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In Vitro and In Vivo Activities of the Nitroimidazole CGI 17341 against *Mycobacterium tuberculosis*

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CGI 17341 (2-ethyl-5-nitro-2,3-dihydro[2-1b]imidazo-oxazole) is a novel orally active representative of the 5-nitroimidazole series of antimicrobial agents. At concentrations ranging from 0.1 to 0.3 μg/ml, CGI 17341 inhibited the drug-susceptible and multi-drug-resistant strains of *Mycobacterium tuberculosis*. CGI 17341 had no cross-resistance with isoniazid, rifampin, streptomycin, or ethambutol. While the in vitro activity of CGI 17341 against *M. tuberculosis* was comparable to those of isoniazid and rifampin, it was superior to those of streptomycin, ciprofloxacin or norfloxacin, and oxazolidinone DuP 721. The MIC of CGI 17341 was not affected when the pH of the medium was decreased from 6.8 to 5.6, while four- to sixfold increases in the MICs of ciprofloxacin and isoniazid were observed. In mice infected with *M. tuberculosis*, the 50% effective dose for CGI 17341 was 7.7 mg/kg of body weight (95% confidence limits, 3.5 and 10.27) when administered on days 11 and 12 postinfection. CGI 17341 gave a dose-dependent ($r = 0.995$) and significant increase in the survival time. Our data indicate that the 5-nitroimidazole CGI 17341 is a promising and novel antituberculosis compound with potent in vitro and in vivo activities. Further investigations on this compound are warranted.

Among infectious diseases, tuberculosis remains a leading cause of illness and death throughout the world despite significant advances in the chemotherapy of the disease in the past 40 years. Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* and is at risk of developing the active disease. It is estimated that 8 million new cases of tuberculosis and 2.9 million deaths from the disease occur each year (14). The trend of increasing incidence of infections due to drug-resistant strains of *M. tuberculosis* poses a serious hindrance to the control of tuberculosis worldwide (4, 7, 8, 18, 23). Therefore, new tuberculocidal drugs, especially ones with novel mechanisms of action, are urgently needed. Since the duration of the treatment of the disease is very long (8 to 9 months), new bactericidal drugs and their combinations are also needed for reducing the treatment period.

Our research has been directed towards the development of a new 5-nitroimidazole derivative as an antituberculosis agent. The 5-nitroimidazoles, such as metronidazole or tinidazole, are valuable therapeutic agents for treating infections due to several anaerobic bacteria and protozoa (5, 6). Because of the novel mechanism of action of these agents, the development of resistance in anaerobic bacteria, especially in the *Bacteroides fragilis* group, is rare (9, 11). In this report, we describe the in vitro and in vivo anti-*M. tuberculosis* activities of CGI 17341 (2-ethyl-5-nitro 2,3-dihydro[2-1b]imidazo-oxazole), a novel lipophilic and orally active representative of the 5-nitroimidazole series (Fig. 1).

MATERIALS AND METHODS

Bacteria and growth. *M. tuberculosis* H37Rv B-216, susceptible to all standard antituberculosis agents, was used. In cross-resistance studies, mutants of *M. tuberculosis* H37Rv resistant to isoniazid (INH), rifampin (RIF), streptomycin (SM), or ethambutol (EMB) were evaluated for their susceptibilities. Ten clinical isolates of *M. tuberculosis* susceptible to all drugs and 15 isolates resistant to INH and RIF were evaluated. In addition, three strains of *M. tuberculosis* simultaneously resistant to INH, RIF, and SM were also tested for their susceptibilities. *M. gordonae* TMC 1318, *M. fortuitum* TMC 1529, *M. avium* K-544 (a gift from F. Kuze from Kyoto University, Kyoto, Japan), *M. avium* ATCC 15769, *M. intracellulare* TMC 1403, *M. kansasii*, and *M. scrofulaceum* (the latter two organisms were gifts from W. Vischer, CIBA-GEIGY, Basel, Switzerland) were also tested. All clinical isolates of *M. tuberculosis* were obtained from Maharashtra State Tuberculosis Hospital, Bombay, India. The strains were identified as *M. tuberculosis* by the procedure described by Vestal (24). The susceptibilities of these strains to INH, RIF, SM, or EMB were determined according to the proportion method described by Vestal (24). All the organisms were grown on Lowenstein-Jensen medium and then subcultivated in Dubos broth containing 0.02% (vol/vol) Tween 80 and Dubos albumin enrichment mixture to obtain log-phase growth. Bacteria were declumped by light sonication. Aliquots of the culture were prepared in ampules and stored at −70°C until testing. CFU per milliliter of the stock solution were determined on Dubos agar.

