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BL-P1654: a Bacteriostatic Penicillin?

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In tube dilution studies, large discrepancies between inhibitory and bactericidal concentrations of BL-P1654 against *Pseudomonas* were observed. To explain these discrepancies which were not observed with carbenicillin, the kinetics of bacterial killing by these two penicillins were evaluated and compared. The kinetics of bacterial killing by both antimicrobial agents were characteristic of a penicillin, with killing initiating simultaneously with growth. Kill curves revealed the presence of a small number of cells resistant to BL-P1654 which were not detectable macroscopically. Studies on microbial resistance also showed the presence of a small but consistent number of cells resistant to BL-P1654 over a broad range of concentrations above its minimal inhibitory concentration. This pattern of resistance was not observed with carbenicillin. Thus, the discrepancies between inhibitory and bactericidal concentrations of BL-P1654 were not due to any unusual bacteriostatic activity but rather due to a small number of resistant cells whose presence could be detected only by sensitive subculturing techniques.

Several investigators have reported BL-P1654 (6-[D-alpha-(3-guanylureido)-phenylacetamido]-penicillanic acid) to be highly active in vitro against ampicillin-resistant, gram-negative bacilli including *Pseudomonas* (1, 3, 7, 10). A few have also reported discrepancies between inhibitory and bactericidal concentrations of BL-P1654 against several genera (3, 6, 9; C. C. Sanders and W. E. Sanders, Jr., Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 369, 1974). These discrepancies were not observed with carbenicillin. This study was designed to explain the discrepancies between inhibitory and bactericidal concentrations of BL-P1654 against *Pseudomonas*. To achieve this purpose, BL-P1654 and carbenicillin were evaluated and compared in regards to (i) frequency and size of discrepancies between inhibitory and bactericidal concentrations, (ii) kinetics of bacterial killing, and (iii) resistance of *Pseudomonas* to these two agents.

MATERIALS AND METHODS

Drug solutions. The drug powders used for preparation of stock solutions were BL-P1654 sodium (Bristol Laboratories) and disodium carbenicillin (Chas. Pfizer and Co., Inc.). Stock solutions of the drugs were prepared by dissolving the appropriate amount of powder (weight adjusted for impurities) in distilled water and sterilizing by microfiltration. No loss in activity was observed for stock solutions of the two penicillins after storage at 4 C overnight or at 25 C for 4 h. Therefore, drug solutions used in the in vitro assays were prepared either the evening preceding or the day of use.

Broth dilution susceptibility testing. All tube dilution tests in this study were performed in Mueller-Hinton Broth (MHB, Baltimore Biological Laboratories) in a volume of 3 ml per tube. Drug solutions of 800 μg/ml in MHB were serially twofold diluted to give final drug concentrations of 400 to 0.4 μg/ml in each test. Appropriate dilutions of an 18-h MHB culture were made to give a final bacterial test population in each tube of 1 to 5×10^4 colony-forming units per ml. Each test after inoculation was incubated at 37 C in air for 18 h. Minimal inhibitory concentrations (MICs) were defined as the lowest concentration of drug that prevented macroscopically visible growth after incubation for 18 h. An aliquot of 0.01 ml was removed from each clear tube and subcultured onto a sheep blood agar plate. Minimal bactericidal concentrations (MBCs) were defined as the lowest concentrations of drug which prevented growth on the blood agar plate subculture.

Kill curves. All experiments to determine the killing kinetics of BL-P1654 and carbenicillin were performed in MHB with incubation at 37 C in air. Inocula were prepared from 18-h MHB cultures. Aliquots were removed at various times intervals and viable numbers were determined by plate counts. In all experiments performed with carbenicillin and BL-P1654 (except at 25 μg/ml against *Pseudomonas*), the antibiotics were inactivated by penicillinase (10^6 U/ml, BBL) before viable counts were determined. In experiments performed with BL-P1654 (25 μg/ml) against *Pseudomonas*, viable counts were determinable down to a lower limit of 100 colony-forming units per ml without any drug inactivation. Control experi-

ments performed showed viable counts to be accurate when performed by these procedures. In all experiments, drug-free controls were inoculated and treated identically as the drug-containing tests. All results given represent averages of determinations performed in duplicate.

