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Activity of Topoisomerase Inhibitors against *Pneumocystis carinii* In Vitro and in an Inoculated Mouse Model

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Five topoisomerase II inhibitors (amsacrine [m-AMSA], two epipodophyllotoxins, and two quinolones) and the alkaloid camptothecin (a topoisomerase I inhibitor) were evaluated to assess their activities against *Pneumocystis carinii*. In vitro, both etoposide (VP-16) and teniposide (VM-26) at 1 μg/ml suppressed *P. carinii* growth. Amsacrine was toxic to *P. carinii* and to the feeder cells in vitro. Camptothecin suppressed the growth of *P. carinii* in vitro only at 100 μg/ml. Studies in immunosuppressed mice demonstrated the efficacy of teniposide against *P. carinii* pneumonia, but successful administration of teniposide was schedule dependent with significant toxicity at therapeutic dosages.

Pneumocystis carinii is an opportunistic pathogen which causes pneumonia (PCP) in many immunocompromised individuals, most notably in patients with AIDS. More than 60% of all patients with human immunodeficiency virus infection who do not receive specific prophylactic antibiotics will develop one or more episodes of PCP during their lifetimes (10, 15). The antibiotics commonly used for the prophylaxis and therapy of PCP in AIDS patients (trimethoprim-sulfamethoxazole [TMP-SMZ] and pentamidine isethionate) are frequently associated with serious adverse reactions necessitating altered therapy (8, 19, 20, 23, 24). Because of the toxicities of these agents, attempts have been made to develop alternative regimens for the prophylaxis and treatment of PCP.

Topoisomerases are essential to the transcription and replication of DNA in prokaryotes and eukaryotes (11, 13). Organisms with defective type I enzymes are still able to complete cell replication. Effective inhibition of topoisomerase II uniformly leads to cell death (11, 13). Because of the universal importance and structural conservation of topoisomerase II enzymes, anti-topoisomerase II agents have been considered potential anti-*Pneumocystis* antibiotics (11, 13). The topoisomerase (I or II)-DNA complex of *P. carinii* may also be the target of pentamidine and related dicationic antibiotics (12). The fluoroquinolones have limited toxicities and broad antibacterial spectra (25). Some quinolones, including pefloxacin and fleroxacin, also have high oral bioavailabilities, concentrations in polymorphonuclear leukocytes in excess of those in plasma, prolonged half-lives in serum, and are actively secreted into pulmonary secretions (1, 18, 25). One study suggested that pefloxacin, but not other quinolones, had efficacy for both prophylaxis and treatment of PCP in the immunosuppressed rat model (9). The present study was designed to test a variety of well-characterized topoisomerase enzyme inhibitors for activity against rat-derived *P. carinii* both in vitro and in vivo.

In vitro drug testing against *P. carinii*. Cultures of *P. carinii* were prepared and evaluated as previously described (2, 22). Drugs were diluted in culture medium and added to cultures to a final concentration of 1, 10, or 100 μg/ml. Separate plates were harvested for analysis at 1, 3, 5, and 7 days after inoculation. At each time point, 10 μl of culture supernatant was taken from each well, air dried onto a 1-cm^2 area of a premarked slide, methanol fixed, and Giemsa stained. Slides were examined as unknowns by two individuals, and organisms were quantitated (at a magnification of ×1,000) as the number of organisms per milliliter in the supernatant (7). Each score is the mean (± standard error of the mean) of eight values, with four wells per parameter. Culture studies were performed with the following drugs: teniposide (VM-26, BMY26604; Bristol-Myers, Wallingford, Conn.), amsacrine (m-AMSA, PD105238; Parke-Davis, Ann Arbor, Mich.), camptothecin (Sigma Chemical Co., St. Louis, Mo.), and etoposide (VP-16, BMY 26600; Bristol-Myers). Trimethoprim-sulfamethoxazole (TMP-SMZ) and pentamidine isethionate (LyphoMed, Rosemont, Ill.) were used as control agents. The diluent (dimethyl sulfoxide) has been shown to have no significant effect on growth at the concentrations present in culture (3).

Drug testing in the *Pneumocystis*-infected mouse model. The inoculated mouse model was used for the in vivo investigation of the agents studied (6). Dexamethasone (4.2 mg/kg of body weight) was administered in drinking water to BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.). After 10 days of immune suppression, mice were intratracheally inoculated with 10^6 trophozoites of *P. carinii* in 0.05 ml of saline. *P. carinii* inoculum was originally prepared from infected SCID mouse lung homogenate as has been previously described (4–6). Mice were studied in groups of 10. Drug treatment was initiated 3 weeks after inoculation with *P. carinii*. Drug dosages were based on manufacturers' recommendations, 50% lethal doses, and previous studies in mice and rats (6, 9, 21). Fleroxacin (Hoffman-La Roche, Nutley, N.J.) was given at 100 or 150 mg/kg (3 mg per mouse) administered subcutaneously daily. Teniposide was administered as follows: (i) a one-time intraperitoneal (i.p.) dose (80 mg/kg, 1.6 mg), followed by 10 mg/kg i.p. every 4 days (days 5, 9, 13, and 17); (ii) 20 mg/kg i.p. (0.4 mg) in a corn oil suspension (days 1, 5, 9, 13, and 17); or (iii) 40 mg/kg i.p. (0.8 mg) every 4 days for three doses (days 1, 5, and 9), followed by 20 mg/kg i.p. (0.4 mg) every 4 days thereafter (days 13 and 17). Pefloxacin (Rhône-Poulenc Pharmaceutical

