3.G Microbiology Summary

Reference NDA Section 7

Note: NDA Application Summary Section 3.G is identical to NDA Application Summary Section 7.

ZITHROMAX (AZITHROMYCIN) MAC TREATMENT SNDA

SECTION 7 MICROBIOLOGY

TABLE OF CONTENTS

A. MICROBIOLOGY (*IN VITRO*, *IN VIVO* AND RESISTANCE)

The *in vitro* and *in vivo* microbiological activities of azithromycin against *Mycobacterium avium* complex (MAC) were recently summarized in the azithromycin prophylaxis application and published (1). Only new publications will be reviewed in this present document.

Macrolides, including azithromycin, have been shown to inhibit bacterial protein synthesis by binding tightly to the 50S ribosomal subunit. This tight binding to the ribosome has been demonstrated specifically for *M. avium* (2). *In vitro* susceptibility studies confirm the previously described *in vitro* and intracellular activity of azithromycin against *M. avium* (3,4). The postantibiotic effect (PAE) for azithromycin against MAC strains grown for 2 h at 2 times their MIC for azithromycin was shown to range from 2.9-5.5 h, depending on the method employed (5). Additional research confirmed that laboratory and clinical resistance to both azithromycin and clarithromycin occurs in MAC isolates through a single step mutation of adenine 2058 or 2059 in the single 23S ribosomal RNA (*Escherichia coli* numbering) with a frequency of 10^{-8}-10^{-10} (6). The efficacy of azithromycin in mouse infection models of *M. avium* complex has been reviewed previously (1). In a study designed to compare the frequency of recovering resistant isolates from animals treated with clarithromycin or azithromycin (200 mg/kg/day for both drugs) in the *M. avium* mouse infection model, there was significantly less resistance emergence in azithromycin vs. clarithromycin treated mice at 8 and 12 weeks of therapy (11). The authors speculated that prolonged high tissue levels of azithromycin hinder the growth of resistant subpopulations.

The rationale for using a macrolide plus ethambutol for *M. avium* complex chemotherapy is based on probable decreased resistance emergence to the macrolide and was recently reviewed (7). However, there is little preclinical data on the subject. In one *in vitro* study, a combination of 4 μg/ml ethambutol plus 23 μg/ml of azithromycin or 2.7 μg/ml of clarithromycin seem to have enhanced activity (8). Unfortunately, the proper controls were not tested (azithromycin at 23 μg/ml and clarithromycin of 2.7 μg/ml as single agents), rendering the data uninterpretable. Another *in vitro* study (9) reported enhancement of activity with a combination of ethambutol plus clarithromycin but not with ethambutol plus azithromycin. However, interpretation of these data is severely compromised because it is impossible to determine the concentration of macrolides evaluated. The best evidence was observed in a mouse infection model. In this experiment, combined treatment with clarithromycin plus ethambutol (200 + 100 mg/kg/day) resulted in a reduction in the frequency of isolation of clarithromycin resistant *M. avium* (MIC ≥32 μg/ml) compared with mice treated only with clarithromycin (10). Combined therapy seemed only to delay resistance emergence, as the isolation frequency of resistant strains after 8 and 12 weeks of therapy was greater than the isolation frequency of resistant strains from untreated animals. Combination therapy did not significantly improve the efficacy compared with clarithromycin monotherapy.

B. PHARMACOKINETICS.

The oral dose of azithromycin administered for MAC therapy (600 mg once a day) results in a mean Cmax of 0.55 μg/ml in serum and 252 μg/ml in leukocytes at day 22 in HIV-seropositive volunteers (Pharmacokinetic section of this Application). The Cmax in leukocytes in the 250 mg/day dosage regimen was 42.4 μg/ml. Serum concentrations increase relatively little between days 15 and 22 following 600 mg daily doses, suggesting that steady state concentrations were nearly achieved by day 15. Azithromycin concentrations in leukocytes also plateaued by day 15. The average concentration in

leukocytes at steady state (AUC0-24/24h) equals 198 μg/ml. The mean minimum concentration (C_0, equal to C_{24} concentrations) at steady state in leukocytes is 146 μg/ml 24 h post dose. The highest MIC_{90} values for azithromycin reported in the literature range from 5.3 to 128 μg/ml varying on test conditions (1,3). Thus, at steady state, the intracellular concentration of azithromycin exceeds the highest MIC values for this intracellular pathogen for the full dosing period. Because of this, a proposed susceptibility breakpoint of ≥256 μg/ml appears reasonable (12).

C. MICROBIOLOGICAL RESULTS FROM CLINICAL STUDY

Methods

A. MIC Values. MICs were determined for Protocols 148, 148B, 162, 162X and 189, in the laboratory of Dr. Lowell Young at the Kuzell Institute, San Francisco by the procedure given in Section 7, Appendix I. Differences between ^{99}MIC and $^{99.9}$MIC values were minimal and did not result in different susceptibility interpretations (e.g. Study Report 066-189 - Appendix V Table, 26, Clinical Section). Thus, only ^{99}MIC values are given in the Tables of data for this Section and are referred to as MIC.

