/TOXICOLOGY NDA/BLA REVIEW AND EVALUATION

Application number: NDA 20-634 S-061
NDA 20-635 S-067
NDA 21-721 S-028

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NDA 20-635 SD no. 556 (eCTD seq. no. 075)
NDA 21-721 SD no. 223 (eCTD seq. no. 072)

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11/4/11 for NDAs 20-635 and 21-721

CDER stamp date: 10/28/11 for NDA 20-634
11/7/11 for NDAs 20-635 and 21-721

Product: Levaquin® (levofloxacin)
NDA 20-634 Tablet
NDA 20-635 Injection
NDA 21-721 Oral solution

Indication: For the treatment of pneumonic plague

Applicant: Johnson & Johnson PRD on behalf of Janssen
Pharmaceuticals

Review Division: DAIP

Reviewer: Amy Nostrandt

Supervisor/Team Leader: Wendelyn Schmidt

Acting Division Director: John Farley

Project Manager: Jane Dean

Template Version: September 1, 2010

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TABLE OF CONTENTS

1	EXECUTIVE SUMMARY	4
1.1	INTRODUCTION	4
1.2	BRIEF DISCUSSION OF NONCLINICAL FINDINGS	4
1.3	RECOMMENDATIONS	6
2	DRUG INFORMATION	6
2.1	DRUG	6
2.2	RELEVANT INDS, NDAs, BLAs AND DMFs	7
2.3	DRUG FORMULATION	7
2.4	COMMENTS ON NOVEL EXCIPIENTS	7
2.5	COMMENTS ON IMPURITIES/DEGRADANTS OF CONCERN	7
2.6	PROPOSED CLINICAL POPULATION AND DOSING REGIMEN	7
2.7	REGULATORY BACKGROUND	7
3	STUDIES SUBMITTED.....	7
3.1	STUDIES REVIEWED.....	7
3.2	STUDIES NOT REVIEWED	8
3.3	PREVIOUS REVIEWS REFERENCED.....	9
4	PHARMACOLOGY	9
4.1	PRIMARY PHARMACOLOGY	9
4.2	SECONDARY PHARMACOLOGY	39
4.3	SAFETY PHARMACOLOGY	39
5	PHARMACOKINETICS/ADME/TOXICOKINETICS	39
5.1	PK/ADME.....	39
6	GENERAL TOXICOLOGY.....	48
7	GENETIC TOXICOLOGY	49
8	CARCINOGENICITY	49
9	REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY	49
10	SPECIAL TOXICOLOGY STUDIES.....	49
11	INTEGRATED SUMMARY AND SAFETY EVALUATION.....	49
12	APPENDIX/ATTACHMENTS	49

1 Executive Summary

1.1 Introduction

The application states that levofloxacin (RWJ-25213-097) is a synthetic fluoroquinolone antibacterial agent that exhibits a wide spectrum of bactericidal activity against both Gram-positive and Gram-negative pathogens. The drug is currently approved and marketed as LEVAQUIN® in the United States [(New Drug Application [NDA] 20-634 (Oral tablet), NDA 20-635 (Injectable), and NDA 21-721 (Oral solution)] and several parts of the world to treat specific infective conditions

The application indicates that *in vitro* microbiological data suggest that levofloxacin should be efficacious for the treatment of pneumonic plague in humans at a dose of 500 mg once daily (QD). That dose is consistent with current labeling.

1.2 Brief Discussion of Nonclinical Findings

NIAID sponsored the nonclinical studies conducted in African Green Monkeys (AGMs). Natural history studies were conducted to characterize the pathogenesis and disease progression for *Y. pestis* in AGMs. These studies confirmed the lethality of aerosolized *Y. pestis* strain CO92 in this species, documented the natural history of the disease in telemetered animals and identified fever as a clinical sign or trigger for antibiotic intervention.

In this model, telemetered monkeys received a target dose of 100 x LD₅₀ (1 x LD₅₀ = [REDACTED]) in a head only aerosol chamber. Actual doses were calculated using sampling data from an all glass impinger in the aerosol chamber and from whole body plethysmography of the animals just prior to aerosol challenge. Actual challenge doses across the studies ranged from 3 to 1150 x LD₅₀, or approximately 1,000 to 400,000 CFU of *Y. pestis* CO92. Four studies were conducted in a total of 36 AGMs. Animals challenged with at least 20 x LD₅₀ developed disease.

In natural history studies, fever and bacteremia were the most prominent features during the course of disease in AGMs. There was strong correlation between fever and bacteremia, but the chronological relationship between fever and bacteremia was less clear. In general, fever and bacteremia were established within 2-3 days. In general, tachypnea and tachycardia coincided with the onset of fever.

Thoracic radiographs were obtained for some animals in two of the natural history studies. An independent review of the radiographs from the two natural history studies concluded that there was a common radiographic pulmonary appearance and progression of the severity of changes over time associated with inhaled *Y. pestis* CO92 in AGMs. All animals exhibited pulmonary infiltrates, beginning as small infiltrates with local involvement noted at time of onset of increased respiratory rate (approximately Day 3). At the time of death of untreated animals, severity had progressed to multiple lobes with opacities consistent with pneumonitis.

Prominent post-mortem changes included lung pathology and dissemination of bacteria to other tissues such as lymph nodes, spleen, liver, and, occasionally, brain (meninges). AGMs died or were euthanized *in extremis* between Day 2 and Day 9 post-challenge. The majority of AGMs died on Day 3 or Day 4.

The animals underwent gross necropsy, with histopathology of selected tissues. Tissues analyzed for histopathology varied by study, but those that were analyzed in all four studies included brain, liver, lung, lymph nodes (bronchial, tracheobronchial) and spleen. Macroscopic findings included pleural effusions, edema of the mediastinum and/or lungs, multiple focal areas of red to purple discoloration with nodules or masses in the lungs. The masses and nodules correlated microscopically with areas of suppurative hemorrhage, inflammation, and edema. Fibrin accumulation was noted in the lung. The most severe lung lesions had progressed to parenchymal necrosis. Bacteria were visible in alveoli and/or in macrophages.

Similar enlargement and/or discoloration of lymph nodes were found in some animals. Microscopic findings included bacteria in the lymph nodes, lymphoid depletion or necrosis, edema, hemorrhage, and neutrophilic inflammation. Spleen and thymus were evaluated in some animals and exhibited findings similar to those seen in lymph nodes. At least one study noted fibrin accumulation in renal glomeruli, which was considered to possibly be consistent with disseminated intravascular coagulation (DIC). At least one animal had meningeal hemorrhage, fibrin exudate, and thrombus affecting the choroid plexuses. Liver findings, where noted, included peracute necrosis of centrilobar hepatocytes; this correlated with clinical chemistry findings and was considered suggestive of agonal hypoxic liver damage.

The independent reviewing pathologist concluded that there is a common pathology associated with lethal infection by inhaled *Y. pestis* Strain CO92 in African Green Monkeys, based on these four studies. Post-challenge infection and dissemination was found to occur quickly in this species, with morphologic changes in the lung appearing to begin as lobar to sublobar serous and fibrinous exudates (edema) with intra-alveolar and intracellular (macrophages) bacteria along with increased numbers of alveolar macrophages. These changes observed in the lung were found to transition quickly to diffuse necrotizing pneumonia characterized by alveoli and airways filled with bacteria, inflammation and hemorrhage. There was dissemination of bacteria to bronchial/tracheobronchial and mediastinal lymph nodes, mediastinal connective tissues and spleen, initiating changes in the tissues such as hemorrhage, inflammation and edema.

The Sponsor concluded that the course of pneumonic plague in the AGM shares many of the features of fatal primary pneumonic plague in humans. The Sponsor determined that the development of persistent fever was the most accurate and reliable clinical signs of established *Y. pestis* infection. The Sponsor concluded that the optimal time to administer an antimicrobial for testing of its efficacy in the treatment of pneumonic plague in this model of disease is when fever is present; approximately 72 hours following exposure.

The application states that data from exploratory pharmacokinetic (PK) studies in AGMs were used to select a daily intravenous dosing regimen of 8 mg/kg followed by 2 mg/kg administered 12 hours later (8/2 mg/kg) that mimics yet provides lower than human exposures at the 500 mg QD dose for levofloxacin. The pivotal efficacy study to support a pneumonic plague (post-exposure) indication was performed in AGMs administered levofloxacin for 10 days using this humanized dosing regimen (8/2 mg/kg).

The pneumonic plague model in AGMs was then used in a study to evaluate the efficacy of levofloxacin in the treatment of that disease. Findings in control animals

were consistent with those in the natural history studies. Treatment with levofloxacin was initiated in animals in the treatment group when a fever of 39°C or more was sustained for over an hour. Fever and tachypnea began to resolve in treated animals. Under the conditions of the efficacy study, levofloxacin administered intravenously for ten days as 8 mg/kg followed by a 2 mg/kg dose 12 hours later each day resulted in a 94% survival rate (16 of 17 animals) compared to a 0% survival rate in untreated control animals. The report states that this dosing regimen in AGMs achieved 98% of the human C_{max} and resulted in an AUC₀₋₂₄ of 19.8 µg·hr/mL, or 41% of the reported AUC₀₋₂₄ in humans. Lung radiographic findings were reported to have returned to normal in the subset of animals filmed at the end of the study (Day 28). Histopathological findings in treated vs. untreated animals demonstrated resolution of the major findings of disease, although treated survivors at Day 28 did exhibit persistence of chronic perivascular inflammation and interstitial/bronchiolar inflammation of the lung. This was considered to be consistent with resolution of changes associated with disease.

1.3 Recommendations

1.3.1 Approvability

The supplement is approvable from a pharmacology/toxicology standpoint.

1.3.2 Additional Non Clinical Recommendations

None at this time

1.3.3 Labeling

There are no new pharmacology/toxicology updates for the label.

2 Drug Information

2.1 Drug

Generic Name

Levofloxacin (pure (-)-(S)-enantiomer of the racemic drug substance ofloxacin)

Chemical Name

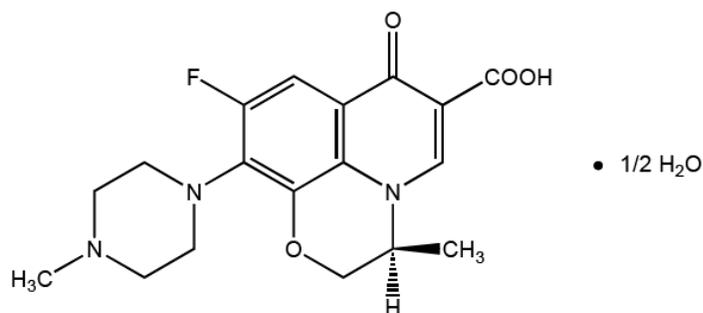
(-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate

Molecular Formula/Molecular Weight

C₁₈H₂₀FN₃O₄ • 1/2 H₂O

MW = 370.38

Structure or Biochemical Description



Pharmacologic Class

Fluorinated carboxyquinolone antibacterial

2.2 Relevant INDs, NDAs, BLAs and DMFs

IND 64,429 from NIAID investigated this drug

(b) (4)

2.3 Drug Formulation

Marketed formulations of Levaquin®

2.4 Comments on Novel Excipients

Not applicable

2.5 Comments on Impurities/Degradants of Concern

Not applicable

2.6 Proposed Clinical Population and Dosing Regimen

For the treatment of pneumonic plague (post-exposure) in adults and pediatric patients > 50 kg ≥ 6 months of age at a dose of 500 mg q24h for 14 days, or
For pediatric patients < 50 kg and ≥ 6 months of age at a dose of 8 mg/kg BID (not to exceed 250 mg per dose) for 14 days

2.7 Regulatory Background

IND 64,429 from NIAID investigated this drug

(b) (4)

3 Studies Submitted

3.1 Studies Reviewed

Pharmacology:

Natural history studies of pneumonic plague in African Green Monkeys (AGM) have been previously reviewed; those reviews or excerpts from them are reproduced here:

1. **USAMRIID Study Report no. F03-09G: Natural History Study of Pneumonic Plague in the African Green Monkey**
2. **Lovelace Respiratory Research Institute Study Report no. FY06-126: A Natural History Study of Inhalational Plague *Y. pestis* Strain CO92 in Adult Telemetered African Green Monkeys**
3. **Battelle Biomedical Research Center Study no. 617-G607610: Natural course of untreated pneumonic plague in African Green monkeys**
4. **Battelle Biomedical Research Center Study no. 875-G607610: Natural course of untreated pneumonic plague in African Green monkeys**
5. **NIAID/DMID report no. NIAID-Yp-NatHis-Path-2011: Independent pathology review of the natural history studies for pneumonic plague in the African Green Monkey**
6. **NIAID/DMID Report Number: NIAID-Yp-NatHis-Rad-2011: Independent radiology review of the natural history studies for pneumonic plague in the African Green Monkey**

Efficacy studies:

1. **Study no. FY07-070: An efficacy study of intravenous infusion of levofloxacin in inhalational plague *Yersinia pestis* strain CO92 in telemetered African Green monkeys**
2. **NIAID/DMID report no. NIAID-Yp-Levo-Path-2011: Independent pathology review of the efficacy study of intravenous infusion of levofloxacin in pneumonic plague in the African Green monkey**
3. **NIAID/DMID report no. NIAID-Yp-Levo-Rad-2011: Independent radiology review of the efficacy study of intravenous infusion of levofloxacin in pneumonic plague in the African Green Monkey**
4. **Report no. UTMB-YpEff-1-8: Compilation of levofloxacin treatment studies in the mouse and rat intranasal challenge models of pneumonic plague**
5. **Report no. RIID-YpEff-2006: Report on the effect of dosing and schedule of levofloxacin against *Yersinia pestis* in and aerosol challenge model in mice.**

Pharmacokinetics:

1. **Study no. FY08-150: A Pharmacokinetic study of intravenous infusion of levofloxacin in African Green Monkeys – Amended final report (6/8/11)**
2. **Study no. B122-03: A pharmacokinetic and toxicity study of levofloxacin following intravenous and oral (nasogastric) administration to African Green Monkeys**
3. **(b) (4) Study no. B465-10: A Pharmacokinetic study of intravenous infusion of levofloxacin in African Green Monkeys**

3.2 Studies Not Reviewed

Microbiology studies were not reviewed here. See the Clinical Microbiology review.

Sixty-four literature references were provided, but are not reviewed. Of those discussed in the nonclinical pharmacology written summary, most are described as studies of other fluoroquinolones, often in models of plague other than pneumonic. None of the published studies as described appeared to be pivotal to support approval of the current application.

3.3 Previous Reviews Referenced

Reviews of previously submitted and reviewed studies are reproduced in the appropriate sections below.