Rates of resistant colonies. Rates of resistant colonies were determined by a modification of a procedure described by Kunin et al. (4). One milliliter of a known bacterial population was placed into 9 ml of Mueller-Hinton agar containing various concentrations of antibiotic. After incubation, the number of colonies growing on each plate were counted. Results were expressed as the ratio of the number of colonies growing in the plates to the number of colonies originally placed in the agar. All results given represent averages of determinations performed in triplicate.

Bacterial isolates. All strains of *Pseudomonas* were clinical isolates from the Shands Teaching Hospital and Clinics, Gainesville, Fla., or the Creighton Memorial Saint Joseph's Hospital, Omaha, Neb.

RESULTS

Discrepancies between MICs and MBCs against Pseudomonas. MICs determined by tube dilution assays showed BL-P1654 to be much more active than carbenicillin against 28 strains of *Pseudomonas* (Fig. 1). However, when MBCs were compared, carbenicillin appeared more active than BL-P1654 against the majority of strains. For BL-P1654, greater than fourfold differences between MICs and MBCs were observed against 27 of 28 strains with the mean difference for all strains being 32-fold. For carbenicillin, greater than fourfold differences between MICs and MBCs were observed for only three strains, with the mean difference being twofold.

Killing kinetics of BL-P1654 and carbenicillin. The first set of kill curves were performed in 25 μg of BL-P1654 per ml with (i) a strain of *Pseudomonas* against which a large discrepancy between MIC and MBC (MIC, 0.78 μg/ml; MBC > 100 μg/ml) for BL-P1654 had been observed, and (ii) a strain of *Escherichia coli* against which no discrepancy between MIC and MBC (MIC, 0.78 μg/ml; MBC, 1.56 μg/ml) for BL-P1654 had been observed. The antibiotic was added at zero hours. Results are shown in Fig. 2. Killing was initiated simultaneously with growth for both strains. However, the rate of growth of the *Pseudomonas* was slower than that of the *E. coli*. Therefore, killing of the *Pseudomonas* was also slower and incomplete at 24 h. It was noticed in these experiments that a population of 10^7 colony-forming units of *Pseudomonas* per ml was not visibly turbid whereas *E. coli* of the same population size was turbid. Kill curves were performed in

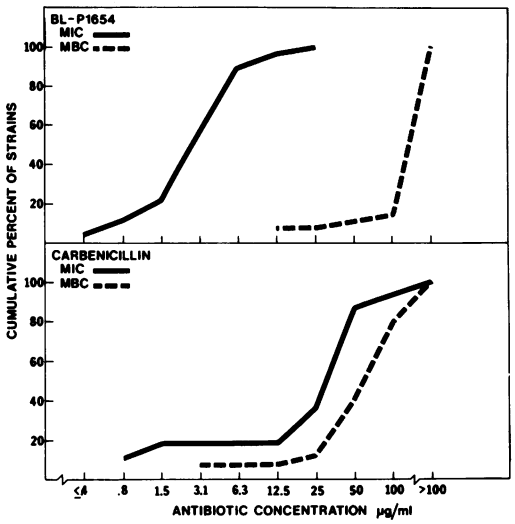

FIG. 1. *Comparative inhibitory and bactericidal concentrations of BL-P1654 and carbenicillin against 28 strains of Pseudomonas.*

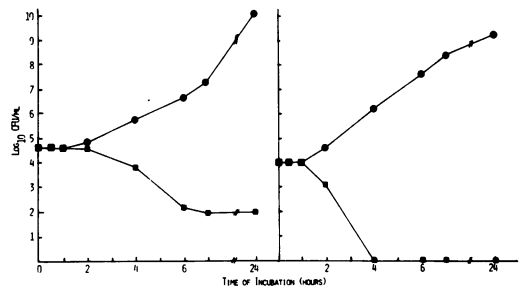

FIG. 2. *Kinetics of bacterial killing by 25 μg of BL-P1654 per ml against Pseudomonas (Lt) and Escherichia coli (Rt). Symbols: control ●; test, ■.*

100 μg of carbenicillin per ml against the same *Pseudomonas* used in the previous experiment. Results given in Fig. 3 show killing kinetics similar to those observed with BL-P1654 except for some regrowth between 7 and 24 h.

