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A Cytotoxicity Assay for Evaluation of Candidate Anti-*Pneumocystis carinii* Agents

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A series of over 60 agents representing several different classes of compounds were evaluated for their effects on the ATP pools of *Pneumocystis carinii* populations derived from immunosuppressed rats. A cytotoxicity assay based on an ATP-driven bioluminescent reaction was used to determine the concentration of agent which decreased the *P. carinii* ATP pools by 50% versus untreated controls (IC_{50}). A ranking system based on the IC_{50} values was devised for comparison of relative responses among the compounds evaluated in the cytotoxic assay and for comparison to in vivo efficacy. With few exceptions, there was a strong correlation between results from the ATP assay and the performance of the compound in vivo. Antibiotics, with the exception of trimethoprim-sulfamethoxazole (TMP-SMX), were ineffective at reducing the ATP pools and were not active clinically or in the rat model of *P. carinii* pneumonia. Likewise, other agents not expected to be effective, e.g., antiviral compounds, did not show activity. Standard anti-*P. carinii* compounds, e.g., TMP-SMX, pentamidine, and dapsone, dramatically reduced ATP levels. Analogs of the quinone and topoisomerase inhibitor groups were shown to reduce ATP concentrations and hold promise for further in vivo investigation. The cytotoxicity assay provides a rapid assessment of response, does not rely on replicating organisms, and should be useful for assessment of structure-function relationships.

Pneumocystis carinii organisms cause a lethal pneumonia in hosts with compromised immune systems, especially in patients with AIDS. Despite prophylactic, therapeutic, and supportive strategies, *P. carinii* pneumonia remains a major concern in this patient population. To date, no drug has been proven to be clinically more effective than trimethoprim-sulfamethoxazole (TMP-SMX) or pentamidine isethionate (26).

The immunosuppressed animal models of pneumocystosis have proven to be valuable tools in the search to identify potential anti-*P. carinii* compounds. However, the use of animal models is limited by the scope and number of compounds that are able to be evaluated. In addition, the methods used by different laboratories to assess efficacy and identify candidate compounds have varied, making direct comparisons of drug responses problematic. In vitro drug screening systems have been an important tool for the initial identification of candidate compounds prior to in vivo testing in other microbial systems, but a continuous in vitro culture system for *P. carinii* remains elusive. Investigators have relied upon short-term cell monolayer or cell-free maintenance systems to screen potential anti-*P. carinii* therapies or conduct metabolic studies (2, 4, 5, 15, 18–20, 28, 37–39, 42–44). There are shortcomings with either approach, and a generally accepted method for in vitro screening has not been established. Most in vitro systems for *P. carinii* drug screening have used a mammalian cell monolayer as a growth matrix and enumeration of organisms as a method of evaluating activity. Disadvantages of such systems include deleterious effects of a drug on the cell monolayer rather than on the organism itself, time-consuming organism enumeration, lack of information on the viability of the organisms, and 5- to 7-day assay length. In most cases, methodical viability assays have not been performed in conjunction with microscopic enumeration of organisms. Dead organisms can maintain tinctorial staining properties during the relatively brief culture period and thus contribute to growth estimations (19). Although cell-free systems permit assaying of a drug's effect directly on the organism, these systems suffer from similar problems of limited replication of organisms and lack of viability assessment. A basic question remains as to the validity of assessing effects of drugs on organisms under suboptimal conditions.

Alternative approaches to screening of anti-*P. carinii* drugs have taken advantage of nonreplicating organisms by evaluating the effects of drugs on enzymes (e.g., dihydrofolate reductase [1]) or on metabolism by lysosomotropic vital dyes (e.g., neutral red [36]) or by incorporation of radiolabeled precursors and compounds (e.g., *para*-aminobenzoic acid [15]). It is our contention that without a continuous in vitro culture system, a suitable screening method should consist of a global assessment of metabolic function rather than a specific target.

An ATP-driven bioluminescence assay has been used for assessing *P. carinii* viability and the effects of drugs and other compounds on the ATP contents of *P. carinii* populations maintained ex vivo for short periods of time (11). ATP drives the reaction of the luciferin-luciferase enzyme-substrate system, resulting in evolution of light, the amount of which is directly related to the concentration of ATP present. As cells die, the amount of intracellular ATP rapidly decreases due to the action of ATPases. Thus, it is possible to assay the immediate effects of inhibitory compounds on organism populations without waiting to observe a decrease or stasis in organism number. Such a method is more similar to a cytoxicity assay than to an assay based on growth inhibition, e.g., MIC.