4 Pharmacology

4.1 Primary Pharmacology

Natural history studies in the African Green monkey (AGM) model of pneumonic plague:

1. Study Report no. F03-09G: Natural History Study of Pneumonic Plague in the African Green Monkey

(previously reviewed by Amy Nostrandt 2/4/09 under IND 64,429, supporting document no. 42)

Conducting laboratory and location: U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD

Date of study initiation: Not provided

GLP compliance: No

QA report: yes () no (X)

Drug, lot #, and % purity: Not applicable

Methods

Three male and three female adult African Green Monkeys (AGM, *Chlorocebus aethiops*) weighing 3.5-6.0 kg were used in the study. All animals were fitted with telemetry devices to monitor body temperature, pulse and blood pressure. The monkeys were anesthetized for aerosol exposure to *Yersinia pestis* (strain CO92). Respiratory minute volumes were measured by whole body plethysmography immediately before challenge. Head-only exposure was accomplished in a dynamic aerosol chamber producing a mass median aerosol diameter of 1.2 μm , generated by a three-jet nebulizer and sampled continuously by an all-glass impinger (AGI). The inhaled dose for each monkey was calculated by equating the ratio of the total dose inhaled to the total collected in the AGI to the ratio of the respiratory minute volume of the animal to the flow rate through the AGI.

The target dose was $100 \pm 50 \times \text{LD}_{50}$. Data from previous investigations revealed the estimated LD_{50} to be [REDACTED]). Actual exposures were $57 \times \text{LD}_{50}$ for first animal and progressively less for subsequent animals. Subsequent investigation implicated the time that the cultured organisms were maintained at room temperature as the reason for inadequate exposure. The first four animals exposed developed disease, but the last two, calculated to have received 9-12 $\times \text{LD}_{50}$ (0.2-0.3 times the previously determined LD_{99}), did not develop signs of disease

or bacteremia. Animals receiving $> 20 \times LD_{50}$ developed clinical signs of disease and bacteremia.

Animals were bled (approximately 5 ml) daily post-exposure for blood culture, CBC, BUN, and creatinine until they were euthanized. They were weighed daily, and clinical signs were documented at least twice per day post-challenge. Respiratory rates were determined manually at the time of clinical assessment. Temperature, blood pressure and heart rate were monitored every 30 minutes (more frequently after evidence of fever). When the presence of clinical signs was confirmed, with emphasis on fever ($\geq 1.5^{\circ}C$) and respiratory rate increase ($\geq 50\%$ increase above baseline), the monkeys were anesthetized, weighed, thoracic radiographs (AP and lateral views) taken, and bled (approximately 5 ml) for culture, CBC, BUN, and creatinine.

Scores were assigned for each animal's activity, behavior, response to stimuli, and breathing to assess their welfare and to determine the appropriate time for euthanasia. Gross and microscopic post-mortem examinations were performed on the four animals that developed disease.

Results:

Mortality: The last two animals exposed did not develop disease and survived. The first four exposed animals developed signs of disease and were euthanized (3) or died (1) between 99.5 and 125 hours post-exposure.

Clinical signs: The four animals that were exposed to $> 20 \times LD_{50}$ of *Y. pestis* developed fevers within 72 hours post-exposure. Heart rates appeared to increase at approximately the time of onset of fever in those animals. A rapid increase in respiratory rate was seen in each of the animals that developed disease, generally beginning approximately 96 hours post-exposure, and appeared to be the beginning of rapid deterioration of clinical condition. Blood cultures confirmed bacteremia at 48 hours post-exposure in one animal and at 72 hours post-exposure in the remaining three, and remained positive until euthanasia.

Hematology: The discussion of CBC results is limited to findings for total leukocyte counts, monocytes, lymphocytes and granulocytes. It is unclear whether or not these were the only parameters evaluated. The report states that all animals had white blood cell counts within the normal range at baseline. Monocyte percentages remain fairly constant throughout the study. In the four animals that developed disease, there was a lymphocyte / granulocyte inversion by Day 3. The report states that there were no significant changes in the two animals that did not develop disease over the same time frame. However, one animal appeared to have a similar change; i.e. increase in lymphocyte percentage over time coincident with a decrease in granulocyte percentage over time, albeit somewhat lower in magnitude.

Clinical chemistry: The only parameters discussed are BUN and creatinine. It is unclear why a full serum chemistry panel was not performed. The report states that the normal ranges are 8-20 mg/dL for BUN and 0.5-1.1 mg/dL for creatinine. Mild increases in both parameters were seen in one of the four animals that developed disease

(increased BUN at 83 and 96 hours, increased creatinine at 24 hours and throughout the study). BUN was within normal limits for the other five animals. Mild increases in creatinine were seen at one time point each for the two animals that did not develop disease.

Gross pathology: All four animals that developed disease had similar gross lesions. Findings included edematous lungs with multiple red to purple areas of discoloration that sometimes involved entire lung lobes, mediastinal edema, blood-tinged frothy fluid in the trachea and bronchi, and enlarged tracheobronchial lymph nodes. Additionally, three of the four animals had edematous tracheobronchial lymph nodes and serosanguinous pleural effusion.

Histopathology: The lesions of pneumonic plague in the four animals consisted of edema, acute hemorrhage, and acute inflammation, either alone or in combination, and always associated with presence of extracellular bacillary bacteria. Occasionally, the bacteria were also present within phagocytic cells. The lungs, bronchial lymph nodes and mediastinal lymph nodes were affected to some degree in all four monkeys that developed disease. Two of the four also had lesions in the larynx and/or trachea, while the other two also had lesions in the connective tissue of the mediastinum.

The spleen of each of the four monkeys contained bacillary bacteria, suggesting that septicemic spread from the respiratory tract had occurred. In three of these animals this was accompanied by mild to moderate splenic inflammation.

Bacillary bacteria were also present within blood vessels of other organs in each of the animals. In one, the presence of these bacteria in the hepatic sinusoids was associated with microscopic fibrin thrombi. In another, bacteria in the hepatic vasculature were accompanied by scattered foci of hepatocellular degeneration and necrosis.

Other: No changes were noted in thoracic radiographs in the two animals that did not develop disease. For the four animals that developed disease, evidence of lung involvement was present at the time that clinical signs were confirmed.

The Sponsor concluded that the course of pneumonic plague in the AGM shares many of the features of fatal primary pneumonic plague in humans. The Sponsor determined that the development of persistent fever and tachycardia were the most accurate and reliable clinical signs of established *Y. pestis* infection. Tachypnea appeared at least 24 hours later and was associated with a more advanced state of disease and rapid clinical deterioration. The Sponsor concluded that the optimal time to administer an antimicrobial for testing of its efficacy in the treatment of pneumonic plague in this model of disease is when fever (≥ 1.5 °C increase over baseline for 2 hours) is present; approximately 72 ± 4 hours following exposure.

2. Study Report no. FY06-126: A Natural History Study of Inhalational Plague *Y. pestis* Strain CO92 in Adult Telemetered African Green Monkeys

(previously reviewed by Simone Shurland 7/12/2011 under IND 64,429, supporting document no. 44.

The current submission contains an “amended final report” for this study; none of the amendments described would have altered the review of this study.)

Conducting laboratory: Lovelace Respiratory Research Institute

The objective was to determine the disease progression in African Green Monkeys (AGMs), infected with *Y. pestis*, that includes the time to onset of fever, respiratory rate, heart rate, electrocardiogram, clinical signs, bacteremia, as well as alterations in laboratory (complete blood count, blood gases, full chemistry panel, and coagulation studies) and pathological findings. The purpose of the study was to compare the progression of disease in AGMs with that in humans and identify a parameter(s) that would be appropriate as a trigger to initiate antibacterial therapy. The study was a non Good Laboratory Practices (non-GLP) study in 10 AGMs (5 males and 5 females) of unknown age (weighing 3 – 6 kg). The AGMs were wild caught on St. Kitts, West Indies and were housed at the National Institutes of Health and Alpha Genesis, Inc. Animals were given a physical examination and screened for B-virus, Simian Immunodeficiency virus (SIV), Simian Retro Virus (SRV), Simian T-cell Leukemia virus (STLV), including tuberculosis tests. The sponsor stated only monkeys of acceptable health were used in the study, however, the criteria used to define acceptable health were not specified nor were the health surveillance reports of each animal included.

Prior to placement in the study, each AGM was implanted with a telemetry monitoring device (T30F, DISS, Inc) using aseptic surgical techniques. Briefly, the telemetry device was inserted into the abdomen and attached to the wall of the abdominal cavity. The telemetry monitoring device measured and recorded body temperature, respiratory rate, heart rate, and basic electrocardiographic signal traces detecting major arrhythmias. The AGMs were allowed to recover and monitored post-operatively for 2 weeks. Each AGM had central venous catheters (CVC; Broviac catheter) inserted into the right femoral vein, which exited through the skin of the upper back of the monkey. All animals wore non-restraining jackets to limit animals’s access to the exit site or CVCs. The monkeys were maintained in individual stainless steel cages in a non-human primate isolation facility and were provided water *ad libitum*; fresh feed was provided twice daily.

Challenge organism

The *Y. pestis* CO92 strain Biovar – Orientalis [REDACTED] (b) (4)

[REDACTED] was originally isolated from a fatal human case of pneumonic plague.

Aerosol challenge

The 50% lethal dose (LD₅₀) used in the study was based on a previously conducted inhalational plague study by Pitt *et al.* (2006) at USAMRIID in AGMs which determined the LD₅₀ to be [REDACTED]. The target aerosol challenge dose of the *Y. pestis*

CO92 strain was 100 LD₅₀. A head-only Automated Bioaerosol Exposure system contained in a Class 3 Biosafety cabinet was used to deliver the target *Y. pestis* aerosol concentrations. AGMs were anesthetized with 2 – 6 mg/kg telazol approximately 15 minutes prior to aerosol exposure. Aerosols were generated using a three-jet Collision nebulizer (MRE-3 jet, BGI Inc., Waltham, MA) with a target particle size of 1 – 3 µm determined using a T Aerodynamic Particle Sizer Spectrophotometer, (Model 3321; TSI Inc.; Shoreview, MN). Aerosol challenges occurred on 2 different days in groups of 5 monkeys on each day.

The starting doses before aerosolization, referred to as *Actual Nebulization Concentration*, ranged from 1.06 to 6.47 x 10⁷ CFU/mL. Effluent air streams were collected directly via an in-line all glass impinger port which was used to calculate the concentration of *Y. pestis* in the test atmosphere at the time of aerosolization. The actual inhaled dose of organisms for each animal was then calculated using the following formula:

$$\text{Dose} = (C \times V),$$

where C is the concentration of viable pathogen in the exposure atmosphere and V was the total volume inhaled as measured using a Buxco plethysmography chamber. The sampling rate in monkeys ranged from 5 to 5.2 L/min, which resulted in aerosol exposure times ranging from 4.5 minutes to 14.7 minutes. The test atmosphere during aerosolization based on the all glass impinger sample, referred to as *CFU recovered*, ranged from 7.98 x 10⁵ to 1.13 x 10⁶. There was an observed 1 to 2 log fold loss from the starting dose and the number of viable bacteria recovered prior to aerosolization. The sponsor states that this may be affected by the temperature and humidity within the chamber at the time of aerosolization; however the actual details of alteration in environmental conditions were not specified. Temperature and humidity changes of the air inside the chamber may affect the virulence of the organism, since virulence factors such as the release of antigens like Yop proteins and F1 antigens are temperature dependent. The number of LD₅₀ equivalents was calculated by dividing the total inhaled dose for each animal by the LD₅₀ (i.e. [REDACTED] CFU). The results showed that the LD₅₀ in group 1 ranged from 160 to 255 which was higher than the target 100 LD₅₀; whereas in group 2 the LD₅₀ ranged from 44 to 129 LD₅₀ which was within the target aerosol dose of 100 LD₅₀. These differences in the calculated LD₅₀ doses between the two groups were due to the variations in the aerosolization procedures such as a lower nebulizer concentration.

Survival Time

Survival was assessed at least twice daily up to the end of the study. All animals succumbed to the plague infection and died within 4 days post-challenge; however, 3 of the 10 animals were found dead, while the remaining 7 animals were euthanized. The survival time in Group 1 (range 86 – 95 hours) was similar to Group 2 (ranged 72- 100 hours) with an overall mean survival time of 90 hours, suggesting the two groups were comparable. The results suggest that all AGMs died after aerosolized administration of 44 to 255 LD₅₀ of *Y. pestis*.

Telemetric parameters

Animals were monitored for onset of fever, changes in respiratory rate, heart rate, and basic electrocardiographic signals using telemetry. Temperature was recorded every minute and averaged hourly. The diurnal temperature patterns were recorded in 9 of the 10 animals at baseline and postchallenge; however, one animal (#790) had technical difficulties with pre-challenge recordings. For the purpose of this study, the investigator's onset of an abnormal body temperature was based on an increase in temperature of $>39^{\circ}\text{C}$ for 2 consecutive hours. However, the basis for this threshold in AGMs is unclear. An increase in temperature based on this definition was observed in all animals averaging at 67 hours (median, 72 hours) post-challenge; the range was from 52 to 80 hours. Though, there was no correlation between time to elevated temperature above 39°C with actual aerosol dose; the interval between onset of fever and euthanasia/death varied from 15 to 38 hours, with a mean interval time of 23 hours (median, 21 hours). The increase in temperature of $> 39^{\circ}\text{C}$ was not persistent in all animals during the course of infection; 6/9 animals (#538, 666, 705, 774, 784 and 789) had fluctuations in body temperature. However, at the time of euthanasia or death, 7 of the 9 animals (#532, 538, 666, 705, 756, 774, and 789) showed a rapid drop in temperature. Heart rates were recorded by counting R waves per minute. Respiratory rates were recorded by intrathoracic (intra-pleural) pressure changes and analyzed as inspirations and expirations per minute. Prior to challenge and immediately after challenge, there were no changes in respiratory depth and heart rates. The onset of tachycardia and tachypnea, defined as departure from the baseline value, appeared to coincide with the onset of fever. During the course of infection, there was significant variation in respiratory and heart rates by animals, which prevented the emergence of any consistent pattern. However, at the time of impending death, the sponsor reported that all AGMs had an increased respiratory rate over 60 breaths per minute or deep labored breaths with obvious excessive work of breathing documented by increased negative (inspiratory) and positive (expiratory) intrapleural pressures in most animals suggesting respiratory distress.