Attempts to increase the rate and completeness of killing of *Pseudomonas* by BL-P1654 were made by (i) shaking the culture during exposure to drug, (ii) increasing the drug concentration, and (iii) changing the time at which the culture was exposed to the drug. No increased rate of growth or killing was observed when cultures were shaken during exposure to 25 μg of BL-P1654 per ml (Fig. 4). Regrowth was observed in both cultures between 7 and 24 h. Increasing the concentration of BL-P1654 added to cultures at zero hours did not increase the rate of killing observed but did increase the completeness of killing as assayed at 24 h (Fig. 5). Changing the time at which 25 μg of

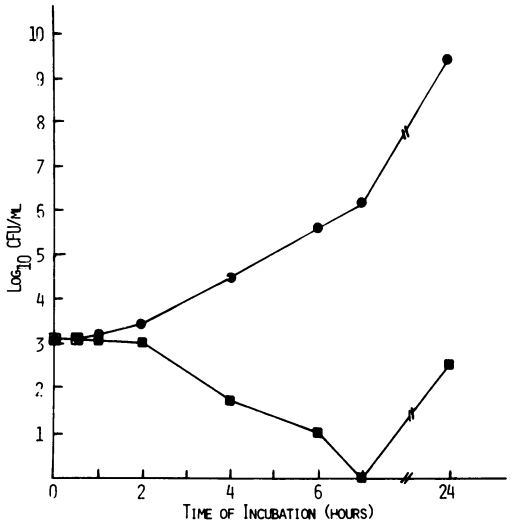

FIG. 3. *Kinetics of bacterial killing by 100 μg of carbenicillin per ml against Pseudomonas. Symbols: control, ●; test, ■.*

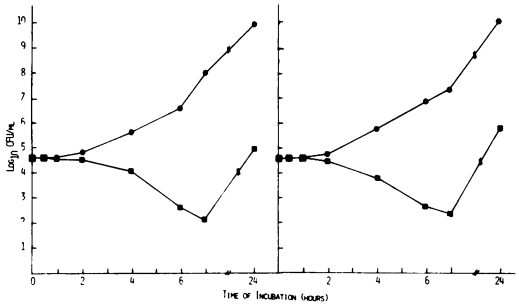

FIG. 4. *Kinetics of bacterial killing by 25 μg of BL-P1654 per ml against Pseudomonas. Cultures incubated still (Lt) and shaking (Rt). Symbols: control, ●; test, ■.*

BL-P1654 per ml was added to the cultures did not alter the rate of killing observed (Fig. 6).

The susceptibility to BL-P1654 of *Pseudomonas* recovered at 24 h in the experiments shown in Fig. 2 (survivors) and 4 (regrowth) was determined in kill curves and broth dilution susceptibility tests. Kill curves were performed as before with 25 μg of BL-P1654 per ml added at zero hours. BL-P1654 showed kinetics of killing against the surviving population similar to those observed previously (Fig. 7). However, BL-P1654 showed very little bactericidal activity against the regrowth population. MICs of both BL-P1654 and carbenicillin against these two population of cells were eightfold higher than that of the parent strain.

Rates of resistant colonies. The number of cells in a known population of *Pseudomonas* that were capable to growing in increasing concentrations of BL-P1654 and carbenicillin was determined. The rates of resistant colonies (calculated by dividing the number of colonies growing by the number of colonies in the

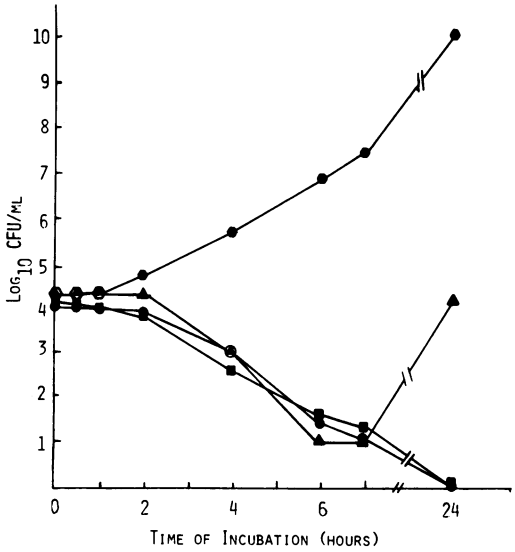

FIG. 5. *Effect of antibiotic concentration on killing kinetics of BL-P1654 against Pseudomonas. Symbols: control, ●; 25 μg/ml, ▲; 40 μg/ml, ●; and 80 μg/ml, ■.*

