Development of a Yeast Assay for Rapid Screening of Inhibitors of Human-Derived *Pneumocystis carinii* Dihydrofolate Reductase

Liang Ma,[†] Qiuyao Jia,[†] and Joseph A. Kovacs*

Critical Care Medicine Department, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892

Received 20 March 2002/Returned for modification 14 May 2002/Accepted 17 June 2002

Human-derived *Pneumocystis carinii* dihydrofolate reductase (DHFR) was expressed in a *Saccharomyces cerevisiae* strain whose growth depends on complementation by this enzyme. We utilized a quantitative assay to measure the sensitivity of this yeast strain to DHFR inhibitors. This assay should be useful for identifying new inhibitors of human-derived *P. carinii* DHFR.

Current treatment of *Pneumocystis carinii* pneumonia (PCP) is complicated by frequent toxic and allergic side effects, emerging drug resistance, and limited therapeutic options. There is a clear need to discover new agents against *P. carinii*. Dihydrofolate reductase (DHFR) is a well-described target for antimicrobial chemotherapy, and its inhibitors, like trimethoprim, pyrimethamine, and trimetrexate, are commonly used drugs for PCP therapy or prophylaxis. Genes encoding DHFR of *P. carinii* from various mammalian host species have been cloned (5, 8, 9). The amino acid sequence of DHFR of human-derived *P. carinii* differs from that of rat-derived *P. carinii* by 38%, with some of the amino acid changes occurring in the active sites of the enzyme (4, 9). Thus, the ideal target for designing potential antifolate drugs for the treatment of humans is human-derived *P. carinii* DHFR (9, 10). The expression of recombinant human-derived *P. carinii* DHFR enzyme has permitted kinetic characterization of the enzyme and re-assessment of the inhibitory potency of several commonly used antifolate drugs by in vitro enzyme assays (10). The goal of the present study was to develop a rapid, complementation-based drug screening system by using the yeast *Saccharomyces cerevisiae* expressing human-derived *P. carinii* DHFR, as has been previously reported for other organisms (3, 7, 15).

The yeast model system used in this study was derived from an *S. cerevisiae* strain (TH5; *Mata ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3*) (kindly provided by Carol Hopkins Sibley) (3) in which the endogenous DHFR gene is inactivated (6). The human-derived *P. carinii* DHFR coding region was amplified by PCR from a plasmid we constructed previously (10), cloned into an expression vector containing *S. cerevisiae* sequences that permit replication of the plasmid and expression of a heterologous gene in yeast (3, 13), and transformed into TH5 cells by the lithium acetate method. The transformants were selected on plates containing tryptophan-deficient synthetic medium supplemented with 100 µg of dTMP (Sigma, St. Louis, Mo.) per ml and plated on rich yeast extract-peptone-dextrose

medium without dTMP to test the function of the construct (13). TH5 yeast strains expressing rat-derived *P. carinii* DHFR or human DHFR have been described previously (3).

To determine the sensitivity of the engineered yeasts to several selected antifolate drugs, assays of the concentration of the drug required to inhibit cell growth by 50% (IC₅₀ assays) were conducted with 1 mM sulfanilamide, as previously described (7, 13). Each drug was tested in triplicate in at least two separate experiments.

Figure 1 and Table 1 illustrate the inhibition patterns of selected DHFR inhibitors for different yeast strains. For the human-derived *P. carinii* DHFR yeast strain, trimethoprim and pyrimethamine were both weak inhibitors, with IC₅₀s in the micromolar range; trimetrexate was about 10-fold and 40-fold more potent than trimethoprim and pyrimethamine, respectively. WR99210, a triazine compound (Jacobus Pharmaceutical Company, Princeton, N.J.), has been found to be highly effective against malaria DHFR (11, 12) but has not been tested for activity against human-derived *P. carinii* DHFR. WR99210 was an even more potent inhibitor, with an IC₅₀ in the 10⁻⁸ M range. In comparison with the rat-derived *P. carinii* DHFR strain, the human-derived *P. carinii* DHFR strain showed very similar sensitivities to pyrimethamine and WR99210 but was about 10-fold more sensitive to both trimethoprim and trimetrexate. WR99210 exhibited excellent selectivity for both human- and rat-derived *P. carinii* DHFRs compared to that for human DHFR. While trimethoprim showed a relatively favorable selectivity, trimetrexate and pyrimethamine appeared not to be selective in this assay system.