Bacteremia

Blood samples for bacteriological culture were collected once daily via catheter (Broviac) or femoral vein on day 7 pre-challenge and days 1, 2, 3 and 4 post-challenge. Additional blood samples were collected for culture at the time any AGM was found dead or moribund post-challenge. Whole blood (approximately 1 mL) was collected in a tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant for quantitative bacteriology. It is important to note that the use of EDTA as an anticoagulant is known to decrease bacterial counts which may increase the incidence of false negative results. In group 1, 3 (#532, 666, and 756) of 5 animals daily blood draws were complicated by obstructed catheters preventing both withdrawal of blood and infusion of saline. The sponsor states that the cultures of the obstructed catheters showed bacterial growth in which the primary organism were consistent with a *Staphylococcus* species (coagulase negative, catalase positive); further characterization of the organism was not performed. The bacterial count of the contaminant was not provided. In the second group, no complications with the catheters were encountered due to the institution of improved catheter management protocols which included swabbing with Betadine before blood draws and daily change of catheter tips. Most of the samples obtained from the

catheter of animals in group 2 did not show signs of contamination, with the exception of 1 animal (#775) that had a blood sample classified as contaminated on day 3; no further information was included (i.e., name of contaminant, bacterial count). Viable *Y. pestis* colonies were observed as early as 43 hours post-challenge in animals; most animals were bacteremic by 68 to 71 hours. The average time to first detectable bacteremia (66 hours) was similar to average time to onset of fever of 67 hours. However, the first detectable bacteremia preceded the onset of fever (based on temperature above 39°C for 2 hours) in 7 of the 10 animals (#532, 538, 666, 705, 774, 789 and 790). There appears to be no correlation between time to bacteremia and the actual concentration dose at the time of challenge. The bacterial counts in each animal over the 4 day-period post-challenge was variable ranging from 10 CFU/mL to $>2 \times 10^5$ CFU/mL, with a median concentration of 270 CFU/mL blood (Table 7). Two animals (#756 and 775) had negative blood culture for 3 days post-challenge; however, the terminal blood culture was positive for *Y. pestis* at the time of euthanasia. During the course of infection, CFUs increased in 6 (#532, 705, 774, 784, 789 and 790) of the 8 remaining animals after the first positive culture. All AGMs' terminal blood cultures were positive for *Y. pestis* as the causative agent. There appears to be no correlation between bacterial load and the inoculum concentration used for challenge.

Radiography

Thoracic radiographs were taken in the animals prior to *Y. pestis* challenge (day 0), at the onset of increased respiration based on telemetry and before euthanasia/death. The changes in lung function based on chest radiographs were variable. Prior to challenge, all animals' radiographic images showed clear lung fields except in one AGM (#666) that had a mid-right caudal lobe opacity on film; the reasons for such changes were not specified. No further information was provided on this animal (#666). At the time of onset of increased respiration (for most animals on day 3), 6/10 animals showed radiographic infiltrates that were small in size, 3 animals showed clear radiographs and 1 animal that was found dead (#790) had complete whiteout. In those animals that showed small radiographic infiltrates, based on the reports, findings were limited to a single lobe thus indicating a localized pulmonary involvement. Radiographs at the time of euthanasia or death showed that the small infiltrates had enlarged, and most animals had multiple lobes that contained opacities, thus suggesting that the localized pulmonary involvement had rapidly progressed to other segments of the lobes, consistent with pneumonitis.

Clinical Observations

Cage side observations, which included activity, posture, nasal discharge, sneezing, coughing, respiratory characteristics, ocular discharge, inappetence/anorexia, stool characteristics, neurologic signs (such as seizures) or other abnormalities were reported. The most commonly observed abnormal behavior was decrease in appetite which ranged from day 1 to day 3 in different animals; however, there was considerable variation in the onset of different abnormal behaviors throughout the study. A correlation with bacterial load could not be evaluated.

Histopathological findings

A gross necropsy was performed in which all animals had external surfaces, body cavities and major organ systems examined. Selected tissues which included liver, spleen, tracheobronchial lymph nodes and lungs were harvested and preserved in 10% neutral buffered formalin for histopathology evaluation. Lung tissues were classified as taken from lesion (discolored region suggestion consolidation) or non-lesion (region that appeared normal without discoloration) areas. Fixed tissue sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Light microscopic evaluation of the tissues was performed and the lesions were graded based on a severity scale correlation with estimates of lesion distribution and extent of tissue involvement. In parallel with fixation of tissues in buffered formalin for histopathological evaluation, a portion of each organ was homogenized in 1 mL of sterile water, serially diluted and cultured on SBA plates. Bacterial colonies were counted and the CFU per gram of tissue or per mL of blood were calculated following 48 hours of incubation at 28°C.

Gross pathologic findings showed that the most commonly observed lesions were discoloration or hemorrhage in tissues such as the lungs, lymph node, liver and spleen. All AGMS had lung lesions that showed multifocal areas of hemorrhage with some areas of unremarkable lung. Microscopic evaluation of the lung lesions showed areas of fibrinosuppurative hemorrhagic pneumonia and inflammation typically composed of septal and alveolar neutrophils and histiocytes. In the lungs, these lesions were also associated with the presence of bacteria, primarily extracellular, that had morphology compatible with *Y. pestis*. The bacteria were present within alveoli or associated with the surface of alveolar macrophages. The pneumonia in the animals was noted to be deep within pulmonary parenchyma and not associated within the airways; similar to many classic naturally acquired inhalation pneumonias (i.e. bronchopneumonia). There was a mild to moderate intravascular leukocyte margination (predominantly neutrophils) in the lung, liver and spleen. Though bacteria were noted (based on H&E staining) in the lung, spleen and tracheobronchial lymph nodes the numbers of bacteria present were not provided. In the spleen, visible bacteria were most commonly found in animals found dead; however splenitis was not a characteristic finding in the study. The tracheobronchial lymph nodes showed bacteria scattered throughout the node, in some areas where there was abundant bacteria the lymphoid tissue showed effacement of normal node architecture. The liver showed peracute necrosis of the centrilobar hepatocytes characterized by hypereosinophilia and loss of differential staining without significant associated inflammation. The pathologist noted that the peracute centrilobular necrosis suggested agonal hypoxic liver damage in the animal. Culture results showed that the tissue burden of *Y. pestis* was high particularly in the lung especially taken from lesion areas (range, 10^7 - 10^9 CFU/g of tissue), however, bacterial load in the range of 10^5 to 10^8 CFU/g of tissue were also reported from the tracheobronchial lymph nodes, liver and spleen.

Overall, the study in 10 AGMs challenged via the aerosol route with *Y. pestis* CO92 strain (ranging from 44 – 255 x LD₅₀) shows a rapid progression to clinical signs of infection with a short incubation period that ranged from 2 – 3 days. The onset of fever averaged about 52 to 80 hours post-challenge. All animals were bacteremic between 43 to 92 hours post challenge. Seven animals were bacteremic at the time of

fever presentation with the exception of 3 AGMs that were bacteremic 5 – 22 hours after the onset of fever. Other parameters such as respiratory rate, heart rate and other clinical signs varied during the course of the disease, however it appears that respiratory distress and increased heart rate were associated with impending death. The interval between onset of fever and death varied from 17 to 40 hours. All AGMs in the study died within 3 – 4 days post-challenge. Gross pathology and radiology findings were consistent of pneumonic plague which included extensive patch multilobar pulmonary pneumonia associated with the presence of bacteria. Tissue samples of the lungs, tracheobronchial lymph nodes, spleen, liver and blood were culture positive for *Y. pestis* organisms.

3. Study no. 617-G607610: Natural course of untreated pneumonic plague in African Green monkeys

(previously reviewed by Amy Nostrandt 10/12/2011 under IND 64,429, supporting document no. 52.

The report included in this submission is labeled “Revised Final Report.” There is a statement indicating that it was a combined document consisting of the original report and a subsequent amendment. None of the amendments or revisions appear to have any effect on the findings in the review below.)

Conducting laboratory: Battelle Biomedical Research Center (BSL-3 laboratory), West Jefferson, Ohio.

Materials and methods:

Test animals were 10 (3 male, 7 female) specific pathogen free (SPF) African Green monkeys (AGM), screened for previous exposure to *Yersinia* species (F1 and V antigens). They were implanted with telemetry devices (D70-PCTP) and monitored for a minimum of 30 seconds every hour for 7 days pre-challenge for baseline body temperature, activity, respiratory function, and cardiovascular function. They continued to be monitored post-challenge until death. ECGs were recorded, but not analyzed or reported as part of this study.

Challenge was performed by head-only aerosol exposure, with the target inhaled dose of 100 LD₅₀ equivalents (The LD₅₀ was previously determined to be [redacted] CFU of *Yersinia pestis*). Whole body plethysmography was performed on each animal during challenge to measure tidal volume, total accumulated tidal volume, and minute volume. The total inhaled dose was calculated from the impinger sample concentration, sampling parameters, and the exposure duration. The actual dose ranged from 106-1150 x LD₅₀, with the mean being 613 x LD₅₀.

Animals were observed twice daily. Appetite was measured by assessing how many biscuits in the daily ration were not eaten. AGM were weighed pre-test and at necropsy.

Blood was collected pre-test and daily after challenge through Day 3 for hematology, clinical chemistry, and bacteriology. Additional samples were taken for animals found dead. Planned serum ELISA analysis to confirm infection was not performed due to early deaths and sufficient evidence of *Y. pestis* infection.

The time of death was determined to within 15 minutes, based on telemetry data. If there was excessive fluid in the respiratory tract, it was cultured. Animals found dead or euthanized in moribund condition were necropsied. The thoracic cavity was examined for excessive fluid accumulation. Brain, heart, kidneys, liver, spleen, lungs, intrathoracic lymph nodes (bronchial and mediastinal), and gross lesions were collected for histopathological examination by a board-certified veterinary pathologist.

Results:

Clinical observations included reduced appetite, lethargy, hunched posture, prostration, bloody discharge from the nose and mouth, respiratory distress, moribundity and death. All ten animals died within 4 days post-challenge. Due to the rapid progression of the disease, individual body weights did not change significantly. Hematology changes were slight, and values were still within normal limits. Of the alterations in platelet counts, a slight decrease in survivors on Day 3 was the only finding considered to be biologically relevant. Statistically significant increases in WBC counts were seen on Days 1 and 2, as well as on Day 3 in 2 of the 3 surviving animals. Increases were attributable to increased lymphocytes on Day 1 and to increased neutrophils on Day 1 and subsequent days, relative to baseline. Most clinical chemistry changes were either within normal limits or considered to not be biologically significant. However, statistically significant increases in AST (Days 1-3), LDH (Days 2-3), and ALT (Day 1) were seen. Of these, only the findings on Day 3 (increased AST and LDH) were considered to be biologically significant.

Telemetry data indicated that fever developed within 1.5-2 days. Body temperature remained elevated until just prior to death. Heart rate was significantly increased by approximately 2 days post-challenge, accompanied by a slight but sustained increase in systolic, diastolic, and pulse pressures. From approximately 2 days post-challenge onward, a marked increase in respiratory rate was evident, with a decrease in inspiratory and expiratory time, i.e. breathing was shallow and rapid; this correlated with lung congestion noted on necropsy.

Pathological lesions were stated to be consistent with pneumonic plague. Gross lesions consisted of presence of thoracic transudate (n=9 of 10) and discoloration of mandibular lymph nodes (n=3) and lungs (n=2). Lung masses and nodules were present in one animal each and correlated with areas of hemorrhage and/or inflammation. Microscopically, bacteria were found in lung macrophages and extracellularly in the lung; this was associated with suppurative inflammation, fibrin accumulation, hemorrhages and occasional edema. Bacteria were also noted in all lymph nodes examined, particularly the bronchial lymph nodes, along with hemorrhage and suppurative inflammation. Fibrin thrombi in renal glomerular capillaries were noted in one male; this is a finding noted in man associated with disseminated intravascular coagulation (DIC). Culture of blood and postmortem nasopharyngeal and lung washes confirmed infection with *Y. pestis*. Bacteremia was found in all surviving animals on Days 2 and 3, but not on Day 1.

The authors of the report hypothesize that the higher than intended challenge doses may have resulted in more rapidly progressing disease than expected. This was attributed to the day to day variation in aerosol generation efficiency. The Sponsor plans to remedy the challenge exposure procedure and to repeat the study.

4. Study no. 875-G607610: Natural course of untreated pneumonic plague in African Green monkeys

(The draft report was previously reviewed by Amy Nostrandt 6/26/09 under IND 64,429 supporting document no. 43.

Although details were not provided, the final report differed from the draft and is re-reviewed below.)

Conducting laboratory and location: Battelle Biomedical Research Center, West Jefferson, OH.

GLP compliance: No

Methods

Species/strain: SPF African Green monkeys, screened by ELISA for previous exposure to environmental *Yersiniae*, e.g. *Y. pseudotuberculosis* and *Y. enterocolita*.

Number/sex/group or time point (main study): 5

Age: Unknown

Weight: 3.3-5.3 kg

Unique study design or methodology: Prior to study initiation, all animals were surgically implanted with D70-PCTP telemetry transmitters.

On Study Day 0, animals were challenged with a targeted aerosol dose of 100 x LD₅₀ *Y.pestis* strain CO92 (approximately [REDACTED] in a head-only aerosol exposure system consisting of a nebulizer, impinger samplers, an aerosol particle size analyzer, temperature and relative humidity monitors, mass flow meters and mass flow controllers. Targeted environmental conditions were 70-75°F and 50-80% relative humidity. The duration of aerosol challenge was based on the estimated CFU concentration and cumulative minute volume measured during challenge. The challenge suspension was quantified before and after exposure. Actual aerosol doses ranged from 24-88 x LD₅₀. The average dose was 48 x LD₅₀. The duration of individual challenges ranged from 10-19 minutes. Challenge time was identified as the time that the nebulizer was turned off.

Fever was defined as body temperature $\geq 1.5^{\circ}\text{C}$ above the baseline average for more than two hours.

Observation and Times:

Clinical signs: Animals in the BSL-3 laboratory were observed twice daily for at least 7 days prior to challenge and 3 times per day (8 hour intervals) after challenge for the first 4 days, then continued at 3 times daily if the animal showed a fever.

Telemetry data was collected for at least 30 seconds every 15 minutes for at least 13 days pre-challenge (baseline) and again for at least 30 seconds every 15 minutes during the post-challenge observation period. Parameters included body temperature, respiratory rate, ECG, heart rate, and systolic and diastolic blood pressure.

Hematology / Clinical chemistry: Blood was sampled for hematology, clinical chemistry, and coagulation analyses from all animals prior to challenge on Day 0. Serum was collected during quarantine for ELISA to rule out prior exposure to *Yersinia* species. While serum samples were collected when possible from animals that were found dead, post-challenge ELISAs were deemed unnecessary based on positive culture results and were not performed. Blood samples were repeated on Days 1, 2, 3, 4, and 5. At each time point, blood for bacteriological culture was obtained from the samples collected for clinical pathology.

Gross pathology: Animals that succumbed to challenge underwent a gross necropsy. Bacterial cultures were made from animals that had frank copious fluid in the respiratory tract.

Histopathology: Tissues collected for histopathological evaluation included the lungs, intrathoracic lymph nodes, and any gross lesions.

Results:

Mortality: All animals succumbed to challenge between Days 3 and 6 post-challenge. All were found dead; none were euthanized. Time of death was estimated using telemetry data. Time to death after challenge averaged 93 hours and ranged from 66-139 hours. The first animal died on Day 3, 5 more died on Day 4, 3 on Day 5, and one on Day 6.

There was no correlation between actual aerosol dose and time to death. The report states that death occurred within 17-57 hours of onset of fever.

Clinical signs: The most frequent signs initially were non specific, e.g. anorexia, hunched posture and lethargy. These were evident as early as 1 day post-challenge in some animals, and were evident in 9 of 10 animals by the second day. The animal that received the lowest aerosol dose did not exhibit signs until the third day. During the later stages of infection, additional signs included rapid breathing, coughing, pale skin, and bloody nasal discharge.

An abnormal clinical observation was the first sign of disease in most animals. In contrast, the report states that fever was the first sign of infection in a published study in cynomolgus macaques.