In the present study, the effects of compounds from several different classes of drugs on *P. carinii* populations were evaluated with the ATP-driven bioluminescence assay. A system for ranking the compounds was devised to facilitate selection of potential compounds for in vivo studies. A strong correlation

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was observed between those compounds that had been both evaluated with the ATP-driven cytotoxicity assay and tested in the rat model of *P. carinii* pneumonia. The cytotoxicity assay offers many advantages over other in vitro systems. It is semiautomated, which alleviates the need for manual counting of organisms and eliminates the possibility of reader bias. It is rapid and reproducible and does not rely on the need for replicating organisms. This assay should permit rapid evaluation of structure-function relationships for analog series, leading to the identification of novel anti-*P. carinii* therapies.

MATERIALS AND METHODS

Sources and preparation of *P. carinii*. *P. carinii* organisms were obtained from two different models of infection. In the first model, *P. carinii* pneumonia was induced by intratracheal inoculation of viral-antibody-negative rats with characterized prototype form 1 *P. carinii* (8, 21). CD rats from a Charles River (Hollister, Calif.) colony were inoculated with 1.0×10^7 to 2.5×10^7 *Pneumocystis carinii* nuclei after 2 weeks of immunosuppression via weekly subcutaneous (s.c.) injections of methylprednisolone (4 mg/kg) and were continued on this regimen for an additional 6 to 10 weeks. All inoculated rats were maintained under strict barrier conditions consisting of shoe box cages fitted with microisolator tops, placed on horizontal flow hoods, and handled aseptically by gowned and gloved personnel. Uninoculated, immunosuppressed animals sacrificed after all other animals were used in the studies served to verify the *P. carinii*-free status of that particular shipment of CD rats. In the second model of infection, *P. carinii* pneumonia was acquired naturally by chronic administration of methylprednisolone (4 mg/kg, s.c.) to Brown Norway or Long Evans rats housed under standard conditions, without barriers and with free exchange of air, at the Cincinnati Veterans Affairs Veterinary Medical Unit. This rat colony has been previously shown to harbor coinfections with form 1 prototype *P. carinii* and variant *P. carinii* (*P. carinii* f. sp. *carinii* and *P. carinii* f. sp. *ratti*, respectively) or single infections produced by either of the two *P. carinii* populations (22).

P. carinii organisms were released from the infected rat lungs by mincing the tissue in RPMI 1640 medium (Gibco BRL, Life Technologies, Inc., Grand Island, N.Y.) supplemented with 20% fetal bovine serum (FBS) (Sigma Chemical Company, St. Louis, Mo.) followed by homogenization for 5 min with a Stomacher 80 Lab Blender as described in our previously published report on the ATP-driven bioluminescence assay (11). The resultant homogenates were sieved through sterile gauze to remove large particulates, treated with 0.85% aqueous ammonium chloride (pH 6.8) for 30 min at 37°C to lyse erythrocytes, washed in RPMI 1640 supplemented with 20% FBS, and centrifuged at $1,000 \times g$ for 20 min to collect the cell pellet. Gravity sedimentation for 30 min at 37°C in RPMI 1640 supplemented with 30% FBS and subsequent low-speed centrifugations ($400 \times g$) were used to reduce host cell debris. The supernatants were collected, pooled, and centrifuged at $2,400 \times g$ for 20 min to pellet the *P. carinii* cells. The pellet was resuspended in 10 ml of the RPMI 1640 with 20% FBS, and the numbers of organism nuclei and host cells per milliliter were enumerated by microscopic analysis of Diff-Quik-stained slides (17). A small sample of the homogenate was evaluated for viability by the dual-fluorescence staining method, which involves the use of calcein acetoxymethyl ester and ethidium homodimer (32). Although this staining method has not proven useful for drug assays, it appears to be the best method for evaluation of the viability of untreated *P. carinii* (34). Preparations with less than 85% viability were not used in the cytoxicity assay.

