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Perspective

New Drug Developments for Opportunistic Infections in Immunosuppressed Patients: *Pneumocystis carnii*

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Introduction

Widespread organ transplantation programs, aggressive chemotherapy of cancer, and the AIDS epidemic have created large numbers of immunosuppressed patients who are at increased risk from opportunistic infections, including those caused by fungi. Fungal infections in AIDS patients include candiasis, cryptococcosis, histoplasmosis, and others, but the most common is pneumonia caused by *Pneumocystis carinii*. Reports of *P. carinii* pneumonia in the pre-AIDS era were sporadic, with fewer than 100 cases reported yearly in the United States,[1] but with the AIDS epidemic, the number of cases of *P. carinii* pneumonia in the United States has been estimated at 50 000 annually, and *P. carinii* pneumonia has been the leading cause of death in AIDS patients in this country.[2] Even with widespread prophylaxis for *P. carinii* pneumonia, the disease is still common in AIDS patients.[3] Because of the seriousness and prevalence of disease caused by *P. carinii*, this review will focus on that organism.

P. carinii was originally discovered in 1909, but was misidentified as a form of *Trypanosoma cruzi*. Although it was later recognized as an independent organism, its exact taxonomic position remained unsettled for 80 years.[4] When modern techniques were applied, *P. carinii* was found to be related more closely to fungi than to protozoans.[3] The full life cycle of the organism is still unclear. Horizontal transmission by the airborne route occurs in animals and probably occurs in humans.[5]

Pathophysiology of *Pneumocystis carinii* Pneumonia. *P. carinii* is an extracellular pathogen that attaches to type 1 pneumocytes, damaging those cells and increasing alveolar capillary permeability. Histologically, the alveoli appear to fill with foamy exudate; mild interstitial pneumonitis is characterized by a corresponding degree of interstitial chronic inflammation, as well as proliferation of type II pneumocytes.[6] Fibrosis is common. Surfactant phospholipids are also altered by the disease, changing the surface tension of the alveolus. These effects of infection progressively impair gas exchange. Clinically, patients with pneumonia caused by *P. carinii* experience increasing difficulty breathing as the disease progresses and more alveoli become blocked with masses of *P. carinii* organisms and the characteristic foamy exudate.[7] Fevers and nonproductive cough are common but pulmonary examination often fails to reveal signs sufficient to establish a diagnosis. Most infections in humans are restricted to pulmonary sites, but disseminated or extrapulmonary infections are possible.[8]

P. carinii pneumonia follows various clinical courses, depending upon the age and immune status of the patient.[7,9] Before 1950, *P. carinii* was a rare cause of clinical disease, mostly in young children. Malnourishment was recognized to predispose patients to *P. carinii* pneumonia.[10] Early reports in children described a disease with a slow, subtle onset characterized by mildly increased respiration rates and poor appetite in its early stages; respiratory distress increased after 7–14 days and about one-fourth of the patients died if untreated.[9] Recovery took 4–6 weeks. The entire disease process could occur without fever. In adults without HIV who develop *P. carinii* pneumonia, nearly all have genetic or drug-induced immunosuppression.[7] These patients and immunosuppressed children develop severe *P. carinii* pneumonia much more rapidly than AIDS patients and are less likely to show interstitial fibrosis and severe alveolar exudate.[7] Among highly immune sup-

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pressed adults or children, mortality of untreated *P. carinii* approaches 100%.[9]

Models for Evaluating Drugs against *Pneumocystis carinii*. Development of drugs to treat *P. carinii* infections has been difficult because models for drug testing are limited. The organism does not grow in continuous culture and does not produce significant infections in animals with intact immune systems. At the start of the AIDS epidemic, the only model of *P. carinii* that had been used to bring drugs into clinical application was an animal model,[11] which was based on early observations that rats immunosuppressed with steroids would spontaneously develop *P. carinii* pneumonia over a period of 3 to 15 weeks.[12,13] If drugs were administered concomitently with immunosuppression, *P. carinii* pneumonia could be prevented in a substantial fraction of animals; this model is therefore a prophylaxis model based upon latently infected rats. The same latently infected rats can be used in a therapy model by immunosuppressing 4–7 weeks before drug treatment is started; therapy may run 14–30 days, with immunosuppression continuing throughout the study.

Latently infected rats from different sources carry *P. carinii* that vary significantly in karyotype.[3] The introduction of an inoculated animal model allowed researchers to propagate a single variant of *P. carinii* and opened up possibilities for assessing biological properties of karyotypically different isolates. The inoculated model was applied both to rats and mice, and both therapy and prophylaxis protocols have been performed.[14–16]

All of the rodent models mentioned above use corticosteroids to immune suppress the animals and allow *P. carinii* pneumonia to develop. A more specific model for immune suppression uses antibody directed to T4 helper cells, creating a mouse model in which animals are specifically depleted of the same cells that are depleted in AIDS patients.[17,18] Nude mice have also been used as models for *P. carinii* pneumonia, but take substantially longer to become infected and infections are generally lighter than with inoculated models.[19]

Drugs have been tested in each of the protocols described above, and evaluation of effect of therapy has included counts of cyst forms of *P. carinii* in lung, counts of trophozoites and cysts in lung, histological grading of lung tissue, and quantitation by DNA probe for trophozoites.[11,12,20–26] No standard method exists in the field but in a direct comparison of the inoculated rat model and a latently infected rat model, similar responses to tested drugs were observed.[27]

All animal models for *P. carinii* pneumonia take weeks and require large quantities of drug. For this reason, many of the first drugs tested were clinical agents already in use for other diseases. In order to explore larger numbers of experimental compounds that were available in small quantities, a number of rapid, higher output systems have been developed. Continuous culture under axenic conditions (i.e., culture of *P. carinii* without coculture of other cells) is not yet possible with this organism, but short-term culture in association with mammalian cells has been used as a model for testing drugs.[21,28–33] Organisms harvested from rats have also been maintained in short-term culture and used in assays to test effects of drugs upon uptake of PABA[34,35] or upon incorporation of radiolabeled amino acid into *P. carinii*-specific proteins.[36] Incubation was for 24 h with incorporation of radiolabeled methionine and 18 h for PABA incorporation; therefore, only rapidly effective agents tend to be active. In contrast, assays based on culture models run up to 10 days and can show effects of more slowly acting agents.

The ability of the various models to predict activity of compounds in humans as been debated. Agents with demonstrated activity against a specific target may fail culture or animal tests because the cell wall and membrane may prevent a drug from reaching its target. In animals, poor pharmacokinetics may prevent activity from being seen. In spite of these caveats, the rodent models of *P. carinii* pneumonia predicted the activity of all the currently used clinical agents; these agents have also shown activity in one or more of the in vitro screens. In the discussion that follows, classes of compounds active against *P. carinii* pneumonia will be discussed, along with the rationale for their selection for testing and comments upon the models in which effectiveness has been demonstrated.

Agents Acting on the Folate Pathway

Rationale for Use for Antifolates. Many microorganisms, including *P. carinii*, cannot take in preformed folates from their environment and therefore must synthesize folates from *p*-aminobenzoic acid, glutamic acid, and a pterin formed from GTP.[35] In contrast, mammalian cells lack the folate biosynthetic enzymes and cannot synthesize folate de novo; therefore mammals must take folate from dietary sources. This fundamental difference in metabolism creates potential targets for species-specific effects. Of these biosynthetic enzymes, dihydropteroate synthase has been most fully exploited as a target for chemotherapy; it is specifically inhibited by sulfonamide antibacterial and antiprotozoal agents.

A second target enzyme is dihydrofolate reductase, which converts dihydrofolate to tetrahydrofolate with the oxidation of NADPH to NADP. Because dihydrofolate reductase functions in folate metabolism in mammals as well as in microorganisms, it might seem to be an unlikely candidate for a selective drug target; however, this enzyme displays exploitable variability in form and function in different organisms.[37,38] On the basis of this variability, researches have developed compounds that preferentially inhibit the enzyme from pathogenic organisms. For example, trimethoprim (1) inhibits bacterial dihydrofolate reductase at concentrations at least four logs below those required to inhibit the mammalian enzyme.[38]

The effects of antifolates on *P. carinii* were discovered empirically in experiments that often included many different types of drugs. Sulfonamides were among the first agents tried against pneumonia caused by *P. carinii* but were generally ineffective when tested alone in the rare patients diagnosed with this condition.[39] The combination of two antifolates, pyrimethamine (**2**) and sulfadiazine, was shown in 1966 to be effective treatment or prophylaxis for *P. carinii* pneumonia in rat models.[12] The rationale for the combination was based upon earlier observations in *Toxoplasma gondii* and in *Plasmodium falciparum*, where sequential blockade of dihydropteroate synthetase and dihydrofolate reductase

by sulfonamides and pyrimethamine, respectively, had shown superior activity to either drug alone. Alone pyrimethamine or sulfadiazine were ineffective or marginally effective in rat *P. carinii* pneumonia, just as they had been in earlier human cases.[12] Pyrimethamine and sulfadoxine were used in 1971 at a 1:20 ratio for propylaxis of *P. carinii* pneumonia in infants;[40] this fixed combination called Fansidar is used for malaria. In 1974 a consistent effect of trimethoprim/sulfamethoxazole was demonstrated in propylaxis or therapy of rat *P. carinii* pneumonia; the choice of this drug combination was again because of superior activity observed against malaria.[11] Application of trimethoprim/sulfamethoxazole in humans quickly followed,[41] and therapy with these two venerable agents remains the standard for most patients today.[3,42]

Trimethoprim/sulfamethoxazole has been widely accepted as standard therapy for *P. carinii* pneumonia, but it is clear from the discussion above that the combination was selected because it was already in use for other diseases; its adoption did not come from attempts to select individual agents with ideal potency and selectivity toward *P. carinii*. In the next two sections, we consider the state of the art in developing antifolates specifically for *P. carinii*.

Inhibitors of Dihydropteroate Synthetase. Sulfonamides. All sulfonamides tested to date in animal models show some activity alone but require relatively high doses. Sulfadiazine alone at 120−160 mg/kg daily for an average of 29 days lowered the incidence of *P. carinii* pneumonia to 50% of control in a therapy model in rats;[12] however, at a dose of 250 mg/kg/day sulfadiazine was extremely effective in a 3 week treatment protocol for *P. carinii* pneumonia in rats, reducing the average number of *P. carinii* cysts from 800 million/lung to 0.7 million/lung, a 99.9% reduction.[43] In this same model sulfamethoxazole alone at 62.5 mg/kg/day reduced the number of cysts by about 99%; at 250 mg/kg/day the cyst counts were reduced more than 99.9%. The lowest dose at which an effect has been observed was 15 mg/kg daily.[23] Sulfadoxine tested alone at 250 mg/kg/day in this model also reduced cyst counts by 99.9%.[43] Acetylsulfisoxazole, tested at 300 mg/kg/day in a prophylaxis model in which rats received drug for 7 weeks from the start of immunosuppression, reduced the incidence of severe *P. carinii* pneumonia to 40% compared to 90% for control groups.[44]

Sulfonamides have shown variable potency against *P. carinii* in vitro. Sulfamethoxazole was a potent inhibitor of PABA uptake, with an IC50 value of 0.1 μM in the presence of 100 μM PABA.[34] Against isolated *P. carinii* dihydropteroate synthetase, sulfamethoxazole had an IC50 value of 0.7 μM and the K_i was 0.6 μM, making it the most potent of the sulfonamides tested.[45] The effectiveness of sulfamethoxazole in these two in vitro systems arises from structural similarities to PABA, but in two other in vitro systems sulfamethoxazole was much less effective. High concentrations (790 μM) of sulfamethoxazole were required to inhibit *P. carinii* growth in culture (Queener, unpublished data) or to inhibit incorporation of [^{35}S]methionine into *P. carinii* proteins (790 μM caused 64% inhibition).[36] Failure to deplete folate pools from *P. carinii* during the time of incubation may explain these results.