The activity of CGI 17341 against *B. fragilis* ATCC 25285 was evaluated in Wilkins-Chalgren agar by the procedure described by Sutter et al. (22).

Media. Dubos broth medium (Difco Laboratories, Detroit, Mich.), pH 6.8, containing 0.02% Tween 80 and Dubos albumin enrichment (Difco Laboratories) was used. For evaluating the antituberculosis activity in acid medium (pH 5.6), a modified Kirchner broth, described previously by Ashtekar et al. (3), was used. The composition of this medium was as follows: solution A, 8.7 g of monopotassium phosphate–0.37 g of disodium phosphate–1,000 ml of dis-

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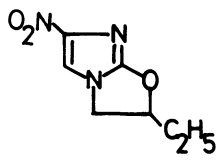

FIG. 1. Structure of CGI 17341 (2-ethyl-5-nitro-2,3-dihydro-[2-1b]imidazo-oxazole).

tilled water; solution B, 0.18 g of magnesium sulfate–0.75 g of sodium citrate–1.5 g of asparagine–6 ml of glycerol–0.015 g of ferric ammonium citrate. The ingredients of solution B were dissolved in 260 ml of solution A and sterilized by autoclaving at 121°C. The medium was cooled to 45°C and 40 ml of Dubos glucose albumin enrichment mixture was added.

Antimicrobial agents. CGI 17341 and DuP 721 were provided by the Chemistry Section of the Hindustan CIBA-GEIGY Pharmaceutical Research Center. The other antibacterial agents were obtained from the following sources: RIF, rifabutin (LM 427), and CGP 29861 were from CIBA-GEIGY (Basel, Switzerland); INH, SM, and EMB were from Sigma Chemical Company (St. Louis, Mo.); norfloxacin was from Merck Sharp & Dohme (West Point, Pa.); and ciprofloxacin was from Miles Inc. (West Haven, Conn.). For in vitro tests, the stock solutions of CGI 17341, DuP 721, RIF, LM 427, and CGP 29861 were made in dimethylformamide and SM and INH were dissolved in water whereas ciprofloxacin and norfloxacin were prepared in 0.1 N NaOH. The working solutions were prepared by diluting the stock solutions with distilled water.

Determination of MIC. After determining the initial susceptibilities to RIF, INH, SM, and EMB by the proportion method, the MICs of various compounds were determined by a standard broth dilution test (19). Twofold serial dilutions of the test compound, starting with 250 μg/ml and ending with 0.01 μg/ml, utilizing 5 ml of Dubos liquid medium or Kirchner broth medium were prepared. Each tube received approximately 2×10^5 CFU of the indicated organisms. Control tubes lacked drugs. The optical density (OD) in each tube was measured at 600 nm (OD_{600}) with a digital spectrophotometer (Spectronic 20; Milton Roy, Rochester, N.Y.). The initial OD (OD at time zero [OD_{t0}]) ranged between 0.025 and 0.027. All tubes were then incubated at 37°C. The growth in the control tubes was monitored daily until it reached an OD_{600} of 0.27, which corresponded to 8×10^6 to 6×10^7 CFU/ml depending on the species tested. The experiment was terminated when the control OD reached 0.28 to 0.3 (OD_{t1}). MIC was defined as the lowest concentration of the drug that prevented visible growth in a tube compared with the drug-free control or an OD_{t0} of ≤0.025 to 0.027.