Table 1 shows a comparison between the IC₅₀s from the yeast assay and those from an in vitro enzyme assay, based upon previously published data (for trimethoprim, pyrimethamine, and trimetrexate) or data generated in this study by using previously described methods and conditions (WR99210) (10). Based on three experiments, the mean IC₅₀ (± standard deviation) of WR99210 for human-derived *P. carinii* DHFR was 10.0 (±1.0) nM. A comparison of the relative inhibition profiles in the yeast assay and those in the in vitro assay supports the usefulness of the yeast assay as an initial step for identifying new inhibitors of human-derived *P. carinii* DHFR. The yeast assay is simple, fast, and inexpensive and could readily be adapted for automation. The usefulness of

* Corresponding author. Mailing address: Building 10, Room 7D43, National Institutes of Health, 10 Center Dr., MSC 1662, Bethesda, MD 20892-1662. Phone: (301) 496-9907. Fax: (301) 402-1213. E-mail: jkovacs@nih.gov.

[†] Present address: Department of Biology, Loyola University at Chicago, Chicago, IL 60626.

FIG. 1. Inhibition of DHFR from human-derived *P. carinii* (■), rat-derived *P. carinii* (△), and humans (○), by using *S. cerevisiae* complemented by the corresponding DHFR genes. The results of a representative IC_{50} assay are shown for each drug tested. The yeast growth in the control without drug was scored as 100%, and the growth of yeast cells at each drug concentration was divided by that of the control to determine the relative growth. Each point represents the mean value of triplicate data.

this system in screening for antimicrobial drugs has been well demonstrated in previous studies (3, 7, 15).

The IC_{50} obtained by the yeast assay is the result of a complex function of the level of expression of the target enzyme in the yeast, the penetration of the drug to the cellular location of the target enzyme, and the intrinsic ability of the drug to inhibit the enzyme (3, 13). Furthermore, the addition of 1 mM sulfanilamide in the yeast assay will also inhibit, to some extent, the growth of the yeast cells (3). Therefore, the actual IC_{50} in the yeast assay cannot be compared directly with that obtained from an in vitro assay, which reflects a direct interaction between the drug and the target enzyme under standardized conditions.

As shown in Table 1, the absolute IC_{50} s of the four drugs tested showed some differences between the two assay systems. In a given recombinant strain, enzyme expression should not account for the relative differences among drugs in the yeast assay and those in the in vitro assay, which suggests that the abilities of the drugs to penetrate the yeast cell wall may differ. Given the uncertainty about the ability of various drugs to penetrate *P. carinii* compared to that of yeast, application of these observations to potential therapeutic benefit in the treatment of PCP must be done cautiously.

It is of particular interest that the human-derived *P. carinii* DHFR yeast strain showed an approximately 10-fold increase in sensitivity to trimetrexate and trimethoprim compared to that of the rat-derived *P. carinii* DHFR yeast strain. Since the

host yeast strains are identical, differences in penetration should not account for the relative differences among drugs demonstrated by the yeast assay and in vitro assay. Furthermore, given that the sensitivities to pyrimethamine and

TABLE 1. Comparison of the IC_{50} s for DHFR inhibitors obtained from the yeast complementation assay with those from the in vitro assay

Inhibitor	Assay ^a	IC_{50} (nM)		
		Human-derived <i>P. carinii</i> DHFR	Rat-derived <i>P. carinii</i> DHFR	Human DHFR
Trimetrexate	Yeast	490	4,200	600
	In vitro	7.5 ^b	26.1 ^c	3.2 ^d
Pyrimethamine	Yeast	20,500	33,200	29,300
	In vitro	240 ^b	2,800 ^c	5,800 ^d
Trimethoprim	Yeast	5,700	81,000	>200,000
	In vitro	4,850 ^b	39,600 ^c	1,300,000 ^d
WR99210	Yeast	9.0	9.55	>742,000
	In vitro	10.0	ND ^e	ND

^a Each value for the yeast complementation assay represents an average of the results of at least two independent experiments.

^b Data from reference 10.

^c Data from reference 1.

^d Data from reference 2.

^e ND, not determined.

WR99210 were similar, it is unlikely that these differences represent differential levels of enzyme expression in the two yeast strains. Rather, as suggested by the in vitro enzyme inhibition data, this likely reflects the effects of the primary sequence differences between human- and rat-derived *P. carinii* DHFRs (8, 9).

DHFR inhibitors have been among the most studied classes of drugs for treatment of PCP. While animal studies (14) suggest that trimethoprim plays a minimal role in the treatment of PCP (consistent with its relatively poor inhibition seen in this and other studies), DHFR inhibitors such as trimetrexate clearly have potent anti-*P. carinii* activity, even as single agents (1). The present assay should facilitate identification of additional potent and selective agents.