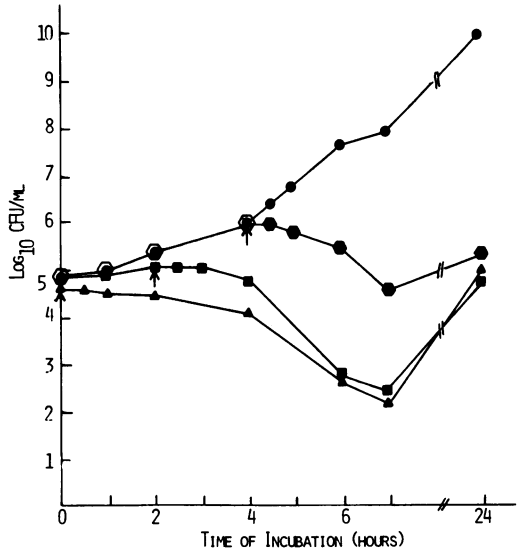

FIG. 6. *Effect of age of culture at time of exposure on killing kinetics of 25 μg of BL-P1654 per ml against Pseudomonas. Time of addition of drug indicated by arrows (↑). Symbols: drug added at zero hours, ▲; drug added at 2 h, ■; drug added at 4 h, ●; control, ●.*

original population) are shown in Table 1. These results indicated that there was a small number of cells capable of growing in very high concentrations of BL-P1654. In contrast, the rates observed for carbenicillin showed a large number of cells capable of growing in concentrations up to 40 μg/ml.

Studies on resistance of Pseudomonas to BL-P1654. A colony growing in each of the antibiotic plates in the previous experiment was subcultured to a blood agar plate, grown overnight in MHB, and used in broth dilution studies. MICs for BL-P1654 and carbenicillin against each of these populations were no different from those of the parent strain. Similar results were obtained when colonies recovered from each clear tube in broth dilution susceptibility tests on the parent strain were used as inocula. These results suggested that the populations recovered after 24 h from concentrations above the MIC of BL-P1654 or carbenicillin against the parent strain were not stable. After two subcultures on drug-free media, they showed the susceptibility characteristics of the parent population from which they had been selected.

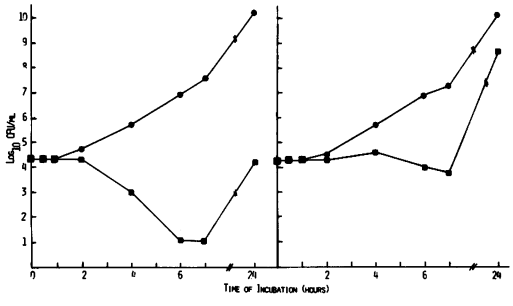

FIG. 7. *Kinetics of bacterial killing by 25 μg of BL-P1654 per ml against Pseudomonas previously exposed to drug for 24 h. Cells which survived previous exposure (Lt), cells which grew during previous exposure (Rt). Symbols: control, ●; test, ■.*

Attempts were made to stabilize these resistant populations by serial exposure of parent cells to concentrations above the MIC of BL-P1654 and carbenicillin in MHB. Daily transfers to fresh MHB containing the same concentration of drug were made. Populations subcultured from each transfer were subjected to broth dilution susceptibility tests. Results are shown in Table 2. Serial daily transfer of

TABLE 1. *Rates of resistant colonies of Pseudomonas to carbenicillin and BL-P1654*

Antibiotic concn (μg/ml)	Rate[a] of resistant colonies	
	BL-P1654	Carbenicillin
0.78	10^{0}	10^{0}
1.25	10^{-1}	10^{0}
2.5	10^{-4}	10^{0}
5.0	10^{-5}	10^{0}
10.0	10^{-6}	10^{0}
20.0	10^{-6}	10^{0}
25.0	10^{-6}	10^{0}
40.0	10^{-6}	10^{-4}
50.0	10^{-6}	10^{-5}
80.0	10^{-6}	10^{-6}
100.0	10^{-6}	10^{-6}
MIC	1.56 μg/ml	50 μg/ml
MBC	400 μg/ml	100 μg/ml

[a] Rate is number of resistant colonies/number in original population.