In vivo testing: determination of ED_{50}. The procedure for determining 50% effective dose (the dose protecting 50% of the mice) (ED_{50}) described previously by Ashtekar et al. (2) was used. Swiss Webster female mice, outbred strain MFA, weighing 16 to 18 g were infected intravenously via the tail vein with *M. tuberculosis* H37Rv B-216. The stocks of the strain were prepared as follows. The organisms were grown on Lowenstein-Jensen medium for 3 weeks. The growth was scraped, suspended, homogenized, and declumped in Dubos liquid medium. The number of CFU in the suspension was determined on Dubos agar medium, and the aliquots were stored in liquid nitrogen. Mice were infected with approximately 6×10^6 CFU contained in 0.2 ml. This inoculum killed 95% of the infected mice within a 24-day (standard deviation, ± 1) period in repeated tests with a mean survival time of 23.6 ± 0.6 days. On day 1 after the infection, five randomly selected animals were sacrificed. The numbers of bacteria in the lungs of the infected mice (baseline) were determined. Each lung was excised aseptically and transferred individually to an aerosol-proof VirTis homogenizing flask (VirTis Company, Gardiner, N.Y.) containing 10 ml of physiological saline. Each lung was homogenized by using a VirTis high-speed homogenizer 23 (VirTis Company) at 32,000 rpm for 10 to 15 min. The suspension was then incubated at 37°C for 2 h. Tenfold serial dilutions of the suspension were performed. Aliquots (1 ml each) of appropriate dilutions were plated on Dubos agar plates. After complete absorption of the inoculum by the agar medium, the plates were individually sealed with Scotch tape and placed in a plastic bag. Bags were then heat sealed. The plates were incubated at 37°C for a period of 4 to 5 weeks, and CFU were enumerated. The viable count of *M. tuberculosis* in the lungs of infected mice on day 1 postinfection was 4×10^5 CFU per lung. The number of viable organisms in the lungs of the randomly selected dead mice ($n = 10$) was determined and was found to be 4×10^8 CFU per lung.

Doses of CGI 17341, INH, and RIF ranging from 1 to 50 mg/kg of body weight were tested. A group of 10 mice was used for each dose level. Drugs were suspended in 0.5% sodium carboxymethyl cellulose, and the desired dose was administered orally in a 0.2-ml volume by gavage. A group of 10 untreated mice served as the control and received 0.2 ml of sodium carboxymethyl cellulose by gavage. Food and water were given ad libitum. Drugs were administered on days 11 and 12 (a total of two doses) after the infection. The number of survivors in each group on day 23 or 24 (when 95% of the infected drug-free control animals died) after the infection was used for calculating ED_{50}. The ED_{50} was determined by using the SAS software package (20) (SAS Institute, Cary, N.C.). The procedure utilizes a dose-effect curve in which the observed and predicted survival rates are plotted against the logarithm of the dose. This program then performs a probit analysis and also computes the confidence intervals using a method of maximum likelihood on $\log_{10}$ doses of drugs. The procedure is described in detail by Finney (10).

Determination of the prolongation of survival time (mean survival time). The test estimates a dose range of a drug within which the survival period of the treated mice is prolonged. The mean survival time of a group of mice is the arithmetic mean of the numbers of days between infection and death for the animals in that group. Mice were infected as described above by utilizing the standard culture suspension. Groups of 10 mice were used for all dose levels and for the untreated controls. The effect of the CGI 17341 on survival time at doses of 20, 40, and 80 mg/kg was monitored. The compound was administered on days 11 and 12 postinfection. Control animals received 0.2 ml of sodium carboxymethyl cellulose by gavage. Food and water were given ad libitum. Death time (the day an animal died) for each mouse in treated and untreated groups was recorded. The survival period from the day of infection to the day of death for each individual animal in a group was determined and was used for calculating the mean survival time of the group (16). The dose-response relationship was analyzed by using linear regression analysis, and the significance of the data was assessed by Student's *t* test (17).

