ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2000, p. 1284–1290 Vol. 44, No. 5
0066-4804/00/$04.00+0

In Vitro Pharmacodynamic Parameters of Sordarin Derivatives in Comparison with Those of Marketed Compounds against *Pneumocystis carinii* Isolated from Rats

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Received 9 July 1999/Returned for modification 14 November 1999/Accepted 28 January 2000

***Pneumocystis carinii* pneumonia remains one of the most serious complications of immunosuppressed patients. In this study, the in vitro pharmacodynamic parameters of four sordarin derivatives (GM 191519, GM 237354, GM 193663, and GM 219771) have been evaluated by a new quantitative approach and compared with the commercially available drugs pentamidine, atovaquone, and trimethoprim-sulfamethoxazole (TMP-SMX). In vitro activities and in vivo therapeutic efficacies of sordarin derivatives against *P. carinii* were also evaluated. In vitro activity was determined by the broth microdilution technique, comparing the total number of microorganisms in treated and drug-free cultures by using Giemsa staining. The in vitro maximum effect (E_{max}), the drug concentrations to reach 50% of E_{max} (EC_{50}), and the slope of the dose-response curve were then estimated by the Hill equation (E_{max} sigmoid model). Sordarin derivatives were the most potent agents against *P. carinii*, with EC_{50}s of 0.00025, 0.0007, 0.0043, and 0.025 μg/ml for GM 191519, GM 237354, GM 193663, and GM 219771, respectively. The EC_{50}s of pentamidine, atovaquone, and TMP-SMX were 0.025, 0.16, and 26.7/133.5 μg/ml, respectively. The results obtained with this approach showed GM 237354 and GM 191519 to be approximately 35- and 100-fold more active in vitro than pentamidine, the most active marketed compound. All sordarin derivatives tested were at least 5,000-fold more active in vitro than TMP-SMX. The three sordarin derivatives tested in vivo—GM 191519, GM 237354, and GM 219771—showed a marked therapeutic efficacy, defined as reduction of cyst forms per gram of lung. GM 191519 was the most potent (daily dose reducing 50% of the *P. carinii* burden in the lungs [ED_{50}], 0.05 mg/kg/day) followed by GM 237354 and GM 219771 (ED_{50}s, 0.30 and 0.49 mg/kg/day, respectively). Good agreement between in vitro parameters and in vivo outcome was obtained when *P. carinii* pneumonia in rats was treated with sordarin derivatives.**

Pneumocystis carinii is an important opportunistic pathogen that remains a significant cause of lethal pneumonia in immunocompromised individuals such as patients with AIDS and patients receiving chemotherapy or immunosuppressive drugs for organ transplantation or other pathological conditions. Patients suffering from *P. carinii* pneumonia (PCP) are usually treated with trimethoprim-sulfamethoxazole (TMP-SMX) or pentamidine (24). However, the relatively high frequency of adverse reactions to these drugs reflects the need for new therapeutic approaches. For this reason, the pharmaceutical industry is investigating more effective and less toxic agents.

Sordarin derivatives are a new class of antifungal agents that target protein synthesis (11), with marked in vitro activity against *P. carinii* (13) and excellent in vivo activity in experimental PCP (E. Dei-Cas, E. M. Aliouat, C. Mullet, E. Mazars, and D. Gargallo, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-65, 1997).

Well-defined mouse, rat, or rabbit experimental models (2, 4, 22) can be used to describe the in vivo anti-PCP activity of new compounds. Several in vitro tests for evaluating compound activity against *P. carinii* have been described using axenic cultures or coculture with feeder cells (8, 10, 14). However, no universally accepted standard method is presently available for the in vitro evaluation of anti-*P. carinii* molecules (10). The anti-*Pneumocystis* activity of any given antimicrobial could be evaluated in terms of its intrinsic activity (in vitro) and serum time profile (in vivo) (12). However, the results obtained with different in vitro assays (8, 10) yield limited information on the intrinsic activity of anti-*Pneumocystis* compounds. Furthermore, comparisons between product activities and extrapolation to in vivo activity remain unreliable.

The Hill equation, which describes sigmoid concentration-effect relationships, has proven its utility by revealing in vitro pharmacodynamic properties of several antibiotics (17, 30). This approach offers at least three parameters which can be used to describe the in vitro activity of antimicrobial compounds (17): the maximum effect (E_{max}) as a measure of efficacy, the 50% effective concentration (EC_{50}) as a parameter of intrinsic activity, and the slope (γ) of the concentration-effect relationship.

The aim of the present work was to establish the experimental conditions allowing definition of the in vitro pharmacodynamic parameters of tested drugs for defining the intrinsic activity of anti-*Pneumocystis* molecules, as well as the relationships between their in vitro activities and in vivo effects on microorganisms.

(This work was presented in part at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 24 to 27 September, 1998 [E. M. Aliouat, P. Aviles, E. Dei-Cas, E. Herreros, L. Dujardin, and D. Gargallo-Viola, abstr. J-15].)