All animals developed sustained fevers that were $> 1.5^{\circ}\text{C}$ higher than baseline. Once fever developed, no body temperature dropped below that threshold prior to death. The average time to onset of fever was 55 hours post-challenge (range 48-62 hours). The average time until the animal succumbed to infection following onset of fever was 38.1 hours. There was no statistically significant correlation between actual aerosol dose and time of onset of fever, nor was there correlation between the aerosol dose and the interval of time between onset of fever and death.

Hematology: After aerosol challenge, the report states that there were no clinically significant changes in red blood cell (RBC) parameters, platelets, percent eosinophils or basophils, or number or percent of monocytes.

The following post-challenge parameters were indicative of active infection. At 3 and 4 days post-challenge, 7 of 9 surviving animals had increased white blood cell

(WBC) counts. At 3 days, the percent of neutrophils for 8 of 9 animals were greater than the upper limit of normal (ULN), and 7 of 9 had neutrophil counts that were above the normal upper limit. Three of 9 had percentages of lymphocytes that were below the normal lower limit, however, one animal had persistently increased lymphocyte counts and three others had transiently increased lymphocyte counts. Six of 9 had increased numbers of eosinophils; at least one of them had increased eosinophils at baseline. The report states that this differs from a study of pneumonic plague in rhesus macaques, where eosinopenia was reported. Four of 9 had increased numbers of basophils.

The one animal that died by Day 3 did not exhibit significant changes in total WBC or any of differential counts.

Evidence of disseminated intravascular coagulation (DIC) was found in all of the animals during the post-challenge observation period. Prothrombin times and partial thromboplastin times were increased in 9 of 10 animals post-challenge. Fibrinogen concentration was increased in 9 of 10 animals, D-dimer was decreased in 7 of 10 animals, and thrombocytopenia was noted in 7 of 10 animals, relative to baseline values. The report notes that hyperfibrinogenemia and evidence of DIC were previously noted in rhesus macaques with pneumonic plague. It also notes that fibrinogen and D-dimer changes were not typical of DIC caused by Gram-negative sepsis, but that hyperfibrinogenemia was consistent with previous findings in rhesus monkeys experimentally infected with pneumonic plague.

Clinical chemistry: Baseline creatinine concentration and sodium concentration were decreased in 10 of 10 and 9 of 10 animals, respectively, but returned to at or near normal post-challenge. All 10 animals had slightly increased serum calcium concentration on Days 1 and 2 post-challenge. On Day 3, 6 of 9 still had increased calcium concentration. Only 1 of 4 surviving AGM had increased calcium concentration at 4 days post-challenge. Serum phosphorus was increased in 6 of 10 on Day 1, but in only 2 of 10 at Day 2. Serum phosphorus concentration was normal in survivors on days 3-5. In general, the deviations from normal for these values were of small magnitude.

Serum AST was increased (130-402% of baseline) in 6 of 9 surviving AGM at 3 days post-challenge. AST remained increased in 3 of 5 surviving animals at Days 4 and 5 post-challenge. The report states that changes in liver function with progressing infection are consistent with previous findings in rhesus macaques.

Gross pathology: Lesions were found on the lungs that were considered to be consistent with a diagnosis of pneumonic plague. The majority of animals (9/10) had fluid in the thoracic cavity and focal discoloration with nodules or masses in the lungs. Enlargement and/or discoloration of lymph nodes, including mediastinal lymph nodes, were noted in some animals. Lesions correlated histologically with inflammation, necrosis, hemorrhage, and edema. The fluid found in the pleural cavity cultured positive for *Y. pestis*.

Additional gross findings included discoloration in the brain of one male, discoloration of the spleen in one male and one female, and fluid in the thymus in one male and one female (see below for microscopic findings).

Histopathology: Lesions primarily involved the lungs and bronchial and mediastinal lymph nodes. A few animals had lesions in other lymph nodes, thymus, spleen or brain. Bacteria were commonly noted in blood vessels, indicating septicemia.

In the lungs, lesions ranged from slightly increased alveolar macrophages, scattered neutrophils and rare fibrin strands in alveolar spaces to focally or regionally extensive parenchymal necrosis with marked inflammation, hemorrhage, fibrin exudation and edema. Rod-shaped bacteria consistent with *Y. pestis*, were visible in the alveoli and small airways of all animals on study, with increased numbers associated with more severe inflammation and necrosis.

Bronchial and mediastinal lymph nodes contained bacteria in all animals on study, usually with concurrent lymphocyte depletion or necrosis. Edema, fibrin exudation, hemorrhage and neutrophilic inflammation were also commonly noted. Other lymph nodes in a few animals had similar lesions to those described for intrathoracic lymph nodes.

Spleen and thymus were evaluated in two animals each and contained bacteria, lymphoid depletion and hemorrhage. Edema was also noted in the thymus of both animals.

The brain of one male animal was examined and had meningeal hemorrhage, fibrin exudation and thrombosis mainly affecting the choroid plexuses. Bacteria were noted within the lesions.

The pathology report concluded that aerosol administration of an average of 48 x LD₅₀ of *Y. pestis* strain CO92 resulted in fatal pneumonia and septicemia in all ten African Green monkeys three to six days after infection. Extrapulmonary lesions were common in lymphoid tissues and, rarely, noted in the brain. It also stated that the lesions were similar to those previously reported in macaques.

Other: All animals became bacteremic. Blood cultures were negative for all animals on the first day post-challenge. Seven of 10 were bacteremic 2 days post challenge, and, at 3 days post-challenge, all survivors were bacteremic. Survivors on subsequent days (4 on Day 4, 1 on Day 5) remained bacteremic. Bacteremia was first noted in 5 of 10 animals prior to the onset of fever and after the onset of fever in the other 5. The report states that this finding differs from those in previous studies in rhesus monkeys that were abacteremic prior to onset of fever.

Concentrations of organisms in blood samples increased with time after challenge. Positive blood cultures were obtained from all terminal blood samples. There was a statistically significant relationship between the log value of the terminal bacterial blood concentration and the time to death.

Samples of fluids from the nares and pleural cavity or trachea were obtained from all animals during necropsy. All yielded positive cultures of *Y. pestis*. There was a statistically significant association between terminal bacterial concentrations (CFU/mL in blood) and the time to death. However, there was no significant association between terminal bacterial concentrations and challenge dose.

5. NIAID/DMID report no. NIAID-Yp-NatHis-Path-2011: Independent pathology review of the natural history studies for pneumonic plague in the African Green Monkey

An independent pathology review was conducted of the above four studies in order to standardize the nomenclature and description of findings. The only tissues evaluated microscopically in all animals from all studies were lungs and bronchial/tracheobronchial lymph nodes. However, the number and/or location of routine sections of lung processed to slide for microscopic evaluation were different for each study, and each study handled processing of gross lesions differently. The report states that, because pneumonic plague in the African Green Monkey appears to be lobar to sublobar in nature, the number and selection of lung tissue samples presented for microscopic evaluation can result in variability in both character and severity of microscopic findings. For the purposes of this review, in cases where different sections from the same animal had the same lesion but differed in severity, the more severe score was used in the study specific Independent Pathology Review Tables.

Bacteria were present in blood vessels in one or more of the organs and tissues evaluated from all animals in all four studies. The independent review pathologist did not consider the intra-luminal presence of bacteria to be a tissue-specific lesion and therefore this is not recorded as a tissue finding by the independent review pathologist.

The report states that the independent review of microscopic slides from the four natural history studies confirms that there is a common pathology associated with inhaled *Y. pestis* Strain CO92 in African Green Monkeys. Intra-alveolar inflammatory infiltrates (neutrophil and or macrophage) were present in lungs of all animals evaluated from all studies. There were, in several animals, remarkable lobar differences in the presence, severity and/or character of the infiltrates within the same animal. The reviewing pathologist indicated the following: "Those areas where edema, macrophages and bacteria predominate appear to represent earlier lesions in the disease process. As the disease progresses, large numbers of neutrophils flood into the affected areas along with hemorrhage and edema which eventually totally efface the normal lung tissue elements. Bacteria are seen in large numbers in the lung and are typically observed in both the alveolar spaces as well as within alveolar macrophages."

Summary of Microscopic Findings in the Lungs

Tissue/Finding	Natural History Study				Total
	BBRC 617	BBRC 875	LRRI FY06-126	USAMRIID F03-09G	
Number Examined	10	10	10	4	34
Lung					
Bacteria	10	10	9	4	33
Edema	10	10	6	3	29
Hemorrhage	8	9	10	4	31
Inflammatory infiltrate, intra-alveolar, macrophage	10	6	10	4	30
Inflammatory infiltrate, intra-alveolar, neutrophil	10	6	10	4	30
Necrosis, multifocal	0	4	0	0	4
Pleura, fibrin	3	8	10	3	24
Pleura, inflammatory infiltrate, macrophage	1	0	0	0	1
Within normal limits	0	0	0	0	0

BBRC = Battelle Biomedical Research Center; LRRI = Lovelace Respiratory Research Institute; USAMRIID = United States Army Medical Research Institute of Infectious Diseases

Microscopic evaluation of the bronchial/tracheobronchial lymph nodes revealed similar findings across studies. The findings included bacterial colonization, edema, hemorrhage, inflammatory infiltrates (primarily neutrophil), and lymphoid hyperplasia or depletion. Similar findings were present in mandibular and mediastinal lymph nodes (in studies where these were evaluated). Lymphoid hyperplasia or sinus histiocytosis was observed in axillary, inguinal and mesenteric lymph nodes (evaluated in only one study of four animals), but no bacteria or inflammatory infiltrates were noted. Bacteria, hemorrhage, inflammatory infiltrates (neutrophil) and/or lymphoid depletion were noted in the spleen of all animals evaluated except for one animal from LRRI FY06-126.

Bacteria, edema, hemorrhage, and neutrophilic inflammatory infiltrates were also observed in the connective tissue of the mediastinum in three of the four animals evaluated (USAMRIID F03-09G). This was stated to be the only other tissue evaluated which showed microscopic lesions clearly attributable to aerosol exposure of *Y. pestis* Strain CO92 in African Green Monkeys.

The independent reviewing pathologist concluded that there is a common pathology associated with lethal infection by inhaled *Y. pestis* Strain CO92 in African Green Monkeys, based on these four studies. Post-challenge infection and dissemination was found to occur quickly in this species, with morphologic changes in the lung appearing to begin as lobar to sublobar serous and fibrinous exudates (edema) with intra-alveolar and intracellular (macrophages) bacteria along with increased numbers of alveolar macrophages. These changes observed in the lung were found to transition quickly to diffuse necrotizing pneumonia characterized by alveoli and airways filled with bacteria, inflammation and hemorrhage. There was dissemination of bacteria to bronchial/tracheobronchial and mediastinal lymph nodes, mediastinal connective tissues and spleen, initiating changes in the tissues such as hemorrhage, inflammation and edema.

6. NIAID/DMID Report Number: NIAID-Yp-NatHis-Rad-2011: Independent radiology review of the natural history studies for pneumonic plague in the African Green Monkey

An independent radiology review was conducted of the two natural history studies for pneumonic plague conducted at Lovelace Respiratory Research Institute (LRRRI) and U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) by a board-certified veterinary radiologist in order to apply consistent terminology to the radiographic interpretation of radiographs from animals in those studies. The independent review of radiographs from the two natural history studies confirms that there is a common radiographic pulmonary appearance and progression of the severity of changes over time associated with inhaled *Y. pestis* Strain CO92 in African Green Monkeys.

Summary of Radiographic Findings in the Lungs (Natural History Studies)

Days Post Challenge (No of Animals in Study)	LRRRI FY06-126			USAMRIID F03-09G		
	Baseline (N=10)	Day 3 (N=10)	Day 4 ^a (N=10)	Baseline (N=6)	80-83hr (N=6)	Terminal (N=6)
No. of Animals Examined	9 ^b	9 ^c	9 ^d	5 ^{e,f}	4 ^{e,f,g}	4 ^{e,f,g}
Lung						
Normal	7	2	1	4	0	0
Pulmonary infiltrate						
Mild	2	4	1	1	3	0
Moderate	0	2	0	0	1	0
Severe (marked)	0	1	7	0	0	4

Hr = hours; LRRRI = Lovelace Respiratory Research Institute; N = number of animals in study; No. = number; USAMRIID = United States Army Medical Research Institute of Infectious Diseases

^a Terminal – Animals on Day 4 were found dead or euthanized in moribund condition.

^b The Independent Review Radiologist did not receive a radiograph for Animal X756 for April 2, 2007 (Day 0).

^c The Independent Review Radiologist did not receive a radiograph for Animal X789 for April 26, 2007 (Day 3).

^d The Independent Review Radiologist did not receive a radiograph for Animal X790 for April 27, 2007 (Day 4); the animal was found dead on Day 3.

^e The Independent Review Radiologist was provided one un-dated radiograph from Animal V521; this was tabulated in the table as a baseline radiograph. Animal V521 received < LD99 challenge dose, showed no clinical signs post challenge, was not anesthetized post challenge so no radiographs were taken post challenge, and survived to study termination.

^f The Independent Review Radiologist was provided two un-dated radiographs from Animal V569. These were tabulated in the table as being from the 80-83 hour and terminal timepoints.

^g Animal V605 received < LD99 challenge dose, showed no clinical signs post challenge, was not anesthetized post challenge so no radiographs were taken post challenge, and survived to study termination.

Efficacy studies:

1. Study title: An efficacy study of intravenous infusion of levofloxacin in inhalational plague *Yersinia pestis* strain CO92 in telemetered African Green monkeys

Study no.:	FY07-070
Study report location:	Electronic submission section 4.2.1.1.
Conducting laboratory and location:	Lovelace Biomedical and Environmental Research Institute, Albuquerque NM
Date of study initiation:	2/15/08
GLP compliance:	Yes, with exceptions
QA statement:	Yes
Drug, lot #, and % purity:	Levofloxacin (Levaquin® Injection Premix in single-use flexible containers (5 mg levofloxacin/mL 5% dextrose)), lot nos. 61-022-JT, 63-262-JT, and 70-23-JT.

Methods

Doses: Vehicle control (5% dextrose in water for injection) or 8 mg/kg levofloxacin (5 mg/mL in 5% dextrose) plus 2 mg/kg levofloxacin

Frequency of dosing: Treatment began within 6 hours (3.4 ± 1.8 h) of the onset of fever. The humanized dose regimen consisted of 2 infusions each 24 hours. First, 8 mg/kg was infused over 30 minutes. A second infusion was administered over 30 minutes within 12 ± 1 hours of the initial infusion, using a dose of 2 mg/kg. Infusions continued for 10 days (total of 20 infusions) or until the animal died.

Route of administration: IV infusion

Dose volume: Not specified

Formulation/Vehicle: 5% dextrose in water for injection, lot P210609

Species/Strain: African Green monkeys (*Chlorocebus aethiops*, wild-caught), jacketed and implanted with T30F (DISS, Inc.) telemeters and intravenous catheters. They were conditioned to a restraint collar, poles, restraint chairs and limb restraints. They were moved into the BSL3 facility at least one week prior to challenge.