TABLE 1. In vitro activities of CGI 17341 compared with the activities of selected antituberculosis agents

Organism	No. of isolates	Agent	MIC (μg/ml)
B. fragilis	1	CGI 17341	250
		Metronidazole	0.25
M. tuberculosis H37Rv	1	CGI 17341	0.06
		INH	0.04
		RIF	0.04
		Ciprofloxacin	0.32
		Norfloxacin	15.6
		SM	0.96
		EMB	0.2
		CGP 29861	0.03
		DuP 721	0.97
		Metronidazole	>250
M. tuberculosis	10	CGI 17341	0.1–0.3
		INH	0.2–0.48
		RIF	0.1–0.24
		CGP 29861	0.06–0.4
		LM 427	0.1–0.4
M. tuberculosis Inh^r	2	CGI 17341	0.16
		INH	30
M. tuberculosis Rif^r	1	CGI 17341	0.08
		RIF	250
		LM 427	250
M. tuberculosis Inh^r Rif^r	15	CGI 17341	0.1–0.3
		LM 427	>250
M. tuberculosis Inh^r Rif^r Sm^r	3	CGI 17341	0.24–0.48
M. tuberculosis Emb^r	1	CGI 17341	0.08
		EMB	50
M. tuberculosis Sm^r	1	CGI 17341	0.08
		SM	100
M. gordonae	1	CGI 17341	31.2
M. avium	3	CGI 17341	>250
M. intracellulare	3	CGI 17341	>250
M. fortuitum	1	CGI 17341	>250
M. kansasii	1	CGI 17341	7.3
M. scrofulaceum	1	CGI 17341	15.6

RESULTS

Table 1 shows that CGI 17341 did not show cross-resistance with any of the antituberculosis drugs tested. CGI 17341 at concentrations of 0.04 to 0.3 μg/ml in Dubos broth inhibited all the standard strains of clinical *M. tuberculosis* isolates which were susceptible or resistant to single or multiple drugs. CGI 17341 inhibited *M. gordonae*, *M. kansasii*, and *M. scrofulaceum* at concentrations of 31.2, 7.3, and 15.6 μg/ml, respectively. CGI 17341 was not active at 250 μg/ml against *M. avium*, *M. intracellulare*, *M. fortuitum*, or *B. fragilis*.

The MIC of CGI 17341 against *M. tuberculosis* H37Rv is independent of the pH of the medium over a pH range of 5.6 to 6.8. This behavior differs from those of INH and ciprofloxacin, whose MICs increased at a lower pH by six- and fourfold, respectively (Table 2).

TABLE 2. Effect of pH on the activities of CGI 17341 and other drugs against *M. tuberculosis* H37Rv

Agent	MIC (μg/ml) at pH:	
	6.8	5.6
CGI 17341	0.06	0.06
INH	0.04	0.16
RIF	0.04	0.04
Ciprofloxacin	0.35	2.1
CGP 29861	0.03	0.03
DuP 721	1.25	1.25

CGI 17341 was protective against *M. tuberculosis* infection in mice. When drugs were administered on days 11 and 12 postinfection, the ED_{50} of CGI 17341 was 7.7 mg/kg (95% confidence limits, 5.27 and 10.27) while the ED_{50} (with 95% confidence intervals in parentheses) of INH and RIF were 3.04 (1.67 to 4.7) and 4.81 (3.5 to 6.69) mg/kg, respectively.

Figure 2 shows that the mean survival time of the animals treated with CGI 17341 increased significantly compared with that of the controls and linearly with increasing doses of the drug ($r = 0.995$). Compared with the mean survival time of the untreated controls (24.5 ± 0.5 days), the survival time for the animals treated with 20 mg of CGI 17341 per kg (30.9 ± 1.9 days) was significantly greater ($P < 0.001$). The prolongation of the mean survival time with 40- and 80-mg/kg doses of CGI 17341 (43.5 ± 4.24 and 61.3 ± 3.9 days, respectively) was highly significant compared with that for the controls ($P < 0.0001$).