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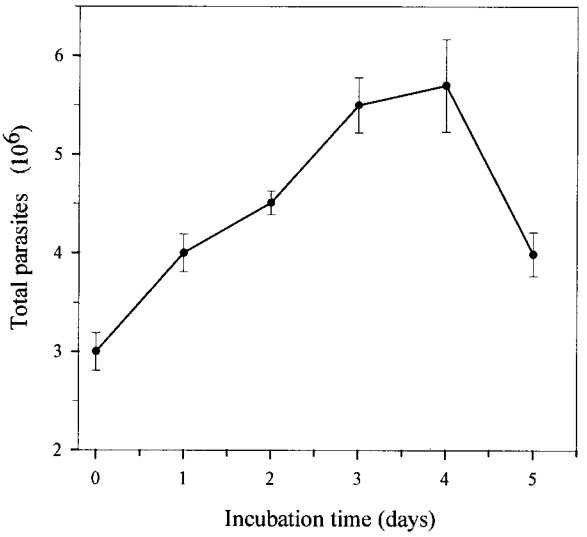

FIG. 1. Typical growth curve of rat-derived *P. carinii* cultivated in DMEM supplemented with 10% FCS.

MATERIALS AND METHODS

Drugs. GM 191519, GM 193663, GM 219771, and GM 237354 are new sordarin derivatives synthesized by GlaxoWellcome, S.A. (Madrid, Spain). TMP-SMX (Sigma Chemical Co., St. Louis, Mo.), pentamidine isothionate (Sigma Chemical Co.), and atovaquone (GlaxoWellcome, Greenford, United Kingdom) were also tested. Sordarin derivatives were dissolved in sterile distilled water at a starting concentration of 10 mg/ml. TMP, pentamidine, and atovaquone were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) to produce a 10-mg/ml stock solution. SMX was also dissolved in DMSO but to a concentration of 30 mg/ml. TMP and SMX solutions were mixed appropriately to obtain a final combination of 1:5. Finally, the drug stock solutions were diluted in Dulbecco's modified Eagle's medium (DMEM) (Bio-Whittaker, Boehringer Ingelheim, Brussels, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO BRL, Life Technologies Inc.) to produce the required drug concentrations. Compound solutions were prepared immediately before use.

Source of *P. carinii*. Corticosteroid-treated rats were used as the source of *P. carinii* organisms. Seven-week-old female Wistar rats (Iffa-Credo, Lyon, France) were immunosuppressed with dexamethasone (Fortecortin; Merck, Darmstadt, Germany) administered in drinking water (2 mg/liter) for approximately 10 weeks (24). Animals had access to sterile standard food (gamma-irradiated rodent maintenance diet) and water ad libitum. At the end of the immunosuppression period the rats were sacrificed and *P. carinii* was recovered from their lungs. The research complied with national legislation, with company policy on the care and use of animals, and with related guidelines (1).

Isolation and quantitation of *P. carinii* organisms. *P. carinii* organisms were isolated as previously described (3), with some modifications. After the immunosuppression period, rat lungs were removed aseptically and cut into small pieces in sterile DMEM. *P. carinii* organisms were extracted by agitation of lung pieces with a magnetic stirrer for 1 h at 4°C. To remove tissue debris, the resulting homogenate was poured through sterile gauze and centrifuged at 2,900 × *g* for 10 min at 4°C. After centrifugation, the pellet was resuspended in a buffered hemolytic solution (9:1 solution of 0.15 M NH_4Cl in 20 mM Tris-HCl, pH 7.4), incubated for 10 min at 4°C, and centrifuged. Then, the pellet was resuspended in DMEM and filtered successively through 250- to 63-μm-pore-size stainless steel filters. Finally, a polysucrose gradient (Histopaque-1077; Sigma Chemical Co.), to obtain purified *Pneumocystis* organisms with a minimum of host contamination, was performed as follows. Polysucrose solution and inoculum suspended in DMEM were prepared 1:1 (vol/vol) in a 15-ml tube (Costar Corporation, Cambridge, Mass.) and centrifuged at 1,000 × *g* for 15 min at 4°C. The band accumulated at the interface between DMEM and polysucrose solution was collected and washed twice with DMEM (2,900 × *g* for 10 min at 4°C). *P. carinii* was quantitated on air-dried smears stained with RAL-555 (Réactifs RAL), a rapid panoptic methanol-Giemsa stain, which stains every *Pneumocystis* life cycle stage. In addition, samples were used to search for putative contaminant organisms. Moreover, final suspension was plated on blood (Difco, Detroit, Mich.) and Sabouraud dextrose agar (Difco) for the detection of bacterial or fungal contamination, respectively. The total numbers of *P. carinii* forms (trophozoites, precysts, and cysts) were calculated as previously described (3): $(n \cdot Sa \cdot R)/Fa$, where n is the average number of microorganisms per oil immersion field (10 fields were counted for each smear), S_a is the 2-μl smear area, R is the ratio between total volume of the microorganism suspension and calibrate smear volume (2 μl), and F_a is the oil immersion field area.

