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Efficacies of Zinc-Finger-Active Drugs against *Giardia lamblia*

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Twenty-nine of 34 (85%) Zn-finger-active compounds at 300 μM or less inhibited the growth of *Giardia lamblia*. The most active compound, disulfiram (Antabuse), was cidal at 1.23 ± 0.32 μM. In the adult mouse model, significant in vivo activity was demonstrated by increased cure rates and decreased parasite burdens.

Giardia lamblia is a protozoan parasite that inhabits the small intestines of humans and other mammals. It is among the world's most common disease-causing parasites, and in the United States (3) it is responsible for epidemics of waterborne diarrhea (17) as well as gastrointestinal illness wherever fecal contamination occurs. In addition to its importance as a pathogen, there is considerable interest in its biology because it is among the most primitive eukaryotes (33). The trophozoite, or growing form of the parasite, is completely covered by one of a family of proteins (variant-specific surface proteins [VSPs]) that undergoes surface antigenic variation (1, 22, 23). VSPs are unique surface cysteine-rich proteins with numerous CXXC motifs (11, 23), a conserved carboxyl terminus (20), and one or more Zn-finger motifs which closely resembles the LIM- and RING-finger motifs found in other Zn binding proteins of higher eukaryotes (22, 25, 35). Zn and Fe have been detected in one VSP (19) predominately expressed in GS/H7, an isolate used here (24), but not in another isolate (28). Most interestingly, no other surface-residing Zn-finger protein exists in any other organism.

Zn-finger proteins are essential to normal cellular function and developmental processes (2). Inhibition of microbe-specific Zn-finger protein activity is a novel approach to chemotherapeutic intervention (26, 29). Zn-finger-active chemotherapeutic agents which inhibit replication of human immunodeficiency virus type 1 (HIV-1) have been designed (26–29). These compounds covalently modify the highly conserved $Cys(X)_2Cys(X)_4His(X)_4Cys$ (CCHC) retroviral Zn-finger domains of the HIV-1 nucleocapsid p7 protein (NCp7) (27) and prevent their essential function. Competitive Zn-finger peptides have also been shown to have a modest effect against the influenza virus (15). Because *Giardia* has abundant surface-located Zn-finger proteins which may be particularly susceptible to Zn-finger-active compounds, a series of compounds (Tables 1 and 2) with known activity toward HIV-1 NCp7 Zn fingers were tested in vitro for their antiparasitic activities, and one of the most active compounds, disulfiram, was also tested for its activity in vivo.

Most of the in vitro studies used *Giardia* isolate WB clone 1267 (WB/1267) (32), but limited assays used isolate GS clone H7 (GS/H7) (24) because this clone was used in vivo. Organisms were maintained as previously described in TYI-S-33 medium with bile and antibiotics (16). Inhibition and cidal activities were determined in 96-well culture plates by methods similar to those reported previously (22). Compounds were supplied at known concentrations in dimethyl sulfoxide (DMSO) by the Drug Synthesis and Chemistry Branch, Development Therapeutics Branch, National Cancer Institute, or, when supplied as dry compound, were dissolved in DMSO at a stock concentration of 100 mM and then added to medium containing various concentrations of cysteine. Typically, 100 to 200 μl of compound in medium was placed in 96-well plates. Depending on the experiment, from 20,000 to 50,000 trophozoites were then added in volumes ranging between 5 to 30 μl. Controls consisted of wells with normal medium, an appropriate concentration of cysteine, and the DMSO solvent, which had no effect on growth at the concentrations used in the study. Plates were incubated anaerobically in sealed bags (22) at 37°C for up to 1 week and were scored visually at various time periods, but the standard period of recording in the present study was at 18 to 20 h. The wells were scored as 0 when no viable organisms were observed, +1/2 when rare motile organisms were present, +1 when a small number of organisms showing movement were present (<20 trophozoites), +2 when moderate growth and adherence of organisms were present, +3 when significant growth that was less than that of untreated controls was present, and +4 when growth equal to that of the control containing the same amount of cysteine was present. Assessment of the cidal effects observed visually at 18 to 20 h was verified in some experiments by quantitative measurement of viable trophozoites after 3 days in culture by a previously described method (22).