Number/Sex/Group: Total of 12/sex; the control group included 3 females and 4 males while the treated group included 9 females and 8 males

Age: At least 2 years old

Weight: 3-8 kg

Satellite groups: None

Unique study design: This efficacy study was conducted in a post-exposure model of pneumonic plague in AGMs challenged via head-only aerosol with a target $100 \pm 50 \times LD_{50}$ (approximately [REDACTED] CFU, target particle size 1-3 μ m) *Yersinia pestis* strain CO92 under Telazol anesthesia, following an overnight fast. The study was conducted in 3 cohorts. The actual challenge dose ranged from 3-145 LD_{50} s (mean 65 ± 47.7). Previous work had determined the LD_{50} to be [REDACTED] CFU. The day of challenge was Day 0. All work was carried out under BSL3 conditions and was as described for the natural history study above.

Deviation from study protocol: None that affected the outcome of the study

Observations and Results

The timetable of experimental procedures is outlined below:

Procedure	Week -2 to -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7-13	Day 14-28
<i>Y. pestis</i> challenge		X								
Arterial Blood Gas ^{b, h}	X			X ^b at moribund euthanasia (not for Cohort 3)						
Thoracic radiograph ^{c, h}		X		On Day 5 (not for Cohort 3) or at moribund euthanasia						
Test/control article infusion		Twice daily (Q12h after fever onset) for 20 infusions or euthanasia								
Levofloxacin levels	X	At infusion 1, 3, 4, 6 and 19								
Quantitative Bacteriology ^{a, g}	X			X	X	X	X	X	X ^g	Days 14, 21, 28
Qualitative Bacteriology ^a		Once prior to first infusion (Cohort 3 only)								
Scrum Chemistry ^a	X			X				X		Day 28
Coagulation Indices ^{a, h}	X			X ^b at moribund euthanasia (not for Cohort 3)						
Hematology ^a	X			X				X		Day 28
Tissue pathogen load ^a		All found dead or euthanized monkeys								
Body Weight ^d	X	X	On day of necropsy or terminal euthanasia							
Echocardiography ^{f, h}	X			X	X	X	X			
Telemetry	X	X	X	X	X	X	X	X	X	X
Detailed clinical observations		X	X	X	X	X	X	X	X	X
Interim necropsy ^a		Dead or moribund monkeys								
Terminal necropsy ^e										Day 28

^a For animals that are alive on Days 2-6, blood samples were collected as noted, for quantitative bacteriology, serum chemistry, and hematology. At interim necropsies (1) prior to euthanasia, if possible, blood was collected for quantitative bacteriology, serum chemistry, hematology and possible serum antibody titer, and (2) tissues were obtained for pathogen load. For Cohort 3, blood was collected for post-challenge quantitative and qualitative bacteriology prior to first infusion.

^b Arterial blood gases and coagulation indices were measured pre-study and, if possible, once prior to euthanasia for moribund animals, except for Cohort 3.

^c Thoracic radiographs were taken in anesthetized animals prior to *Y. pestis* challenge on Day 0, at the time prior to moribund euthanasia and necropsy.

^d Body weight also was obtained on the day of necropsy.

^e Terminal necropsies were performed on any surviving animals on Day 28 post-challenge.

^f Performed 1-2x pre-study, Study Days 2-5, prior to moribund euthanasia (if possible), and Day 14 ± 1 and Day 27 ± 1, except for Cohort 3.

^g Blood culture (quantitative bacteriology) may have been performed on Study Days 7-27. Blood culture was performed on animals that continue to show positive blood culture, show signs of illness/infection, and at weekly intervals as long as collected blood volumes are within IACUC guidelines

^h Not performed for Cohort 3

Animals were randomized to control or levofloxacin treatment prior to challenge. The trigger for initiation of treatment was fever, defined as a mean body temperature greater than 39°C for more than one hour. Treatment was initiated within 6 hours (mean 3.4±1.8) of the onset of fever. There was no statistically significant trend relating challenge dosage with time of onset of fever. Infusions were administered twice daily as described above to approximate the pharmacokinetics of levofloxacin in humans

using an indwelling catheter in the femoral vein. Doses were based on the body weight measured at the time of aerosol exposure.

Mortality

Animals were observed twice daily (morning and afternoon) on each day of the study for mortality and morbidity. All seven control animals died of severe pneumonic plague and disseminated infection or were euthanized in moribund condition by 4 or 5 days of challenge. All levofloxacin-treated animals survived beyond that point. One of the levofloxacin-treated animals was euthanized on Day 9 due to gastrointestinal problems. There were no signs in that animal that would attribute the cause of death to plague. The rest survived to termination of the study at 28 days.

Clinical Signs

Clinical observations were made prior to exposure, then twice daily cageside observations were made, at least 4 hours apart. The animals were monitored for up to 28 days post-challenge.

The main clinical signs noted included reduced activity, changes in posture, inappetence, and stool changes. Additional signs reported included reduced grooming, respiratory changes, limited neurological signs, and nasal/ocular discharge.

The treated animal that was euthanized at 9 days was found to be vomiting tissue and unable to retain food beginning on Day 7. Prior to that, it had shown no significant adverse clinical signs.

Body Weights

Animals were weighed pre-test (within one week prior to aerosol challenge), on the day of aerosol challenge, and at necropsy.

Most animals lost weight over the course of the study. The weight loss was statistically significant in control animals post-challenge. In treated animals that survived to Day 28, weight loss was not significant when compared to baseline body weights.

Feed Consumption

Not measured

Telemetry

Continuous recording via telemetry of body temperature, intrathoracic pressure, respiratory rate, heart rate, and ECG was performed. Body temperature was recorded every 5 minutes and was averaged for hourly temperatures at the end of the study. Respiratory rate was determined by recorded changes in intrathoracic pressure. Heart rate was determined by counted R waves per minute, and the ECG was recorded as raw data.

Baseline telemetry data were recorded. Data were inspected at least twice daily, and every 4-6 hours during the clinically critical phase of the study (i.e. Days 2-7). These data were used to determine if and when a moribund animal should be euthanized, as well as when fever was sufficient to initiate treatments.

The onset of fever ranged from 53-93 hours post-challenge in most animals. However, in three animals, fever was delayed up to 165 hours post-challenge. The report states that there was no significant relationship between challenge dose and the time of onset of fever. Increased respiratory rate appeared at the same time or slightly after the increase in body temperature and was followed by increased heart rate in most animals.

The increases in heart rate and respiratory rate were statistically significant, beginning on Day 2 in all animals. By Day 3, the mean respiratory rate in control animals had rapidly increased while that of the treated group stabilized at ≤ 10 /min. The body temperature increase became statistically significant beginning on Day 2 in all animals and was equally high in control and treated animals on Day 3, but began to diminish in treated animals by Day 4. Fever tended to resolve in treated animals prior to resolution of tachypnea. Heart rates were more subject to fluctuations, but generally reflected respiratory rate changes. A statistically significant increase in nocturnal heart rate persisted to Day 14, but the physiological significance of that finding was uncertain. Inspection of telemetry data after the end of treatment revealed no evidence of clinical relapse.

The report states that levofloxacin treatment reduced increases in respiratory rate, but not the fever, in the first 2 days of treatment.

Hematology / Clinical Chemistry

Blood was collected via femoral venous catheter for hematology, clinical chemistry, and coagulation analysis. Baseline samples were collected prior to pathogen exposure. Additional samples were taken on Days 2, 6, and 28 (or at time of euthanasia or spontaneous death). Coagulation data were determined at baseline and at moribund euthanasia for Cohorts 1 and 2.

Total white blood cell (WBC) count was increased in the control group on Day 5, but not in the treated group. Differences were primarily in neutrophil and monocyte fractions; the report states that this is indicative of the effect of relatively early antibiotic treatment on inflammation.

Increased hematocrit in the control group was consistent with hemoconcentration due to dehydration. In the treated group, hematocrit was decreased, as was hemoglobin concentration, reticulocyte and erythrocyte counts. The report states that those findings would be consistent with the physiological impact of this systemic disease on erythropoiesis. Nevertheless, changes in red cell indices were considered not to be physiologically significant.

Coagulation data were only obtained from Cohort 1 and 2 animals pre-study and moribund euthanized animals post-challenge. No statistical comparisons were made.

Clinical chemistry findings included statistically significant increases in ALT, alkaline phosphatase, AST, GGT, LDH, and total bilirubin. Decreases in total protein, cholesterol and triglycerides were also found in control animals prior to euthanasia, compared to pre-study values. Of the increases in ALT, AST, LDH, total bilirubin, total protein and triglycerides in the test article group on Day 6 relative to pre-study values, only AST and LDH were considered to be biologically significant; these were attributed to lung inflammation. Values in treated animals returned to pre-study values by the end of the study.

Blood glucose was decreased, and BUN was increased in both treated and control groups, and creatinine was increased in the control group. Changes were only significant in the control group and were stated to be consistent with blood volume decreases associated with more severe disease.

Urinalysis

Not performed

Gross Pathology

Gross necropsy was performed following euthanasia or spontaneous death. Lung (lesion and non-lesion) and liver from all animals, plus spleen, kidneys, and brain from animals found dead or euthanized in moribund condition were collected for bacterial quantitation and histopathological examination.

The main macroscopic lesions in control animals were large deep purple regions in multiple lung lobes that were consistent with parenchymal hemorrhage and enlarged/discolored tracheobronchial lymph nodes. Discolored liver and spleen (without enlargement) and enlarged heart in were found in two animals, and fluid on the brain was noted in one. The appearance of lungs in surviving treated animals was characterized by a discolored surface. The report states that this appearance is consistent either with anesthesia artifact or with resolution of plague pneumonia.

Histopathology

H&E slides of lung (lesion and non-lesion), liver, spleen, tracheobronchial lymph nodes, brain, and gross lesions were examined by a veterinary pathologist.

The treated animal that was euthanized on Day 9 exhibited necrosis of the gastric epithelium and no evidence of *Y. pestis* infection in any organs. The cause of death was unexplained.

All control animals were found dead or moribund sacrificed. Marked fibrinosuppurative pneumonia was observed in all of them.

In levofloxacin-treated animals sacrificed at the end of the study, chronic lesions of histiolympocytic infiltrates within the pulmonary parenchyma were seen in over half of the animals and were considered to be related to ongoing resolution of plague pneumonia. Mixed liver inflammation was also considered to be associated with resolving lesions.

The report concluded that control animals died of overwhelming pneumonic plague and that the levofloxacin-treated animals recovered from established pneumonic plague.

Special Evaluation

Radiology

Digital radiographs were taken of the entire chest and abdomen at the time of anesthesia prior to aerosol exposure on Day 0. Normal or interstitial patterns observed were considered to be an artifact of not taking film at full inspiration. For animals surviving by Day 5, a thoracic radiograph was taken under anesthesia on Day 5 or 6 for Cohorts 1 and 2. Another radiograph was taken at the time of anesthesia for euthanasia from a total of 6 surviving animals in Cohorts 1 and 2. Radiographs were reviewed by a veterinary radiologist.

The animal euthanized on Day 9 from Cohort 3 had films on Day 9 with patchy multifocal pulmonary infiltrates.

In the three control animals radiographed post-challenge, large multilobar infiltrates involving most of lung fields were seen and considered to be consistent with severe plague pneumonia. These correlated with gross findings of extensive hemorrhage and palpable indurated masses in multiple lobes.

In the nine levofloxacin-treated animals in Cohorts 1 and 2 radiographed on Day 5 or 6, abnormalities seen were consistent with mild or moderate pulmonary infiltrates. It was considered that plague pneumonia had progressed in those animals to become radiographically apparent. Images were as late as 2.5 days after start of treatment, yet abnormalities were still apparent.

In radiographs taken 28 days after challenge (5-17 days after the end of therapy) in five animals were normal. The sixth still had an interstitial pattern (as did nearly half of the animals prior to challenge).

Transthoracic echocardiography (TE)

TE was performed on 2 unanesthetized animals from each treatment group from Cohorts 1 and 2 pre-test, on Days 2-5, prior to moribund euthanasia, and on Days 14 and 27. The data were not audited and not intended to contribute to the analysis of antibiotic efficacy.

The report stated that pre-exposure ejection fraction was 60-85%, corresponding to healthy human adults. Early depression of that parameter was seen, and it was as low as 20% immediately prior to euthanasia in one untreated animal. The report states that ejection fraction changes were less dramatic and/or variable in the other animals examined. No conclusions were drawn from these data.

Arterial blood gases

Arterial blood was drawn from unanaesthetized restrained animals pre-study from Cohorts 1 and 2 only, and proved to be too difficult to be of utility. Samples could not be obtained from moribund animals due to decreased blood pressure. No comparisons were made, and the data were not used in evaluation of efficacy.

Quantitative bacteriology

Quantitative bacteriology was performed on venous whole blood sampled as per the schedule in the Methods table above. Additionally, tissues [lung (lesion and non-lesion), liver, spleen, and tracheobronchial lymph nodes] were sampled at necropsy and analyzed for presence of *Y. pestis* by quantitative culture.

Control animals had bacteremia and marked bacterial concentrations in analyzed tissues. The highest tissue concentrations were in the lungs and tracheobronchial lymph nodes.

In levofloxacin-treated animals, 13/17 (76%) were bacteremic prior to infusion. The report goes on to state that no bacteremia was detected in treated animals after the start of infusions, and that, at study termination, no detectable significant *Y. pestis* concentrations were reported in blood samples. The report also states that nine in-life blood samples from Cohort 1 contained "contaminant bacteria" that appeared to be *Y. pestis*, seemingly contradicting the previous statement. According to the report, in two tissues and two blood samples from animals in Cohort 1 taken at terminal necropsy,

colonies were recorded that appeared to be *Y. pestis*. These were attributed to contamination by the Sponsor, and are discussed in more detail in the Clinical Microbiology review.

In the treated animal that was euthanized on Day 9, blood cultures were positive for *Y. pestis* only prior to initiation of treatment on Day 3. Daily blood cultures after treatment was initiated and tissues cultured at necropsy were negative.

Pharmacokinetics

Blood was collected via femoral venous catheter for levofloxacin concentration determinations. Samples were obtained pre-study, at infusion 1, infusion 3, infusion 4, infusion 6, and infusion 19. Samples were drawn 10-30 minutes before the start of infusions 3, 6, and 19 for trough levels, and at 5-15 minutes after the termination of infusions 1, 3, 6, and 19 for peak levels. Blood was also sampled 5-15 minutes following infusion 4. Plasma was separated and centrifuge-filtered prior to removal from the BSL-3 facility for analysis. For Cohorts 1 and 2, analysis was by a validated LC/MS method, and for Cohort 3, analysis was by a validated fluorescence method. There were procedural problems and accuracy limitations with the former analysis method. Data from Cohort 1 were not consistently within the validated range of the assay, and there were similar issues with Cohort 2. After the analytical method was changed for Cohort 3, the data were less variable, and only Cohort 3 data were used for statistical analysis. The report states that there were still some procedural failures in the analysis for Cohort 3.