MIC values were obtained on all positive cultures at the beginning of treatment and any breakthrough cultures, defined as those cultures that went from sterile to positive . Susceptibility determinations were also done if there was a progressive rise in cfu (2-fold increase or minimum increase of 10 cfu/ml).

B. Colony forming unit (cfu) determination. This was also done at the Kuzell Institute and the procedure is given in Appendix II of this Section. The limit of detection of the assay is approximately 7 cfu per 10 ml blood specimen (0.7 cfu/ml, rounded up to 1 cfu/ml, or <0.6 cfu/ml rounded down to 0 cfu/ml). The influence of peak serum concentrations of azithromycin and clarithromycin on recovery of viable *M. avium* cell from serum specimens was also performed in Dr. L. Young's laboratory (Appendix III).

Results

The $MIC_{50/90}$ for azithromycin against the baseline cultures from both the 600 mg and 250 mg azithromycin dosage arms is 16/32 μg/ml (Table 3.1). The $MIC_{50/90}$ for the 62 baseline isolates in Protocols 148,148B, 162 and 162X is 16/64 μg/ml (Tables 4 and 5). The clarithromycin $MIC_{50/90}$ for baseline isolates in the clarithromycin arm was 2/4 μg/ml (Table 3.2). Less than 2% of the strains were resistant to the macrolides at baseline in all protocols (Table 2, MIC >256 μg/ml azithromycin and >32 μg/ml clarithromycin; Table 4, MIC >128 μg/ml azithromycin). The overall susceptibility of the baseline strains in the 3 arms of Protocol 189 to both azithromycin and clarithromycin was very similar (Table 3.1, 3.2). In the 600 mg azithromycin dosage arm there were two baseline cultures with an azithromycin MIC of >32 - ≤64 μg/ml, zero with an azithromycin MIC of >64 - <128 μg/ml and one at >256 μg/ml (Table 1a). Although these patients were infected with strains with high azithromycin MIC values, all had a Positive Microbiological Response (PMR = 1 $\log_{10}$ reduction of cfu/ml or sterile blood culture). In the 250 mg azithromycin dosage arm there were two strains with MIC values of >32 - ≤64 μg/ml; one had a PMR (Table 1b). In the clarithromycin arm (dosage of 500 mg bid) there were zero strains with a MIC of >4 - ≤16 μg/ml to clarithromycin and two isolates with a MIC of >16 μg/ml; neither of these had a PMR (Table 1c). The overall PMR in the 600 mg and 250 mg azithromycin dosage arms was 81% and 77%, respectively, compared to 74% for the clarithromycin arm. The median

time to a PMR was equivalent in the azithromycin 600 mg and clarithromycin arm (24 and 23 days respectively), but was higher in the 250 mg azithromycin arm (41 days, Table 1a, 1b, 1c). The mean reduction in cfu over time occurred slightly sooner in the clarithromycin arm compared with the azithromycin 600 mg dose arm (Figure 1; in plotting Fig 1, 0 cfu/ml is defined as log = -1 and 1 cfu/ml is defined as log = 0).

The azithromycin arms of the study had a number of blood samples that yielded 1 cfu/ml during the 24 week therapy period (25 and 16 for the 600 mg and 250 mg dosage arms respectively, Appendix V Table 17.2, Clinical Section). The clarithromycin arm had only 15 single cfu/ml specimens through the 24-week end point. One cfu/ml specimens were observed during the first month of therapy at a rate of 32% and 13% for the 600 mg and 250 mg azithromycin dosage arms, respectively, compared with a rate of 40% in the clarithromycin arm.

Correlation of microbiological response and clinical response is tenuous because of the large number in the "Missing" column (Table 1a, 1b. 1c). An improved clinical response was observed in 71%, 91% and 74% of the patients in the three arms (azithromycin 600 mg, 250 mg and clarithromycin respectively).

All isolates were susceptible to azithromycin in the 600 mg or 250 mg dose arms through week 24 of therapy (Table 2). Resistance emerged in one isolate in the 250 mg azithromycin arm at week 38 (Study Report 066-189, Appendix V Table 17.2 and Table 26 and case report form, Subject ID). However, this patient was put on clarithromycin therapy at week 9 (0 cfu/ml in week 9 blood sample) and then was positive for *M. avium* at week 31 (110 cfu/ml). Thus, this patient relapsed and resistance developed during clarithromycin therapy. Two resistant isolates were obtained during primary clarithromycin therapy period at weeks 16 and 20 (Table 2). One became resistant after the 24 week time point at week 43 (Table 2). Only one of the isolates that emerged resistant had an initial MIC greater than its MIC_{50} for the MAC strains in this study (4 µg/ml clarithromycin, subject in Study Report 066-189, Appendix V Table 26). All resistant strains isolated in the study arms are resistant to both macrolides (Study Report 066-189, Appendix V, Table 26).

D. DISCUSSION

Data generated from *in vitro* experiments and animal model studies provide evidence for the microbiological efficacy of azithromycin and support the tentative high susceptibility breakpoint of ≥256 µg/ml for azithromycin. The clinical data provide support for efficacy of azithromycin (600 mg) and clarithromycin in reduction of *M. avium* cfu in the patients.