The presence of the usual 11.3 mM cysteine in medium blunted the activities of these compounds. For the most active compounds, cidal activity was decreased from 28- to 250-fold in 11.3 mM cysteine compared to that in 2.8 mM cysteine. In studies with GS/H7 a dose-response inhibition of activity was demonstrated with compound 1, disulfiram ($y = 3.183 - 0.032$; $r = 0.996$). The minimal amount of cysteine required to yield +4 growth in control wells varied and was likely due to the differences in the oxidation of the added cysteine and to the amount of cysteine in the serum added to TYI-S-33 medium. Block titrations indicated that 2.8 mM prepared daily and added immediately to the medium gave +4 growth and reproducible cidal levels of drug when the WB isolate was used.

The most active compounds were the thiuram derivatives. Among the 34 compounds tested, 29 of 34 (85%) were cidal or showed some degree of growth inhibition when the compound was used at 300 μM (Tables 1 and 2). Of the 12 nonthiuram compounds screened at 300 μM, four exhibited cidal effects at 300 μM, four caused a reduction of growth, and four did not

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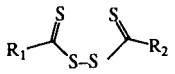
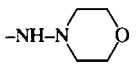
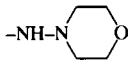
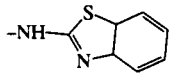
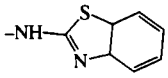
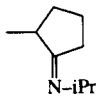
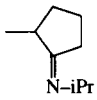
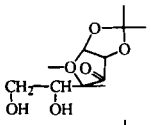
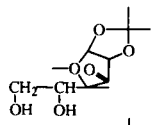
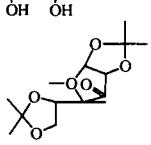
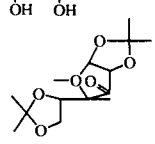
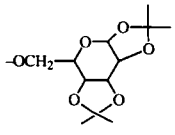
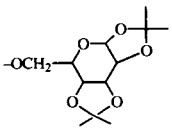
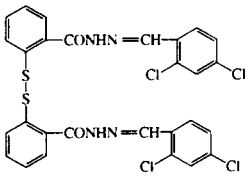
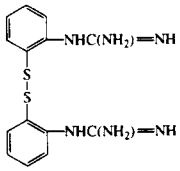
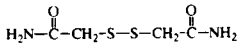
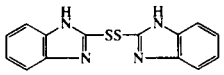
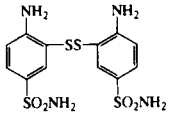
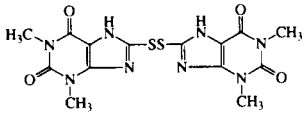
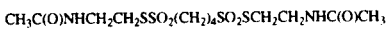
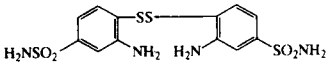
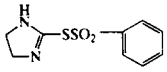
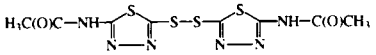
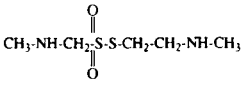
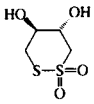
TABLE 1. Compound identification, structure, and cidal activity against *G. lamblia* for thiuram compounds[a]



Compound	NSC no.	R_1	R_2	Cidal concn (μM)
1	25953	-N(Et)$_2$	-N(Et)$_2$	1.2
2	1771	-N(Me)$_2$	-N(Me)$_2$	1.2
3	27320	-NHNH$_2$	-NHNH$_2$	>300*
4	608475	-N(CH$_2$Ph)$_2$	-N(CH$_2$Ph)$_2$	>300Δ
5	527035			1.2
6	402538			11.1
7	403854			11.1
8	327377			100
9	290661			33.3
10	290662			>300Δ
11	1339	-OCH(Me)$_2$	-OCH(Me)$_2$	>300*
12	402561	-OEt	-OEt	>300*
13	5239	–OBu	–OBu	>300*
14	317926	-OPh	-OPh	>300*
15	93059			>300*
16	93057			>300*
17	93058			>300*
18	14560	-NHMe	-NH(CH$_2$)$_3$NHC(S)SSC(S)NHMe	>300*
19	14561	-OMe	-NH(CH$_2$)$_3$NHC(S)SSC(S)NHMe	100
20	20871	-OMe	-NH(CH$_2$)$_2$N[C(S)SSC(S)OMe][(CH$_2$)$_2$NHC(S)SSC(S)OMe]	>300*
21	20866	-OBu	-NH(CH$_2$)$_2$NHC(S)SSC(S)OBu	>300*
22	20867	-O-iPr	-NH(CH$_2$)$_2$NHC(S)SSC(S)OiPr	>300*

[a] Asterisks indicate that a recognizable degree of inhibition of growth occurred when the compound was used at 300 μM. The delta symbol indicates that the compound at 300 μM had no effect on growth. Compound 1 is disulfira. Me, methyl; Bu, butyl; Et, ethyl; Ph, phenyl; Pr, propyl.