The report states that the human dose for Gram-negative pneumonia is 500 mg IV q24h, resulting in a peak concentration of 6.2 µg/mL and an AUC of 48.3 µg*hr/mL. For these animals, peak C_{max} after the first and third doses of 8 mg/kg ranged from 2.4-4.5 µg/mL. Only 3 of 16 infusions attained a level within 2 standard deviations of the target 6.2 µg/mL. Trough levels were said to all exceed the MIC for this strain.

Conclusions

In this study, multifocal pneumonia, bacteremia, and dissemination to liver and spleen were found in the African Green monkey model of pneumonic plague. The disease underwent a rapid course with fever onset within 53-93 hours of exposure. Bacteremia was found in most animals at the time of onset of fever. Radiographically detectable pneumonia was seen in all animals examined. Actual aerosolized exposure to the pathogen fell into a wide range above and below targeted exposure, but did not affect the progression of disease or the outcome of treatment, although the report states that there was a borderline trend for relationship between lower exposure and delayed onset of fever. Initiation of treatment with IV levofloxacin, using the onset of fever as a trigger, and continued treatment for 10 days in this model was considered to be efficacious for pneumonic plague. Plasma levels achieved were considered to be relevant to those established for human dosing.

2. NIAID/DMID report no. NIAID-Yp-Levo-Path-2011: Independent pathology review of the efficacy study of intravenous infusion of levofloxacin in pneumonic plague in the African Green monkey

An independent pathology review was conducted of the microscopic slides from animals in Lovelace Respiratory Research Institute (LRRI) study number FY07-070 entitled, 'An Efficacy Study of Intravenous Infusion of Levofloxacin in Inhalational Plague *Yersinia pestis* Strain CO92 in Telemetered African Green Monkeys.'

All control animals were found dead or sacrificed in a moribund condition on Days 4/5. A total of eight sections of lung were processed to slide for microscopic evaluation for each animal. Microscopic evaluation of the tissues provided from the control animals was said to confirm the common pathology associated with lethal infection by inhaled *Y. pestis* Strain CO92 in African Green Monkeys as described in the Natural History studies (reported in NIAID-Yp-NatHis-Path-2011). Intra-alveolar inflammatory infiltrates (neutrophils and/or macrophages) were present in lungs of all control animals evaluated. There were often lobar differences in the presence, severity and/or character of the infiltrates within the same animal. Morphologic changes in the lung appeared to begin as lobar to sublobar serous and fibrinous exudates (edema) with intra-alveolar and intracellular (macrophages) bacteria along with increased numbers of alveolar macrophages. These lung changes appear to transition quickly to diffuse necrotizing pneumonia characterized by alveoli and airways filled with bacteria, inflammation (characterized by large numbers of neutrophils) and hemorrhage that eventually obliterates the normal lung tissue elements. Bacteria were seen in large numbers in the lung and were typically observed in both the alveolar spaces as well as within alveolar macrophages.

In contrast, the changes in the lungs of the animals treated with levofloxacin under the conditions of this study were reported to be consistent with ongoing resolution to the lesions associated with the challenge-induced infection with *Y. pestis*. All treated animals survived to the scheduled study termination on Day 28 with the exception of one animal. The character of the changes in the treated animals was more chronic than that seen in the control animals. The inflammatory infiltrates in the treated animals were interstitial and/or perivascular in location and consisted of mononuclear cells rather than neutrophils and macrophages. In addition, other pulmonary changes observed in control animals, such as edema and hemorrhage, were not present in the treated animals. Bacteria were not observed in alveoli or macrophages in the sections of lung evaluated from the treated animals.

Microscopic findings in the lungs of animals in this study are summarized in the Sponsor's table below:

Tissue/Observation	LRRF FY07-070	
	Control Animals	Treated Animals ^a
Number Examined	7	17
Lung		
Bacteria	7	0
Cellular debris, bronchiolar	0	1
Edema	7	0
Fibrosis	0	1
Hemorrhage	7	0
Inflammation, chronic, perivascular	0	13
Inflammatory infiltrate, interstitial and/or bronchiolar, mononuclear cell	0	16
Inflammatory infiltrate, intra-alveolar, macrophage	6	0
Inflammatory infiltrate, intra-alveolar, neutrophil	7	0
Pleura, fibrin	4	8
Thrombus, organizing	0	1
Within normal limits	0	1

LRRF = Lovelace Respiratory Research Institute

^aLevofloxacin treated

In natural history studies, there was dissemination of bacteria to bronchial and tracheobronchial lymph nodes and spleen followed by changes in the tissues such as hemorrhage, inflammation and edema. Microscopic evaluation of the tracheobronchial lymph nodes and spleen from the control animals in LRRF FY07-070 revealed similar findings. In the tracheobronchial lymph nodes, bacterial colonization, edema, hemorrhage, inflammatory infiltrates (primarily neutrophil), and lymphoid hyperplasia or depletion were seen. In the spleen of most control animals, bacteria, hemorrhage, inflammatory infiltrates (neutrophil) and/or lymphoid depletion were seen.

Microscopic evaluation of tracheobronchial lymph nodes and spleen from treated animals in LRRF FY07-070 revealed essentially normal tissues in 15 of 17 treated animals. Changes, when present, were reported to be minimal to mild in severity and reflected an ongoing recovery process.

Microscopic findings in the lymph nodes and spleen of animals in this study are summarized in the Sponsor's table below:

		LRRR FY07-070	
Tissue/Observation		Control Animals	Treated Animals^a
	Number Examined	7	17
Lymph Node, Tracheobronchial			
Bacteria		7	0
Edema		5	0
Hemorrhage		2	0
Inflammatory infiltrate, neutrophil		4	1
Lymphoid depletion		4	0
Lymphoid hyperplasia		0	1
Within normal limits		0	15
	Number Examined	7	17
Spleen			
Bacteria		5	0
Congestion		1	0
Hemorrhage		1	0
Inflammatory infiltrate, macrophage		2	1
Inflammatory infiltrate, neutrophil		2	0
Lymphoid hyperplasia		0	1
Lymphoid depletion		3	1
Within normal limits		1	15

LRRR = Lovelace Respiratory Research Institute

^aLevofloxacin treated

Microscopic evaluation of the brain did not reveal any changes in any control or treated animals with the exception of a minimal lymphocytic infiltrate in the meninges in one male treated with levofloxacin. The cause and significance of this finding is unknown. Microscopic evaluation of the liver reportedly did not reveal any unexpected or marked changes in any control or treated animals. The liver was described as normal in 3/7 control animals and 15/17 treated animals. In 4/7 control animals, changes in the liver included bacteria and/or inflammatory and cellular infiltrates (neutrophil, lymphoplasmacytic). The changes observed in the liver of two treated animals were minimal chronic perivascular inflammation and minimal periportal cellular infiltrates (lymphoplasmacytic).

The only treated animal from LRRR FY07-070 that died prior to the scheduled study termination (Day 28) was euthanized on Day 9 due to moribund condition from repeated vomiting. Microscopic evaluation of tissues from this animal revealed resolving pulmonary lesions similar to those described for other treated animals. At necropsy, a gross lesion was recorded for the stomach which was preserved for microscopic evaluation. The stomach lesion was diagnosed as submucosal inflammatory infiltrates of mononuclear cells with associated marked edema. The cause of the lesion could not be determined from the microscopic evaluation of the tissue sections provided and bacteria consistent with *Y. pestis* were not observed in the tissue sections evaluated.

3. NIAID/DMID report no. NIAID-Yp-Levo-Rad-2011: Independent radiology review of the efficacy study of intravenous infusion of levofloxacin in pneumonic plague in the African Green Monkey

This report indicates that a board-certified veterinary radiologist reviewed the data from the levofloxacin efficacy study and applied terminology consistent with that used in the evaluation of radiographs from animals in the natural history studies. The Sponsor's table below summarizes radiographic findings in that study.

Summary of Radiographic Observations in the Lungs

Days Post Challenge	Control (n=7)			Levofloxacin Treated (n=17)			
	Baseline	Day 5 ^a	Day 28	Baseline	Day 5 or Day 6 ^b	Day 9 ^c	Day 28
No. of Animals Examined Lung	7	3	0	17	9	1	6
Normal	7	0	--	17	0	0	6
Pulmonary Infiltrate							
Mild	0	0	--	0	4	0	0
Moderate	0	0	--	0	5	1	0
Severe (marked)	0	3	--	0	0	0	0

-- = not applicable; n = number of animals in group; No. = number

^a Terminal—If possible, radiographs were performed at the time of anesthesia for the purpose of euthanasia for animals in moribund condition.

^b Radiographs were performed on Day 6 instead of Day 5 for Animal X437, Animal X523, Animal X648, Animal X662 and Animal X663. These animals were inadvertently not fasted for anesthesia prior to Day 5.

^c Animal Y160 was euthanized in moribund condition on Day 9 due to repeated vomiting.

4. Report no. UTMB-YpEff-1-8: Compilation of levofloxacin treatment studies in the mouse and rat intranasal challenge models of pneumonic plague

A total of eight studies were performed, four in Swiss Webster mice (outbred), three in Brown Norway rats, and a single study in both mice and rats. In all of the rodent studies, treatment with levofloxacin was administered for six days by the intraperitoneal (IP) route, usually beginning 24 hours following intranasal challenge with *Y. pestis* CO92 (20 µL for mice, 25 µL for rats of a stock culture; numbers of organisms in the challenge inoculum were not determined in all experiments). In three of the studies in mice, surviving animals were rechallenged with *Y. pestis* between 21 and 34 days following the initial challenge. In the fourth mouse study, the effect of delay in initiating therapy following *Y. pestis* exposure was investigated. In rats, surviving animals were rechallenged with *Y. pestis* at 43 or 34 days post-challenge in two studies, respectively. The effect of delayed initial treatment was also examined in the second rat study. A third study in rats examined the efficacy of a high dose (20 mg/kg) administered by the IP route compared to the oral route, using the injectable formulation for both routes of administration.

Doses of 1-150 mg/kg/day administered IP for 6 days beginning 24 hours post-challenge were effective in improving survival in mice, with survival of 100% seen at 5-10 mg/kg/day in most studies. Decreased survival was seen if treatment initiation was delayed until more than 36 hours post-challenge. In the studies in which mice were rechallenged, surviving animals succumbed to the second challenge, with no clear

relationship to prior dose. The Sponsor theorizes that this species failed to develop a protective immune response following first exposure to the organism.

In rats, doses of 10 mg/kg/day IP for 6 days beginning 24 hours post-challenge resulted in 100% survival. If treatment was initiated later than 42 hours post-challenge, protection was decreased. Survival in the rat following re-challenge was higher than in mice, indicating that this species may be more capable of developing an immune response following initial infection that was effective in resisting subsequent infection. In the study comparing IP and oral doses of 20 mg/kg/day, that dose by both routes resulted in 100% survival.

Finally, a study comparing a dose of 10 mg/kg/day IP for 6 days beginning 24 hours post-challenge in both rats and mice resulted in 100% survival in both species. However, when these animals were re-challenged 30 days after the initial challenge, 100% of the rats survived without additional treatment, but only 10% of the mice survived.

In all of the studies, control animals died by 3-4 days post-challenge, with the exception of unexplained 20% survival in one of the rat studies. When treatment was initiated in treated groups, no clinical signs were noted. Clinical signs in animals where treatment was delayed included hunched posture, ruffled fur, lethargy, and crusty eyes.

In summary, doses of levofloxacin of 5-10 mg/kg IP for 6 days, initiated by no more than 36 hours after *Y. pestis* exposure was effective in treating pneumonic plague in mice, resulting in 100% survival in at least one of the experiments. In rats, doses of 10-15 mg/kg/day for 6 days, initiated by no more than 42 hours post-infection were similarly effective. Rats were resistant to re-challenge.

5. Report no. RIID-YpEff-2006: Report on the effect of dosing and schedule of levofloxacin against *Yersinia pestis* in and aerosol challenge model in mice.

A report from (b) (4) was provided summarizing several non-GLP experiments in mice that evaluated dosing and dose schedule under normal and neutropenic conditions. Six to eight week old female Balb/c mice were challenged with 20-25 x LD₅₀ (6.8 x 10⁴ CFU) by whole body aerosol exposure. Treatment was initiated either as post-exposure prophylaxis (PEP) at 24 hours post-challenge or as treatment at 48 hours post-exposure, when bacteremia was considered to be established. Treatment was administered IP for five days. Control animals received saline only. Levofloxacin was administered at escalating doses of 1.5 to 15 mg/kg q12h in the initial experiment in the PEP model, with a dose of 30 mg/kg/day (15 mg/kg q12h) used in subsequent experiments. The latter dose was based on a previously determined AUC₂₄ of 30 (units not provided) and an AUC:MIC ratio of 922 to approximate a human oral dose of 500 mg/day with an AUC₂₄ of 47 (units not provided). Levofloxacin treatment was compared to treatment with gentamicin (12 mg/kg q6h) or ciprofloxacin (30 mg/kg q12h). In another experiment, mice were made neutropenic by injection of cyclophosphamide prior to challenge. Ten animals per group were used in all experiments. Spleens were removed and cultured for *Y. pestis* at the end of each experiment.

All animals administered levofloxacin survived in the PEP model at all doses ranging from 1.5-15 mg/kg. One 15 mg/kg animal died after 30 days, apparently from

other causes, since the spleen culture was negative for *Y. pestis*. Results for levofloxacin in the PEP model were equivalent to those achieved with gentamicin and ciprofloxacin and were significantly different from control (100% mortality).

In the treatment model, survival in the levofloxacin group (15 mg/kg q12h) was 7/10. This was statistically equivalent to that in the gentamicin and ciprofloxacin-treated groups.

In the neutropenic mouse model, neutropenia had no effect on the efficacy of levofloxacin for PEP. In contrast, lower survival was seen with PEP using gentamicin in neutropenic animals relative to normal animals.

Culture results for all surviving animals at the end of the experiments were negative for *Y. pestis*.

4.2 Secondary Pharmacology

No additional secondary pharmacology data were submitted.

4.3 Safety Pharmacology

No safety pharmacology data were submitted.

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

1. Study no. FY08-150: A Pharmacokinetic study of intravenous infusion of levofloxacin in African Green Monkeys – Amended final report (6/8/11)

This study was conducted by [REDACTED] (b) (4) and was not GLP-compliant.

Three female African Green monkeys (AGM) were fitted with IV Broviac catheters in the femoral vein for blood withdrawal. The animals were trained to poles and collars, chairs and limb restraints. Levofloxacin (Levaquin® 5 mg/mL in 5% dextrose; Lot nos. 61-022-JT and 63-262-JT) infusions were made through a temporary catheter in the saphenous vein. The dose regimen was designed to approximate human pharmacokinetics and consisted of infusion of levofloxacin 8 mg/kg (1.6 mL/kg) over 30 min followed by a second infusion of 2 mg/kg (0.4 mL/kg) over 30 min approx 12 hours later.

Blood samples for pharmacokinetics were collected prior to dosing and at 5 min, 1, 3, 6, 9, and 12 hours post-infusion. Plasma was separated and stored at -80°C until analyzed using a validated HPLC/floiuorescence detection method.