Sulfones. Dapsone and 4,4′-sulfonylbisformanilide (DFD) have been tested against *P. carinii*. Dapsone had an IC50 value of 1.5 μM when tested against isolated dihydropteroate synthetase[45] and was also effective at 0.4 μM against *P. carinii* in a culture model.[46] Dapsone at 10 μM had no effect on incorporation of [^{35}S]methionine into *P. carinii* proteins. The lack of effect of sulfones in this assay has been ascribed to the lack of folate depletion over the short time of incubation.[36] Variable results have been observed with sulfones in vivo. In a rat prophylaxis model, dapsone at 25 mg/kg daily completely prevented *P. carinii* pneumonia and at 5 mg/kg prevented the disease in 40% of rats.[22] In a rat therapy protocol, dapsone at 25 or 125 mg/kg in food reduced organisms to undetectable levels, but at 5 mg/kg *P. carinii* pneumonitis was still detected in half the animals;[22] others reported only a modest effect with 125 mg/kg dapsone daily.[47] DFD completely prevented *P. carinii* infection in a rat prophylaxis model at doses of 25 or 125 mg/kg daily; for therapy, 25 mg/kg daily lowered the numbers of rats with extensive disease to 12% of control.[48]

DFD has not been tested in humans, but dapsone has been used in several trials. Dapsone was effective for prophylaxis in humans and well tolerated at doses of <200 mg daily or intermittently.[49,50] Dapsone was less effective alone for therapy of *P. carinii* pneumonia in a small trial of seven patients, two of whom died while on dapsone.[51] Therapy trials in AIDS patients in which dapsone was used with trimethoprim were more successful, showing comparable effectiveness to the standard combination of trimethoprim/sulfamethoxazole and less serious toxicity.[52] Success of the trimethoprim/dapsone combination may arise in part from a pharmacokinetic interaction that increases serum levels of both drugs.[53]

Systematic Screening. Assays of dihydropteroate synthase directly in extracts of *P. carinii* are limited by the numbers of *P. carinii* that can be obtained.[45,54,55] A more systematic approach to screening sulfones, sulfonamides, and other PABA analogues would be possible if the target enzyme, dihydropteroate synthase, were more freely available. The gene encoding *P. carinii* dihydropteroate synthase has been cloned;[56] this folic acid synthesis (*fas*) gene contained an open reading frame from which a protein of 740 amino acids was predicted. Expression of the gene was not possible in *Escherichia coli* but was achieved in a baculovirus/insect cell system, although the expressed protein appeared on SDS polyacrylamide gel electrophoresis as 71.5 kDa/69 kDa products rather than the predicted 84 kDa. Activity of dihydropteroate synthase was observed under conditions where the 71.5 kDa/69 kDa polypeptide was overexpressed. The entire open reading frame is divided into four domains, in which the *fas D* domain must be expressed for dihydropteroate synthase activity, the *fas B* domain is associated with dihydropterin aldolase activity, and the *fas A* domain whose enzyme function is undiscovered must be expressed to maximize activities of products of the *fas B* and *fas D* domains. The *fas C* domain encoding 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase is less influenced by the other domains of the *fas* gene.[56]

The activities coded by the *fas* gene are required by *P. carinii* but are absent from mammalian cells. This obviously attractive set of targets for drugs of potentially

Table 1. Summary of Activity of Inhibitors against *P. carinii* Dihydrofolate Reductase (DHFR)

DHFR inhibitor	IC50, μM[a]	selectivity[b]	ref	K_i, nM[c]	selectivity[d]	ref
antibacterial						
trimethoprim	12–43	9.8–21	63, 64, 68, 70	152	1.3	66
tetroxoprim	>63	ND	Queener, unpublished			
epiroprim	3	200	63	17.4	3.4	66
(Ro11-8958)	2.6	28 (rat liver) 44 (human)	Queener, unpublished			
antiprotozoal						
pyrimethamine	2.8–13	0.6–0.9	64, 68, 70	9.7–700	0.12	66
cycloguanil				23.3	0.063	66
anticancer						
trimetrexate	0.026–0.066	0.07–0.22	64, 68, 70	0.71	0.003	66
piritrexim	0.019–0.032	0.05	70, Queener, unpublished	0.143	0.054	66
methotrexate	0.0013–0.0014	1.0–1.9	64, 68	0.008	ND	66
edatrexate				0.0039	0.26	66

[a] Determined at 37 °C for dihydrofolate reductase from extracts of *P. carinii* harvested from rats. [b] Ratio: IC50 for mammalian DHFR/IC50 for *P. carinii* DHFR. [c] Determined at 20 °C with purified recombinant *P. carinii* DHFR. [d] Ratio: K_i for human recombinant DHFR/K_i for recombinant *P. carinii* DHFR.

high selectivity has driven attempts to establish enzyme screening, but expression of the proteins in high yields has not been reported.

Dihydrofolate Reductase Inhibitors. Tests with Established Compounds. Inhibitors of dihydrofolate reductase with clinical uses against other organisms have been tested against *P. carinii* using in vitro models. Trimethoprim had little effect alone at 340 μM on growth of *P. carinii* in in short-term culture,[57] but pyrimethamine inhibited at 4 μM;[28] in contrast, piritrexim (**3**) and trimetrexate (**4**) were effective at 0.22 and 0.27 μM, respectively.[57] Incorporation of tritiated PABA, used as an index of drug effect, was much less sensitive to trimetrexate than was growth in culture. Trimetrexate at 100 μM inhibited incorporation of PABA over 24 h by about 65%, whereas 100 μM trimethoprim produced 30% inhibition.[35] Piritrexim was also a relatively weak (IC50 value of 100 μM) inhibitor of PABA uptake,[34] but effectively inhibited [^{35}S]methionine incorporation by *P. carinii*, producing 83% inhibition at 0.1 μM, whereas trimethoprim had no effect even at 100 μM.[36]

Several established inhibitors of dihydrofolate reductase had minimal activity against *P. carinii* when used alone in animal models. In a rat treatment model, pyrimethamine alone at 18.75 mg/kg was toxic, whereas trimethoprim or diaveridine at 100 mg/kg were tolerated but were ineffective.[24,43] Tetroxoprim (**5**), an antibacterial inhibitor of dihydrofolate reductase, was tested in the same rat model; at 50 mg/kg the drug lowered the median cyst count 8-fold, but the combination of 50 mg/kg tetroxoprim with 100 mg/kg sulfamethoxazole was only marginally better than sulfamethoxazole alone.[58]

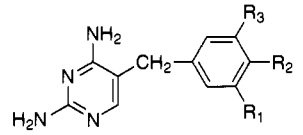
Prophylaxis models for *P. carinii* are generally considered to be more sensitive and likely to reveal activity of weakly effective compounds because the burden of organisms is initially smaller than in therapy models. In prophylaxis models, pyrimethamine was variably effective at 3–4.5 mg/kg/day when given with folinic acid; trimethoprim at doses of 3–50 mg/kg/day was marginally effective or ineffective.[23] Prophylaxis with trimetrexate at 3 or 4.5 mg/kg/day, given by subcutaneous injection, was not effective,[23] but doses of 7.5 mg/kg/day divided into two doses and given by intraperitoneal injection prevented infection in 9 of 10 rats.[57] Piritrexim was also highly effective at 5 mg/kg/day when given intraperitoneally in two divided doses, preventing infection in 8 of 10 rats.[57] In a treatment protocol, 25 mg/kg/day of trimetrexate in feed had no significant effect on degree of infection, but at 7 mg/kg/day in a single subcutaneous injection the drug was effective.[59] Because trimetrexate has a half-life of 1 h in rats, questions have been raised as to whether the drug has been adequately tested in these rodent models.[59]

Isolation of dihydrofolate reductase from *P. carinii* made possible a direct comparison of established and experimental inhibitors (Table 1). Among the established dihydrofolate reductase inhibitors, tetroxoprim and trimethoprim were the least potent when tested directly against the drug target. Epiroprim (**6**) and pyrimethamine were slightly more potent than the antibacterial compounds, but the best potency was observed with the anticancer compounds trimetrexate, piritrexim, and methotrexate (**7**). Significant selectivity toward *P. carinii* dihydrofolate reductase was observed only with trimethoprim and epiroprim (Table 1).

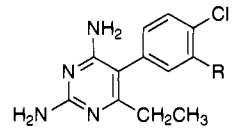
The studies cited above suggest that none of the established inhibitors of dihydrofolate reductase is ideal for *P. carinii*. Pyrimethamine, trimethoprim, and related compounds have low potency in most tests, and must be combined with a sulfonamide to be effective. Trimetrexate and piritrexim are potent and may be somewhat effective alone, but because these drugs are not selective inhibitors of *P. carinii* dihydrofolate reductase and significantly inhibit mammalian dihydrofolate reductase, their utility is limited by toxicity. Most evaluations of these two agents included leucovorin treatment to minimize direct drug effects on host cells. This addition to therapy is expensive and experience in humans suggests failures or relapses are common.[60,61] Development of a single, highly potent agent capable of inhibiting the pathway by blocking dihydrofolate reductase has been a goal of several laboratories. As the following sections illustrate, potency against *P. carinii* dihydrofolate reductase has been relatively easy to achieve, but design of selective compounds has been more difficult.

Screening for Inhibitors of *P. carinii* Dihydrofolate reductase. With the availability of *P. carinii* dihydrofolate reductase, classical SAR (structure–activity relationship/research) techniques could be used to develop new agents, the biological results being used to guide synthesis toward better inhibitors.[62] For most of the studies discussed below, SAR has been used without the benefit of crystal structural data on *P. carinii* dihydrofolate reductase. The goal was to design

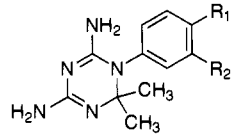
Chart 1. 2,4-Diaminopyrimidines and *s*-Thiazine Analogues



1: $R_1 = R_2 = R_3 = OCH_3$ (trimethoprim)
5: $R_1 = R_3 = OCH_3$, $R_2 = OCH_2CH_2OCH_3$ (tetroxoprim)
6: $R_1 = R_3 = OCH_2CH_3$, R_2 = pyrrole (epiroprim, Ro 11-8958)



2: R = H (pyrimethamine)
8: R = NO_2 (NSC302325)
9: R = N_3 (NSC319947)
10: R = N_2 (NSC330463)
11: R = N=NN$(CH_3)_2$ (NSC330465)
12: R = NCHN$(CH_3)_2$ (NSC372950)



13: R_1 = Cl, R_2 = H (Cycloguanil, NSC 3074)
14: R_1 = H, $R_2 = (CH_2)_8CH_3$ (ref 38)

a compound as potent as trimetrexate or piritrexim and as selective as trimethoprim or epiroprim, i.e. nanomolar IC50 values and selectivity in excess of 10-fold. In the discussion that follows, compounds that approach some aspect of this ideal are described.

Trimethoprim Analogues. Trimethoprim (1, Chart 1) and its analogues have typically shown selectivity for *P. carinii* dihydrofolate reductase and micromolar IC50 values. Epiroprim (**6**) was the best of a series of trimethoprim analogues based on the combined criteria of potency toward purified recombinant *P. carinii* dihydrofolate reductase and selectivity.[63] Most compounds in this series, with IC50 values ranging from 3 to 180 μM, were no more potent than pyrimethamine or trimethoprim. The series exhibited selectivity because most compounds had very low activity toward human dihydrofolate reductase. Selectivity for epiroprim was reported to be 200, but unfortunately the conditions for assay were not reported.[63] When epiroprim was retested in a standard screening assay, the selectivity using rat liver enzyme as reference was 28 and selectivity using human recombinant dihydrofolate reductase was 44 (Table 1). Because of its favorable selectivity, epiroprim was tested further for antipneumocystis activity. In an in vitro assay measuring [^{35}S]methionine incorporation into protein, epiroprim at 100 μM gave 86% inhibition.[36] Epiroprim used with 100 mg/kg sulfamethoxazole dropped cyst counts below the levels seen with sulfamethoxazole alone, suggesting some synergy with the combination, but epiroprim alone at the high dose of 100 mg/kg in a rat therapy protocol had no effect on cyst counts.[58] The disappointing performance of epiroprim in the methionine uptake studies using whole cells and in the whole animal studies may reflect poor transport and/or poor pharmacokinetics.

Pyrimethamine Analogues. Pyrimethamine (**2**, Chart 1) has an IC50 value in the micromolar range, but unlike trimethoprim, pyrimethamine is not selective for *P. carinii* dihydrofolate reductase.[64] Pyrimethamine analogues with improved selectivity have been created, but potency is not greatly enhanced over the parent compound.

The structure–activity relationships of pyrimethamine analogues were examined by measuring inhibition of dihydrofolate reductase obtained from extracts of *P. carinii*. Compounds differing from pyrimethamine by a non-halogen substitution at the meta position of the phenyl ring (**8**–**12**) showed IC50 values from 0.19 μM (**10**) to 10.6 μM (**12**).[64] The most selective compound in this series was 11; the IC50 value of 2.8 μM for *P. carinii* dihydrofolate reductase was similar to that of pyrimethamine (IC50 3.65 μM), but 11 was less active against rat dihydrofolate reductase (IC50 value 18.9 μM vs 2.3 μM for pyrimethamine).[64] Thus, 11 was 7-fold selective for the *P. carinii* enzyme. Compound 11 inhibits *P. carinii* growth in culture with an IC50 value of 5.5 μM.[65] The close correlation of the IC50 value for enzyme activity and for growth in culture suggests that the target in intact *P. carinii* is freely accessible to this agent.