Axenic in vitro culture of *P. carinii*. In order to determine the in vitro drug susceptibility of *P. carinii*, axenic cultures of the organism were produced as follows. All the experiments were carried out in 24-well plates (Nalge Nunc International, Roskilde, Denmark) with a final volume of 2 ml of DMEM supplemented with 10% FCS containing a final inoculum of 1.5×10^6 organisms per ml. Plates with organisms were incubated for 5 days in an atmosphere of 5% CO_2 at 37°C, and the kinetic patterns of in vitro *P. carinii* development were determined. Daily for 5 consecutive days, the total volume of each well was removed and centrifuged for 10 min at 2,900 × *g*, and the pellet was resuspended with 200 μl of phosphate buffer solution Dulbecco (Sigma Chemical Co.). Two-microliter smears were obtained from each suspension. *P. carinii* organisms were stained with RAL-555 and were quantified as described above. All experiments were performed in triplicate.

In vitro susceptibility studies. The above method was validated by performing three independent experiments involving GM 237354 as reference compound. Concentration-response curves were calculated, and the corresponding E_{max}, EC_{50}, and slope were obtained.

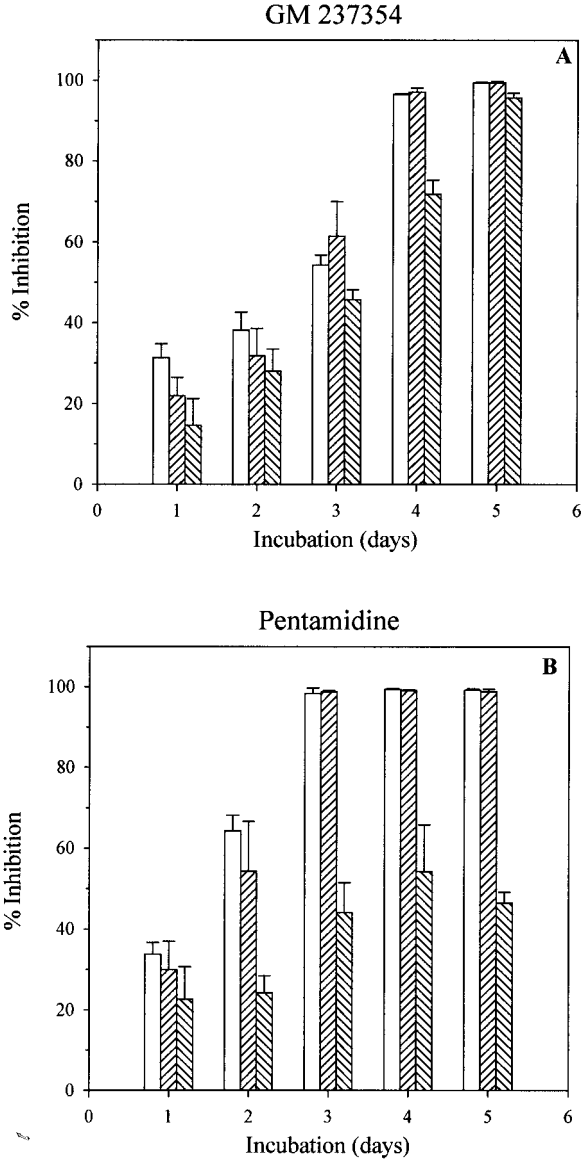

FIG. 2. Influence of incubation time on the in vitro activities of GM 237354 and pentamidine for 5 consecutive days of culture. Both compounds were tested at three different concentrations. □, 10 μg/ml; ▨, 1 μg/ml; ▧, 0.01 μg/ml. The effect of each compound concentration on *P. carinii* was expressed as percent inhibition versus the drug-free control.