Cytotoxicity assay. After enumeration, organisms were added to 24-well plates containing 1 to 2 ml of RPMI supplemented with 20% FBS, vitamins, minerals, and other additives (11) at a standard density of 10^8/ml with or without a test compound. At least three concentrations of each compound were evaluated (10, 1.0, and 0.1 μg/ml) by incorporation into the medium. Triplicate wells were used for each concentration of test compound evaluated and for control groups. Each drug was evaluated at least two times with different *P. carinii* preparations. If compounds were not miscible in aqueous solutions, the vehicle used was also evaluated for direct effects on the organism and for quenching of the luciferin-luciferase reaction. The diluents used in these assays were dimethyl sulfoxide and ethanol, neither of which exceeded 0.2% (vol/vol). The highest concentration of a test compound was assessed for quenching of the bioluminescence reaction. For determination of quenching, the drug or vehicle was added to a sample tube in the series of tubes containing 10^{-8} M ATP for standardization of the assay. Quenching would be observed as a decrease in light output compared to that of untreated tubes. None of the compounds tested in the series directly inhibited this reaction. To sample the wells, 1/10 volume was withdrawn from each well 24, 48, and 72 h after inoculation and extracted with 3.5% trichloroacetic acid (TCA) to release cytoplasmic ATP pools, and 10 μl of the extract was added to the luciferin-luciferase monitoring reagent to measure the light evolved (11). To stabilize the pH and ATP levels of the *P. carinii* populations maintained ex vivo, 60% of the culture medium was removed on a daily basis and replaced with fresh medium containing the compound being tested after centrifugation ($1,000 \times g$) of the entire multiwell plate by use of a plate adapter. This prevented reduction of *P. carinii* viability due to a change in pH and ensured daily exposure to the test drug without its dissipation from the culture medium. Besides the vehicle control, a series of control compounds were included in every in vitro assay. Pentamidine isethionate (500 to 1,000 ng) functioned as the positive control, and ampicillin (10 μg/ml) served as a negative control. As previously described, pentamidine dramatically reduces the ATP contents of *P. carinii* populations (>90%) in this concentration range, while the organism populations are unaffected by ampicillin at 10 μg/ml, the highest concentration at which any drug is tested.

In some cases, organism number was calculated by manual counting of stained nuclei during the assay time period (17).

Assessment of drug activity. Ten-microliter aliquots of the TCA extracts were placed in 940 μl of Tris-acetate buffer (0.1 M Tris-acetate, 2 mM EDTA, pH 7.75) and measured with an AutoLumat LB 953 luminometer (Wallac Inc., Gaithersburg, Md.) after automatic injection of 50 μl of the ATP monitoring agent (luciferin-luciferase, 50 mg of bovine serum albumin, 0.5 mmol of magnesium acetate, 0.1 μmol of inorganic pyrophosphate). For each sample, the sum integral of the detected light emission (in relative light units [RLU]) is output as a result. The molar ATP concentration was calculated according to the following formula: $[(RLU_S - RLU_B)/RLU_I] \times$ moles of ATP standard added, where S = sample, I = internal calibration, and B = background light emission. At least 10 separate reactions are run to standardize and calibrate the instrument prior to each usage.

The ATP contents of *P. carinii* populations in wells containing experimental compounds or vehicle controls were compared to that of untreated *P. carinii* at the same time point, expressed as percentages of the control, and graphed against the log of the drug concentration to calculate the concentration at which the ATP content was decreased by 50%, i.e., the 50% inhibitory concentration (IC_{50}) (23). The effects of the drugs on the *P. carinii* populations followed the same trend over the 3-day testing period, and the IC_{50}s were based on the 24-h time point, with the exception of the sulfa compounds. These drugs were found to exert their effects after a 24-h incubation period, and so the IC_{50} was based on the 48-h time point for this series.

Agents evaluated for *P. carinii* cytotoxicity. Filter-sterilized aqueous or solvent-based stock solutions (1 mg/ml) were prepared immediately prior to use in the assays. At least three concentrations of each compound were evaluated in the supplemented RPMI 1640 medium: 0.1, 1.0, and 10.0 μg/ml. Some compounds, e.g., pentamidine, were further titrated.