As observed in study 066-189, and consistent with findings from two MAC prophylaxis studies (1, 15, 16) and a MAC lung disease therapy study in HIV negative patients (14), and a murine treatment study (11), clarithromycin treatment appears to select for macrolide resistance at a greater frequency compared with azithromycin. Amsden (13) proposed that the higher sustained concentration of azithromycin achieved in monocytes/macrophages compared with clarithromycin leads to less resistance emergence during azithromycin treatment. The pharmacokinetic data presented in that paper regarding intracellular clarithromycin (500 mg bid dosage) concentrations certainly suggest that non-compliance in the clarithromycin dosage regimen for a couple of days will result in intracellular levels below the MIC. For azithromycin, the intracellular leukocyte concentration exceeds 100 µg/ml 2 days after a 600 mg dose at steady state and still exceeds the MIC_{90} 8 days post a 600 mg dose (Pharmacokinetic Section, day 22 data). Thus, non-compliance for

even a week after reaching steady state on azithromycin therapy would not allow for growth of MAC.

The preponderance of serum specimens with 1 cfu in the azithromycin arms compared to the clarithromycin arms in Protocol 189 is somewhat perplexing. The peak azithromycin serum level (0.6 μg/ml) is low in comparison to its MIC values for *M. avium*, and was shown not to affect *M. avium* viability in test serum samples over a 24 hour exposure, meant to replicate conditions involved in shipment and processing (Appendix III). The peak serum levels of clarithromycin (2-3 μg/ml) are above its MIC_{90} for *M. avium* isolates (Table 3.2, clarithromycin package insert and 13). But these high serum levels did not significantly affect viability of *M. avium* cells, even at low burdens of organisms, in the test serum samples (Appendix III). Clarithromycin reaches steady state concentration in white blood cells soon after initiation of therapy as compared to 15 days for azithromycin. This could explain some of the difference in rate of resolution of bacteremia, as presented in Figure 1. However, specimens with 1 cfu/ml are not inordinately concentrated in the azithromycin arm in the first month of therapy as one might expect if insufficient intracellular levels were the cause. A third possible explanation is that the very high concentrations of azithromycin in the leukocytes result in a "paradoxical effect". A phenomena of a reduced bactericidal effect at higher antibiotic concentrations (17). Experiments measuring the influence of high concentrations of azithromycin on the rate of bactericidal activity have not been reported. As discussed in the clinical section, there appear to be little, if any, clinical consequences of these bacteremias at densities $\leq$ 1 cfu/ml.

E. SUMMARY

The mode of action of the azalide, azithromycin, against *M. avium* complex is inhibition of protein synthesis by binding to the 50S ribosomal subunit; this is its mode of action against bacteria in general. Laboratory and clinical macrolide resistance in MAC arises through a single step mutation in adenine 2058 or 2059 of the 23S ribosomal RNA of the 50S subunit. A broad range of MIC_{90} values (5.3 - 128 μg/ml) has been reported for azithromycin against susceptible MAC. The method of MIC determination does influence the observed *in vitro* potency. An MIC_{90} of 32 μg/ml was obtained for the 163 baseline isolates in Protocol 189. Extracellular concentrations of azithromycin, as low as 3% of the *in vitro* MIC, have produced good intracellular killing of *M. avium* in macrophages. This activity correlates with the 100-fold concentration of azithromycin in macrophages reported in *ex vivo* studies. Azithromycin has also demonstrated good prophylactic and therapeutic activity in beige mouse and immunosuppressed rat *M. avium* infection models. Azithromycin was as efficacious as clarithromycin *in vivo* and showed less resistance emergence than did clarithromycin. The *in vivo* efficacy of azithromycin correlates with its high-sustained tissue concentrations, especially phagocyte intracellular concentrations measured *in vivo*. At steady state with the 600 mg/kg/day dose, leukocyte concentrations of azithromycin in HIV+ patients exceeded the highest azithromycin MIC values for *M. avium* for the full dosing period. In clinical study 066-189, azithromycin therapy was similar to clarithromycin in producing a reduction in cfu. Higher azithromycin MIC values for the infecting isolate did not correlate with decreased reduction in cfu in the azithromycin 600 mg arm. Emergence of resistance during therapy did not occur in the two-azithromycin arms, but was observed in the clarithromycin arm. This is consistent with previous laboratory, prophylaxis and therapy observations comparing resistance emergence to azithromycin and clarithromycin.