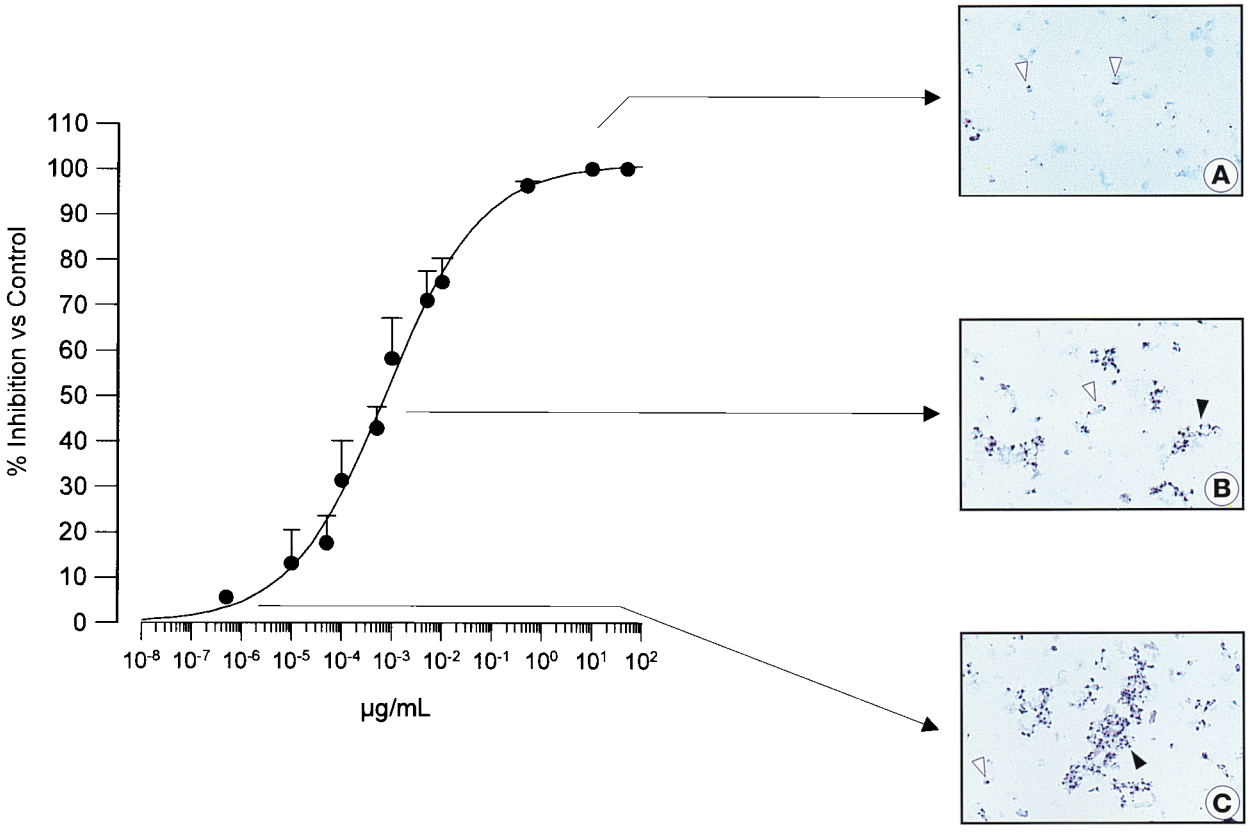

FIG. 3. In vitro activity of GM 237354 against *P. carinii*. (A) Culture medium with 0.5 µg of GM 237354 per ml. A dramatic reduction in the number of microorganisms was observed. (B) Culture medium with 5×10^{-4} µg of GM 237354 per ml. A marked decrease in the number of microorganisms was observed. (C) Drug-free control culture. Trophozoites (open arrowhead) and trophozoite clusters (solid arrowhead) were observed. RAL-555 staining was used. Magnification, ×1,100.

In vitro susceptibility studies were performed using the twofold broth microdilution technique. Final drug concentrations ranged from 5×10^{1} to 5×10^{-7} µg/ml for GM 191519, GM 193663, GM 219771, GM 237354, pentamidine, and atovaquone. The TMP-SMX combination was tested from 150/750 to 1×10^{-5}/5×10^{-5} µg/ml. Plates were incubated for 4 days in an atmosphere of 5% CO_2 at 37°C. One drug-free control was included in each assay. Microorganisms were quantified on homogenate smears as described above. All susceptibility assays were performed in triplicate.

Analysis of results. The anti-*Pneumocystis* activity of a single concentration of compound may be expressed in terms of percent inhibition, defined as the decrease (expressed as percentage) in *P. carinii* forms in antifungal-treated cultures with respect to the total microorganism count in compound-free culture. Once all the differences between drug-treated and untreated wells were calculated, the concentration-effect relationship was established by means of the Hill equation (17, 30): $E_R = E_{R,max} \cdot C^S/[(EC_{50})^S + C^S]$, where E_R is the effect of each drug concentration (C) upon percent inhibition estimated from experimental results, S is a parameter reflecting the steepness of the concentration-effect relationship curve, and EC_{50} is the concentration of the compound at which 50% of the maximum effect ($E_{R,max}$) is obtained.

Once the E_R values were fitted, the parameters of the pharmacodynamic model were calculated by nonlinear least-squares regression techniques using commercial software (WinNonlin; Scientific Consulting, Inc.).

In vivo study. An in vivo pilot study which involved PCP in rats was performed to explore whether in vitro results reflect in vivo efficacy. PCP was established by using a previously described method. Briefly, animals were immunosuppressed with dexamethasone (Fortecortin; Merck) at a concentration of 2 mg/liter in the drinking water for 9 weeks. Tetracycline (Terramicine; Pfizer Laboratories) at 1 g/liter was added as antibacterial prophylactic agent. All animals remained on immunosuppressive therapy with dexamethasone throughout the study. Before treatment, PCP was microscopically verified as previously reported (28). Animals were divided into groups of six, and then, sordarin derivatives (GM 191519, GM 237354, and GM 219771) were dosed at 0.1, 1.0 and 5.0 mg/kg by subcutaneous route. The drugs were given twice a day for 10 consecutive days. Control animals were dosed with sterile water. Twenty-four hours after the last dose all animals were sacrificed by overdosing of sodium pentobarbital (Euthalender; Normon). Lungs were aseptically removed and weighted. *P. carinii* extractions were performed by using a previously described method (2). *P. carinii* cystic forms were quantitated by Toluidine Blue-0 staining (Sigma-Aldrich, S.A.). The number of cysts were determined by visual assessment by light microscopy (20 microscopic fields). All results were expressed as $\log_{10}$ of the number of cysts per gram of lung (logQ/g). Daily dose (milligrams per kilogram per day) and logQ/g were plotted and adjusted to an E_{max} model by nonlinear least-squares regression techniques using commercial software (WinNonlin). Then, ED_{50} (defined as the daily dose which reduces 50% the *P. carinii* burden in lungs) was calculated.