The following compounds were obtained from Sigma Chemical Company: acridine hydrochloride; albendazole [methyl-5-(propylthio)-2-benzimidazolecarbamate], amantadine (adamantanamine hydrochloride), amsacrine [4-(9-acridinylamino)-*N*-(methanesulfonyl)-*m*-anisidine], AZT-glucoronide (3′-azido-3′-deoxythymidine β-D-glucoronide), amphotericin B, ampicillin (sodium salt) [D(-)-α-aminobenzylpenicillin], antimycin A, camptothecin, cholera toxin, clindamycin hydrochloride [7(*S*)-chloro-7-deoxylincomycin), chloroquine (diphosphate salt), chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl)phenothiazine], dapsone (diaminodiphenyl sulfone), diaveridine ([5-(3,4-dimethoxyphenyl) methyl]-2,4-pyrimidinediamine), diminazine aceturate (Berenil), 2,4-dinitrophenol, ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide), etoposide, euflavin hydrochloride, foscarnet (phosphonoformic acid [trisodium salt: hexahydrate], gentamicin sulfate, G418 (geneticin; disulfate salt), hygromycin B, imipramine hydrochloride [10,11-dihydro-*N*-methyl-*N*-(methyl-d_3)-5H-dibenz(b,f)azepine-5-propanamine], isoniazid (isonicotinic acid hydrazide), mebendazole (5-benzoyl-2-benzimidazolecarbamic acid methyl ester), minocycline hydrochloride, ofloxacin, oligomycin B, pertussis toxin, DL-phosphinothricin [glufosinate, bialophos; methyl(homoalanin-4-yl)phosphinic acid], primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinolone], pyrimethamine [5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidine diamine], sulfadiazine (sodium salt) (4-amino-*N*-2-pyrimidinylbenzenesulfonamide), sulfamethoxazole [4-amino-*N*-(5-methyl-3-isoxazolyl)benzenesulfonamide], thiabendazole [2-(4-thiazolyl)benzimidazole], trifluoperazine dihydrochloride, and trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine].

The remaining compounds were obtained from the following vendors and laboratories: LyphoMed, Inc., Melrose Park, Ill. (pentamidine isethionate); the National Cancer Institute (through Chris Lambros, National Institutes of Health) (the NSC series and acedapsone); Steve Meshnick, University of Michigan, Ann Arbor (gifts of salicylhydroxamic acid [SHAM] and menoctone); Marion Merrell-Dow Research Institute, Strasbourg, France (gifts of DL-α-difluoromethylornithine and DL-α-difluoromethylarginine); Ben Yenue Laboratories, Inc. (trimetrexate); Fisher Scientific, Cincinnati, Ohio (potassium cyanide); and Hoffman-LaRoche, Nutley, N.J. (sulfadoxine).

RESULTS

The responses of *P. carinii* populations to over 60 compounds were evaluated by the cytotoxicity assay based on an ATP-driven bioluminescence method. Standard anti-*P. carinii* drugs (e.g., pentamidine), metabolic inhibitors (e.g., SHAM), and representatives of 18 classes of compounds were tested. The results, expressed as IC_{50}s after 24 h, were assigned activ-

TABLE 1. Comparison of ATP-driven cytotoxicity assay responses with in vivo activity