F. LITERATURE CITED.

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Table 1a
Azithromycin Minimum Inhibitory Concentration (MIC) at Baseline Vs. Investigator Defined Clinical Response and Time to Positive Microbiologic Response
ITT Subjects - For Subjects Treated with Azithromycin 600 mg
Azithromycin Protocol 189

		Positive Microbiological Response (PMR)		Investigator Clinical Response at Week 24		
MIC	N#	N+	Median Time to PMR	Improved*	Deterioration or No Improvement**	Missing
MIC <=4	6	4 (66.7%)	24.5	0 (0.0%)	0 (0.0%)	6
>4 MIC <=8	14	13 (92.9%)	22.0	7 (63.6%)	4 (36.4%)	3
>8 MIC <=16	24	19 (79.2%)	22.0	6 (85.7%)	1 (14.3%)	17
>16 MIC <=32	17	13 (76.5%)	26.0	7 (70.0%)	3 (30.0%)	7
>32 MIC <=64	2	2 (100.0%)	62.5	0 (0.0%)	0 (0.0%)	2
MIC > 256	1	1 (100.0%)	22.0	0 (0.0%)	0 (0.0%)	1
Missing	4	3 (75.0%)	41.0	2 (66.7%)	1 (33.3%)	1
Total	68	55 (80.9%)	24.0	22 (71.0%)	9 (29.0%)	37

Number of ITT subjects
+ Number of subjects having positive microbiological response
Note that post study cultures only affect week 24.
* Improved = Marked or moderate or mild improvement
** Deterioration or no improvement = Marked, moderate or mild deterioration or no change

Database Cutoff:
Source Data: Appendix III Tables 9.3, 9.5, 9.11. Date of Data Extraction: Date of Table Generation:

Table 1b
Azithromycin Minimum Inhibitory Concentration (MIC) at Baseline Vs. Investigator Defined Clinical Response and Time to Positive Microbiologic Response
ITT Subjects - For Subjects Treated with Azithromycin 250 mg
Azithromycin Protocol 189

		Positive Microbiological Response (PMR)		Investigator Clinical Response at Week 24		
MIC	N#	N+	Median Time to PMR	Improved*	Deterioration or No Improvement**	Missing
MIC <=4	1	1 (100.0%)	22.0	1 (100.0%)	0 (0.0%)	0
>4 MIC <=8	8	6 (75.0%)	24.5	3 (100.0%)	0 (0.0%)	5
>8 MIC <=16	18	17 (94.4%)	42.0	3 (75.0%)	1 (25.0%)	14
>16 MIC <=32	15	9 (60.0%)	43.0	3 (100.0%)	0 (0.0%)	12
>32 MIC <=64	2	1 (50.0%)	43.0	0 (0.0%)	0 (0.0%)	2
Missing	3	2 (66.7%)	21.5	0 (0.0%)	0 (0.0%)	3
Total	47	36 (76.6%)	41.0	10 (90.9%)	1 (9.1%)	36

Number of ITT subjects
+ Number of subjects having positive microbiological response
Note that post study cultures only affect week 24.
* Improved = Marked or moderate or mild improvement
** Deterioration or no improvement = Marked, moderate or mild deterioration or no change

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Table 1c
Clarithromycin Minimum Inhibitory Concentration (MIC) at Baseline Vs. Investigator Defined Clinical Response and Time to Positive Microbiologic Response
ITT Subjects - For Subjects Treated with Clarithromycin 500 mg
Azithromycin Protocol 189

		Positive Microbiological Response (PMR)		Investigator Clinical Response at Week 24		
MIC	N#	N+	Median Time to PMR	Improved*	Deterioration or No Improvement**	Missing
MIC <=1	26	21 (80.8%)	22.0	8 (72.7%)	3 (27.3%)	15
>1 MIC <=2	23	18 (78.3%)	26.0	7 (77.8%)	2 (22.2%)	14
>2 MIC <=4	4	2 (50.0%)	22.0	1 (50.0%)	1 (50.0%)	2
>16 MIC <=32	1	0 (0.0%)		0 (0.0%)	0 (0.0%)	1
MIC > 32	1	0 (0.0%)		0 (0.0%)	0 (0.0%)	1
Missing	2	1 (50.0%)	23.0	1 (100.0%)	0 (0.0%)	1
Total	57	42 (73.7%)	23.0	17 (73.9%)	6 (26.1%)	34

Number of ITT subjects
+ Number of subjects having positive microbiological response
Note that post study cultures only affect week 24.
* Improved = Marked or moderate or mild improvement
** Deterioration or no improvement = Marked, moderate or mild deterioration or no change

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Table 2
Pathogen (MAC) Resistance to Study Drug - Central Lab Data
All Isolates at Baseline and Break Through Isolates Post Baseline
Azithromycin Protocol 189

	Azithromycin 250 mg			Azithromycin 600 mg			Clarithromycin 500 mg		
	N	n	Observed Rate	N	n	Observed Rate	N	n	Observed Rate
Baseline	44	0	0.0%	64	1	1.6%	55	2	3.6%
Week 3	6	0		10	0		2	0	
Week 6	1	0		2	0		1	0	
Week 9	4	0		1	0		2	0	
Week 12	4	0		2	0		1	0	
Week 16	3	0		2	0		1	1	
Week 20	3	0		5	0		1	1	
* Week 24	1	0		2	0		1	0	
> Week 24	1	1		0	0		1	1	

* Primary timepoint

N=number of isolates at baseline or number of break through isolates post baseline
n=number of resistant isolates at baseline or number of resistant break through isolates post baseline
Resistance is defined as MIC >= 256 mcg/ml for azithromycin or >=16 mcg/ml for clarithromycin.
For post baseline, only the first break through isolate is used for a subject.
Study drug was administered only through week 24.