TABLE 2. *MIC of BL-P1654 and carbenicillin against Pseudomonas serially transferred in superinhibitory concentrations of antibiotic*

Concn (μg/ml) in transfer medium	No. of transfers									
	MIC BL-P1654[a]					MIC carbenicillin[b]				
	2	3	4	5	6	2	3	4	5	6
BL-P1654										
5	25	25	25	25	50	P[c]	P	P	P	P
10	50	25	25	50	50	P	P	P	P	P
20	50	25	25	50	50	P	P	P	P	P
40	25	100	50	200	200	P	P	P	P	P
50	50	100	50	100	100	P	P	P	P	P
Carbenicillin										
80	6.25	25	50	25	200	200	>400	>400	>400	>400
100	12.5	50	25	100	200	200	>400	>400	>400	>400
200	12.5	100	50	50	400	200	>400	>400	>400	>400

[a] MIC against parent = 3.12 μg/ml.
[b] MIC against parent = 50 μg/ml.
[c] Same as parent.

parent cells in concentrations from 5 to 50 μg of BL-P1654 per ml resulted in stabilization of resistant populations but the magnitude of the resistance usually did not increase with every transfer. No concomittant increase in resistance to carbenicillin was observed. Serial transfers of parent cells in carbenicillin also resulted in stabilization of resistant populations to both BL-P1654 and carbenicillin. The magnitude of the resistance usually increased with sequential transfers. The resistance to BL-P1654 and carbenicillin of populations recovered after six transfers in each concentration of each drug (column 6, Table 2) has been found to be stable after six successive transfers on drug-free media.

The killing kinetics of BL-P1654 and carbenicillin against the most resistant populations recovered in this experiment included parent cells, cells recovered after six transfers in 40 μg of BL-P1654 per ml, and cells recovered after six transfers in 200 μg carbenicillin per ml. MICs and MBCs of BL-P1654 and carbenicillin against these populations are given in Table 3. All kill curves were performed with a drug concentration of 400 μg/ml. This concentration was selected because it represented the MBC of BL-P1654 against each population.

The killing kinetics of 400 μg of BL-P1654 and carbenicillin per ml were similar against the parent cells. Both achieved complete killing by 24 h (Fig. 8). Similar kinetics of growth and killing were observed during the first 7 h with the cells recovered after six transfers in BL-P1654 (Fig. 9). However, regrowth in 400 μg of carbenicillin per ml occurred between 7 and 24 h. Cells recovered after six transfers in carbenicillin showed growth kinetics similar to parent cells, but were not affected at all by 400 μg of carbenicillin per ml (Fig. 9). BL-P1654 at 400 μg/ml appeared to be bacteriostatic during the first 7 h, after which regrowth occurred.

Cell-free filtrates were made at 24 h from each test and control for each population. Residual antibiotic activity was determined by a disk diffusion technique using a susceptible *E. coli* indicator strain and freshly prepared drug solutions for controls. No detectable loss in activity of either drug in any filtrate was observed. All activity of each filtrate was destroyed by incubation with penicillinase.

TABLE 3. *Susceptibility of Pseudomonas and resistant progeny to carbenicillin and BL-P1654*

Pseudomonas population	BL-P1654		Carbenicillin	
	MIC	MBC	MIC	MBC
Parent	3.12[a]	400	50	100
B6[b]	200	400	100	200
C6[c]	400	400	>400	>400

[a] All values in micrograms per milliliter.

[b] Recovered after six transfers in 40 μg of BL-P1654 per ml.

[c] Recovered after six transfers in 200 μg of carbenicillin per ml.