Drug class	Activity in cytotoxic assay[a]	IC_{50} (ng/ml)	In vivo immunosuppressed rat model activity[b] and reference(s)
Acridines			
Euflavine	Marked	105.0	ND[c]
Acridine	Marked	297.0	ND
Aminoquinolones			
Primaquine	None	>10,000	None 6, 31, 38, 39
Chloroquine	None	>10,000	None 31
Antibiotics			
Ampicillin	None	>10,000	None 45
Clindamycin	None	>10,000	None 39, 45
Gentamicin	None	>10,000	None 45
G148	None	>10,000	ND
Hygromycin B	None	>10,000	ND
Minocycline	None	>10,000	ND
Ofloxacin	None	>10,000	None 9
TMP-SMX	Marked	104.4	Very marked 45
Antifungal agent amphotericin B	None	>10,000	None 29, 45
Antiparasitic agents			
Albendazole	None	>10,000	—[d]
Mebendazole	None	>10,000	ND
Thiabendazole	None	>10,000	None 45
Antipsychotics-antidepressants			
Trifluoperazine	Marked	153.0	ND
Imipramine	Marked	184.4	None 46
Chlorpromazine	Marked	173.5	None 45
Antiviral agents			
AZT	None	>10,000	None 45
Acyclovir	None	>10,000	None 45
Foscarnet	None	>10,000	ND
Amantadine	None	>10,000	Slight 45
DHFR inhibitors			
Diaveridine	None	>10,000	None 45
Pyramethamine	None	>10,000	None 45
Ro 11-8958	None	>10,000	None 45
Trimetrexate	None	>10,000	Slight 45
TMP	None	>10,000	None 45
Diamidines			
Diminazine	Marked	656.0	Marked 45
Pentamidine	Marked	293.0	Moderate 45
Metabolic and miscellaneous inhibitors			
SHAM	Moderate	1,355	ND
Isoniazid	None	>10,000	None 45
DL-Phosphinothricin	None	>10,000	ND
Nitrofuran furazolidone	None	>10,000	None 45
Phenanthridine ethidium bromide	Marked	110.0	None 45
Polyamine biosynthesis inhibitors			
DFMO	None	>10,000	None 45; moderate 12, 14
DFMA	None	>10,000	ND
Quinones			
Menoctone	Marked	560.3	ND
NSC 112922	Marked	120.23	ND
NSC 92203	Marked	121.0	ND
NSC 92074	Marked	213.8	ND
NSC 113452	Moderate	1023.2	ND
NSC 117252	Moderate	858.0	ND
NSC 108700	Moderate	1446.0	ND
NSC 306389	None	>10,000	ND
Respiratory inhibitors			
Antimycin A	Marked	312.7	ND
2,4-Dinitrophenol	Marked	331.3	ND
Oligomycin B	None	>10,000	ND
Potassium cyanide	Very marked	11.97	ND
Sulfonamides-sulfones			
Sulfadiazine	Marked	—[e]	Very marked 45
Sulfadoxine	Moderate	2,407	Very marked 45
Dapsone	Marked	737.0	Very marked 31, 45
SMX	Marked	356.0	Very marked 45
Acedapsone	None	>10,000	ND
Topoisomerase I and II inhibitors			
Camptothecin	Very marked	68.8	ND
Amsacrine	Marked	334.8	ND
Etoposide	Marked	743.1	Active[f]
Toxins			
Cholera toxin	None	>10,000	ND
Pertussis toxin	None	>10,000	ND

[a] Activity in the ATP assay was ranked according to the IC_{50} of the compound: very marked, <100 ng/ml; marked, 100 to 999 ng/ml; moderate, 1,000 to 9,999 ng/ml; or none, >10,000 ng/ml.

[b] Activity in the immunosuppressed rat model of *P. carinii* pneumonia was ranked according to the system of Walzer (45): very marked, ≥1,000-fold reduction in cyst counts of homogenized lungs from treated rats versus counts in untreated rats; marked, 100- to 999-fold reduction; moderate, 10- to 99-fold reduction; slight, 5- to 9-fold reduction; or none, <5-fold reduction.

[c] ND, not determined.

[d] Albendazole was tested in an intratracheally inoculated mouse model of *P. carinii* pneumonia and found to inhibit the infection (7).

[e] Decreases in ATP pools were greater than 50% at all concentrations evaluated, and an IC_{50} could not be calculated.

[f] An analog of etoposide, teniposide, was tested in these studies (28). The method of assessment of infection level, semiquantitative evaluation of lung touch preparations, could not be correlated with that of the quantitative cyst counts of Walzer (45) and was thus simply noted as active.

ity ranks based on the decrease in ATP and are summarized in Table 1.

Both TMP-SMX and pentamidine effectively reduced the ATP content at nanogram concentrations (IC_{50}s of 104 and 293 ng/ml, respectively). In contrast to pentamidine and TMP-SMX, ampicillin and several other compounds were ineffective at reducing the ATP pools of *P. carinii*, even at the highest concentration of 10 μg/ml. Several classes of compounds markedly reduced the ATP pools of *P. carinii* populations. All of the compounds evaluated in the acridine, antipsychotic-antidepressant, diamidine, and topoisomerase inhibitor groups were active at concentrations below 1,000 ng/ml. Most of the compounds in the quinone, respiratory inhibitor, and sulfonamide groups likewise exerted a moderate to very marked effect on ATP pools. Some individual compounds were active at lower concentrations, while other members of the same class were

TABLE 2. ATP content and number of *P. carinii* nuclei exposed to etoposide[a]

Time (h)	No. of nuclei/ml (10^8)		ATP content (fmol/10^8 cells/ml)	
	Untreated *P. carinii*	Etoposide-treated *P. carinii*	Untreated *P. carinii*	Etoposide-treated *P. carinii*
24	1.90	1.70	83.70	30.40
48	1.72	1.07	64.20	15.00
72	5.13	1.30	126.50	1.20

[a] Medium was replenished every 24 h. The initial mean density of *P. carinii* was 1.00×10^8/ml. Etoposide was added to the medium at a concentration of 1 μg/ml.

ineffective. TMP-SMX was the only active member in the antibiotic class. SHAM exerted a moderate effect, while the other metabolic inhibitors had no effect. Ethidium bromide, the only phenanthridine tested, showed a marked reduction of ATP pools.