Database Cutoff:
Source Data: Appendix III Table 9.11. Date of Data Extraction: Date of Table Generation:

Table 3.1
Summary of MIC Results - Susceptibility to Azithromycin at Baseline
All Isolates
Azithromycin Protocol 189

	Minimal Inhibitory Concentration (MIC) (ug/ml)**									
	<=1	<=2	<=4	<=8	<=16	<=32	<=64	<=128	<=256	>256
Azithro 250mg	0(0.0%)	0(0.0%)	1(2.3%)	9(20.5%)	27(61.4%)	42(95.5%)	44(100.0%)	44(100.0%)	44(100.0%)	44(100.0%)
Azithro 600mg	0(0.0%)	0(0.0%)	6(9.4%)	20(31.3%)	44(68.8%)	61(95.3%)	63(98.4%)	63(98.4%)	63(98.4%)	64(100.0%)
Clarithro 500mg	0(0.0%)	0(0.0%)	3(5.5%)	21(38.2%)	36(65.5%)	48(87.3%)	53(96.4%)	54(98.2%)	54(98.2%)	55(100.0%)
All Subjects	0(0.0%)	0(0.0%)	10(6.1%)	50(30.7%)	107(65.6%)	151(92.6%)	160(98.2%)	161(98.8%)	161(98.8%)	163(100.0%)

** Note: Numbers are cumulative from left to right.

Database Cutoff:
Source Data: Appendix III Table 9.11. Date of Data Extraction: Date of Table Generation:

Table 3.2
Summary of MIC Results - Susceptibility to Clarithromycin at Baseline
All Isolates
Azithromycin Protocol 189

	Minimal Inhibitory Concentration (MIC) (ug/ml)**						
	<=1	<=2	<=4	<=8	<=16	<=32	>32
Azithro 250mg	21(47.7%)	44(100.0%)	44(100.0%)	44(100.0%)	44(100.0%)	44(100.0%)	44(100.0%)
Azithro 600mg	36(56.3%)	60(93.8%)	63(98.4%)	63(98.4%)	63(98.4%)	63(98.4%)	64(100.0%)
Clarithro 500mg	26(47.3%)	49(89.1%)	53(96.4%)	53(96.4%)	53(96.4%)	54(98.2%)	55(100.0%)
All Subjects	83(50.9%)	153(93.9%)	160(98.2%)	160(98.2%)	160(98.2%)	161(98.8%)	163(100.0%)

** Note: Numbers are cumulative from left to right.

Database Cutoff:
Source Data: Appendix III Table 9.11. Date of Data Extraction: Date of Table Generation:

Table 4
Summary of MIC Results - Susceptibility to Azithromycin at Baseline
All Isolates
Azithromycin Protocol 148

	Minimal Inhibitory Concentration (MIC) (ug/ml)**						
	<=4	<=8	<=16	<=32	<=64	<=128	>128
Azithromycin 600 mg	2(7.7%)	9(34.6%)	18(69.2%)	23(88.5%)	23(88.5%)	26(100.0%)	26(100.0%)
Azithromycin 12000mg	1(3.0%)	6(18.2%)	15(45.5%)	26(78.8%)	32(97.0%)	32(97.0%)	33(100.0%)
All Subjects	3(5.1%)	15(25.4%)	33(55.9%)	49(83.1%)	55(93.2%)	58(98.3%)	59(100.0%)

** Note: Numbers are cumulative from left to right.

Source Data: Appendix V Table 22. Date of Data Extraction: Date of Table Generation:

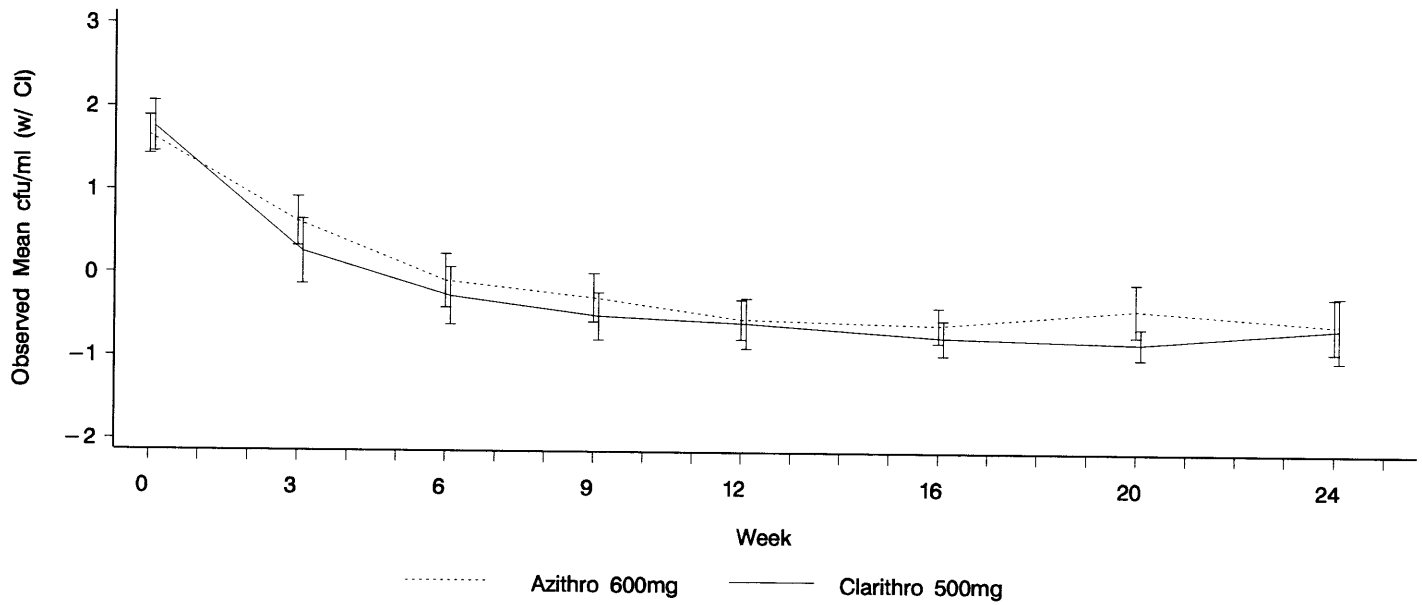
Table 5
Summary of MIC Results - Susceptibility to Azithromycin at Baseline
All Isolates
Azithromycin Protocols 162, 162X, and 148B