We thank Carol Hopkins Sibley, Department of Genetics, University of Washington, Seattle, for providing yeast cells and plasmids and helping to establish the yeast assay, in addition to critically reviewing the manuscript. We also thank David Jacobus, Jacobus Pharmaceutical Company, Princeton, N.J., for providing WR99210, and Carmen J. Allegra, National Institutes of Health, Bethesda, Md., for providing trimetrexate.

REFERENCES

- Allegra, C. J., J. A. Kovacs, J. C. Drake, J. C. Swan, B. A. Chabner, and H. Masur. 1987. Activity of antifolates against *Pneumocystis carinii* dihydrofolate reductase and identification of a potent new agent. *J. Exp. Med.* **165**: 926–931.
- Allegra, C. J., J. A. Kovacs, J. C. Drake, J. C. Swan, B. A. Chabner, and H. Masur. 1987. Potent in vitro and in vivo antitoxoplasma activity of the lipid-soluble antifolate trimetrexate. *J. Clin. Invest.* **79**:478–482.
- Brophy, V. H., J. Vasquez, R. G. Nelson, J. R. Forney, A. Rosowsky, and C. H. Sibley. 2000. Identification of *Cryptosporidium parvum* dihydrofolate reductase inhibitors by complementation in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **44**:1019–1028.
- Cody, V., N. Galitsky, J. R. Luft, W. Pangborn, A. Gangjee, R. Devraj, S. F. Queener, and R. L. Blakely. 1997. Comparison of ternary complexes of *Pneumocystis carinii* and wild-type human dihydrofolate reductase with coenzyme NADPH and a novel classical antitumor furo[2,3-d]pyrimidine antifolate. *Acta Crystallogr. Sect D* **53**:638–649.
- Edman, J. C., U. Edman, M. Cao, B. Lundgren, J. A. Kovacs, and D. V. Santi. 1989. Isolation and expression of the *Pneumocystis carinii* dihydrofolate reductase gene. *Proc. Natl. Acad. Sci. USA* **86**:8625–8629.
- Huang, T., B. J. Barclay, T. I. Kalman, R. C. von Borstel, and P. J. Hastings. 1992. The phenotype of a dihydrofolate reductase mutant of *Saccharomyces cerevisiae*. *Gene* **121**:167–171.
- Lau, H., J. T. Ferlan, V. H. Brophy, A. Rosowsky, and C. H. Sibley. 2001. Efficacies of lipophilic inhibitors of dihydrofolate reductase against parasitic protozoa. *Antimicrob. Agents Chemother.* **45**:187–195.
- Ma, L., L. Borio, H. Masur, and J. A. Kovacs. 1999. *Pneumocystis carinii* dihydropteroate synthase but not dihydrofolate reductase gene mutations correlate with prior trimethoprim-sulfamethoxazole or dapsone use. *J. Infect. Dis.* **180**:1969–1978.
- Ma, L., H. Imamichi, A. Sukura, and J. A. Kovacs. 2001. Genetic divergence of the dihydrofolate reductase and dihydropteroate synthase genes in *Pneumocystis carinii* from 7 different host species. *J. Infect. Dis.* **184**:1358–1362.
- Ma, L., and J. A. Kovacs. 2000. Expression and characterization of recombinant human-derived *Pneumocystis carinii* dihydrofolate reductase. *Antimicrob. Agents Chemother.* **44**:3092–3096.
- Rieckmann, K. H. 1973. The in vitro activity of experimental antimalarial compounds against strains of *Plasmodium falciparum* with varying degrees of sensitivity to pyrimethamine and chloroquine, p. 58. In *Chemotherapy of malaria and resistance to antimalarials*. WHO Tech. Rep. Ser. No. 529. World Health Organization, Geneva, Switzerland.
- Rieckmann, K. H., A. E. Yeo, and M. D. Edstein. 1996. Activity of PS-15 and its metabolite, WR99210, against *Plasmodium falciparum* in an in vivo-in vitro model. *Trans. R. Soc. Trop. Med. Hyg.* **90**:568–571.
- Sibley, C. H., V. H. Brophy, S. Cheesman, K. L. Hamilton, E. G. Hankins, J. M. Wooden, and B. Kilbey. 1997. Yeast as a model system to study drugs effective against apicomplexan proteins. *Methods* **13**:190–207.
- Walzer, P. D., C. K. Kim, J. M. Foy, M. J. Linke, and M. T. Cushion. 1988. Inhibitors of folic acid synthesis in the treatment of experimental *Pneumocystis carinii* pneumonia. *Antimicrob. Agents Chemother.* **32**:96–103.
- Wooden, J. M., L. H. Hartwell, B. Vasquez, and C. H. Sibley. 1997. Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **85**:25–40.