The majority of compounds evaluated were found to be ineffective against *P. carinii*. The two aminoquinolones tested, primaquine and chloroquine, were evaluated individually and found to produce no effect on ATP pools at the concentrations assayed. Likewise, *P. carinii* populations failed to respond to all single-component antibiotics, amphotericin B, the antiparasitic drugs, the antiviral agents, the dihydrofolate reductase inhibitors, isoniazid, DL-phosphinothricin, furazolidone, the polyamine biosynthesis inhibitors, NSC 306389, oligomycin B, acedapsone, and the cholera and pertussis toxins.

Generally, there was good correlation between the in vitro results and the available in vivo data. However, in some cases, the in vitro assay detected cytotoxic effects for some compounds that were not effective in the animal models of the pneumonia. These compounds were members of the antipsychotic-antidepressant class and ethidium bromide. In contrast, a few compounds did not reduce the ATP pools of *P. carinii* but were found to reduce the organism burden in the animal model. Trimetrexate and amantadine had slight effects on the pneumonia in vivo but no effects in vitro.

The Merrell-Dow compounds DFMO and DFMA did not reduce ATP pools of *P. carinii* populations. These agents are polyamine biosynthesis inhibitors; DFMA is thought to target pathways unique to plants or trypanosomes. The efficacy of DFMO against *P. carinii* pneumonia in the animal model and in human beings has been controversial, with some studies showing a reduction in organism burden (12, 14) and others revealing apparent toxicity and a lack of therapeutic effects (45).

Two classes of compounds that appeared to be quite active in vitro were the quinones and the topoisomerase inhibitors. A previous study reported that an analog of etoposide, teniposide, was efficacious against the infection in vivo (28). Topoisomerase inhibitors are thought to exert their effects at the replication forks as the enzymes relax the DNA strands prior to transcription. Since the organisms held in our in vitro assay were assumed to replicate only at low levels, the action of these compounds on *P. carinii* populations was investigated further by enumeration of organisms. As shown in Table 2, a modest increase in organism number occurred over the 72-h assay period, with a concomitant small increase in ATP levels. Treatment with etoposide dramatically decreased the ATP levels (by >60% after 24 h, >80% after 48 h, and >99% after 3 days of exposure). Treatment with etoposide did not decrease organism number but rather maintained it at inoculum levels. This study underscores the need for a viability assay concomitant with microscopic analysis of organism number. It is difficult to discern from the number of *P. carinii* alone whether the drug is a cytostatic one or the organisms are killed and yet retain the capacity to be stained.

A time course study with pentamidine was conducted to observe the response time of this standard anti-*P. carinii* therapy on the ATP pools. Although the precise mechanism of action of pentamidine is not known, some evidence indicates that inhibition of topoisomerases is involved (25). In the study whose results are shown in Table 3, *P. carinii* populations were exposed in vitro to 100 ng of pentamidine per ml for up to 3 days. Samples were taken at early time points to evaluate the onset of response as determined by ATP levels. A 50% inhibition was achieved by ~4 h. When *P. carinii* were enumerated after exposure to pentamidine for 72 h, a decrease in number from inoculum levels was not observed (data not shown), again reinforcing the need for a concomitant viability assay.

DISCUSSION

The in vitro assay presented here differs from all previous drug evaluation systems for *P. carinii* in the brevity of the assay period, the target of assessment, and quantitative measurement. The evaluation time period, 24 to 72 h, was chosen because the organism populations could reliably maintain their ATP pools for this length of time. The medium was changed every day, affording continuous exposure to the test compounds. Short-term assays have been used to screen compounds on parasitic protozoa (23), as well as for evaluation of cytotoxic and cytostatic chemotherapeutic agents on cancer cell lines (33, 40). The choice of ATP level as a parameter for assessment provides an exquisitely sensitive indicator of the effect of an agent on the overall metabolism of the organism. This in turn eliminates the need for replicating organisms, which even in the best of circumstances rarely increase more than five- to sevenfold in number in any in vitro system. The addition of instrumentation (a luminometer) to evaluate the responses of the organism populations provides an objective method for measurement and reduces the reliance on tedious manual counting methods. Due to the short assay period and the measurement of overall cellular metabolism, the ATP assay presented here is more similar to the cytotoxic studies performed on cancer cell lines than to the standard microbial evaluations reliant upon growth inhibition (40).