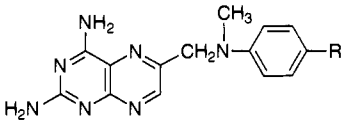
Analogues differing from pyrimethamine by larger or more electronegative groups at position 6 of the pyrimidine ring were clearly inferior to pyrimethamine. IC50 values ranged from 17 to 490 μM and selectivity was unsuitable for an antipneumocystis agent.[64] Generally speaking, 3′,4′-dichloro analogues were equivalent to pyrimethamine or slightly inferior and compounds in which the para chloro group in pyrimethamine was replaced by various bulky substituents retained activity toward *P. carinii* dihydrofolate reductase but showed greatly enhanced activity toward mammalian dihydrofolate reductase.[64]

Cycloguanil Analogues. Dihydrofolate reductase inhibitors that have an *s*-triazine rather than a pyrimidine ring have received attention (Chart 1). The lead compound in this series is cycloguanil (**13**). With a K_i value of 23.3 nM, cycloguanil was a less potent inhibitor of *P. carinii* dihydrofolate reductase than pyrimethamine (K_i value 9.7 nM); moreover, cycloguanil was not selective.[66] Cycloguanil, tested under conditions previously reported,[64] showed an IC50 value of 0.45 μM for *P. carinii* dihydrofolate reductase but the IC50 for rat liver dihydrofolate reductase was 0.11 μM [Queener, unpublished data]; these studies therefore confirm that cycloguanil is not selective for *P. carinii*.

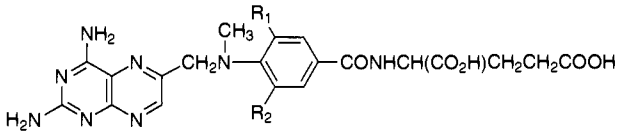
Several cycloguanil analogues have been tested against *P. carinii* dihydrofolate reductase. Compounds with *p*-phenyl substituents other than chlorine mostly exhibited IC50 values between 0.3 and 6.1 μM, but substitution of an amino group or a carboxylic acid group at the para position lowered potency (IC50 value >52 μM).[64] Occupation of both ortho positions, both meta positions, or both meta positions and the para position of the phenyl ring lowered potency (IC50 value >13 μM).[64,67] None of these cycloguanil analogues were selective for *P. carinii* dihydrofolate reductase. A derivative bearing a naphthyl ring in place of the phenyl ring was also potent (IC50 0.16 μM), but substitution of chlorine groups on the naphthyl ring lowered potency.[64,67]

Forty-six meta-substituted 4,6-diamino-2,2-dimethyl-1-phenyl-*s*-triazines were analysed by QSAR.[38] K_i values for the compounds ranged from ca. 70 μM to 3 nM. The hydrophobic parameter accounted for three-

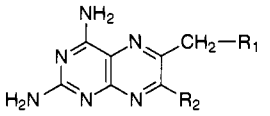
Chart 2. Pteridine Analogues[a]



7: R = CONH**C**H(COOH)CH_2CH_2COOH (methotrexate)
15: R = CONHCH(COOH)CH_2CH_2COOH (NSC117356)
16: R = CO[NHCH(COOH)$CH_2CH_2CO]_4OH$ (NSC341077)



17: $R_1 = R_2$ = Cl (NSC29630)
18: R_1 = Cl, R_2 = Br (NSC98579)
19: $R_1 = R_2$ = F (NSC136736)
20: R_1 = I, R_2 = H (NSC136735)



21: $R_1 = CH_2$-2-quinoline, R_2 = H (ref 69, compound **8c**)
22: $R_1 = C_6H_4$-4-nC_4H_9, $R_2 = CH_2C_6H_4$-4-nC_4H_9 (ref 67)
23: $R_1 = C_6H_{11}$, $R_2 = CH_2C_6H_{11}$ (ref 67)
24: R_1 = NH(CH_3)-1-naphthyl-4-CONH**C**H(COOH)CH_2CH_2COOH, R_2 = H (NSC174121)
25: R_1 = NH-1-naphthyl, R_2 = H (NSC235777)
26: R_1 = S-phenyl, R_2 = H (NSC232965)

[a] Carbons shown in boldface (**C**) have L configuration.

quarters of the variance in the equations that successfully predicted K_i values for 43 of the compounds, suggesting that hydrophobic interactions are more important for *P. carinii* dihydrofolate reductase than for most other forms of the enzyme. Several highly lipophilic compounds in this series were selective for *P. carinii* dihydrofolate reductase; the best combination of selectivity and potency was **14**, but 3-methoxy-1-naphthyl and 3-methoxyadamantyl derivatives were also potent and selective.

Hydrophilic *s*-triazine analogues with multiply substituted phenyl or naphthyl groups at N-1 have not shown adequate potency or selectivity for *P. carinii*, but very highly lipophilic *s*-triazine analogues may have promise, based upon K_i values in the nanomolar range for *P. carinii* dihydrofolate reductase and K_i values 1–2 orders of magnitude higher for the human enzyme.[38] Culture or animal results with these compounds have not been reported.

Methotrexate Analogues. As an inhibitor of *P. carinii* dihydrofolate reductase, methotrexate (**7**, Chart 2) is manyfold more potent than the most potent analogues of trimethoprim and pyrimethamine.[64,68] Some analogues of methotrexate have shown activity against *P. carinii* dihydrofolate reductase at subnanomolar concentrations and a few have also shown interesting selectivity.

Methotrexate analogues that lacked the amino acid amide in the phenyl ring para to the point of attachment were less potent than methotrexate.[64] Configuration of the amino acid substitution was not critical for activity; the IC50 values for methotrexate and for **15**, which differ only in configuration of the asymmetric carbon in the glutamic acid residue, were 1.4 and 0.88 nM, respectively.[64] Polyglutamylation increased potency; for example, **16** with four glutamic acid residues had an IC50 value of 0.035 nM. High potency was also observed in a series of compounds in which one or both ortho positions on the phenyl ring were occupied by a halogen, a CF_3 group, or a OCH_3 group; IC50 values ranged from 1.09 to 0.035 nM, the latter being for the dichloro analogue **17**.[64] In addition to nanomolar IC50 values, several methotrexate analogues were also 7–16-fold selective for *P. carinii* dihydrofolate reductase (**15**–**20**).

Methotrexate enters cells via specific transport systems. Unfortunately, *P. carinii* cells lack those systems.[68] For this reason, methotrexate activity against *P. carinii* in culture is relatively weak; the IC50 value for culture is 4.3 μM, or roughly 3300-fold higher than the IC50 value for free enzyme.[65] Many methotrexate analogues show even greater disparity between culture and enzyme IC50 values. For example, compounds **17**, **18**, and **20** required concentrations over 20 000 times higher than the enzyme IC50 values to inhibit *P. carinii* in culture.[65]

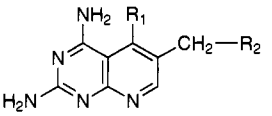
Several compounds that retain the pteridine ring but differ from methotrexate in substituents at position 6 (Chart 2) differ widely in potency toward *P. carinii* dihydrofolate reductase. A series of 2,4-diaminopteridines with 2-carbon bridges to various aromatic substituents had IC50 values similar to pyrimethamine. One compound **21** had a selectivity ratio of 12 but the other compounds in the series were not selective.[69] A series of 2,4-diamino-6,7-disubstituted pteridines had IC50 values in the micromolar range for *P. carinii* dihydrofolate reductase and included selective compounds; selectivity ratios for **22** and **23** (Chart 2) were 11 and 10, respectively.[67]

A naphthyl analogue of methotrexate **24** was more potent than methotrexate (IC50 values 0.19 and 1.3 nM, respectively) and more selective (ratio 13.2 and 1.9, respectively).[64] A naphthyl analogue lacking the *N*-methyl and glutamic acid substituents **25** retained selectivity but was 1% as potent as methotrexate.[64] Compound **26** with an *S*-phenyl substituent was also less potent (IC50 9.5 μM) than methotrexate but was 26-fold selective toward *P. carinii* dihydrofolate reductase.[64] Compounds **24**–**26** were also effective against *P. carinii* in culture;[65] culture IC50 values were 8-fold higher than enzyme IC50 values for **26**, 28-fold higher for **25**, and 520-fold higher for **24**. These results suggest that penetration of **24**–**26** into *P. carinii* may occur more readily than for methotrexate itself. These methotrexate analogues that combine potency in the nanomolar range, selectivity toward *P. carinii* dihydrofolate reductase in excess of 10-fold, and show the ability to inhibit growth of *P. carinii* in culture are excellent candidates for further testing in animal models.

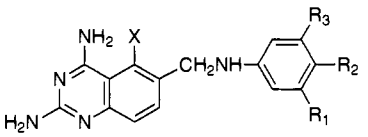
Piritrexim Analogues. Piritrexim (**3**, Chart 3) is a 5-deazapteridine with high potency toward *P. carinii* dihydrofolate reductase (31 nM IC50 value) but even higher potency toward mammalian dihydrofolate reductase (1.5 nM IC50 value);[64,70] selectivity therefore highly favors the mammalian enzyme. Analogues of this compound have been synthesized by several groups and tested against an array of dihydrofolate reductases in an attempt to improve selectivity while retaining potency of the parent compound.

A naphthoyl analogue of 5-deazaaminopterin **27** that retained the glutamate substitution analogous to that of methotrexate was highly active against *P. carinii* dihydrofolate reductase (IC50 value 0.53 nM) and was

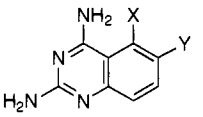
Chart 3. 5-Deazapteridines and 5,8-Dideazapteridines[a]



3: $R_1 = CH_3$, R_2 = 2, 5 dimethoxyphenyl (Piritrexim)
27: R_1 = H, R_2 = NH-1-naphthyl-4-CONHCH(COOH)CH_2CH_2COOH (compund **23b**, ref 71)
28: $R_1 = CH_3$, R_2 = NH-3,4,5-trimethoxyphenyl (compound **7**, ref 72)
29: $R_1 = CH_3$, R_2 = N(CH_3)-3,4,5-trimethoxyphenyl (compound 5a, ref 74)
30: $R_1 = CH_3$, R_2 = NH-2-methyl-5-methoxyphenyl (compound 5, ref 72)



4: X = CH_3, $R_1 = R_2 = R_3 = OCH_3$ (Trimetrexate)
31: X = CH_3, R_1 = H, R_2 = Cl, R_3 = CONH**C**H(COOH)CH_2COOH (NSC132483)
32: X = CH_3, $R_1 = R_3$ = H, R_2 = CONHCH(CH_2COOH)$_2$ (NSC129516)



33: X = Cl, Y = H (entry 4, ref 67)
34: X = H, Y = Cl (entry 17, ref 67)

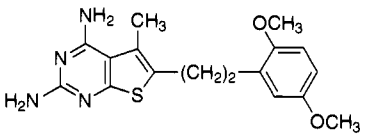
[a] Carbons shown in boldface (**C**) are in L configuration.