RESULTS

In vitro *P. carinii* growth. Figure 1 shows the *P. carinii* growth curve obtained after 5 days of incubation. The curve displays two well-defined portions: a first segment (from day 0 to 3 or 4), where *P. carinii* gradually increases in number, with doubling times of 85.3 h (up to day 3) and 102.7 h (up to day 4), and a second segment, reflecting *P. carinii* population decrements after the fourth day of incubation. Moreover, the formation of large, typical trophozoite clusters was observed throughout the incubation period, while the number of cystic forms gradually decreased in proportion, to less than 2% of total microorganisms after 3 days of incubation.

Optimal assay setup conditions. Preliminary experiments were performed using pentamidine and GM 237354. High (10-µg/ml), medium (1-µg/ml), and low (0.01-µg/ml) concentrations of compound were tested against *P. carinii* for 5 days of culture. Percent inhibition compared to drug-free control was determined (Fig. 2). When *P. carinii* was incubated for 1 to 3 days with three concentrations of GM 237354, relatively low growth inhibition was observed, even when the highest con-

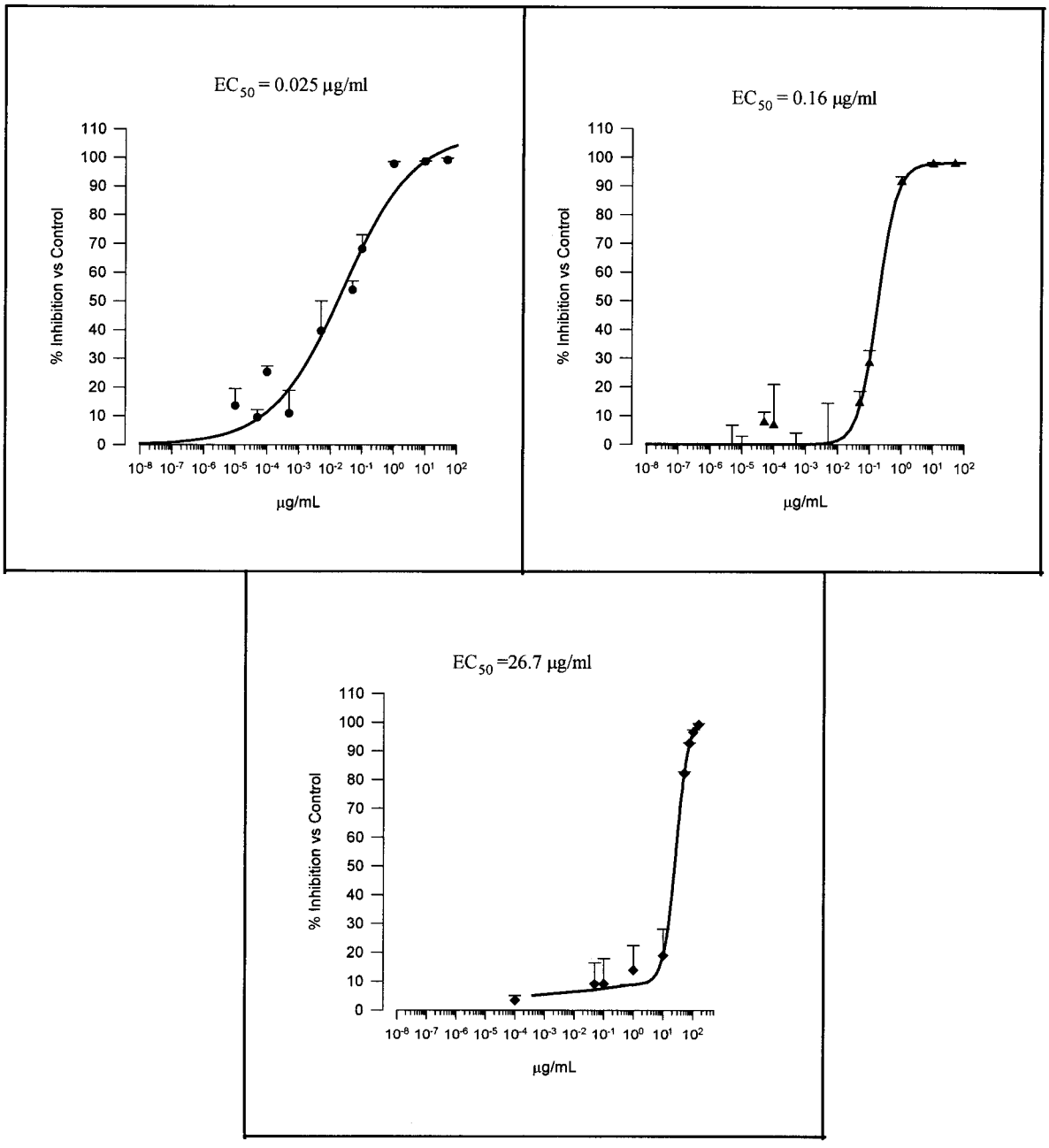

FIG. 4. Concentration-in vitro activity relationships of the three commercial compounds against *P. carinii*. ●, pentamidine; ▲, atovaquone; ◆, TMP-SMX. Results were calculated after 4 days of incubation.