The 29 evaluable cases (Table 1) which had both in vitro and in vivo data were analyzed for predictive values, where *a*, *b*, *c*, and *d* were the numbers of test results corresponding to activity detected by both the in vitro and in vivo assays, no activity detected by either assay, activity detected in vitro but not in vivo, and no activity detected in vitro but activity detected in vivo, respectively. Analysis resulted in a 73% true-positive rate

TABLE 3. Time course of in vitro response of *P. carinii* populations to pentamidine

Time (h)	Response of pentamidine-treated *P. carinii* (fmol of ATP/10^8 *P. carinii* nuclei)	% Decrease vs untreated *P. carinii*
0	41.0	0
0.5	41.2	0
1	34.8	15
2	32.5	21
3	27.1	34
4	22.3	44
5	19.9	51
6	21.4	48
7	20.4	50
24	17.4	57
28	11.5	72
72	2.5	94

(a/a + *c*) and an 89% true-negative rate (*b*/*b* + *d*), with a specificity of 84% (*b*/*b* + *c*) and a sensitivity of 80% (*a*/*a* + *d*) (40). Deletion of the data for the two cases in which no in vitro effect was observed and only a slight effect on organism burden was detected (i.e., amantadine and trimetrexate) increases the true-negative rate to 100% and the sensitivity to 100% while the other values remain the same. These values are comparable to those reported for other in vitro screening assays that focus on assessment of large numbers of compounds. For example, the Ames test is a standard method used to assess the mutagenic potential of a wide variety of agents. Depending on the modification of the assay used, the correlation with animal studies ranged from 63 to 92% (10).

Examination of the activities of individual classes of compounds was also revealing. Dihydrofolate inhibitors alone had no effect on the organisms, supporting the concept that SMX is the more active drug against *P. carinii* in this combination. However, a difference between the SMX IC_{50} value, 356 ng/ml, and the value for the TMP-SMX combination, 104.4 ng/ml, was detected, suggesting a role for TMP in the observed reduction of ATP. Importantly, the effective concentrations of test compounds were physiologically appropriate. For example, the maximum concentrations in the plasma of patients treated with pentamidine parenterally have been reported to range from 164 to 1,360 ng/ml (16, 24, 35), while for patients receiving aerosolized pentamidine, levels of pentamidine in bronchoalveolar lavage specimens varied widely (26 to 1,200 ng/ml) depending on the length of treatment (41). The ~300 ng/ml required to reduce the ATP pool of rat *P. carinii* by 50% is well within these ranges.

The respiratory chain inhibitor potassium cyanide dramatically decreased the ATP in the *P. carinii* populations (IC_{50}, ~12 ng/ml). Other respiratory inhibitors, i.e., antimycin A and 2,4-dinitrophenol, were also effective at low concentrations. Ethidium bromide has also been reported to target yeast mitochondria by induction of petite mutants, which are deficient in respiration (27). Ethidium bromide had a marked ability to reduce the ATP pools of organism populations but was quite toxic in the animal model of infection, with little apparent reduction of organism burden at the concentrations used (45). The ATP synthase inhibitor oligomycin B did not decrease ATP pools, indicating that *P. carinii* may be resistant to this compound. Since host cells and other yeasts are sensitive to this antibiotic (30), the lack of activity observed is indicative of the purity of the *P. carinii* preparations. Had contaminating cells contributed to the ATP pool, a reduction in the ATP level would have been evident. In spite of this single exception, targeting of *P. carinii* mitochondria seems to be a useful approach for identification of new therapies.