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TABLE 1. Activities of topoisomerase inhibitors against *P. carinii* in vitro

Expt, drug, and concn (μg/ml)	Mean no. of *P. carinii*[a] on day:				% of day 7 control
	1	3	5	7	
Expt 1					
None (control)	5.1 (0.6)	12.8 (1.0)	14.1 (1.2)	15.2 (2.5)	
Pentamidine (2)	3.0 (0.4)	3.4 (0.5)	2.4 (0.4)	1.2 (0.2)*	8
Camptothecin					
10	4.6 (0.6)	12.1 (1.1)	7.7 (1.2)	15.5 (2.2)	102
100	4.9 (0.4)	11.2 (0.9)	3.4 (0.5)	6.2 (1.0)*	41
Amsacrine					
10	3.0 (0.5)	7.1 (0.6)	5.6 (0.9)	7.2 (0.7)*	47
100	2.1 (0.3)	2.9 (0.2)	1.1 (0.4)	0.7 (0.2)*	5
Teniposide					
10	3.9 (0.3)	5.6 (0.7)	4.0 (0.5)	4.2 (0.4)*	28
100	4.8 (0.6)	4.4 (0.5)	1.7 (0.3)	2.0 (0.3)*	13
Expt 2					
None (control)	1.9 (0.2)	3.8 (0.2)	4.2 (0.5)	7.4 (0.6)	
TMP-SMX (50 and 250, respectively)	1.0 (0.2)	1.5 (0.6)	0.4 (0.1)	0.6 (0.1)*	8
Amsacrine					
1	1.7 (0.3)	2.4 (0.3)	2.8 (0.4)	4.1 (0.6)*	55
10	0.9 (0.2)	1.2 (0.2)	0.8 (0.1)	0.5 (0.1)*	7
Teniposide					
1	1.0 (0.1)	1.5 (0.3)	1.6 (0.3)	1.4 (0.3)*	19
10	1.1 (0.2)	1.1 (0.2)	1.3 (0.2)	0.7 (0.2)*	
Etoposide					
1	1.3 (0.2)	3.0 (0.2)	3.1 (0.4)	4.3 (0.6)*	58
10	1.4 (0.2)	1.8 (0.3)	1.7 (0.2)	1.1 (0.1)*	15

[a] Counts are expressed as the number of organisms per ×1,000 field of culture supernatant. Values can be converted to the number of organisms per milliliter by multiplying by 3.9×10^5 (dilution and magnification factor). Each measurement is the mean for four wells read in duplicate, with the standard error of the mean reported in parentheses. The asterisks indicate significant differences ($P < 0.01$ by two-tailed t test) compared with day 7 control values.

Co., Antony, France) was administered by injection (either 100 or 300 mg/kg daily i.p.) in water. TMP-SMZ (50 mg of TMP per kg and 250 mg of SMZ per kg) was administered in drinking water. A control group was not treated with antibiotics.

After 4 weeks of treatment, anesthetized mice were exsanguinated by cardiac puncture, and standard portions of aseptically removed lung tissue were used to prepare two impression smears for Giemsa and Gomori's methenamine-silver nitrate staining. All slides were reviewed as unknowns by three examiners scoring at least 10 fields per slide (each field at a magnification of ×1,000) according to the following scale: 5+, >100 organisms per field; 4+, 11 to 100 organisms per field; 3+, 1 to 10 organisms per field; 2+, 2 to 9 organisms in 10 fields; 1+, ≤1 organism in 10 fields; and 0, no organisms in 50 fields. Scoring was determined as the mean value for each group.

Statistical analyses of data were performed by two-tailed t test (Table 1) and by one-way analysis of variance (Table 2) with Systat software (Evanston, Ill.). Histologic scoring uses a nonparametric, discontinuous scale. However, one-way analysis of variance has been used to estimate the likelihood that animals receiving treatments were derived from the same population as that of the control subjects.

In vitro studies. In the culture model, at least a threefold increase in the number of *P. carinii* nuclei occurred over 7 days in the absence of antibiotic (Table 1). Both pentamidine and TMP-SMZ inhibited this growth; the number of countable nuclei fell to less than half of that in the initial inoculum and to 8% of control (untreated) growth by day 7 of incubation. Camptothecin had no significant effect on the growth of *P. carinii* at 10 μg/ml. At higher concentrations of camptothecin (100 μg/ml), cytolytic effects were observed in the WI-38 feeder cell monolayer and decreased numbers of *P. carinii* were observed. At 100 μg/ml, amsacrine gave inhibition comparable to that observed with either pentamidine or TMP-SMZ, but significant injury to the feeder cell layer was also noted at this concentration. Teniposide decreased *P. carinii* replication at concentrations of 1 to 100 μg/ml. Significant reductions of *P. carinii* numbers were observed at both the 1 and 10 μg/ml levels without apparent feeder cell injury. Less inhibition was observed with the same concentrations of etoposide.