centration (10 μg/ml) was tested. Maximum inhibition rates of 38 and 60% were observed after 2 or 3 days of culture, respectively (Fig. 2A). However, after 3 days of culture, pentamidine (10 and 1 μg/ml) reached 100% inhibition (Fig. 2B). Both concentrations, i.e., 10 and 1 μg of pentamidine and GM 237354 per ml, had the maximum effect by the fourth day of incubation. The inhibitory effect of pentamidine at 0.01 μg/ml showed similar levels (44 to 54% inhibition) after 3 to 5 days of culture, whereas the GM 237354 inhibitory effect reached 70 and 96% inhibition at 4 and 5 days postinoculation, respectively (Fig. 2A).

Considering both these results and the behavior of the *P. carinii* population, 4 days of culture was selected as the best assay duration for in vitro anti-*Pneumocystis* drug susceptibility studies under our experimental conditions. In further studies a wider range of drug concentrations was used (5×10^{1} to 5×10^{-7} μg/ml).

In vitro susceptibility studies. The robustness of the method was assayed by performing three different experiments with GM 237354. All the three resulting concentration-response curves obtained essentially overlapped (data not shown). Mean calculated values for E_{max} (99.06%), EC_{50} (0.00076 μg/ml), and the slope (0.53) showed variation coefficients of 2, 20, and 11.5%, respectively.