SHAM (without glycerol) exerted a moderate decrease in ATP. This compound targets an enzyme, *sn*-glycerol phosphate oxidase, in a pathway unique to African trypanosomes (13). SHAM induces anaerobic conditions in these parasites by inhibiting the oxidation of NADH. Addition of glycerol to the medium containing the bloodstream trypanosomes results in lysis due to inhibition of a compensatory enzyme, glycerol kinase, and total loss of ATP synthesis. The effect of this compound on *P. carinii* may be indicative of the existence of a similar pathway, and this line of study could be pursued to provide further insights into the metabolism of *P. carinii.*

A compound targeted at a unique pathway thought to exist in *P. carinii* due to cloning of genes specific to this pathway did not decrease ATP pools. The herbicide phosphinothricin (glufosinate, bialophos) is thought to inhibit an early step in the shikimic acid pathway. We reasoned that this compound might be inhibitory to *P. carinii* since the 5-enolpyruvyl shikimate phosphate synthase gene has been cloned from *P. carinii* (3). Other compounds shown to exhibit antifungal properties, e.g., hygromycin, were also ineffective at decreasing ATP pools of organism populations. Cholera and pertussis toxins, which have been shown to target G proteins in other cells, did not affect *P. carinii.*

Evidence for use of the ATP-driven cytotoxicity assay for candidate drug selection was provided by the group of quinone analogs obtained from the National Institutes of Health, the NSC series. A structure-function response was apparent, as three of the seven drugs were as effective at reducing ATP pools as pentamidine; three showed a moderate decrease, while one compound had no effect on the *P. carinii* populations. Like the topoisomerase inhibitors, compounds of this class offer alternative therapies to be studied in the animal models.

Topoisomerase inhibitors are thought to exert their inhibitory action during the S phase of the cell cycle and prevent these enzymes from unwinding the DNA during replication. It is clear from the ATP-driven cytotoxicity assay that the members of this class of compounds could dramatically decrease the ATP pools of *P. carinii*, suggesting that active replication is occurring or that these inhibitors also affect other metabolic functions. Similar findings were reported in a previous study (28). Amsacrine and etoposide were found to inhibit the growth of *P. carinii* in a cell monolayer-based in vitro assay at higher concentrations, 10 and 1 μg/ml, respectively (28). In the same study, an analog of etoposide, teniposide, was found to have some activity against the infection when evaluated by a semiquantitative scoring system. Investigation of the in vitro conditions used in the present studies showed a modest increase in organism number which did not appear to account for the dramatic reduction in ATP pools, suggesting that there may be an alternative effect of these compounds in this assay or on the organisms. Further investigation of this class of compounds seems warranted, and with the current ATP-driven cytotoxicity assay, structure-function testing could occur at a rapid pace with reduced compound quantities.

Likewise, the action of pentamidine on *P. carinii* populations appears to involve more than inhibition of topisomerases, since a reduction in ATP pools was observed soon after exposure of the organisms to the drug. It is conceivable that pentamidine has an immediate effect on ATP biosynthesis or that it causes an increase in ATP degradation followed by inhibition of the topoisomerases.

There may be compounds that cannot be detected by the cytotoxicity assay, such as agents targeted to interrupt the adhesion of the organism to host cells. A monolayer-based system in which adherence of the organism to the cells has been documented (e.g., A549 cells) (17, 19) would then be needed to assess the effects of the antibodies or other compounds blocking adhesion on organism growth or replication. It is also possible that agents targeting replication would not be identified by this assay, although camptothecin and other topoisomerase inhibitors were found to decrease ATP pools.

It is likely that a continuous culture system for *P. carinii* will not be found until further breakthroughs are made in the understanding of the life cycle and metabolic capacities of this organism. It is our contention that a global assessment of viability, e.g., ATP content, is a better approach for screening drugs than enumeration of organisms or use of a specific enzyme or target molecule. Results from the ATP-driven cytotoxicity assay showed a strong correlation with in vivo activity in the rat model of *P. carinii* pneumonia. With a viability indicator (ATP level) already available, we are able to discern any discrepancies between microscopic counts of the *P. carinii*

organisms and viability of the *P. carinii* populations. Compared with microscopic enumeration, the response of the compounds can be assessed within hours. For example, we found that pentamidine begins to decrease the ATP pools of *P. carinii* populations within 1 h of exposure. This rapid response time can be exploited to reduce the length of assay and ultimately should be applicable to evaluation of drugs against clinical *P. carinii* isolates. The use of multiwell plates reduces reaction volumes and the need for large numbers of *P. carinii* while decreasing the time needed for setup, sampling, and analysis. Addition of the quantitative measurement of ATP by instrumentation for assessment of organism responses reduces the need for tedious microscopic counting of organisms, which in turn eliminates operator bias, and offers a method that should be directly comparable among laboratories.

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