	Minimal Inhibitory Concentration (MIC) (ug/ml)**						
	<=4	<=8	<=16	<=32	<=64	<=128	>128
162/162X/148B	1(33.3%)	1(33.3%)	2(66.7%)	3(100.0%)	3(100.0%)	3(100.0%)	3(100.0%)
Oral Only	1(33.3%)	1(33.3%)	2(66.7%)	3(100.0%)	3(100.0%)	3(100.0%)	3(100.0%)
IV with or without Oral	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)

** Note: Numbers are cumulative from left to right.

Source Data: Appendix V Table 17.3. Date of Data Extraction: Date of Table Generation:

Figure 1
MAC Colony Count (cfu/ml, log base 10) – Observed Mean Over Time – ITT Subjects (Observed Data)
Azithromycin 600 mg versus Clarithromycin 500 mg
Azithromycin Protocol 189



No of Subjects at Week ==>	0	3	6	9	12	16	20	24
Azithro 600mg	65	63	51	47	45	40	39	32
Clarithro 500mg	57	43	41	38	38	31	32	24

log(0 cfu/ml) = −1 = No growth; log(1 cfu/ml) = 0)
Database Cutoff: 23Sep1998
Source Data: Table 5.3.1 Date of Data Extraction: Date of Figure Generation:

APPENDIX I SUSCEPTIBILITY TESTING

Mycobacteria colonies were harvested from the Middlebrook 7H11 plate, suspended in Hank's Balanced Salt Solution. Azithromycin was dissolved and diluted in a 50% ethanol solution to yield a stock solution and stored at -70° C. The concentrations (4 µg/ml to 32 µg/ml) of the drug were tested for each baseline *M. avium* isolate. If the isolate susceptibility was greater than 32 µg/ml, the assay was repeated using concentrations of azithromycin up to 256 µg/ml. Follow-up isolates were also tested at the higher range. Clarithromycin was dissolved and diluted in a 50% ethanol solution to yield a stock solution and stored at -70 ° C. The concentrations (1 µg/ml to 8 µg/ml) of the drug were tested for each baseline isolate. If the isolate susceptibility to the compound was greater than 8 µg/ml, the assay was repeated using concentrations up to 32 µg/ml. Follow-up isolates are tested at the higher range.

Bactec™ 12B vials (1 vial / concentration) with and without drug were inoculated with approximately 104 cfu/vial. The controls included: (i) no drug; (ii) inoculum diluted 1:100; and (iii) inoculum diluted 1:1000. The 1:100 control follows the convention of measuring the activity of an antimycobacterial agent against *M. tuberculosis* to a breakpoint of 99% inhibition. The 1:1000 control more closely follows the convention used with rapidly growing aerobic bacteria (starting with an inoculum of 104 cfu, the endpoint of agar dilution is the absence of growth which is interpreted here as < 10 cfu); 10 cfu is the limit of sensitivity for the Bactec™ method as described in this procedure. The vials were incubated at 37° C and read daily. Inderlied et al.* devised a method for analyzing the Bactec™ Growth Index (GI) reading which has advantages over the method of analysis described by Becton Dickinson Microbiology Systems, Sparks, MD. Cumulative daily GI values (ΣGI24hr+ ... GIthr) are plotted semi-logarithmically as a function of time and as such approximate a standard growth curve. A value, termed T100, is defined to facilitate the analysis of growth inhibition: the T100 is the time necessary for a culture to produce a ΣGI value of 100. Thus, the T100 value is a measure of both the lag time and the steady-state rate of 14CO2 production. The MIC of azithromycin was measured by interpolation from the dose (µg/ml)-response (T100) curve at the point of intersection with the T100 values for the controls (1:100 and 1:1000) expressed as the 99MIC or 99.9MIC, respectively. The advantage of this method is that the MIC is a discrete value (as opposed to a doubling dilution) and information about the mode of action or the mechanism of resistance may be deduced from the kinetics of inhibition and the nature of the dose-response curve.