TABLE 2. Compound identification, structure, and cidal activity against *G. lamblia* for nonthiuram compounds[a]

Compound	NSC no.	Structure	Cidal concn (μM)
23	4493		33-100
24	20625		>300*
25	28727		>300Δ
26	35825		>200*
27	58950		>300
28	71895		>300
29	76302		300
30	83217		>300
31	112801		>300*
32	327174		300
33	342031		>300Δ
34	624152		>300Δ

[a] See footnote *a* of Table 1 for definitions of symbols.

inhibit growth. On further testing, only compound NSC 4493 was cidal at concentrations of <100 μM. Eleven of 22 thiuram compounds that scored from 0 to +1/2 growth (i.e., effective growth inhibition) when they were used at 300 μM were analyzed further. As shown in Fig. 1, a wide range of activities among these compounds was observed, but disulfiram (NSC 25953; compound 1) was among the most active, yielding a mean total cidal concentration of 1.23 μM. Four different experiments yielded mean cidal levels of 1.17 ± 0.32 μM (standard deviation). Quantitative assessment showed no growth at concentrations of 0.88 μM or higher and a graded increase in organisms with decreasing drug concentration (5), which is consistent with analysis by visual assessment (data not shown). Compound 5 (NSC 527035) showed cidal activity at 1.23 and 1 μM in two separate experiments.

The in vivo efficacy of compound NSC 25953 (disulfiram) against *G. lamblia* was tested because it was among the most active compounds in vitro, was available in large quantities

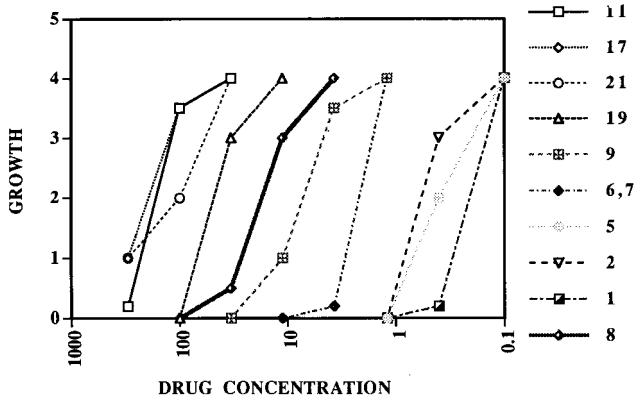


FIG. 1. Sensitivity of *G. lamblia* to thiuram compounds. The following compounds have the indicated NSC numbers: 1 (disulfiram), NSC 25953; 2, NSC 1771; 5, NSC 527035; 6, NSC 402538; 7, NSC 403854; 9, NSC 290661; 17, NSC 93058; 19, NSC 14561; and 21, NSC 20866. Growth was scored visually at 18 to 20 h as described in the text. 0 signifies no growth, and +4 signifies growth equal to that in the control wells.

commercially (Aldrich Chemical Company), and is used for the treatment of alcoholism in humans (8). The commercial drug showed the same activity in vitro as the supplied drug. Since GS/H7 is used in the adult mouse model of *G. lamblia* infection, the activity of disulfiram was tested against this isolate, and it was found to have sensitivity comparable to that of disulfiram in 2.8 mM cysteine when the sensitivities were compared at the same time. Adult C57B female mice were obtained from Takonic Labs and were housed as described previously (4). The mice were inoculated with 500,000 trophozoites by gavage on day 0, treated on days 3 through 6, and then killed on day 7. Disulfiram or metronidazole was administered by gavage as a fine suspension in 200 μl of water. Small intestines were minced in 10 ml of ice-cold medium, allowed to cool for 30 min, and then warmed to 37°C. The number of motile trophozoites in five random fields at all depths not obscured by intestines were counted at a magnification of ×200. If no organisms were noted, then five counts at a magnification of ×25 were performed. Cure was defined as the failure to detect any trophozoites. All but 1 of 30 untreated control mice were infected. In contrast, cure rates of 40, 40, and 21% were found in the three groups of treated animals, respectively (Table 3). The parasite burden was significantly decreased in all three experiments and in the mice treated with the largest dose of disulfiram (25 mg twice daily for 4 days), an average of 2.03 ± 3.1 trophozoites were found in the treated animals, whereas 78.2 ± 28.6 trophozoites were found in the control group. To confirm this model as a valid measure of chemotherapeutic efficacy for anti-*Giardia* compounds, the effectiveness of metronidazole, a drug that is known to be active and that is commonly used for the treatment of giardiasis, was evaluated. All of the metronidazole-treated mice were cured, whereas 1 of 10 of the control mice were cured.