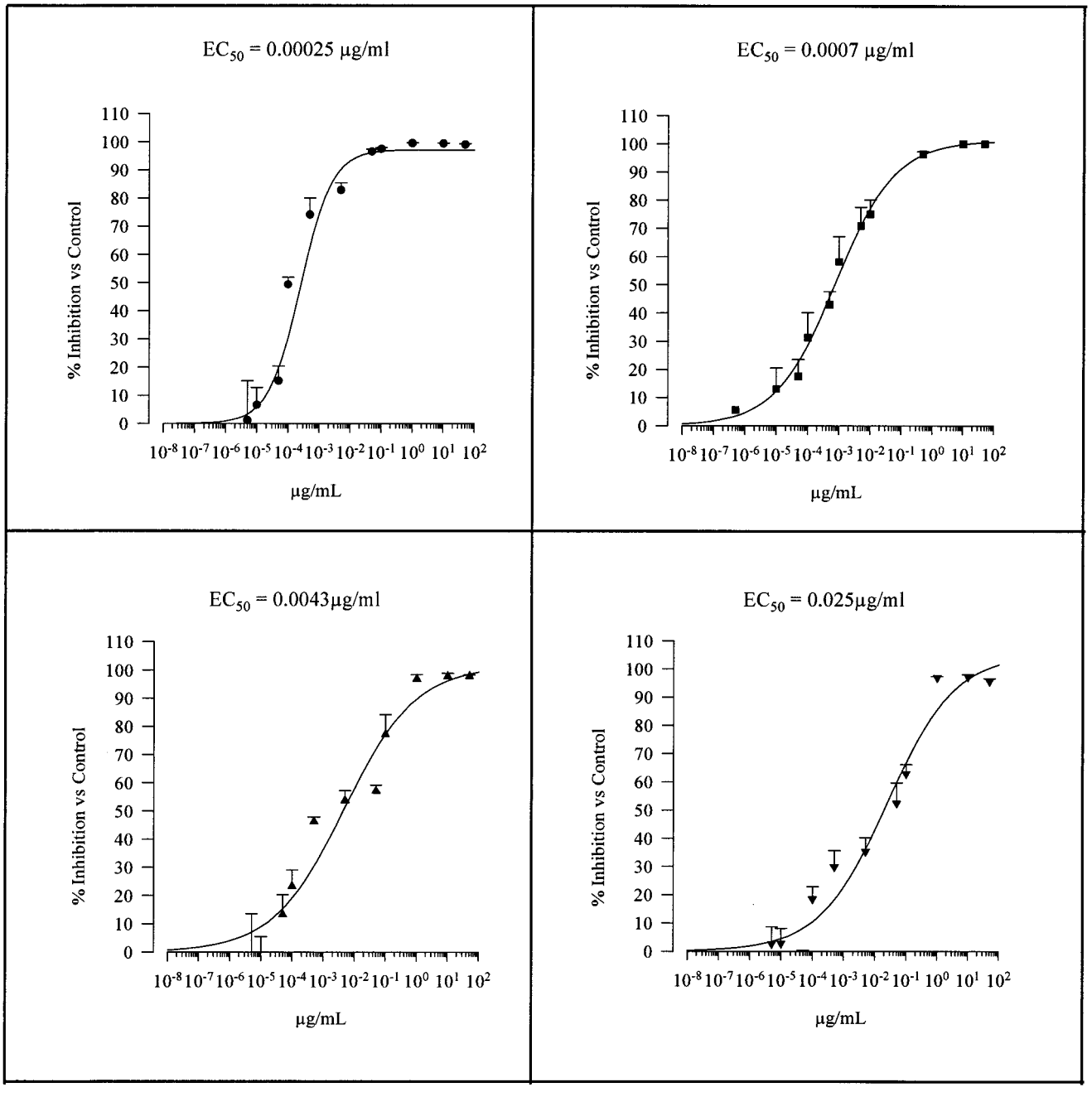

FIG. 5. Concentration-in vitro activity relationships of the four sordarin derivatives against *P. carinii*. ●, GM 191519; ■, GM 237354; ▲, GM 193663; ▼, GM 219771. Results were calculated after 4 days of incubation.

Figure 3 shows a concentration-response curve obtained after 4 days of incubation of *P. carinii* with GM 237354 (concentration range, 5×10^{1} to 5×10^{-7} μg/ml). The reduction in the number of microorganisms detected per field was gradual and concentration dependent. Figures 3A and B show an evident decrease in *P. carinii* forms when the culture was incubated with 0.005 and 0.5 μg/ml, respectively. These concentrations correspond to the upper and middle portion of the concentration-response curve. Figure 3C shows the characteristic appearance of a drug-free culture.

Figures 4 and 5 show the concentration-response curves obtained for sordarin derivatives and marketed compounds, respectively. All tested compounds inhibited *P. carinii* growth compared with drug-free control cultures. TMP-SMX demonstrated the lowest intrinsic activity, followed by atovaquone and pentamidine (EC_{50}s, 26.7/133.5, 0.16, and 0.025, respectively). In terms of efficacy, both pentamidine and atovaquone reached about 100% inhibition at a concentration of 1 μg/ml. However, TMP-SMX reached the same inhibition level (100%) at high concentrations (75/375 μg/ml).

Sordarin derivatives exhibit a differential potency (EC_{50}) ranging from 0.00025 μg/ml (GM 191519) to 0.025 μg/ml (GM 219771) (Fig. 5). In fact, the less potent sordarin derivative (GM 219771), which was 100 times less active than the most

TABLE 1. In vitro susceptibilities of *P. carinii* to marketed compounds and sordarin derivatives

Antifungal agent	EC_{50} (μg/ml)
Pentamidine	0.025
Atovaquone	0.16
TMP-SMX	26.7/133.5
Sordarins	
GM 191519	0.00025
GM 237354	0.0007
GM 193663	0.0043
GM 219771	0.025

efficient derivative, exhibits a potency equal to that of pentamidine, which in turn was the most potent of the marketed compounds. This differential in vitro activity was also reflected when sordarin derivatives were used for the treatment of experimental PCP in rats.

In vivo study. The number of *P. carinii* cysts per gram of lung in untreated animals which develop PCP reaches $10^{7.6}$ at the end of the treatment period (73 days after the start of dexamethasone immunosuppression). All the three sordarin derivatives caused a marked reduction in the number of *P. carinii* cyst forms per gram of lung. Dose-response curves obtained after sordarin derivative treatment of experimental PCP in rats are displayed in Fig. 6. Good agreement between experimentally observed and calculated values was obtained (r^2, 0.999, 0.980, and 0.998 for GM 191519, GM 237354, and GM 219771, respectively) for each therapeutic regimen used. Calculated ED_{50}s for sordarin derivatives demonstrated that GM 191519 was the most potent of the three compounds (ED_{50}, 0.05 mg/kg/day) followed by GM 237354 (ED_{50}, 0.30 mg/kg/day) and GM 219771 (ED_{50}, 0.49 mg/kg/day). These in vivo potencies are in the same order as those observed for in vitro results.

DISCUSSION

A quantitative and reproducible in vitro susceptibility axenic model for evaluating pharmacodynamic parameters of anti-*Pneumocystis* compounds is described in the present study. The results obtained indicate that in vitro EC_{50} could be an index of the expected anti-PCP in vivo effect, at least for sordarin derivatives. Pharmacodynamic parameters of anti-*Pneumocystis* drugs were calculated using the Hill equation, which has been previously used to describe in vitro concentration-effect relationships of antibacterial compounds (17, 30). Antimicrobial agents were evaluated by comparing *P. carinii* growth in drug-treated versus drug-free control cultures after a 4-day incubation period. Microorganism growth was assessed on dry smears stained by methanol-Giemsa-like staining (RAL-555), which allows total microorganism quantitation. Most *P. carinii* microorganisms developing in this system are vegetative forms. In agreement with other reports (5, 16), we found that in vitro *Pneumocystis* proliferation leads to the formation of clusters in which more than 98% are trophozoite forms.