3-fold selective.[71] A similar compound with a methyl group at carbon 5 and lacking the glutamate was much less active (IC50 value 220 nM) and not selective.[72] A series of compounds containing substituted indolines were also less active than piritrexim, with IC50 values from 110 to 410 nM.[72,73]

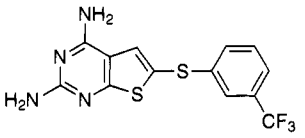
The effect of substitutions on the bridge nitrogen varying from H to methyl to CHO was tested using 3′,4′,5′-trimethoxyphenyl, 3′,4′-dichlorophenyl, and 3′,4′,5′-trichlorophenyl substitutions. For all three sets, compounds containing N(CHO) in the bridge were least active (IC50 value 510 to 550 nM).[74] The most active compounds in the series contained the 3′,4′,5′-trimethoxyphenyl group with either a hydrogen or methyl substitution on the bridge nitrogen (**28** and **29**); the IC50 value for *P. carinii* dihydrofolate reductase was near 13 nM for both compounds.[71,74] A similar compound with a 2′,5′-dimethoxyphenyl substituent was also highly potent (IC50 value 11 nM).[72] Modest selectivity was shown by three compounds bearing 2′,5′-disubstituted phenyl groups, the best having an IC50 value of 38 nM and a selectivity of 3.9 (**30**).[72] In a series of 17 compounds with varying positions of substitution on the phenyl ring, 2′,5′- or 3′,5′-dimethoxy substitutions had potency and selectivity similar to **30**, but other combinations were less effective.[75]

Trimetrexate Analogues. Several analogues of trmetrexate (**4**, Chart 3) have been tested against *P. carinii* dihydrofolate reductase. A series of 2,4-diamino-5-chloroquinazolines had IC50 values from 12 nM to 95 μM, and none were selective. The most potent compound in this series had a selectivity ratio of 1.[76] Quinazolines containing amino acid substituents at the 3′ or 4′ position on the phenyl ring had IC50 values from 9.7 to 0.22 nM, making them more potent than trimetrexate (IC50 value 42 nM).[64] Several members of this series were somewhat selective (e.g., **31** selectivity ratio 2.8) for *P. carinii* DHFR, whereas trimetrexate was highly selective toward mammalina DHFR (ratio of

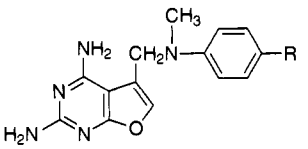
Chart 4. 2,4-Diaminothieno[2,3-*d*]pyrimidines and 2,4-Diaminofuro[2,3-*d*]pyrimidines Tested against *P. carinii* Dihydrofolate Reductase



35: compound **5**, ref 78



36: Ro 15-4672, ref 63



37: R = CONHCH(COOH)CH_2CH_2COOH (compound **2**, ref 80)

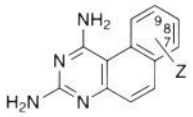
0.07). Compound **31** was active in culture against *P. carinii*, with an IC50 value of 1.4 μM, which was 260-fold higher than the enzyme IC50. A large series of 2,4-diaminoquinazolines bearing nonaromatic substitutions on the quinazoline ring included several compounds with potency in the range of 0.28 to 3.6 μM and selectivity ratios from 8 to 12.[67] In this series, the best combination of potency and selectivity was with compounds bearing halogens at the 5 or 6 position and no other ring substitutions (e.g. **33** and **34**). A series of 2,4-diamino-5-substituted quinazolines bearing aromatic substitutions at the 5 position were largely inactive and none were selective.[77]

Trimetrexate analogues have the potential for high potency and selectivity, although both qualities have yet to be incorporated into a single molecule. Nevertheless, this potential coupled with relative ease of penetration into whole cells make this class appealing for development.

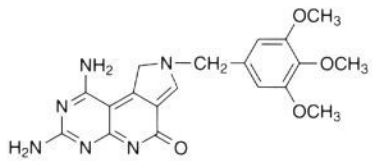
Novel Ring Systems. Molecular modeling studies done before publication of the crystal structure of *P. carinii* dihydrofolate reductase suggested additional ring systems that might inhibit *P. carinii* dihydrofolate reductase (Chart 4). The rationale for a series of 2,4-diaminothienopyrimidines was that the sulfur atom was nearly isosteric with C7–C8 in trimetrexate and with C7–N8 in piritrexim.[78] All the thieno compounds were manyfold less potent than piritrexim and trimetrexate, the compounds they were intended to model. The best 2,4-diaminothienopyrimidine tested was a 2,5-dimethoxyphenyl compound **35**, with an IC50 value of 1.2 μM and almost 5-fold selectivity toward *P. carinii* DHFR.[78] The 2,5-disubstituted phenyl compounds among the 5-deazapteridine series (for example, **30**, Chart 3), also tended to be more selective than other patterns of substitution. Another thieno compound **36** that showed 3-fold selectivity was not potent (IC50 value of 120 μM for *P. carinii* dihydrofolate reductase).[63]

In a series of 2,4-diaminofuro[2,3-*d*]pyrimidines the spatial orientation of the substituents on C5 and C6 were similar to the positioning of substituents on analogous atoms in pyridopyrimidines or tetrahydroquinazolines.[79,80] The best of this series **37** was potent

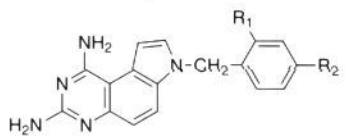
Chart 5. Tricyclic 2,4-Diaminopyrimidines Tested against *P. carinii* Dihydrofolate Reductase



38: Z = 9-chloro (NSC137191)
39: Z = 8-bromo (compound **2**, ref 28)
40: Z = 8-chloro (compound **3**, ref 28)



41: compound 4, ref 81



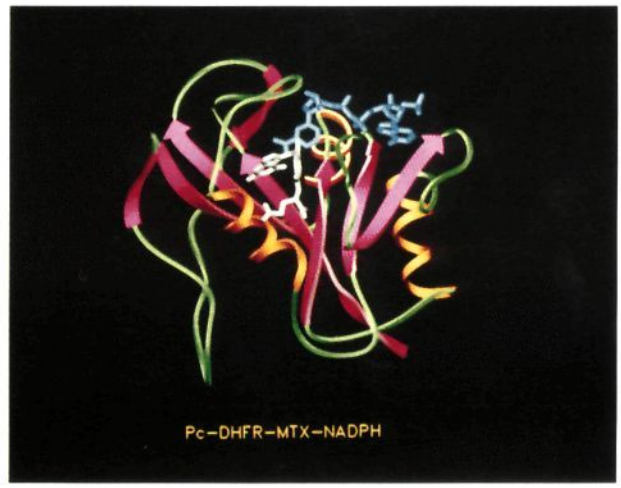
42: $R_1 = R_2 = H$ (Ro 18-4802, ref 63)
43: $R_1 = R_2 = Cl$ (RI 18-5771, ref 63)

(IC50 35 nM) and 12-fold selective but the presence of glutamate in this molecule makes it likely that uptake into *P. carinii* will be limiting.[80]

A series of tricyclic 2,4-diaminopyrimidines can be seen as fixed plane analogues of pyrimethamine or as fused ring analogues of quinazolines (Chart 5).[28] The best of this series (**38**) had an IC50 value of 0.12 μM for *P. carinii* dihydrofolate reductase but was not selective.[64] Compounds **38**–**40** were tested in culture for inhibition of *P. carinii* growth; all were active at 3.5–4.1 μM, but only the 8-chloro- and 8-bromo-substituted compounds were effective at 0.7–0.8 μM.[28] This level of activity in culture suggests that these compounds freely penetrate to their target in *P. carinii*.

Another compound with three fused rings was designed to mimic the spatial orientation of substituents of trimetrexate (**41**, Chart 5); this compound had an IC50 value of 3.1 μM which is far higher than values obtained for piritrexim, trimetrexate, or methotrexate,[81] but the compound was 7-fold selective (IC50 value for rat liver DHFR was 20.3). Compounds with less aromaticity in the ring system were much less active.[81] Two related compounds **42** and **43** containing the quinazoline nucleus failed to show selectivity for *P. carinii* dihydrofolate reductase, but the dichloro compound **43** showed a remarkable increase in potency (IC50 value 6 nM) relative to the unsubstituted phenyl (IC50 value 4 μM).[63]

Modeling Based on Crystal Structure of *P. carinii* dihydrofolate Reductase. Several groups are now using X-ray crystallography to study *P. carinii* dihydrofolate reductase cocrystallized with specific inhibitors.[82,83] The structure of *P. carinii* dihydrofolate reductase contains an eight-stranded β-sheet like other dihydrofolate reductases, but one of the strands is interupted by an α-helical insert; the *P. carinii* enzyme also contains longer, more flexible loops away from the active site (Figure 1a). The active site is conserved but not identical to other species (Figure 1b). Three key residues influencing binding are shown in Figure 1b; a furo analogue **37** of methotrexate that has shown good



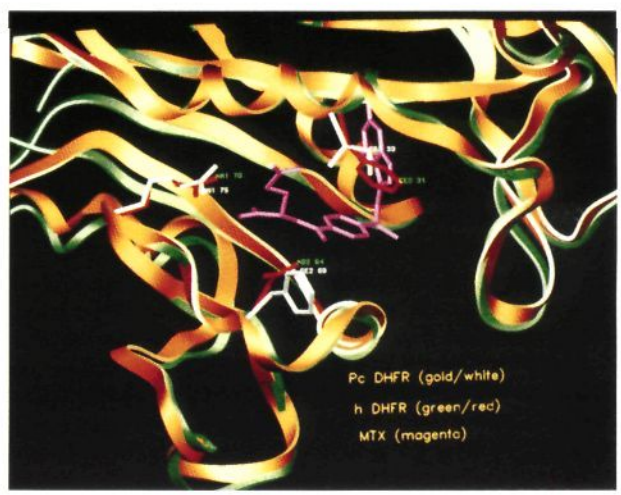


Figure 1. *Pneumocystis carinii* dihydrofolate reductase. Computer generated models supplied by Dr. Vivian Cody; recombinant *P. carinii* dihydrofolate reductase was expressed as soluble protein and crystallized by established techniques.[83] (a, top) The tertiary structure of *P. carinii* dihydrofolate reductase is shown, with secondary elements indicated (β-sheets are violet arrows, helices are in yellow, and loops are in green). NADPH is represented in blue; a furo analog of methotrexate (**37**) is shown in white. Both compounds are docked, based upon the human structure. (b, bottom) Active sites of the human and *P. carinii* enzyme are compared. *P. carinii* dihydrofolate reductase is shown as a gold ribbon with white amino acid side chains. Human dihydrofolate reductase is represented as a green ribbon with red amino acid side chains. The furo analogue of methotrexate (**37**), shown in magenta, is docked as predicted for the human enzyme.

selectivity for *P. carinii* dihydrofolate reductase[80] is shown docked to the mammalian enzyme, for the purposes of comparison. The change of phenylalanine at position 31 of the mammalian enzyme to isoleucine in the *P. carinii* enzyme, as well as the substitution in *P. carinii* dihydrofolate reductase of a phenylalanine (F69) for asparagine (N64), may significantly influence binding of **37** and contribute to the selectivity of this agent.

Analysis of crystallographic data showed significant difference between trimethoprim and piritrexim binding in the active site of *P. carinii* dihydrofolate reductase. The 2,5-dimethoxybenzyl moiety in piritrexim lies closer to amino acids 65–69 than does the trimethoxybenzyl moiety in trimethoprim; moreover, when piritrexim binds, Phe69 moves relative to its position when trime-

thoprim binds. The high potency of piritrexim in direct enzymatic assays supports its good fit into the active site revealed by X-ray crystallography; piritrexim interacts at 20 different positions in the active site, whereas the weaker inhibitor trimethoprim interacts at 15 positions.[82]

Structural influences on binding to the active site have been deduced from the potency and selectivity of the many compounds tested in vitro against *P. carinii* dihydrofolate reductase. The enzyme assays suggest that methotrexate and trimetrexate are well accommodated in the active site of the *P. carinii* enzyme. Analogous rationalizations of data on the naphthyl analogue of methotrexate **24** that was both potent and selective, the *S*-phenyl analogue of methotrexate **26** that was highly selective, and the halogenated quinazolines **33** and **34** will be interesting. These studies should allow assessment of which residues are critical for selectivity, as well as potency. One caveat to these studies is that selective, high-affinity binding to the active site of the target enzyme is only one step in the action of an effective antimicrobial agent. Drug design should include the critical elements of entry of the drug into the target organism and predicted pharmacokinetics of the drug in man.

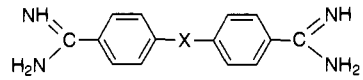
Agents Acting by DNA Binding or Inhibition of Topoisomerases

Rationale. Drugs that bind DNA may block interaction of DNA with specific, required proteins or may prevent the DNA from adopting conformations required for biological function. For some of the drugs discussed below, the details of how DNA binding is related to the antipneumocystis effect remain to be elucidated. Other compounds are known to act on topoisomerases. Topoisomerases catalyze reactions that change conformation of DNA; activity of these enzymes is required for survival of all cells. Topoisomerase I enzymes break one DNA strand, introduce one positive turn into double-stranded DNA, and are independent of ATP. Topoisomerase II enzymes break both DNA strands, introduce two negative twists into double stranded DNA, and are dependent upon ATP for energy to drive the reaction. The breaks in DNA introduced by both enzymes are closed in the last step of the reaction, but interference with the enzyme activity may cause fragmentation of DNA. Inhibitors of topoisomerases can show dramatic species selectivity because topoisomerases from mammalian cells differ significantly from the enzymes from bacteria or lower eukaryotes.