The major findings of the present study are that many Zn-finger-active compounds have activity against *G. lamblia* in vitro and one of the most active compounds in vitro, disulfiram, showed efficacy in vivo. Additionally, the adult mouse model of infection proved to be a convenient and viable system for testing the efficacies of drugs in vivo.

The compounds tested had various degrees of activity against *G. lamblia*, and a majority of the most active compounds were thiuram derivatives. Despite the various effects of cysteine on the activities of these compounds, the results were reproducible and relatively consistent. The mode of action of Zn-finger-active compounds and disulfiram (12) against HIV infection is destruction of Zn-finger motifs in NCp7; the mode of action against *G. lamblia* is unclear. However, there was a strong correlation between the ability of these compounds to eject Zn from the NCp7 protein of HIV-1 and the in vitro activities of the same compounds against *G. lamblia* (extracted from reference 29). Five of 5 Zn-releasing compounds were cidal at levels of ≤10 μM, while 12 of 12 compounds unable to release Zn effectively were cidal at levels of ≥50 μM and most were cidal at ≥300 μM.

The in vivo activity of disulfiram was clearly demonstrated and was modest compared to the high degree of activity demonstrated in vitro. At the highest dosage, 25 mg twice daily for 4 days, 40% of the mice were cured, whereas 0% of the controls were cured, and the remaining mice had a dramatically decreased parasite burden. Despite the differences in the numbers of parasites in the control mice, which is a variability seen with this animal model, the results of all three experiments were consistent and showed the efficacy of disulfiram. It is likely that the activity of disulfiram would have been greater if the solubility of disulfiram could have been increased to raise the concentration of drug able to affect the parasite and facilitate administration of the drug.

The biological effects of disulfiram and its metabolism are complex and are reviewed elsewhere (5–10). Disulfiram and/or its immediate metabolites have been shown to be active in vitro against a number of organisms, including *Plasmodium falciparum* (31), *Trypanosoma cruzi* (18), *Trichomonas vaginalis* (14), and *Entamoeba histolytica* (25a); additionally, disulfiram has in vivo efficacy against *Trichomonas muris* (13) and inhibits *Candida albicans* in immunosuppressed mice (34). Neither the mode(s) of action of this drug nor the active metabolites that

TABLE 3. In vivo efficacy of disulfiram

Expt no.	Drug (dosage)[a]	Treated mice		Control mice	
		No. cured/total no. (*P* value)	No. of trophozoites/high-power field (*P* value)	No. cured/total no.	No. of trophozoites/high-power field
1	Disulfiram (25 mg q.d., 4 days)	4/10 (0.003)	2.87 ± 7.85 (0.049)	0/10	6.74 ± 9.30
2	Disulfiram (25 mg, q.d., 5 days)	3/14 (0.082)	5.23 ± 7.33 (0.011)	1/10	13.16 ± 6.78
	Metronidazole (5 mg, q.d., 5 days)	15/15	0	1/10	13.16 ± 6.78
3	Disulfiram (25 mg, b.i.d., 4 days)	4/10 (0.003)	2.03 ± 3.12 (<0.001)	0/10	78.20 ± 28.69

[a] q.d., every day; b.i.d., twice a day.

are cidal toward these organisms are known. Of interest, *Giardia* has a bifunctional alcohol dehydrogenase-coenzyme A-dependent acetaldehyde dehydrogenase which could be a target enzyme (30).

These types of compounds are potentially useful in the treatment of giardiasis. Disulfiram has been given to humans for decades and is relatively safe (8). In addition, newer agents are needed because patients infected with *Giardia* strains resistant to standard courses of therapy are being more frequently recognized (personal observations).