*Inderlied CB, Young LS, Yamada JK. Determination of *in vitro* susceptibility of *Mycobacterium avium* complex isolates to antimycobacterial agents by various methods. Antimicrobial Agents and Chemotherapy 1987; 31:1697-1702.

Appendix II Isolation and Quantification of Mycobacteria from Blood Specimens.

1. Collection and Shipment of Blood Specimens.
2. Receiving and Testing of Blood Specimens.

M E M O

TO: All Investigators
Pfizer Protocol No. 066-189/189B

FROM: Terry Coghlan
Manager, Clinical Supplies
Pharmaco LSR

DATE: September 11, 1994

RE: Specimen Shipments To Kuzell Institute

Please find below specific instructions on preparation and shipping of specimens.

1) Draw specimen into 10 mL Isolator Tube;

2) Place Isolator Tube into 50 mL Falcon Tube;

3) Place absorbent squares into 50 mL Falcon Tube and close;

4) Complete required information on specimen label and affix to 50 mL Falcon Tube;

5) Place 50 mL Falcon Tube into metal canister and close;

6) Place metal canister into styrofoam box;

7) Insert styrofoam box into fiberboard box and close;

8) Place into fiberboard box into shipping box;

9) Place misc. shipping materials into shipping box to occupy unused/empty space and insert completed Laboratory Test Requisition form inside and tape closed;

10) Complete Federal Express Airbill and Shipper's Declaration For Dangerous Goods form (see highlighted attachments);

11) Affix airbill pouch to outside shipping box and insert forms mentioned in step 10;

12) Affix Shipping/Address Label, Infectious Substance Label and Infectious Substance Affecting Humans (HIV)/UN2814 Label, Danger Label and This Side Up Arrow Label to outside shipping container.

If you have any questions, please contact me or Ron Piper at (512) 448-8866. I have enclosed the following items:

Pre-printed Federal Express Airbills, Airbill Pouch, Pre-printed Shipper's Declaration For Dangerous Goods, Infectious Substance Label, Infectious Substance Affecting Humans (UN 2814), Large Plastic Falcon Tubes, Shipping Labels, Specimen Labels.

Please use these items for future shipments.

RECEIVING AND TESTING OF BLOOD SPECIMENS

I. Receiving Specimens

Upon arrival, all specimens (isolator tubes) should be checked for cracks and leakage. All information on the label is recorded on the log-in form.

II. Quantitative Culture

A. Centrifugation

Volume of blood in the Isolator tube (~9-10 ml) is measured by comparison with a standard graduated tube and recorded on the culture data sheet. The tube is centrifuged for 30 minutes at 4,000 rpm in a fixed angle centrifuge. Upon completion, the tube is checked for leakage and spilled blood is disinfected thoroughly with a 10% bleach solution.

B. Concentration

Isolator tube is placed in the cap-press device. Septum is swabbed with iodine solution and wiped off. A sterile cap is placed on the tube and pressed into place. The supernatant is drawn off with a supernatant pipet and discarded. The volume of the remaining concentrate (~2 ml) is measured by comparing with a standard graduated tube and recorded on the culture data sheet. Then tube is vortexed for 30 seconds to produce a uniform suspension.

C. Dilution

The concentrate is transferred from the Isolator tube to a sterile 13 X 100 mm screw-capped culture tube using a disposable transfer pipet. An aliquot of 0.1 ml of the concentrate is removed and added to 0.9 ml of sterile normal saline in a 12 X 75 ml culture tube, resulting in a 1:10 dilution. An aliquot (0.1 ml) of the 1:10 dilution is removed and diluted in sterile normal saline as above, resulting in a 1:100 dilution of the original concentrate.

D. Plating

An aliquot of 0.1 ml of the concentrate and each dilution is pipetted onto 3 Middlebrook 7H11 agar plates/concentrate or dilution and then spread evenly on the plates with a glass spreader. Plates are labeled with the patient initials, number, date and dilution plated.

E. Incubation

Plates are incubated at 37 degrees Celsius for up to four weeks and examined at least once per week for growth.

F. Counting and Calculations

Plates are counted manually using the dark-field plate viewer and the number of colonies is recorded on the culture data sheet. The CFU/ml in the patient sample is calculated as follows:

> #CFU/ml = mean # colonies/plate X concentrate volume/total volume X dilution factor X 10

Thus, using the above equation, 1 cfu on one of 3 plates spread with the concentrate equals 0.67 cfu/ml in the original blood specimen. This is rounded up to 1 cfu/ml. The presence of one or two colonies of possible contaminants such as coag neg staph diphtheriods and alpha-streptococci are noted but otherwise ignored. Any colonies of growth are sampled and stained with both Gram's and AFB stains. Pathogens or potential pathogens, other than AFB, are reported to the submitting center.