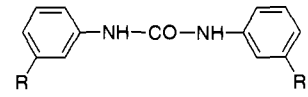
Diamidines. Pentamidine. Because of their broad antiprotozoal activity, diamidines were among the first agents used in patients with *P. carinii* pneumonia. Both pentamidine (**44**, Chart 6) and stilbamidine (**45**) were used in early trials but pentamidine was superior. By 1963 excellent results were reported with pentamidine; mortality observed in a university children's clinic in the 2 years prior to adopting pentamidine was 19% and 20%, but in the first 2 years of pentamidine use, mortality was 2.3% and 3.5%.[84] By 1970 pentamidine was considered the drug of choice for *P. carinii* pneumonia in the United States.[85]

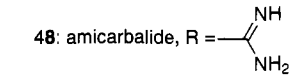
Experience with pentamidine in patients is now extensive for both treatment and prophylaxis in AIDS patients and others.[60] Toxicity is high with this agent

Chart 6. Antitrypanosomal Amidines and Imidazolines with Activity against *P. carinii*

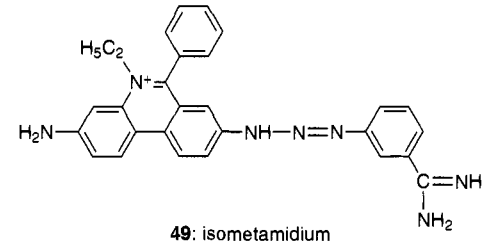


44: pentamidine, X = $O(CH_2)_5O$
45: stilbamidine, X = CH=CH
47: diminazene, X = NHN=N

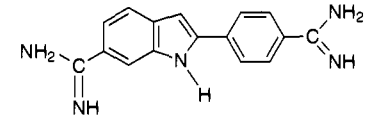




48: amicarbalide, R =



49: isometamidium



46: DAPI

when used systemically. Aerosolized pentamidine has been used for prophylaxis to allow the drug to be inhaled and deposited at the site of action without distribution to the rest of the body. Unfortunately, experience has shown that protection is not complete. *P. carinii* infections in portions of the lung not fully exposed to pentamidine or at sites elsewhere in the body have been reported in patients receiving aerosolized pentamidine.[60,86,87]

Diamidines are appealing drugs, in spite of difficulties encountered in their clinical use. One attractive feature is high potency. For example, pentamidine was highly effective against cultured *P. carinii* at 0.8 μM,[88] inhibited PABA incorporation (IC50 value 7.3 μM), but at 100 μM produced 82% inhibition of [^{35}S]methionine incorporation into *P. carinii* protein.[34,36] Diamidines also have a broad antimicrobial spectrum.[89] Pentamidine and/or pentamidine analogues are active against *Toxoplasma gondii* in cell cultures,[90] against *Cryptosporidium parvum* in neonatal mice,[91] against the enteric protozoan *Giardia lamblia* in culture,[89] and against *Leishmania mexicana amazonensis* and *Plasmodium falciparum* in vitro.[92] These considerations have driven drevelopment of several types of analogues, as discussed below, and have prompted studies designed to clarify their mechanism of action.

Mechanism of Action of Pentamidine. Establishing the mechanism of action of pentamidine has been difficult. At one time pentamidine was proposed to inhibit thymidylate synthetase, trypsin, dihydrofolate reductase, or DNA polymerase II but these enzymes were ruled out as likely mechanisms for antipneumocystis effects.[25,93,94] One current hypothesis relates to diamidine binding in the minor groove of DNA with a preference for AT-rich regions.[95] Several diamidines with strong affinity for DNA were active against *P. carinii*, but binding affinities did not directly correlate

with antipneumocystis activity in rats.[95,96] Correlations may be difficult to establish in animal models because pentamidine and presumably other diamidines are highly metabolized in rats.[97] In contrast, binding of these compounds to calf thymus DNA or poly(dA)·poly(dT) correlated well with in vitro activity against *Giardia lamblia* when thymidine uptake was used as an index growth.[89]

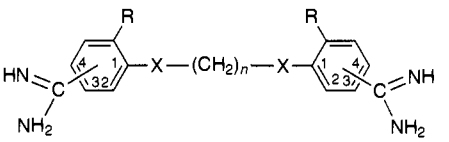
Pentamidine was suggested to inhibit topoisomerase from susceptible organisms, but pentamidine inhibition of topoisomerases from *P. carinii* was not measurable; in contrast, two pentamidine analogues inhibited *P. carinii* ATP-dependent topoisomerase at micromolar concentrations.[94] A related compound **46**, 4′,6-diamidino-2-phenylindole (DAPI), also inhibited *P. carinii* ATP-dependent topoisomerase[94] and bound to *P. carinii* DNA.[98]

Screening of Diamidine Analogues of Pentamidine. Most early studies focused on pentamidine, but a few related compounds were also studied. In rats, pentamidine was effective at daily doses of 20–40 mg/kg for 10–14 days, but hydroxystilbamidine had minimal effects.[11,12] Diminazene (**47**) and amicarbalide (**48**) were active in a rat therapy protocol,[47,99] but the monoamidine isometamidium (**49**) was only moderately active.[99] More recent studies have systematically examined the diamidine family, looking at a series of analogues with 3, 4, 5, or 6 carbons between the oxygens in the ether bridge.[25,93] The high toxicity of pentamidine was retained by most pentamidine analogues, with the exception of a dichloro compound that was inactive; likewise the hexamidine series was toxic, inactive or both.[25,93] Propamidine (Table 2, **50**) was active but toxic. In contrast, propamidine analogues (**51**–**53**), butamidine (**54**), and butamidine analogues (**55**–**57**) were without toxicity; all the metal-substituted analogues (**51** and **55**) were less active than pentamidine. Butamidine (**55**) and the dimethoxy analogue of propamidine (**52**) were both much superior to pentamidine. Butamidine was compared directly to pentamidine in a dose response study in rats; both compounds were effective at 10 mg/kg, but ineffective at 1 mg/kg when given by daily iv injection.[100]

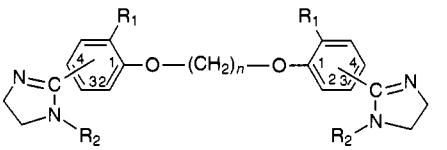
Imidazolines. When the amidine moieties in certain diamidines were replaced with imidazolines, activity against *P. carinii* was retained or enhanced.[25,93,100] The most active diimidazoline was DIMP (**58**), which was highly effective at 2.5 mg/kg by daily iv injection; DIMP retained significant activity at 1 and 0.5 mg/kg, but not at 0.25 mg/kg.[100] The next most active diimidazoline was DIPP (**59**). In a dose–response therapy study in rats DIPP was completely effective at 10 mg/kg when given by daily iv injection and was significantly superior to pentamidine at the same dose.[100] Both pentamidine and DIPP were active at 5 mg/kg but not at doses of 1 mg/kg or below. The optimal position of imidazoline substituents was para; the meta derivatives were inactive (e.g., **60**) or highly toxic (e.g., **61**).

One limitation of the diamidines has been the need for iv administration. To circumvent this problem, the possibility of administering some of the new compounds orally was explored.[100] DIPP was without effect when given daily by gavage at doses of 20 mg/kg, although it was effective by iv injection at 5 mg/kg. In contrast, DIMP was highly effective by gavage at 40 or 25 mg/kg daily or at 25 mg/kg every other day for 2 weeks. Pentamidine at 10 mg/kg given by gavage was without any effect at all. These studies suggest that less toxic and more potent relatives of pentamdine can be developed, and moreover that oral administration may be a possibility with certain members of this class of agents.[93]

Table 2. Diamidine and Dimidazoline Analogues Tested in Rat Models of *P. carinii* Pneumonia[44,130]



compound	*n*	R	or[a]	effectiveness[b]	toxicity
50, propamidine	3	H	4	1.2	+
51		H	3	0.7	0
52		OCH_3	4	1.8[d]	0
53		NH_2	4	1.1	0
54, butamidine	4	H	4	2.2	0
55		H	3	0.6	0
56		OCH_3	4	1.2[d]	0
57		NH_2	4	1.1	0
44, pentamidine	5	H	4	1.0	++



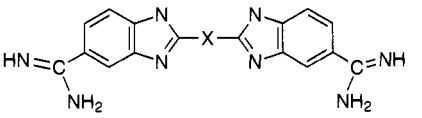
	compound[c]	R_1	R_2	or[a]	effectiveness	toxicity
58	(DIMP, no. 3)	OCH_3	H	4	1.3[e]	0
59	(DIPP, no. 1)	H	H	4	2.4	0
60	(no. 2)	H	H	3	0.6	0
61	(no. 4)	OCH_3	H	3	1.7[d]	+++

[a] "or" refers to orientation of diamidine or imidazoline substituents on the rings. [b] Effectiveness at 10 mg/kg iv, unless otherwise indicated, relative to pentamidine (1.0 = pentamidine, <1.0 = less effective than pentamidine, >1.0 = more effective than pentamidine). [c] Parentheses contain compound designation in ref 100. [d] Tested at 5 mg/kg intravenously. [e] Tested at 2.5 mg/kg intravenously.

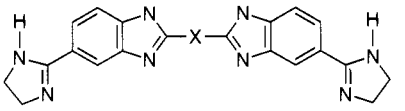
The extended structure and large size of diamidines allow many conformations. To investigate the optimal conformation of the diamidine or diimidazoline molecule for antipneumocystis activity, semirigid congeners of butamidine were synthesized.[101,102] These compounds contained four carbons in the ether bridge between the rings, and a double bond was inserted between carbons 2 and 3 to create cis and trans isomers. Both cis and trans isomers of diamidines or diimidazolines were more active and less toxic than pentamidine; there was no significant difference in antipneumocystis activity among the geometric isomers, but the cis isomers bound calf thymus DNA or poly(dA)·poly(dT) significantly better than the trans isomers. Studies with more rigid, highly restricted analogues will be needed to establish the optimal configuration for compounds of this type.

Bis-benzimidazoles. Benzimidazoles were selected for study because they bind DNA more avidly than the diamidines or diimidazolines described above. A series of diamidine or diimidazoline bis-benzimidazoles was synthesized, differing in the length of the carbon bridge between the ring systems (Table 3). All of these compounds showed significant antipneumocystis activity in the animal model, but those with four carbon

Table 3. Activity of Bis-benzimidazoles against *P. carinii* Pneumonia[a]



compound	X	mean score[b]	cysts/g of lung % control[c]	toxicity
62	$(CH_2)_4$	0.6	0.3	0
64	$(CH_2)_3$	3.0	32	+
66	$(CH_2)_2$	0.7	1	++
68	CH=CH	1.5[d]	3	+++



compound	X	mean score[b]	cysts/g of lung % control[c]	toxicity
63	$(CH_2)_4$	0.5	0.1	0
65	$(CH_2)_3$	1.4	4	+
67	$(CH_2)_2$	0.6[d]	2	+++

[a] Data from ref 20. [b] Histologic score reflecting severity of *P. carinii* pneumonia. No treatment yielded a mean score of 3.6, whereas treatment with pentamidine at 10 mg/kg gave a scores of 1.2. [c] *P. carinii* cysts per gram of lung tissue were determined; values are expressed as percent of saline-treated control. [d] Tested at 5 mg/kg by intravenous injection in a therapy rat model protocol; all others tested at 10 mg/kg.

bridges (**62** and **63**) were superior, showing extremely strong activity with no detectable toxicity (cf. **64**–**68**). Compound **63** given intravenously was active down to 1 mg/kg. When given orally at 25 mg/kg, significant activity was observed, but it was less than that observed with 2.5 mg/kg given intravenously.[20]

An extended series of bis-benzimidazoles and bis-aminoindoles has been tested for ability to bind DNA.[103] All of the bis-amidinobenzimidazoles and bis-imidazolinobenzimidazoles, including those in Table 3, strongly bound DNA and showed preferential binding to poly(dA)·poly(dT) homopolymer. The binding of the bis-aminoindoles was weaker toward the poly(dA)·poly(dT) homopolymer and showed less preference for AT-rich regions. Modeling suggested that, like pentamidine and its analogues, the bis-benzimidazoles bind to the minor groove of DNA.[103] Correlation of antipneumocystis activity of bis-benzimidazoles with DNA binding was not possible with the limited series available, because antipneumocystis activity and DNA binding were both relatively high for most analogues. This series of bis-benzimidazoles has been tested against *Giardia lamblia* using an in vitro system and against *G. lamblia* topoisomerase II activity; binding to calf thymus DNA or poly(dA)·poly(dT) homopolymer correlates strongly with antigiardial activity and with the ability to inhibit giardial topoisomerase II.[104]

This same series of bis-benzimidazoles was tested for the ability to inhibit topoisomerases from *P. carinii*.[105] Compounds **63** and **66**–**68** inhibited *P. carinii* topoisomerase I with IC50 values below 10 μM; the best of these compounds (**63**) was more than 40-fold selective for *P. carinii* topoisomerase I relative to calf thymus topoisomerase I. Compounds **66**, **63**, **68**, and a diisopropylamidine derivative of **63** inhibited *P. carinii* topoisomerase II with IC50 values from 0.31 to 1.25 μM; these compounds were all highly selective (80-fold to >160-fold) for the *P. carinii* enzyme. All of the highly selective inhibitors of *P. carinii* topoisomerases were also very effective against *P. carinii* in the rat therapy model, but correlation of topoisomerase inhibition with antipneumocystis activity was not demonstrated. Until these compounds are studied in vitro where pharmacokinetics will not confound comparisons, it is impossible to determine how much of the antipneumocystis activity of these compounds derives from inhibition of topoisomerase activity.