DISCUSSION

Our data indicate that CGI 17341 exhibits very potent in vitro and in vivo activities against *M. tuberculosis*. While the in vitro activity of CGI 17341, as indicated by MICs, was comparable to those of INH and RIF, it was superior to those of SM, ciprofloxacin, and DuP 721. CGI 17341 displays no cross-resistance with conventional antituberculosis agents. This suggests that the compound will be very valu-

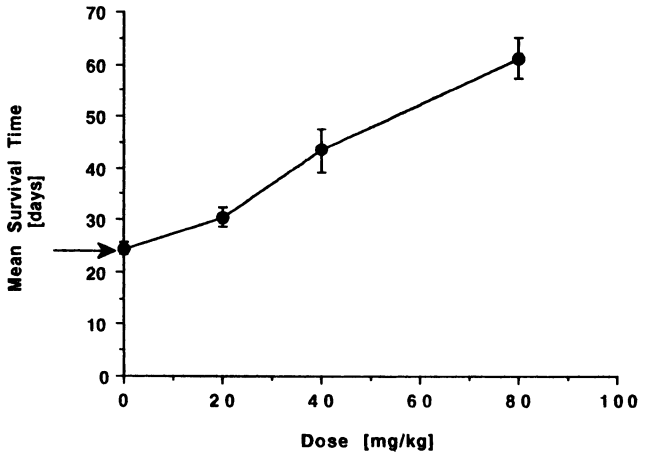

FIG. 2. The effect of CGI 17341 administered on days 11 and 12 postinfection on the survival time of mice infected with *M. tuberculosis*. The data are means for groups of 10 mice each. Arrow, the mean survival time of control animals. Bars, standard errors of the means.

able in the treatment of drug-resistant cases. Attempts to select mutants resistant to CGI 17341 by a single-step process showed that *M. tuberculosis* strains resistant to CGI 17341 occurred at a frequency of 1 in 10^9 at 1 μg/ml (unpublished observation).

The sterilizing activity of an antituberculosis agent depends on the ability to kill both extracellular and intracellular bacteria. The intracellular activity of a drug depends on both the penetration of a drug into the macrophages and the extent of the antagonistic effect of low intracellular pH on the activity of the drug. A decrease in the activity of a drug at a low pH may have far-reaching consequences on sterilizing activity. Since the MIC of CGI 17341 was not altered at pH 5.6 and since the combination of CGI 17341 and RIF was synergistic at a low pH (1), CGI 17341 may play a significant role in improving the overall sterilizing activity of drug regimens against *M. tuberculosis*.

One unanticipated finding that emerged from this study was the increase in the MIC of INH at pH 5.6 (Table 2). The mechanism by which the activity of INH decreases at pH 5.6 is unknown. Also, the information available on this subject is meager. Nevertheless, our observation on the activity of ciprofloxacin at pH 5.6 is concordant with the earlier findings that the MICs and bactericidal rates of ciprofloxacin against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were significantly impaired in an acid environment (12, 21). Because the activity of ciprofloxacin is impaired at a lower pH, it is important to evaluate the activities of newer quinolones in an acidic environment.

It is well established that the MICs of many drugs are lower when tested in a medium containing Tween 80. However, Tween 80 was necessary in our studies in order to prevent the bacterial clumping which would affect the OD measurements and the subsequent MIC estimations.

Regarding the in vivo activity of CGI 17341, the review of the published literature suggests that ED_{50} and the survival rates of the infected mice treated with CGI 17341 were 10-fold lower than those for oxazolidinone DuP 721 and significantly lower than those for one of the most active new quinolones, sparfloxacin (2, 13).

The prolongation of survival time of the infected animals directly reflects the in vivo killing of bacteria. Kradolfer has demonstrated that the increase in the prolongation of survival time strongly correlates with the decrease in the viable counts of *M. tuberculosis* in lungs (15). The dose-dependent increase in the survival time of the infected animals suggests that bacterial killing increases with increasing dose. Such an effect depends on bactericidal activity, dosage, frequency of administration, tissue penetration, and residual activity of the test compound.

In conclusion, CGI 17341 shows promise in the treatment of tuberculosis, especially for treating drug-resistant cases. Studies leading to the development of new regimens of CGI 17341 are continuing in our laboratory.

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