No adverse clinical signs were reported. Baseline samples were analyzed, and levofloxacin levels were determined to be below the LLOQ (30 ng/mL). After the first infusion, mean plasma levels were 3173 ng/mL at 5 minutes and 88 ng/mL at 12 hours. After the second infusion, mean plasma levels were 710 ng/mL at 5 minutes and 55 ng/mL at 12 hours.

The figure and tables below, from the amended report, illustrate the time course of levofloxacin in the blood and the pharmacokinetic parameters after each of the two doses.

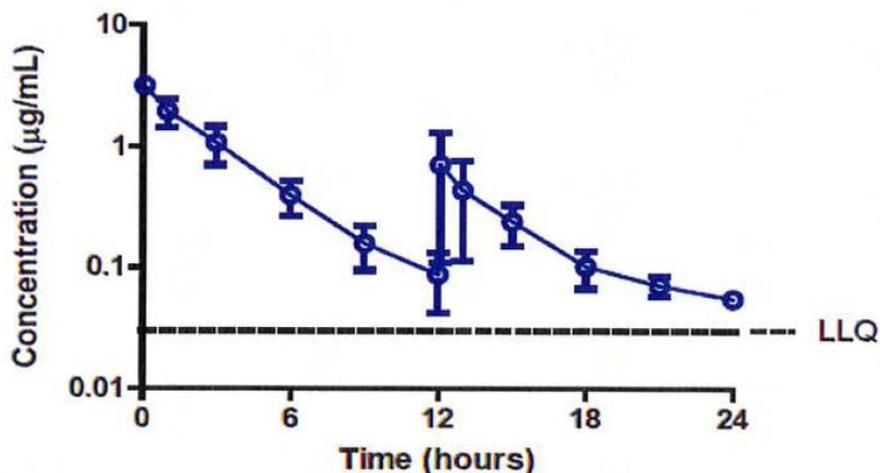


Figure 2 Mean ± SD plasma drug concentrations in AGMs given 8 mg/kg followed by 2 mg/kg

Table 1: Individual and mean ± SD pharmacokinetic parameters following a single 30 minute infusion of 8 mg/kg (revised)

<i>Animal ID</i>	<i>T_{1/2} (h)</i>	<i>T_{max} (h)</i>	<i>C_{max} (ng/mL)</i>	<i>V_{dss} (mL/kg)</i>	<i>Cl (mL/h/kg)</i>	<i>V_z (mL/kg)</i>	<i>AUC (ng/mL*h)</i>
W425	2.63	0.08	3178.20	2504.37	717.58	2719.23	11148.61
X440	2.52	0.10	2936.12	3059.53	1085.84	3940.67	7367.59
X510	2.81	0.08	3406.30	3004.58	992.77	4018.31	8058.25
N	3	3	3	3	3	3	3
Mean	2.649	0.089	3173.540	2856.156	932.062	3559.401	8858.151
SD	0.146	0.010	235.125	305.896	191.488	728.649	2013.432

Table 2: Individual and mean ± SD pharmacokinetic parameters following a single 30 minute infusion of 2 mg/kg at 12 hours (revised)

<i>Animal ID</i>	<i>T1/2 (h)</i>	<i>T_{max} (h)</i>	<i>C_{max} (ng/mL)</i>	<i>V_{dss} (mL/kg)</i>	<i>Cl (mL/h/kg)</i>	<i>V_z (mL/kg)</i>	<i>AUC (ng/mL*h)</i>
W425	NC	12.73	163.94	NC	NC	NC	NC
X440	6.21	13.15	1353.35	2595.96	463.72	4154.87	4312.98
X510	4.13	12.75	612.84	3834.90	700.79	4178.19	2853.93
N	2	3	3	2	2	2	2
Mean	5.172	12.877	710.043	3215.430	582.253	4166.526	3583.455
SD	NC	0.237	600.633	NC	NC	NC	NC

NC- not calculable from existing data

The report concluded that the plasma concentration did not exceed the predicted concentration in the AGM or the predicted concentration in man after a 500 mg IV dose. The estimated AUC₀₋₂₄ in AGM was 11.2 µg*hr/mL, or approx 23% of the reported AUC in man (48.3 µg/mL*hr after a 500 mg IV dose).

The following two of the three nonclinical PK studies in AGMs submitted were conducted in accordance with Good Laboratory Practice (GLP) guidelines.

2. Study title: A pharmacokinetic and toxicity study of levofloxacin following intravenous and oral (nasogastric) administration to African Green Monkeys

Study no.: (b) (4) study no. B122-03
 (b) (4) study no. 1599-20
 Study report location: Pharmacokinetics section of electronic submission
 Conducting laboratory and location: (b) (4)
 Date of study initiation: 6/25/2003
 GLP compliance: Yes
 QA statement: Yes
 Drug, lot #, and % purity: Levaquin® (levofloxacin) injection (25 mg/mL), lot nos. F1246 and E1211

Key Study Findings

Levofloxacin, given to African green monkeys as a single oral (nasogastric [NG]) dose or single or 14-day repeated IV infusions at doses up to 25 mg/kg PO or 20 mg/kg IV, was generally well tolerated. There were no levofloxacin-related changes in food consumption, body weight, hematology, coagulation or urinalysis parameters. Post-dose

observations of decreased activity in 2 of 6 monkeys, or brown urine and facial swelling (around the right eye and upper and lower eyelids) in a single monkey were consistent with anticipated effects from administration of levofloxacin.

Target plasma levels of levofloxacin were achieved after IV infusion but not following oral dose administration. Following repeated daily IV infusion dosing, plasma levels of levofloxacin increased in a dose-dependent fashion with extensive extravascular distribution and rapid clearance rates. Transient and mild levofloxacin-related increases in serum ALT, AST and/or LDH activities were suggestive of reversible hepatocellular injury. These changes coincided with dose-dependent extravascular accumulation of levofloxacin and decreased after 14 days of treatment, concurrent with the increased rate of levofloxacin clearance.

Methods

Doses: Phase I - Ascending single oral (via nasogastric tube) doses of 15, 20, or 25 mg/kg
Phase II - Single IV infusion of 15 mg/kg
Phase III - Repeated IV infusions of 20 mg/kg/day for 14 days

Frequency of dosing: Single doses of IV or oral test article, followed by IV infusions once daily for 14 days

Route of administration: IV infusion over 20 minutes, or PO via nasogastric tube

Dose volume: 6 mL/kg

Formulation/Vehicle: 0.9% sodium chloride for injection, USP

Species/Strain: African Green monkeys

Number/Sex/Group: A single group of 3/sex was used for each dosing regimen, with at least 2-week washout periods in between.

Age: Estimated 2.4-6.4 years

Weight: 3.2-6.4 kg

Satellite groups: None

Unique study design: See below

Deviation from study protocol: None noted that affected the outcome of the study. Phase II was intended to be an escalating dose phase, but the target C_{max} was achieved with the first dose, so higher doses were not attempted in that phase of the study.

The timetable of experimental procedures is outlined in the table below:

Group No.	No. of M/F ^a	Dose Volume (mL/kg)	Dose Day	Dose Level (mg/kg)	Dose Conc. (mg/mL)	Dose Day	Dose Level (mg/kg)	Dose Conc. (mg/mL)	Dose Day	Dose Level (mg/kg)	Dose Conc. (mg/mL)
Phase I (Escalating Single-Dose Nasogastric Administration)											
1	3/3	6	1	15	2.5	15	20	3.33	29	25	4.17
Phase II (Escalating Single-Dose 20-Minute Intravenous Infusion Administration)											
1	3/3	6	43	15	2.5	N/A	N/A ^b	N/A	N/A	N/A	N/A
Phase III (14-Day Repeat Dose 20-Minute Intravenous Infusion Toxicity Study)											
1	3/3	6	N/A	N/A	N/A	N/A	N/A	N/A	85-98	20	3.33

M = male; F = female; Conc. = concentration of levofloxacin; N/A = not applicable

^a The same monkeys were used for each phase of the study.

^b Intravenous infusion dose administration with 20 mg/kg was not performed because the targeted maximal levofloxacin concentration of 6.2-11 µg/mL was achieved with the 15-mg/kg dose.

Observations and Results

Mortality

None

Clinical Signs

Animals were monitored twice daily (AM and PM) plus continuously during infusion and at 1 and 2 hours post-dose on each dosing day.

In Phase I, after nasogastric dosing, decreased activity was noted at 1 hour post-dose in two males (Day 1, 15 mg/kg). Salivation was noted at 2 hours post-dose in one female monkey (Day 15, 20 mg/kg). During Phase II, one of the same males that had decreased activity on Day 1 (C23323M) exhibited brown urine at 2 hours post-dose (Day 43, 15 mg/kg). During Phase III, that same male had brown urine at 2 hours post-dose (Day 85, the first day of the 14 day treatment period, 20 mg/kg/day), swollen periorbital area around the right eye with clear discharge from the right eye (Day 87), slightly swollen upper and lower right eyelids (Day 88), and decreased activity at 2 hours post-dose (Day 98). In general, these were considered to be consistent with anticipated effects of administration of levofloxacin. Other findings were considered to be incidental.

An additional male (C23324M) had ulcerated areas on the head and neck and erythema on Day 21. These findings were considered to be possibly due to exposure to the disinfectant used to clean the room (TBQ). This animal did not receive the 25 mg/kg nasogastric dose. The animal was dosed again on Day 43 with a single IV infusion of 15 mg/kg. The disinfectant exposure may have had a confounding effect on clinical pathology parameters.

Body Weights

Body weights were monitored weekly. Food was withheld before weighing. The fluctuations observed were considered to be within normal limits (WNL). Slight weight loss was noted for the male with possible chemical burns.

Feed Consumption

Changes in food consumption were monitored daily. No levofloxacin-related changes were reported.

Ophthalmoscopy

Not performed

ECG

Not performed

Hematology

Blood was sampled from overnight-fasted animals pre-study and according to the following schedule for hematology, serum chemistry and coagulation parameters:

- Phase I - Escalating Nasogastric Dose: Days 5, 11, 19, 25, 33 and 39 (4 and 10 days after each dose administration)
- Phase II - Escalating Intravenous Infusion Dose: Days 47, 53 (4 and 10 days after dose administration) and 67
- Phase III - 14-Day Repeat-Dose IV infusion: prior to initiation of dosing (Day 85), before infusion on Days 86, 89, 91 and 95 (1, 4, 6, and 10 days after the first dose) and the day after the last dose (Day 99; approximately 24 hours after the completion of the last infusion).

No levofloxacin-related changes in hematology or coagulation were reported. Increased absolute neutrophil counts, band neutrophil counts (Day 19), and fibrinogen values (Days 19 and 25) in male C23324M were considered to be secondary to TBQ exposure.

Clinical Chemistry

In Phase I, animals began the single ascending dose phase on Day 1. Two-fold increases in AST were seen in three animals on Day 5, four days after a dose of 15 mg/kg. AST and LDH were increased by 1.1- to 4-fold. After doses of 20 and 25 mg/kg, the report indicates that there was not convincing evidence of hepatotoxicity. The exception was the animal with possibly confounding chemical burns, which exhibited the most dramatic hepatic enzyme increases seen in this phase.

In Phase II, animals were dosed on Day 43. Three animals exhibited approximately 2-3-fold increases in ALT on Day 47 (4 days after a single intravenous dose of 15 mg/kg). Two of those animals also exhibited increases in Phase 1. Concurrent increases in AST and LDH activity ranged from approximately 1.5- to 6-fold. On Day 53 (10 days after dosing) two of those animals continued to exhibit somewhat increased ALT (approximately 2-fold), while AST and LDH had decreased nearly to

baseline. These increases, followed by return toward baseline are suggestive of levofloxacin-related hepatocellular injury, followed by recovery.

In Phase III, where animals were dosed daily on Days 85-98, all six animals exhibited an approximate 2-15-fold increase in ALT on most of Days 86 through 95. The most pronounced increases were on Day 89 and declined through Day 95 and were accompanied by increased AST and LDH activities. Again, these rises in serum enzyme activity during the early part of the 14-day dosing period, following by declining enzyme activity during the later part of the dosing period, were considered to be strongly suggestive of transient levofloxacin-related hepatocellular injury.

Urinalysis

Samples (5 mL) were collected in a cage pan on Day 99 (the day following the last IV infusion at 20 mg/kg) over up to 5 hours. No levofloxacin-related findings were reported.

Gross Pathology / Organ Weights / Histopathology

Not performed. Animals were transferred back to the (b) (4) colony at the end of the study for use in future studies.

Toxicokinetics

Blood was collected from a peripheral vein at the following timepoints:

Phase I - Escalating Nasogastric Dose: on Days 1, 15 and 29; predose, at 30 and 60 minutes postdose, and at 2, 5, 8, 12 and 24 hours postdose

Phase II - Escalating Intravenous Infusion Dose: on Day 43; predose and at the following timepoints after start of infusion: 5, 10, and 20 minutes (at end of infusion) and at 35, 50, 80, 140, 320, 500, and 740 minutes

Phase III - 14-Day Repeat-Dose IV infusion: on Days 85 and 98; predose and at the following timepoints after start of infusion: 5, 10, and 20 minutes (at end of infusion) and at 35, 50, 80, 140, 320, 500, and 740 minutes.

Plasma was collected from the samples and stored at -70°C until analysis by HPLC/fluorescence detection.

Levofloxacin was absorbed after an oral dose of 15, 20 or 25 mg/kg. The target C_{max} of 6.2-11 µg/mL was not achieved after oral administration. AUC increased in proportion with dose, but C_{max} did not. Bioavailability ranged from 74-80% at the doses examined. After IV infusion, the target C_{max} was achieved, and both plasma concentration and AUC appeared to be dose-proportional. Repeated IV dosing resulted in lower C_{max} and AUC values toward the end of the dosing period, as well as a shorter half-life. Half life after a single dose was slightly longer after oral administration than after IV administration, although plasma clearance was considered to be rapid for both routes. The volume of distribution was large after administration by both routes, indicating extensive distribution. Clearance increased after 14 days repeated dosing. No gender differences were noted.

Pharmacokinetic parameters are shown in the table below:

Dose (mg/kg)	C _{max} (µg/mL)	T _{max} (hr)	AUC _{0-∞} (µg*hr/mL)	T _{1/2} (hr)	V _z (mL/kg)	Cl _s (mL/hr/kg)	F
Phase I Oral							
15	5.00	1.5	24.16	4.74	3317.48	504.32	0.8
20	5.22	1.5	32.56	5.77	4084.37	503.54	0.8
25	5.82	2.6	36.01	4.24	3145.57	528.38	0.74
Phase II IV infusion							
15	13.36	0.33	30.55	3.30	2334.88	503.89	---
Phase III IV infusion							
20 (1 st day)	16.92	0.30	45.35	3.21	2570.44	566.99	---
20 (14 th day)	11.83	0.25	30.44	2.52	3036.95	836.34	---

Dosing Solution Analysis

The report states that all dose formulations had concentrations within 10% of the intended concentrations. The relative standard deviation in homogeneity analyses was ≤ 2%, within the acceptance criterion of ≤ 5%. Dosing solutions were considered to be stable for up to 21 days when stored refrigerated or at room temperature.