Fluoroquinolones. Fluoroquinolones effectively inhibit bacterial topoisomerase II and also have excellent pharmacokinetics and low toxicity. These qualities led to tests against *P. carinii*. Pefloxacin was reported to be effective against *P. carinii* for both therapy and prophylaxis in rats.[106] Several other fluoroquinolines were tested at 100 mg/kg given by oral gavage in a prophylaxis study with pefloxacin; temafloxacin, ofloxacin, ciprofloxacin, sparfloxacin, and norfloxacin did not prevent development of *P. carinii* pneumonia from latent organisms in male rats. Pefloxacin and sparfloxacin were present at levels exceeding 7 μg/g of lung tissue in this study, but the other drugs were present at much lower concentrations.[106] Pefloxacin and the closely related compound fleroxacin tested in an inoculated mouse model for therapy of *P. carinii* pneumonia were ineffective at doses of 100 mg/kg by intrapertoneal injection and 150 mg/kg by subcutaneous injection, respectively; at 300 mg/kg pefloxacin killed all the test mice.[30] The high doses required and the variable results in different models suggest that antibacterial fluoroquinolones are not likely to be useful clinically against *P. carinii*.

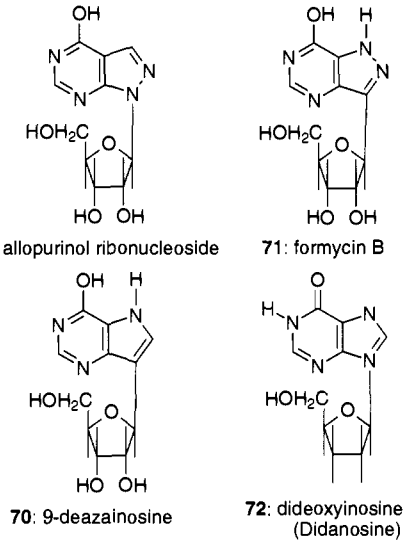
Cytotoxic Topoisomerase Inhibitors. Amsacrine, camptothecin, etoposide, and teniposide were evaluated against *P. carinii* in culture.[30] Camptothecin and amsacrine damaged the cell feeder layer and were therefore considered too toxic for further testing. Etoposide and teniposide were each effective at 1 and 10 μg/mL, but teniposide was more potent and was therefore chosen for animal testing. In the inoculated mouse model, teniposide was active only when given at 80 mg/kg for a loading dose, followed by 10 mg/kg.[30] The results suggested that useful activity might be found with agents of this class, but neither etoposide nor teniposide was the ideal agent.

Agents Affecting Purine Pathways

Rationale. Many parasitic organisms depend heavily on salvaging materials from the hosts to supply their own needs. For example, parasitic protozoa cannot synthesize purines de novo and must salvage them from outside sources.[107] The purine metabolism of *P. carinii* has not been fully characterized, but several compounds known to interfere with purine synthesis or salvage have been tested empirically.

Inosine Analogues. Inosine analogues have been explored previously for antiprotozoal, anticancer, and antiviral activities. Allopurinol ribonucleoside, 9-deazainosine, and formycin B (**69**, **70**, **71**, Chart 7) inhibit purine salvage pathways in pathogenic hemoflagellates.[108,109] In early culture studies in *P. carinii* with these three compounds, the most active was 9-deazainosine; at 10 μg/mL, 9-deazainosine was equivalent to

Chart 7. Inosine Analogues Tested against *P. carinii*



50/250 μg/mL trimethoprim/sulfamethoxazole and at 1 μg/mL the drug inhibited growth by 25%.[110] Formycin B was strongly inhibitory to *P. carinii* growth at 40 μg/mL, with less effect at 10 μg/mL and no effect at 1 μg/mL; allopurinol ribonucleoside had no effect on growth in culture.[110] Allopurinol at 50 mg/kg per day failed to prevent development of *P. carinii* pneumonia in rats in earlier studies.[22]

The high activity of 9-deazainosine in culture against *P. carinii* led to tests in which rats received 6 mg/day (ca. 60 mg/kg, divided into two doses) given intraperitoneally for 7 days, then 3 mg/day in two doses for 8 days.[111] With this regimen, 9-deazainosine was highly effective and electron microscopy showed early and progressive effects on morphology of *P. carinii* trophozoites. The drug was also tested in a similar rat therapy model, where 9-deazainosine was effective when given intraperitoneally at 25 or 50 mg/kg/day for 5 days/week; the drug was not effective at 15 to 7.5 mg/kg/day given subcutaneously.[23] This inosine analogue was classed as having marked activity in a survey of agents tested in that rat model.[24]

Antiviral inosine analogues have also been tested against *P. carinii*. Dideoxyinosine (**72**), an approved antiretroviral agent, was not effective in a rat model.[24] Isoprinosine was also inactive in the animal model for therapy,[24] even though the drug inhibited PABA incorporation by *P. carinii* in short-term culture.[55] Isoprinosine is composed of inosine and the *p*-acetamidobenzoic acid salt of *N,N*-dimethyl-2-aminopropanol (pADA) in a 1:3 ratio.[55] Inosine and pADA were tested for effects on PABA incorporation and on dihydropteroate synthetase activity; inosine was inactive, but pADA was as active an inhibitor of PABA incorporation as isoprinosine itself.[55] In addition, pADA had an IC50 value of 29 ± 48 μM toward *P. carinii* dihydropteroate synthetase, whereas isoprinosine had an IC50 value of 20 ± 8.4 μM; these results suggested that the antipneumocystis activity of isoprinosine resided in the pADA contained in the mixture.[55] In spite of these activites in vitro, pADA was totally inactive in a prophylaxis study in rats, even at extremely high doses,[55] and was also ineffective in rats for therapy at 250–500 mg/kg.[24] The pharmacokinetics of this compound in rats and in humans have not been explored.[55]

Inhibitors of Purine Synthesis. Inhibitors of inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) prevent conversion of IMP to XMP. This inhibition can starve the cell for guanine nucleotides, unless adequate salvage of guanine or guanosine occurs. Empirical tests showed mycophenolic acid, a known inhibitor of IMPDH, to be an extremely potent inhibitor of *P. carinii* growth in culture;[112] another IMPDH inhibitor, pyrazofuran, was also active in culture (Queener, unpublished data). The toxicity of these compounds precluded adequate testing in animal models but the strong potency of two compounds with this mechanism of action suggests that inhibitors of IMPDH should be further explored as antipneumocystis agents.

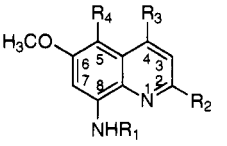
Agents That Affect Electron Transport or Redox Processes

Rationale. Electron transport has been the target of several classes of antimalarial agents. Hydroxynaphthoquinones bind to an iron–sulfur protein at the ubiquinone oxidation site of the cytochrome bc1 complex in many organisms.[113] This complex is also linked to dihydroorotate dehydrogenase in eukaryotes; interference with this complex can deplete UMP.[114] Primaquine and other 8-aminoquinolines can be metabolized to quinone structures that are active redox agents, generating superoxide from oxygen.[115] The exact mechanism of action of any of these agents against *P. carinii* has not been elucidated but is being actively studied in several laboratories.

8-Aminoquinolines and Related Agents. Because *P. carinii* was originally believed to be a protozoan, many of the drugs first tested for *P. carinii* pneumonia were antiprotozoal agents. In one early study the antimalarials primaquine (an 8-aminoquinoline) and chloroquine (a 4-aminoquinoline) were given orally in a fixed combination of 5.62 mg of primaquine and 37.5 mg of chloroquine per kilogram.[22] On a weekly basis, this dosage was 60 times higher than human doses effective for malaria, but no antipneumocystis effect was observed. Chloroquine alone was ineffective for therapy when given in food (12 mg/100 g of food).[12] Chloroquine was effective in culture at 2 or 20 μg/mL,[88] but pilot studies with chloroquine in rats again showed the drug to be ineffective.[116] A related bis-aminoquinoline was highly effective in culture at 0.1 μg/mL, but was ineffective in animals at the doses that could be achieved with this relatively insoluble compound.[116] Because of repeated failure to act against *P. carinii* in whole animal models, the 4-aminoquinolines were abanadoned and further tests centered on 8-aminoquinolines.

Primaquine was tested in combination with clindamycin.[21] This unusual combination was adopted because the combination of primaquine and mirincamycin, an analog of clindamycin, had been reported to be synergistic in animal models of malaria.[117] Alone in doses up to 2 mg/kg daily, primaquine was without effect in a therapy model of *P. carinii* pneumonia; clindamycin was also without effect when given at 225 mg/kg daily, divided into three subcutaneous injections 8 h apart.[21] The combination of 2 mg/kg primaquine and 225 mg/kg clindamycin was effective treatment for *P. carinii* pneumonia, and at lower doses the drugs also were extremely effective for prophylaxis of *P. carinii* pneumonia.[21]

Table 4. 8-Aminoquinolines Tested against *P. carinii*



compound	R_1	R_2	R_3	R_4	culture model, μM MEC[a]	rat model score[b]
73, primaquine	$CH(CH_3)(CH_2)_3NH_2$	H	H	H	0.02	4.3
74, WR182234	$CH(CH_3)(CH_2)_3NH_2$	CH_3	H	H	0.03	1.4
75, NSC305805	$CH(CH_3)(CH_2)_3NH_2$	H	CH_3	H	0.02	0.2
76, WR242511	$CH(CH_3)(CH_2)_3NH_2$	H	CH_3	$O(CH_2)_5CH_3$	0.2	0.2
77, WR225448	$CH(CH_3)(CH_2)_3NH_2$	H	CH_3	$O(C_6H_4)$-*m*-CF_3	0.2	0
78, WR238605	$CH(CH_3)(CH_2)_3NH_2$	OCH_3	CH_3	$O(C_6H_4)$-*m*-CF_3	0.2	0.9
79, NSC305835	$(CH_2)_4CH(CH_3)NH_2$	H	CH_3	H	0.2	0.1
80, WR6026	$(CH_2)_6N(CH_2CH_3)_2$	H	CH_3	H	2.3	0.3

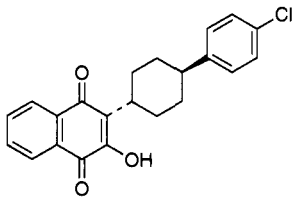
[a] MEC = minimal effective concentration (μM) inhibiting growth of *P. carinii* in culture.[119] [b] Score is based upon microscopic evaluation of numbers of organisms in impression smears prepared from lungs, with a score of 5 indicating the heaviest infection and 0 indicating no organisms were detected in 50 random 1000× fields; untreated controls in these studies had scores of 4.3 to 4.6 ± 0.2 SEM.[27,118,119]

Demonstration of activity of primaquine (**73**) against *P. carinii* in culture led to a survey of other 8-aminoquinolines **74**–**80**. Three compounds (Table 4, **76**, **78**, **80**) were identified that were similar to primaquine in activity against *P. carinii* in culture but more active in animal models; at 2 or 4 mg/kg all three compounds were as effective as 50/250 mg/kg trimethoprim/sulfamethoxazole in a therapy model in rats.[27] Doses as low as 0.5 mg/kg for **78** and **80** or 0.25 mg/kg for **76** retained significant activity for treatment, and at 0.57 mg/kg the drugs were equivalent to trimethoprim/sulfamethoxazole for prophylaxis.[27] Equivalent effects were obtained with daily doses or higher doses given every 4 days.[118]

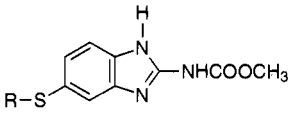
A larger series of 8-aminoquinolines was studied in both culture and animal models to further define structural requirements for antipneumocystis activity.[119] The series differed from primaquine in the 8-(aminoalkyl)amino side chain or in ring substituents at positions 2, 4, and 5. Compounds with less than four carbons in the 8-(aminoalkyl)amino side chain were devoid of activity.[119] Ring substitutions had less effect on activity in culture, but activity was influenced in the rat model where drug metabolism may play a role. For example, **74** and **75** (Table 4), which differ from primaquine only in the presence of a methyl group at position 2 or 4 on the ring were significantly more potent in the rat model than was primaquine. Analogues of **75** that contained complex substituents at position 5 on the ring **76**–**78** were all much more potent than primaquine in the rat model. Among the compounds with (aminoalkyl)amino side chains different from primaquine, the two that were most active in rats were **79** and **80**; both of these compounds had methyl substituents at position 4 on the ring. The activity of 8-aminoquinolines in animal models may relate to the high degree of metabolism of these compounds; primaquine, for example, has an extremely short half-life in rats[118] and some of the metabolites are active antimalarials.[115] The role of metabolism of 8-aminoquinolines in the rat model of *P. carinii* pneumonia has not been fully explored.