APPENDIX III RECOVERY OF *MYCOBACTERIUM AVIUM* FROM BLOOD SPECIMENS WITH AND WITHOUT AZITHROMYCIN CLARITHROMYCIN

Introduction:

Examination of the isolate data from the Pfizer Protocol #066-189/189B
A Randomized, Double Blind, Comparative Study of Azithromycin versus Clarithromycin Combination with Ethambutol for the Treatment of Disseminated *Mycobacterium avium* Complex (MAC) Infection in AIDS Patients, suggested that there was a higher occurrence of low isolate recovery in the clarithromycin versus the azithromycin group of the study. To determine if the peak serum level of Clarithromycin or Azithromycin could affect the recovery of MAC in the Isolator™ tube, a number of isolates from AIDS patients were inoculated into blood, which was processed for quantitative culture.

Method:

90 ml of blood from a healthy male volunteer was collected for each experiment. Aliquots were inoculated with MAC suspensions of different patient isolates, all with MIC<2 µg/ml of clarithromycin. One hour after blood infection, clarithromycin at a final concentration of 4 µg/ml or azithromycin at a concentration of 0.4 µg/ml or Hank's buffered salt solution (HBSS) were added to each sample. The blood was then placed in Isolator™ tubes and stored at room temperature in darkness for 24 hours. Blood was then processed for quantitative culture as per standard protocol, (see attached protocol) with only the undiluted sample plated.

Results:

In the first experiment, isolates from ALM and ORI were used. The levels of infection were 97 and 33 CFU/ml, respectively, as quantitated from the control (HBSS- containing) sample (Table 1). The second experiment used isolates from LAP and SDM (370 and 91 CFU/ml, respectively). Quantitative culture showed no reduction in the number of MAC recovered between samples containing azithromycin or clarithromycin, compared with control.

A further set of isolates (LAP, SDM and VB) were tested by the same method, though at a much lower concentration in the blood (5, 7 and 5 CFU/ml, respectively). Again, there was no difference beyond sampling error beween the azithromycin or clarithromycin-containing samples and control.

Isolation and Quantification of Mycobacteria

I. Receivingspecimens

Upon arrival, all specimens (isolator tubes) should be checked for cracks and leakage. All information on the label is recorded on the log-in form.

II. Quantitative Culture

A. Centrifugation

Volume of blood in the Isolator tube is measured by comparison with a standard graduated tube and recorded on the culture data sheet. The tube is centrifuged for 30 minutes at 4,000 rpm in a fixed angle centrifuge. Upon completion, the tube is checked for leakage and spilled blood is disinfected thoroughly with a 10% bleach solution.

B. Concentration

Isolator tube is placed in the cap-press device. Septum is swabbed with iodine solution and wiped off. A sterile cap is placed on the tube and pressed into place. The supernatant is drawn off with a supernatant pipet and discarded. The volume of the remaining concentrate is measured by comparing with a standard graduated tube and recorded on the culture data sheet. Then tube is vortexed for 30 seconds to produce a uniform suspension.

C. Dilution

The concentrate is transferred from the Isolator tube to a sterile 13 X 100 mm screw-capped culture tube using a disposable transfer pipet. An aliquot of 0.1 ml of the concentrate is removed and added to 0.9 ml of sterile normal saline in a 12 X 75 ml culture tube, resulting in a 1:10 dilution. An aliquot (0.1 ml) of the 1:10 dilution is removed and diluted in sterile normal saline as above, resulting in a 1:100 dilution of the original concentrate.

D. Plating

An aliquot of 0.1 ml of each dilution is pipetted onto each of 3 Middlebrook 7H11 agar plates and spread evenly on the plate with a glass spreader. Plates are labeled with the patient initials, number, date and dilution plated.

E. Incubation

Plates are incubated at 37 degrees Celsius for up to four weeks and examined at least once per week for growth.

F. Counting and Calculations

Plates are counted manually using the dark-field plate viewer and the number of colonies is recorded on the culture data sheet. The CFU/ml in the patient sample is calculated as follows:

#CFU/ml = mean # colonies/plate X concentrate volume/total volume X dilution factor X 10

The presence of one or two colonies of possible contaminants such as coag neg staph diphtheriods and alpha-streptococci are noted but otherwise ignored. Any colonies of growth are sampled and stained with both Gram's and AFB stains. Pathogens or potential pathogens, other than AFB, are reported to the submitting center.

Table 1.

Isolate		ALM	ORI	LAP	SDM	LAP	SDM	VB
	Plate#							
Control (HBSS)	1	52	18	200	40	4	4	2
cfu/plate	2	45	17	160	50	1	4	4
	3	48	14	195	46	4	3	3
	4					2	4	1
Calculated cfu/ml		**97**	**33**	**370**	**91**	**5**	**7**	**5**
Clarithromycin	1	62	18	200	60	2	3	0
4µg/ml	2	65	20	192	58	1	3	6
cfu/plate	3	55	15	190	55	2	5	4
	4					3	4	2
Calculated cfu/ml		**121**	**35**	**388**	**115**	**4**	**7**	**6**
Azithromycin	1	58	15	185	58	1	4	3
0.4µg/ml	2	56	15	200	48	1	4	6
cfu/plate	3	50	16	190	48	1	1	5
	4					2	1	2
Calculated cfu/ml		**109**	**31**	**383**	**103**	**3**	**5**	**8**

Conclusion:

Neither azithromycin nor clarithromycin at the concentrations tested have an effect on recovery of MAC isolates having low MIC's against the respective agents.