The application states that the bioanalytical methods used for the non-GLP PK study no. FY08-150 and the GLP efficacy study in AGMs were not completely in compliance with the FDA Guidance on Bioanalytical Method Validation (2001), and generally accepted bioanalytical practices. An additional GLP-compliant PK study, B465-10, reviewed below, was conducted in AGMs that was said to have followed FDA's guidance and to have adhered to generally accepted practices. This confirmatory GLP PK study was said to use a similar high-performance liquid chromatography with fluorescence detection (HPLC/FLD) method as the initial non-GLP PK study (FY08-150).

3. ^{(b) (4)} Study no. B465-10: A Pharmacokinetic study of intravenous infusion of levofloxacin in African Green Monkeys

This study was conducted by ^{(b) (4)} to confirm the exposure to levofloxacin (Levaquin 5 mg/mL in 5% dextrose lot no. 94-083-JT) in AGMs using a humanized dose regimen and to demonstrate that the exposure in AGM is lower than that in humans after a 500 mg IV dose of levofloxacin.

AGMs (3/sex) with venous access ports (VAPs) in the left saphenous vein for blood withdrawal and trained to poles and collars, chairs, and limb restraints were used

for the study. In each individual animal, an infusion of test article (levofloxacin, 8 mg/kg [high dose]) was administered over 30 ± 5 minutes beginning at T=0, followed by a second infusion of levofloxacin (2 mg/kg [low dose]) administered over 30 ± 5 minutes beginning at T=12 hours after the high dose infusion, using a separate vein from that used for taking samples. Blood samples (1.5 ml per sample) for levofloxacin plasma concentration analyses were collected prior to dosing and at 5 minutes, 1, 3, 6, and 9 hours after completion of the 8 mg/kg infusion and prior to the start of the 2 mg/kg infusion, 5 minutes after completion of the infusion, 1, 3, 6, 9, and 12 hours after the 2 mg/kg dose. Plasma was separated and frozen at -80°C until analysis by HPLC / fluorescence detection. The data were used to generate pharmacokinetic parameters using noncompartmental analysis. Additional blood samples were obtained at 1 and 13.5 hours post-first dose for evaluation of plasma protein binding. Urine was collected in cage pans pre-dose, over 0-8 hours post-dose, and over 8-48 hours post-dose for evaluation of urinary excretion of the parent drug.

Peak levofloxacin plasma levels after the first infusion (0.083 hr post dose) were 3.25 ± 0.36 and 3.34 ± 0.21 $\mu\text{g/ml}$ for males and females, respectively. There was log-linear decay of plasma levels after each infusion and males generally had higher levels than females. After the second infusion, peak plasma levels at 0.083 hr post dose were 0.90 ± 0.22 and 0.72 ± 0.10 $\mu\text{g/ml}$ for males and females, respectively. Levofloxacin levels declined rapidly after the second infusion, and the decline continued up to ~ 9 hr after the second infusion (~ 21.5 hr elapsed) for females and up to 12 hr after the second infusion (~ 24.5 hr elapsed) for males.

The time course of levofloxacin plasma concentrations are shown in the Sponsor's figure below:

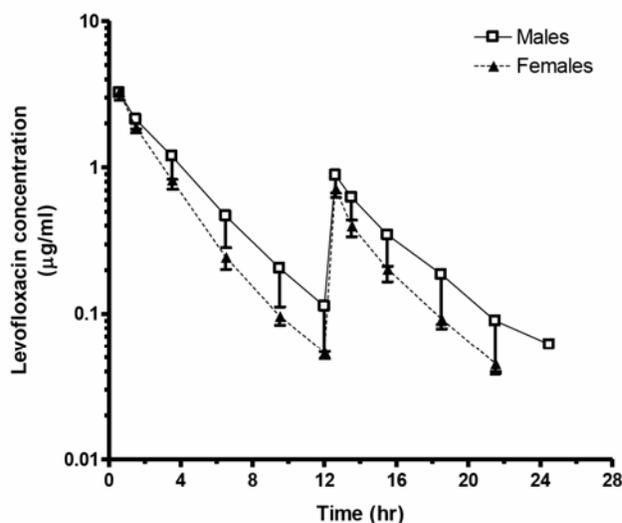


Figure 1. Levofloxacin plasma concentration-versus-time profile.

Male and female African Green monkeys were administered 8 mg/kg of levofloxacin by intravenous infusion followed by a 2 mg/kg infusion 12 hr later. Each point represents the mean plasma levels (\pm SD) of three animals, except for the final time point for males (n=1). The collection times displayed were nominal times adjusted for the 0.5 hr infusion time.

Systemic exposure was determined for the time intervals after each infusion, for the entire study period, and extrapolated to infinity. The majority of systemic exposure occurred between 0 and 12 hours after the first infusion. AUC values and other pharmacokinetic parameters are shown in the Sponsor's table below:

Sex		Pharmacokinetic Parameter Values (Mean \pm SD) of Levofloxacin															
		1st infusion (8 mg/kg)						2nd infusion (2 mg/kg)						AUC ₀₋₂₄		AUC _{0-inf}	MRT _{last}
		C _{max}	T _{max} ^a	AUC ₀₋₁₂	t _{1/2}	V	Cl	C _{max}	T _{max} ^a	AUC ₁₂₋₂₄	t _{1/2}	V	Cl	(hr· μ g/ml)	(hr· μ g/ml)	(hr)	
		(μ g/ml)	(hr)	(hr· μ g/ml)	(hr)	(L/kg)	(ml/hr/kg)	(μ g/ml)	(hr)	(hr· μ g/ml)	(hr)	(L/kg)	(ml/hr/kg)	(ml/hr/kg)	(hr· μ g/ml)	(hr· μ g/ml)	(hr)
M	Mean	3.25	0.60	10.30	2.36	1.98	602.15	0.90	12.8	3.53	2.81	2.50	661.62	13.83	14.05	6.00	
	SD	0.36	0.01	2.33	0.44	0.30	179.28	0.22	0.1	1.33	0.61	0.71	340.76	3.61	3.70	0.95	
F	Mean	3.34	0.60	7.95	2.01	2.30	793.83	0.72	12.6	2.04	2.57	3.60	964.32	9.96	10.13	4.90	
	SD	0.21	0.01	0.69	0.01	0.11	37.93	0.10	0.1	0.32	0.17	0.69	130.89	0.48	0.50	0.40	
All animal mean		3.30	0.60	9.13	2.19	2.14	697.99	0.81	12.7	2.79	2.69	3.05	812.97	11.90	12.09	5.45	
All animal SD		0.27	0.01	2.01	0.34	0.27	156.38	0.18	0.1	1.19	0.42	0.86	284.23	3.13	3.19	0.88	

^a Actual times are based on the start of the first infusion, which lasted 0.5 hr.

Volume of distribution was ~2-2.3 L/kg, indicating appreciable tissue distribution. Mean clearance was ~600-800 ml/hr/kg, and the half life was relatively short, averaging 2-3 hours. Males had a slightly lower clearance and slightly longer half life than females. Males consistently showed a trend for higher exposure than females for each time interval. Although the data analysis indicated possible gender differences in levofloxacin PK parameters following IV infusion, statistical significance was not achieved. The report states that levofloxacin exposure in male and female African Green Monkeys after two IV infusions was lower in this study than that reported in humans following IV administration of 500 mg.

Significant urinary output of levofloxacin occurred up to 8 hr post dose with concentrations higher than those in plasma (345 \pm 187 and 415 \pm 14 μ g/ml for males and females, respectively). Mean levofloxacin concentrations in urine were 48.6 \pm 35.4 and 8.5 \pm 4.2 μ g/ml from 8 to 48 hours post-dose for males and females, respectively. The percent of total administered dose recovered in the urine up to 48 hours was 29.3 \pm 15% and 26.5 \pm 1.4% for males and females, respectively.

Males showed unbound concentrations of levofloxacin in plasma of 1.49 \pm 0.21 and 0.450 \pm 0.126 μ g/ml at 1.5 and 13.5 hr, respectively. Unbound concentrations of levofloxacin in plasma for females averaged 1.52 \pm 0.12 and 0.317 \pm 0.067 μ g/ml at 1.5 and 13.5 hr, respectively. The mean fraction of levofloxacin not bound to plasma proteins was 0.70 \pm 0.01 and 0.72 \pm 0.04 for males at 1.5 and 13.5 hr, respectively, and 0.81 \pm 0.05 and 0.78 \pm 0.06 for females at 1.5 and 13.5 hr, respectively. The overall mean values for fraction unbound for the entire group at both timepoints was 0.75, leaving approximately 25% bound to plasma proteins. The fraction of drug unbound to plasma proteins ranged from 0.68-0.85 for all animals and there was a trend towards higher protein binding in females.

6 General Toxicology

No new general toxicology data were submitted.

7 Genetic Toxicology

No new genetic toxicology data were submitted.

8 Carcinogenicity

No new carcinogenicity data were submitted.

9 Reproductive and Developmental Toxicology

No reproductive and developmental toxicology data were submitted.

10 Special Toxicology Studies

No special toxicology data were submitted.

11 Integrated Summary and Safety Evaluation

The proposed clinical dose and dose regimen are consistent with the current approved labeling for Levaquin®. In the efficacy study in the AGM model of pneumonic plague, treatment resulted in a statistically significant increase in survival and findings consistent with resolution of disease.

The Sponsor provided the following table comparing the clinical dose with the humanized dose regimen used in the AGM efficacy study:

Table 2: Comparison of Levofloxacin Pharmacokinetic Parameters in Human and African Green Monkey

Species	I.V. Dose	AUC/MIC ₉₀ Ratio ^a	% Human		AUC _{0-24 h} (µg·h/mL)	% Human AUC (500 mg)	Trough Conc. (µg/mL)
			C _{max} (µg/mL)	C _{max} (500 mg)			
Human Volunteer ⁴	500 mg i.v. QD Single	402	6.2	NA	48.3	NA	0.5
Human Volunteer ⁴	500 mg i.v. QD Multiple	455	6.4	NA	54.6	NA	0.6
African Green Monkey (Mod4.2.2.2\B465-10)	8/2 mg/kg i.v. ^b Single	53	3.3	53	11.9	25%	<0.03-0.06 ^c

^a MIC₉₀ value used in calculations is 0.12 µg/mL. MIC₉₀ values determined for levofloxacin against sets of *Y. pestis* isolates (n = 12 to 100) range from <0.03 to 0.012 µg/mL and are listed in the Pharmacology Written Summary, Mod2.6.2\Table 1.

^b First dose 8 mg/kg, followed by a second dose of 2 mg/kg 12 h later.

^c Trough concentration in females and males.

AUC = area under the curve; NA = Not applicable; QD = once daily

12 Appendix/Attachments

None

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

AMY C NOSTRANDT
04/13/2012

WENDELYN J SCHMIDT
04/13/2012

I concur with Dr. Nostrandt's conclusions on the adequacy and interpretation of the pharmacology/toxicology data.

**Division of Anti-Infective Drug Products (HFD-520)
Pharmacology/Toxicology Forward Planning Meeting**

NDA Number: 20-634 (S-061) (oral tablet) **Date:** 11/28/2011
20-635 (S-067) (injectable)
21-721 (S-028) (oral solution)

Drug Name: Levaquin

Reviewer: Amy Nostrandt

CAS Number: Not provided

Drug Type: New indication

Drug Class: Fluoroquinolone

Indication: Treatment of pneumonic plague

Route of Administration: Oral and IV

Date CDER Received: 10/28/2011

User Fee Date: 4/28/2011

Expected Date of Draft Review: 2/28/2011

Sponsor: Johnson & Johnson on behalf of Janssen Pharmaceuticals

Fileability:

On initial overview of the NDA application:

YES NO

(1) On its face, is the pharmacology/toxicology section of the NDA organized in a manner to allow substantive review to begin? X
Comments? Electronic submission

(2) Is the pharm/tox section of the NDA indexed and paginated in a manner to allow substantive review to begin? X
Comments?
There is no overall index or nonclinical index for electronic submissions.

(3) On its face, is the pharm/tox section of the NDA legible so that substantive review can begin? X
Comments?

(4) Are all required (*) and requested IND studies completed and submitted in this NDA (carcinogenicity, mutagenicity, teratogenicity*, effects on fertility*, juvenile studies, acute studies*, chronic studies*, maximum tolerated dosage determination, dermal irritancy, ocular irritancy, photocarcinogenicity, animal pharmacokinetic studies, etc)?
Comments?
Not applicable. This supplement is for the addition of a new clinical indication. The proposed treatment duration should be supported by the existing toxicology data to support the approved product.

(5) If the formulation to be marketed is different from the formulation

- used in the toxicology studies, has the Sponsor made an appropriate effort to either repeat the studies using the to be marketed product or to explain why such repetition should not be required? _____
- Comments? _____
- Not applicable.
- (6) Are the proposed labeling sections relative to pharm/tox appropriate (including human dose multiples expressed in either mg/m² or comparative serum/plasma levels) and in accordance with 201.57? _____
- Comments? _____
- Not applicable. The only changes to the label will be related to the efficacy of the drug for the new indication.
- (7) Has the Sponsor submitted all special studies/data requested by the Division during pre-submission discussions with the Sponsor? _____
- Comments? _____
- Not applicable.
- (8) On its face, does the route of administration used in the animal studies appear to be the same as the intended human exposure route? If not, has the Sponsor submitted a rationale to justify the alternative route? _____ X
- Comments? _____
- A single efficacy study was performed in African Green monkeys under the Animal Rule using the intravenous route. Two of the applications supplemented here are for oral formulations. It is possible that referenced articles from the scientific literature may address the oral route of administration.
- (9) Has the Sponsor submitted a statement(s) that all of the pivotal pharm/tox studies have been performed in accordance with the GLP regulations (21 CFR 58) or an explanation for any significant deviations? _____ X
- Comments? _____
- Without an indexed section, it is difficult to find such a statement. There is a statement in the pharmacology summary that the nonclinical efficacy study was performed under GLP conditions. Toxicology data would have been addressed in the original applications.
- (10) Has the Sponsor submitted the data from the nonclinical carcinogenicity studies, in the STUDIES electronic format, for the review by Biometrics? _____
- Comments? _____
- Not applicable.
- (11) Has the Sponsor submitted a statement(s) that the pharm/tox studies

have been performed using acceptable, state-of-the-art protocols
which also reflect agency animal welfare concerns? _____ X
Comments?

Without an indexed section, it is difficult to find such a statement.
It is likely that this issue is addressed in one of the narrative overviews or
summaries. Toxicology data would have been addressed in the original
applications.

(12) From a pharmacology perspective, is this NDA fileable? If "no",
please state below why it is not. X _____

(13) If the NDA is fileable, are there any issues that need to be conveyed to
Sponsor? If so, specify: _____ X

(14) Issues that should not be conveyed to the Sponsor:
None at this time.

Reviewing Pharmacology Officer

Pharmacology Supervisor

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

AMY C NOSTRANDT
11/30/2011

WENDELYN J SCHMIDT
12/02/2011