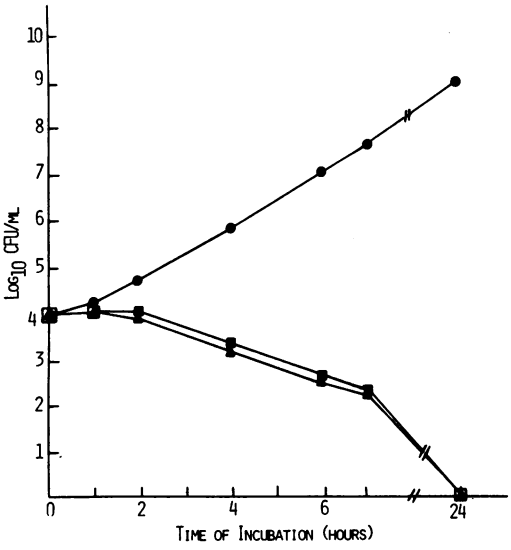

FIG. 8. *Kinetics of bacterial killing by 400 μg of BL-P1654 (■) and carbenicillin (▲) per ml against Pseudomonas parent strain. Control (●).*

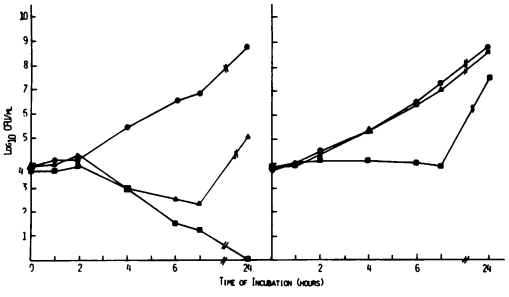

FIG. 9. *Kinetics of bacterial killing by 400 μg of BL-P1654 (■) and carbenicillin (▲) per ml against Pseudomonas selected for resistance by six transfers in 40 μg of BL-P1654 per ml (Lt) and 200 μg of carbenicillin per ml (Rt). Control (●).*

DISCUSSION

The results of this study indicated that the discrepancies between MICs and MBCs of BL-P1654 against *Pseudomonas* could not be explained by any unusual kinetics of bacterial killing. The kinetics of bacterial killing by BL-P1654 were those characteristic of a penicil-

lin, i.e., killing was initiated simultaneously with growth, and the rate of killing was dependent on the rate of bacterial growth not the drug concentration. Similar kinetics of killing were observed for carbenicillin which did not show such large and frequent discrepancies between MICs and MBCs against *Pseudomonas*.

The results of this study did indicate that the discrepancies between MICs and MBCs could be explained by the presence of cells resistant to BL-P1654 in numbers too small to be detected macroscopically but large enough to be detected by more sensitive subculturing procedures. The presence of these cells was suggested by the regrowth detected only by subculture during the kill curves and confirmed by the observation of very small but consistent rates of colonies resistant to BL-P1654 over a broad range of concentrations. Such small rates were not observed for carbenicillin over a similar range of concentrations.

The small number of resistant cells in a susceptible parent population could be selected for and stabilized by successive transfer in a single concentration of BL-P1654 above the MIC against the parent population. No concomitant increase in resistance to carbenicillin was noted. Similar procedures to select for cells resistant to carbenicillin resulted in stabilization of cells resistant to both carbenicillin and BL-P1654. These results appear to conflict with those of Price et al. which demonstrated that cells selected for resistance to BL-P1654 were also resistant to carbenicillin (7). However, it should be noted that the selection procedure used by those authors involved serial transfer in increasing concentrations of drug, not a single high concentration.

The data in this study do not indicate a precise mechanism for the resistance observed. However, extracellular destruction of the two penicillins was shown not to occur. These data indirectly support beta-lactamase production as a possible mechanism of resistance since gram-negative beta-lactamases are cell bound (2, 5) and Price et al. have shown that BL-P1654 is susceptible to hydrolysis by crude beta-lactamase preparations from *Pseudomonas* (7). Several results of this study suggest that this beta-lactamase, if present, may be inducible rather than constituitive. First, increasing the concentration of BL-P1654 used to select resistant cells did not significantly change the number of resistant cells nor the level of resistance observed. Second, a minimum of two serial exposures to a high drug concentration was required to stabilize the resistant cells. An inducible beta-lactamase from *Pseudomonas* has been reported by Sabath and co-workers (8). Further studies are needed to fully answer these questions.

The results of this study emphasize the necessity of performing broth dilution assays to determine both MICs and MBCs of any new antibiotic against a large variety of bacterial genera early in its evaluation. Any large discrepancies between MICs and MBCs of a bactericidal agent should be further evaluated by studies on killing kinetics and microbial resistance. Invalid conclusions concerning qualitative aspects of activity and predictions of clinical efficacy can occur if these studies are not performed.

ACKNOWLEDGMENTS

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