Three 8-aminoquinolines have been proposed or used for *P. carinii* pneumonia in humans: primaquine, WR6026, and WR238605. The use of primaquine with clindamycin has become an accepted treatment for mild to moderate *P. carinii* pneumonia, especially in patients

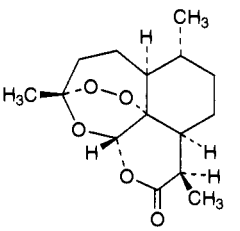
Chart 8. Miscellaneous Compounds with Activity against *P. carinii*



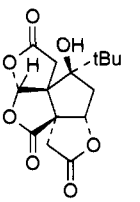
81: atovaquone (566C80; 2-[*trans*-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone)



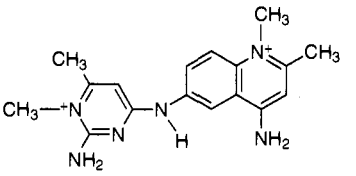
82: albendazole, R = $CH_2CH_2CH_3$
83: fenbendazole, R = phenyl



84: artemisinin



85: bilobalide



86: quinapyramine

intolerant to pentamidine or trimethoprim/sulfamethoxazole.[120,121] Primaquine/clindamycin has also been successful for salvage therapy, i.e. for treating patients who have failed other therapy.[122] Compounds WR6026[123] and WR238605 are being developed for other diseases (6026 for leishmaniasis, 238605 for malaria). Unfortunately, trials in humans with *P. carinii* pneumonia have been slow in developing.

Atovaquone. Atovaquone (Chart 8, **81**) is a hydroxynapthoquinone that moved very quickly from animal studies into full clinical trials. Already under development as an antimalarial agent, in 1990 atovaquone was reported to have activity for prophylaxis of *P. carinii* in a rat model at doses above 50 mg/kg and was also

effective for treatment at 100 mg/kg.[124] An open-label (uncoded) trial of atovaquone in 34 AIDS patients with untreated pneumocystis pneumonia was reported in 1991,[125] and the full double-blind multicenter trial comparing atovaquone with standard therapy in 322 AIDS patients with mild or moderately severe *P. carinii* pneumonia was reported in 1993.[126]

Atovaquone was tested against *P. carinii* because of previously demonstrated activity against *Plasmodia*, but the family of naphthoquinones to which atovaquone belongs has a much broader antiprotozoal spectrum that includes trypanosomes, *Theileria* parva, and *Eimeria*.[127] Atovaquone was also effective against *Toxoplasma gondii* in vitro and in vivo[128] and quickly moved into clinical trials for *T. gondii* infections in AIDS patients.[129]

Atovaquone was well-tolerated by AIDS patients, but was less effective than standard therapy with trimethoprim/sulfamethoxazole; 20% of patients receiving atovaquone and 7% of patients receiving trimethoprim/sulfamethoxazole failed to respond.[126] A key element in determining efficacy was pharmacokinetics. Absorption of this oral agent, although improved by food, was still variable; the steady-state plasma concentration achieved was a strong predictor of success of therapy.[126] AIDS patients with significant diarrhea had impaired absorption and did not respond as well to atovaquone. Atovaquone is currently approved for mild to moderate *P. carinii* pneumonia in patients intolerant to trimethoprim/sulfamethoxazole.

A prodrug form of atovaquone has been developed to improve bioavailability and make absorption more uniform.[130] This compound contains a carbamate group in place of the hydroxyl group of atovaquone. The carbamate is water soluble, efficiently absorbed, and cleaved to atovaquone by a rapid nonenzymatic pH-dependent process.[130] The prodrug gave higher plasma levels of atovaguone than did direct dosing with atovaquone itself and the prodrug was more efficacious than atovaquone in a mouse model of *P. carinii* pneumonia.[131]

Agents Acting on Microtubules

Benzimidazoles. Benzimidazole derivatives are active against microtubules in a variety of eukaryotic organisms, including *Trichomonas vaginalis* and *Giardia lamblia*, and are used clinically against a variety of helminths.[132] The mechanism of action of these drugs had not been exploited for *P. carinii*. In an effort to seek drugs that act toward new targets in *P. carinii*, 10 benzimidazoles with varying activities toward fungi, helminths, and protozoa were tested for the ability to inhibit growth of *P. carinii* on human lung fibroblasts.[29] The highest potency was seen with compounds bearing a carbamate at position 2; within the carbamate series, substitutions at position 5 also influenced potency, with the best activity being produced by the simple *n*-butyl substitution in parbendazole. Albendazole (**82**) with an *S*-*n*-propyl side chain was also highly active, but oxibendazole with an ether *n*-propyl side chain was somewhat less active. Many of these same compounds were also tested for ability to inhibit in vitro growth of *Cryptococcus neoformans*, a fungus that causes grave illness in immune suppressed hosts. Against *C. neoformans*, fenbendazole (**83**) was 10-fold more active than the other benzimidazoles and was more active than amphotericin B.[133]

One appeal of the benzimidazoles was that they were already used clinically and therefore could be more rapidly applied to *P. carinii* infections in patients than could compounds arising from new synthesis programs. With this consideration in mind, albendazole, parbendazole, and mebendazole were carried into animal tests. In a therapy model using dexamethazone immunosuppressed mice, albendazole at 600 mg/kg was effective, but the other two drugs were ineffective.[134] Absorption of drug was difficult with all three compounds and the amount of drug actually absorbed in unknown. Albendazole was more effective at 600 mg/kg when tested in mice immunosuppressed with the L3T4 antibody.[134] Clinical trials of albendazole for *P. carinii* pneumonia are being planned jointly by groups in Italy and the United States (C. Atzori, personal communication). Albendazole has been used for hydatid disease in humans at doses up to 800 mg daily for 28 days. Absorption of albendazole is difficult in humans, as well as in rodents. Comparison of blood levels will aid in assessing whether blood levels effective in animal models can be produced in man.

Miscellaneous Compounds Displaying Activity against *P. carinii*

In previous sections, large groups of compounds have been discussed to allow some evaluation of structure–activity relationships. In this section, smaller groups or single compounds with activity against *P. carinii* are discussed. A list of compounds that were ineffective in rat models for *P. carinii* pneumonia has been published.[24] In general, arsenicals, antibacterial agents, and antiviral agents were inactive.

Acridone Alkaloids. Acridone alkaloids are natural products isolated from plants of the family *Rutaceae*. Various members of this family, as well as derivatives and analogues, have cytotoxic effects and antimicrobial activity. Antiplasmodial activity was observed with seven compounds out of a series of 30 acridone alkaloids tested in a rodent model.[135] Because many antimalarial compounds have shown activity against *P. carinii* in a variety of models, six of these acridone alkaloids were selected for testing against *P. carinii*.[136] All six of the alkaloids were active against *P. carinii* in culture at concentrations of 17–34 μM, but only two were active at lower concentrations: atalaphillinine (2.7 μM) and glycobismine A (1.7 μM). Atalaphillinine was cytotoxic to L1210 cells (ED50 value of 3.4 μM), which raised the question of whether the antipneumocystis activity could be ascribed to cytotoxicity of the mammalian cell feeder layer in culture. The fact that the most cytotoxic agent in the series, 5-hydroxy-*N*-methylseverifoline, lacked antipneumocystis activity at 2.6 μM suggested that cytotoxicity was insufficient to explain antipneumocystis activity. Studies of these compounds in animals have not been reported.

Artemisinin. Artemisinin, or qinghaosu (Chart 8, **84**), is an antimalarial agent derived from an herbal remedy used for centuries in China. Activated oxygen may play a role in the antimalarial activity.[137] Qinghaosu and several derivatives were also active against another protozoan, *Toxoplasma gondii*.[138] These activities led to tests of artemisinin against *P. carinii* in a short-term culture system.[31] Artemisinin at 100 μM gave inhibition of growth equivalent to 15 μM penta-

midine in pilot studies. Evaluation of a dose response showed that artemisinin was a potent inhibitor of growth, producing more than 50% inhibition at 3 days with 0.5 μM concentrations.[31] Animal tests have not been reported.

Bilobalide. Bilobalide (Chart 8, **85**) is a sesquiterpene originally isolated from the leaves of *Ginkgo biloba*. In a culture model, bilobalide at 50 μg/mL inhibited growth of *P. carinii* and in a rat model bilobalide at 10 mg/kg was effective therapy for *P. carinii* pneumonia.[32] Extracts from the leaves of *G. biloba* have been used as a herbal remedy for 5000 years in chinese medicine; in recent years extracts of the leaves have been tested in humans in Europe, but controlled tests with bilobalide have not been reported.[32]

Deferoxamine and Other Iron Chelators. Deferoxamine is used in humans to treat iron overload but is also active against malaria.[139] Because of this antimalarial activity, the compound was tested in a rat model for therapy of *P. carinii* pneumonia; deferoxamine effectively reduced cyst counts in the lungs of rats receiving 250−1000 mg/kg in a single daily intraperitoneal injection.[140] Deferoxamine was less active in another report in which 800 mg/kg was given to rats with *P. carinii* pneumonia, but parallel studies in a mouse showed doses of 400 mg/kg deferoxamine were as effective as trimethoprim/sulfamethoxazole (50/250 mg/kg).[33] Deferoxamine was also effective against cultured *P. carinii* at concentrations of 7.5 or 30 μM,[33] and the effect of the drug was blocked by prior saturation of deferoxamine with iron, suggesting that the mechanism of action against *P. carinii* involved withholding iron from the organism.[33] Other metals did not block the antipneumocystis effect.[33] Direct studies of the antipneumocystis activity of deferoxamine in humans has not been reported.

A series of hydroxypyridinones explored as oral chelating agents have also been tested in culture against *P. carinii*.[33] These agents showed only modest activity at 18 μM but were strongly inhibitory to *P. carinii* growth at 72 μM.

Eflornithine (DFMO). DFMO is a suicide inactivator of ornithine decarboxylase, an enzyme crucial for the synthesis of polyamines required by all cells. The compound is active against trypanosomiasis in humans and against *Eimeria tenella* and *Plasmodium berghei* in experimental systems. For these reasons, the drug was explored for *P. carinii* but the results were not encouraging. DFMO was effective in rats only at very high doses (3.4−7.5 grams/kg).[22,47,140] In one study, similar doses of DFMO were ineffective in a rat treatment model either alone or in combination with pentamidine or diminazene.[99] In a culture model for *P. carinii*, DFMO was effective only at 1 mM.[141] Studies with ornithine decarboxylase isolated from *P. carinii* showed that DFMO was a much poorer inhibitor of the enzyme from the parasite than from the host; the average IC50 for rat liver ornithine decarboxylase was 1.6 μM but the IC50 for *P. carinii* orthithine decarboxylase was 110 μM.[142]

DFMO was used in patients who had failed conventional therapy for *P. carinii* pneumonia as early as 1984.[42] Controlled trials reported in 1992 showed that 49% of patients failed on DFMO versus 21% for trimethoprim/sulfamethoxazole; the study was terminated early, based upon this high failure rate.[143] The drug is currently not approved for use in *P. carinii* pneumonia.