In vivo studies. Teniposide was selected for use in animal studies, because it was the most potent drug tested in vitro and least toxic to the feeder cell layer. Pefloxacin was tested because of previous reports of efficacy in rats (9, 21). Fleroxacin was tested as another long-acting quinolone with good tissue distribution. TMP-SMZ exposure suppressed infection of *P. carinii* in the lungs to nearly undetectable levels (Table 2). Teniposide was toxic to mice in experiment 1 (Table 2), in which teniposide was given in corn oil; however, animals surviving initial therapy with 80 mg of teniposide per kg (followed by 10 mg/kg every fourth day) had lower levels of infection than those of control animals or animals receiving lower drug doses without the initial 80 mg/kg loading dose (Table 2). Animals receiving teniposide administered without corn oil in experiment 3 had infections similar to those of controls and no toxicity; however, the drug may not have been absorbed. The quinolone agents had different effects. Pefloxacin at 300 mg/kg per day, a dosage level established based on the effective dose reported for rats (21), resulted in the death of all exposed mice; a lower dose (100 mg/kg per day), previously reported to be effective for prophylaxis in rats (9), was not toxic but ineffective thera-

TABLE 2. Activities of topoisomerase inhibitors against *P. carinii* in vivo

Expt, drug, and concn (mg/kg)	Giemsa-stained slides		Silver-stained slides	
	Mean infectivity score (SEM)[a]	No. of infected animals/total no.	Mean infectivity score (SEM)	No. of infected animals/total no.
Expt 1				
None (control)	4.1 (0.3)	12/12	3.5 (0.2)	12/12
Teniposide				
80, 10	2.8 (0.6)*	4/4	2.1 (0.4)*	4/4
20	4.8 (0.1)	3/3	3.3 (0.3)	3/3
Fleroxacin (100)	3.4 (0.6)	7/9	3.0 (0.3)	9/10
Pefloxacin (300)		—[b]		—[b]
TMP-SMX (50 and 250, respectively)	0.1 (0.1)*	2/8	0.1 (0.1)*	2/8
Expt 2				
None (control)	3.5 (0.5)	9/10	2.2 (0.5)	7/9
Pefloxacin (100)	3.6 (0.5)	9/10	3.1 (0.5)	9/10
Expt 3				
None (control)	4.2 (0.4)	10/10	3.4 (0.3)	10/10
Teniposide (40, 20)	4.3 (0.1)	10/10	3.4 (0.1)	10/10
Fleroxacin (150)	3.8 (0.3)	9/9	3.4 (0.1)	9/9
TMP-SMX (50 and 250, respectively)	0.4 (0.2)*	3/10	0.4 (0.1)*	4/10

[a] Each unit on the infectivity scale represents a 90% decrease or increase in the number of organisms detected. The asterisks indicate statistically significant differences from the controls by one-way analysis of variance.
[b] All mice given perfloxacin died.

peutically in mice. Fleroxacin (100 or 150 mg/kg per day) was neither toxic nor effective.

Short-term, successful antibiotic therapy in in vitro cultures and in in vivo studies in virus-free, intratracheally infected rats or mice have been predictive of clinical efficacy for the treatment of PCP (4–6). Results from the in vitro culture system suggest that inhibitors of topoisomerase I or II may have efficacy against *P. carinii*. In vitro, the epipodophyllotoxins teniposide and etoposide inhibited the growth of *P. carinii*, with teniposide being more potent and less toxic to the required mammalian feeder cells. Amsacrine was ineffective at doses that were not toxic to the feeder cells. The plant alkaloid camptothecin also demonstrated some anti-*Pneumocystis* properties in vitro at higher concentrations; these levels would be expected to be toxic in vivo.

The in vivo investigations confirmed the anti-*Pneumocystis* activity of teniposide seen in vitro. However, the data suggest that a specific dosing schedule of teniposide or related drugs may be necessary. Treatment with a high initial loading dose (80 mg/kg) followed by lower maintenance doses (10 mg/kg) resulted in over a 90% (1.3 log units) reduction in countable *P. carinii* from the lungs (i.e., less than 10 organisms per high-power field) compared with untreated controls. The efficacy of this modified dosage regimen correlates with the schedule dependence of the epipodophyllotoxins used for cancer chemotherapy. In oncologic studies, a prolonged low-level maintenance phase of teniposide after higher initial levels is associated with greater therapeutic efficacy (16, 17).

Teniposide-related drugs may have several potential advantages as alternatives for the treatment of *P. carinii*-infected patients intolerant of other agents. First, teniposide is a representative drug of an entirely new class of anti-*Pneumocystis* compounds with novel specificities and resistance patterns. Epipodophyllotoxins remain the least toxic of all antitumor agents (14). Second, the unusual pharmacokinetics of teniposide might be advantageous for the prophylaxis of AIDS-related infections. This investigation supports future studies involving a potential role for teniposide and related agents in the development of novel classes of antibiotics for the treatment of *P. carinii* infection.

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