In vitro susceptibility tests are a basic step in any pharmacological screening for new anti-infective drugs. Several factors influence the outcome and reproducibility of susceptibility tests (6), including medium composition, inoculum size, incubation time, and the nature of the microorganism. In the present study, *P. carinii* organisms were cultivated by means of an axenic culture rather than the usual coculture methods. The main reasons for adopting this approach can be summarized as follows. (i) Mammalian cell growth produces extracellular catabolic products and depletes broth nutrients—processes capable of affecting *P. carinii* growth and influencing the susceptibility patterns. (ii) Developing cocultured cells could influence the stability of tested compounds. (iii) New compounds can display toxicity against monolayer cells. This fact could affect *P. carinii* growth and hence the susceptibility results obtained. (iv) Microorganisms attached to cell monolayers could acquire susceptibility patterns different from those of unattached microorganisms in suspension due to several factors, such as different growth rates, metabolic processes, or drug accessibilities. (v) Interactions between unattached microorganisms and compounds are probably easier than those found when microorganisms are attached to target cells. All the above considerations could also negatively affect interlaboratory reproducibility. Furthermore, in order to minimize the presence of a biological matrix in the culture medium, FCS should be replaced by a synthetic substitute, since the presence of serum could have multiple effects in susceptibility testing. Some authors have reported compound inactivation as a result of drug binding to serum components (9), where only the free fraction shows antimicrobial activity. Moreover, considering the behavior of *P. carinii* in in vitro cultures, some authors have attempted to improve culture performance by using cocultured cells to evaluate susceptibility. However, the presence of mammalian cells in the system could add a poorly controlled variable and potentially result in a lack of assay reproducibility.

Another variable with specific weight in drug susceptibility test outcomes is incubation time. Our results show that this parameter must be considered to establish an effective compromise between microorganism growth in drug-free controls and microorganism inhibition in drug-treated microculture wells.

In the antibacterial field, the MIC is the most widely used parameter for determining susceptibility. For this reason, internationally accepted guidelines have been published (18) for performing the tests so as to avoid or minimize interlaboratory variability. Similar initiatives have been proposed by the international scientific community for other tests which offer complementary information on the in vitro activities of antibacterial drugs (20, 21). More recently a standard method was proposed for pathogenic yeast (19). *P. carinii* has recently been included in the fungal kingdom, though it may be regarded as

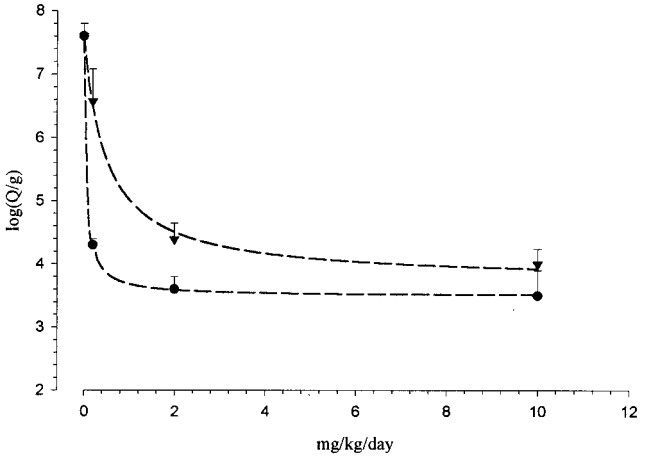

FIG. 6. In vivo efficacies of GM 191519 (●) and GM 219771 (▼) on the *P. carinii* burden in lung. The data are means with standard deviations for six animals. The dose-effect relationships were calculated according a simple E_{max} model.

an atypical fungus (29): it lacks ergosterol (a target for most antifungals) and refuses to grow well in vitro (25). Recent efforts have been made to improve this situation (16), however.

Antigenic and genomic host species-related differences have been reported among *P. carinii* isolates, yielding a strong host species specificity (4). However, it has not been proven whether *Pneumocystis* strains from different mammal hosts can exhibit different drug susceptibility patterns. The development of an in vitro pharmacodynamic model affords an improved tool for the evaluation of susceptibility patterns of *Pneumocystis* strains from different host species.

Mouton et al. have offered a pharmacological explanation for the parameters in the Hill equation (17). They also suggested that the Hill model could be very useful for understanding changes in susceptibility, relating EC_{50} values to classical MICs. The findings described herein provide sufficient information for considering EC_{50} to be an accurate indicator of in vitro activities of anti-*Pneumocystis* compounds. Such an approach will provide a new tool for selecting new compounds and establishing therapeutic protocols. By using this pharmacodynamic approach, the results obtained revealed a high in vitro anti-*Pneumocystis* activity on the part of sordarin derivatives which belong to a new family of antifungals targeting fungal protein synthesis (11). Moreover, EC_{50} seems to reflect in vivo anti-*Pneumocystis* activity, as sordarin derivative in vivo ED_{50}s suggest. Future experimental in vivo work is warranted to further establish the performance of this new in vitro approach with other anti-*Pneumocystis* compounds.

ACKNOWLEDGMENTS

The Pasteur Institute team was supported by the French Ministry of Research (PRFMMIP program).

We thank María José Guillén and Elena Jiménez for excellent technical assistance.

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