Quinapyramine. Quinapyramine (Chart 8, **86**) is active against trypanosomes and on this basis was tested in a rat model for treatment of *P. carinii* pneumonia.[99] The drug was highly effective at 4 mg/kg daily by subcutaneous injection and had detectable activity at 0.5 mg/kg daily by the same route. Quinapyramine is one of the more potent compounds tested and also one of the most effective.[24] Further studies of this compound have not been reported.

Sulfonylurea Compounds. Carbutamide and tolbutamide are hypoglycemic agents chemically related to sulfonamides. On this basis they were studied in a rat model for *P. carinii* pneumonia.[144] Carbutamide was more effective than tolbutamide for prophylaxis; carbutamide was found effective for therapy at 100 mg/kg and effective for prophylaxis at 50 mg/kg.[144] Although carbutamide has been used in man and is known to have limited hypoglycemic activity, studies of the drug for *P. carinii* pneumonia in man have not been reported.

Antifungal Agents. Infections caused by *Pneumocystis carinii*, now classed with the fungi, have been the most common diagnostic indicator for AIDS. Another common fungal infection in AIDS patients has been mucosal or mucocutaneous candidiasis. The appearance of oral candidiasis is an early sign of progression of HIV disease to AIDS. In one study, 59% of HIV-positive patients with oral candidiasis developed AIDS within 23 months of followup, whereas a control group of HIV-positive patients without oral candidiasis had no patients who progressed to AIDS.[145] Esophageal candidiasis in HIV-positive patients is considered diagnostic for AIDS.[146] *Cryptococcus neoformans* causes meningitis, pneumonia, or disseminated disease in up to 7% of AIDS patients and is the third leading cause of neurological disease in these patients, after HIV encephalopathy and toxoplasmosis. Other fungal infections are more geographically restricted; disseminated histoplasmosis, which is diagnostic for AIDS, is more common in the midwest,[147] whereas coccidioidomycosis is more common in the desert southwest.[148]

Antifungal agents are currently being developed at a rapid rate, with new broad-spectrum and relatively nontoxic agents having the potential for replacing older potent but toxic agents. The need for effective antifungal therapy has never been greater, as the number of patients with impaired immune systems increases not only from HIV-infected persons but also from the increasing numbers of patients being immunosuppressed by medical interventions for organ transplantation and treatment of neoplastic disease. In the sections below, major classes of antifungal agents are considered, with emphasis on those that have potential for use against *P. carinii* infections. A review of therapeutic indications and pharmacology of antifungal agents has appeared elsewhere.[149]

Studies with Established Antifungal Agents. Azole antifungal agents inhibit cytochrome P450-dependent lanosterol 14-α-demethylase, the enzyme than converts lanosterol to ergosterol in fungal cells. In susceptible fungi, ergosterol is the primary sterol of the cell membrane and without its stabilizing effect the fungal cell membrane cannot maintain differential concentrations of ions and small molecules. Some

members of this class of compounds may also influence sterol biosynthesis in mammalian tissues, a source of potential toxicity.

Several azole antifungal agents have been tested against *P. carinii*. Ketoconazole at 25 mg/kg did not prevent *P. carinii* pneumonia in rats.[22] Several azoles were compared at 10 μg/mL for their ability to inhibit growth of *P. carinii* in culture;[150] miconazole was somewhat inhibitory, but no effect was seen with itraconazole, fluconazole, ketoconazole, etanidazole, or SCH 39304.[150] These compounds were also ineffective in a rat model for therapy of *P. carinii* pneumonia. Most of the compounds were tested at 40 mg/kg given by oral gavage, but etanidazole was given intravenously at 500 mg/kg for 7 days followed by 1000 mg/kg for 7 days; even at these massive doses, no effect was seen on the numbers of trophozoites or cysts in the lungs of treated animals.[150] The ineffectiveness of azoles correlates with the lack of ergosterol in cell membranes of *P. carinii*.[150-153]

Flucytosine is taken into fungal cells and converted to 5-fluorouracil, which replaces uracil in pyrimidine pools in the fungal cell; in addition, 5-fluorouracil is further metabolized to the 5-fluoro analogue of UMP and in that form inhibits the activity of thymidylate synthetase. As a result of these actions, both protein and DNA synthesis are impaired. The drug has been used in combination with amphotericin B for treatment of cryptococcal meningitis, and for systemic infections caused by *Candida albicans* or *Aspergillus fumigatus*.[149] Flucytosine at an oral dose of 150 mg/kg was ineffective either alone or combined with amphotericin B or miconazole in a rat model for therapy of *P. carinii* pneumonia.[24] The mechanism of resistance of *P. carinii* to flucytosine has not been explored.

Griseofulvin inhibits mitotic spindle formation in fungal cells. The drug is used to treat fungal infections of the skin that fail to respond to topical agents. Griseofulvin at 100-300 mg/kg given orally was inactive for therapy against *P. carinii* pneumonia in rats.[12,24]

The polyene antifungal agent amphotericin B is the most effective drug for systemic fungal infections.[149] The drug is accepted for therapy of invasive infections caused by *Candida* species or *Aspergillus fumigatus*, and for disseminated infections caused by *Histoplasma capsulatum*. Meningitis caused by *Cryptococcus neoformans* is also treated with amphotericin B either alone or with flucytosine. Clinical experience in treating fungal infections such as these suggested that *P. carinii* infections could progress while amphotericin B was being used. Amphotericin was not effective against *P. carinii* in culture at 0.5 μg/mL, but inhibited growth at 5 μg/mL,[88] a concentration higher than peak serum concentrations expected in humans. In a rat treatment protocol for *P. carinii* pneumonia, amphotericin B was inactive either alone or combined with other antifungal agents such as flucytosine or miconazole.[24] The lack of effect of amphotericin B relates to the absence of ergosterol, the primary target of the polyenes, from the membranes of *P. carinii*.[150-153]

Terbinafine (**87**, Chart 9) is a topical antifungal agent that inhibits squalene epoxidase, an early enzyme in sterol biosynthesis that is required for cholesterol as well as ergosterol biosynthesis. Terbinafine was effective against *P. carinii* in culture and highly effective at doses of 5-20 mg/kg in a rat model for treatment of *P. carinii* pneumonia.[154] The efficacy of this compound suggests that sterol biosynthesis in *P. carinii* may be an exploitable target in spite of the lack of ergosterol.

Experimental Antifungal Agents. In contrast to the poor activity displayed by most traditional antifungal agents, newer agents directed at different targets have shown promising activity against *P. carinii*. The most promising classes of agents are directed toward cell wall biosynthesis, and include echinocandins, papulacandins, and pneumocandins. The rationale for applying these agents to *P. carinii* is that the compounds are known to inhibit 1,3-β-glucan synthesis in certain yeasts and cell walls of *P. carinii* cysts are known to contain similar structures.[155]

Early studies with natural products of the echinocandin or papulocandin class showed that reduction in cysts occurred within 4 days of therapy, whereas pentamidine or trimethoprim/sulfmethoxazole therapy produced a gradual fall in cysts beginning about 1 week after the start of therapy and progressing for up to 3 weeks.[156,157] Trophozoites were not eliminated by therapy with these compounds but prophylaxis with them blocked the appearance of trophozoites.[157] Compounds were subsequently tested in an acute therapy model in rats where 90% cyst reduction in four days (ED90) was the goal. The naturally occurring pneumocandin B_0, L-688786, (**88**, Chart 9) was active against *P. carinii* with an ED90 value of 0.08 mg/kg.[157]

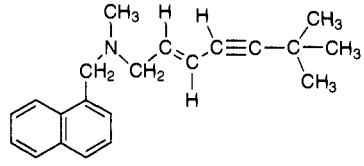
Compound **88** and related natural products were poorly soluble in water, which led to the necessity of administering the compounds in dilute DMSO or in polyethylene glycol.[157] More water-soluble versions prepared from the natural products retained activity against *P. carinii* and other fungi.[157-159] One of these water-soluble compounds (**89**, L-693,989) reduced cyst counts by 90% in four days when used at a dose of 0.15 mg/kg;[157] trophozoite counts did not parallel the fall in cyst counts, again suggesting that echinocandins are not rapidly lethal to trophozoites. LY302146 (**90**) at 2 mg/kg was highly effective for therapy in mouse models of *P. carinii* pneumonia.[159] LY303366 (**91**), a semisynthetic echinocandin was highly active against *P. carinii* in rats for therapy at a dose of 5 mg/kg for 4 days, or for prophylaxis at a dose of 4 mg/kg for 28 days.[158] A semisynthetic pneumocandin (**92**) was effective for therapy at 9 μg/kg twice daily for 4 days;[155] **92** was also highly active against candida and asperigillus in animal models.[26]

A different class of compound acting against cell walls of *P. carinii* is represented by benanomicin A (**93**). This compound binds to mannose in *P. carinii* cell walls; it does not inhibit β-glucan synthesis in *Aspergillus fumagatus*, suggesting a different mechanism of action than echinocandins or pneumocandins. Benanomicin A was active at 1-100 mg/kg for treatment in a nude mouse model of *P. carinii* pneumonia.[19]

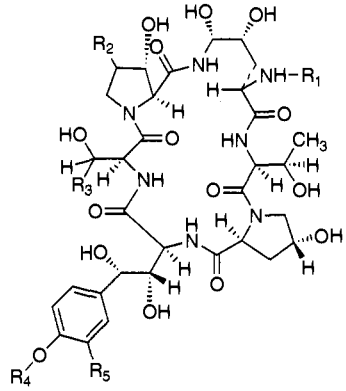
Future Directions

At the start of the AIDS epidemic in the early 1980s, therapeutic options for *P. carinii* pneumonia included pentamidine given intravenously or trimethoprim/sulfamethoxazole given orally or intravenously. Much progress has been made since that time. Drug families with potent antipneumocystis activity have been dis-

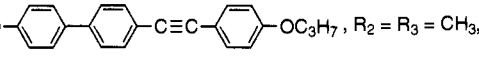
Chart 9. Antifungal agents active against *P. carinii*

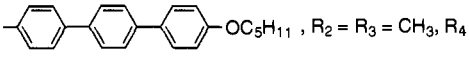


87: terbinafine

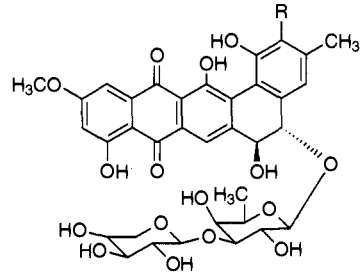


88: R_1 = 10,12-dimethylmyristoyl, R_2 = H, R_3 = CH_2CONH_2, R_4 = R_5 = H (L-688, 786)
89: R_1 = 10,12-dimethylmyristoyl, R_2 = H, R_3 = CH_2CONH_2, R_4 = PO_3Na, R_5 = H (L-693,989)

90: R_1 =  , R_2 = R_3 = CH_3, R_4 = R_5 = H (LY302146)

91: R_1 =  , R_2 = R_3 = CH_3, R_4 = R_5 = H (LY303366)

92: R_1 = $CO(CH_2)_8CH(CH_3)CH_2CH(CH_3)CH_2CH_3$, R_2 = H, R_3 = $CH_2CH_2NH_2$, R_4 = R_5 = H (L733, 560)



93: R = $CONHCH(CH_3)COOH$ (benanomicin A)

covered, including 8-aminoquinolines (primaquine, WR6026, WR238605), a hydroxynaphthoquinone (atovaquone), and β-glucan synthesis inhibitors. Some of these classes are beginning to be exploited clinically but sponsorship to bring promising leads to clinical trial has been slow to develop for several excellent candidate compounds. Recognition that *P. carinii* pneumonia will remain a significant clinical problem for the foreseeable future may stimulate drug development efforts. In addition, many of the new antipneumocystis agents have significant activity against other pathogens, potentially increasing the market and hence the appeal for developing for new agents.

Successful drug design for *P. carinii* can now begin to rely on sophisticated modeling to maximize binding to drug targets. New drug targets are being identified and studied by several laboratories, which may broaden development efforts. In addition, a second level of drug evaluation is required to assess the ability of the designed or discovered compounds to enter intact *P. carinii* in an in vitro model such as short-term culture. Pharmacokinetics of the compounds must also be assessed in one or more of the available animal models that have been successfully employed in the past. This array of tested tools should facilitate development of agents with a high likelihood for clinical success.

Successful control of *P. carinii* in the future will depend on development of an array of agents operating by a variety of mechanisms. In part this strategy is driven by the necessity of dealing with the intolerance to certain drugs that is displayed by AIDS patients. More fundamentally, we must prepare to deal with the inevitable development of resistance to established drugs by creating alternate modes of therapy today.

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