REFERENCES

1. **Adam, R. D., A. Aggarwal, A. A. Lal, V. F. de la Cruz, T. McCutchan, and T. E. Nash.** 1988. Antigenic variation of a cysteine-rich protein in *Giardia lamblia*. J. Exp. Med. **167:**109–118.
2. **Berg, J. M., and Y. G. Shi.** 1996. The galvanization of biology: a growing appreciation for the roles of zinc. Science **271:**1081–1085.
3. **Bryan, R. T., R. W. Pinner, and R. L. Berkelman.** 1994. Emerging infectious diseases in the United States, improved surveillance, a requisite for prevention. Ann. N. Y. Acad. Sci. **740:**346–361.
4. **Byrd, L. G., J. T. Conrad, and T. E. Nash.** 1994. *Giardia lamblia* infections in adult mice. Infect. Immun. **62:**3583–3585.
5. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 344–345. Chapman & Hall, London, United Kingdom.
6. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 137–203. Chapman & Hall, London, United Kingdom.
7. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 95–136. Chapman & Hall, London, United Kingdom.
8. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 295–305. Chapman & Hall, London, United Kingdom.
9. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 7–9. Chapman & Hall, London, United Kingdom.
10. **Gessner, P. K., and T. Gessner.** 1992. Disulfiram and its metabolite diethyldithiocarbamate, p. 167–193. Chapman & Hall, London, United Kingdom.
11. **Gillin, F. D., P. Hagblom, J. Harwood, S. B. Aley, D. S. Reiner, M. McCaffery, M. So, and D. G. Guiney.** 1990. Isolation and expression of the gene for a major surface protein of *Giardia lamblia*. Proc. Natl. Acad. Sci. USA **87:**4463–4467.
12. **Hathout, Y., D. Fabris, M. S. Han, R. C. Sowder II, L. E. Hendeson, and C. Fenselau.** 1996. Characterization of intermediates in the oxidation of zinc fingers in human immunodeficiency virus type 1 nucleocapsid protein P7. Drug Metab. Dispos. **24:**1395–1400.
13. **Hill, D. E., and R. H. Fetter.** 1997. The effect of disulfiram on egg shell formation in adult *Trichuris muris*. J. Parasitol. **83:**938–942.
14. **Jeney, E., and T. Zsolnai.** 1964. Versuche zur chemotherapeutischen Beeinflussung der durch *Trichomonas vaginalis* hervorgerufenen Infektionen. Zentbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **193:**542–547.
15. **Judd, A. K., A. Sanchez, D. J. Bucher, J. H. Huffman, K. Bailey, and R. W. Sidwell.** 1997. In vivo anti-influenza virus activity of a zinc finger peptide. Antimicrob. Agents Chemother. **41:**687–692.
16. **Keister, D. B.** 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. **77:**487–488.
17. **Kramer, M. H., B. L. Herwaldt, G. F. Craun, R. L. Calderon, and D. D. Juranek.** 1996. Surveillance for waterborne-disease outbreaks—United States, 1993–1994. Morbid. Mortal. Weekly Rep. **45:**1–33.
18. **Lane, J. E., R. R. Ribeiro, C. C. Suarez, B. J. Bogitsh, M. M. Jones, P. K. Singh, and C. E. Carter.** 1996. In vitro trypanocidal activity of tetraethylthiuram disulfide and sodium diethylamine-*N*-carbodithioate on *Trypanosoma cruzi*. Am. J. Trop. Med. Hyg. **55:**263–266.
19. **Luján, H. D., M. R. Mowatt, J. J. Wu, Y. Lu, A. Lees, M. R. Chance, and T. E. Nash.** 1995. Purification of a variant-specific surface protein of *Giardia lamblia* and characterization of its metal-binding properties. J. Biol. Chem. **270:**13807–13813.
20. **Mowatt, M. R., A. Aggarwal, and T. E. Nash.** 1991. Carboxy-terminal sequence conservation among variant-specific surface proteins of *Giardia lamblia*. Mol. Biochem. Parasitol. **49:**215–218.
21. **Nash, T.** 1992. Surface antigen variability and variation in *Giardia lamblia*. Parasitol. Today **8:**229–234.
22. **Nash, T. E., and A. Aggarwal.** 1986. Cytotoxicity of monoclonal antibodies to a subset of *Giardia* isolates. J. Immunol. **136:**2628–2632.
23. **Nash, T. E., A. Aggarwal, R. D. Adam, J. T. Conrad, and J. J. Merritt.** 1988. Antigenic variation in *Giardia lamblia*. J. Immunol. **141:**636–641.
24. **Nash, T. E., T. McCutchan, D. Keister, J. B. Dame, J. D. Conrad, and F. D. Gillin.** 1985. Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals. J. Infect. Dis. **152:**64–73.
25. **Nash, T. E., and M. R. Mowatt.** 1993. Variant-specific surface proteins of *Giardia lamblia* are zinc-binding proteins. Proc. Natl. Acad. Sci. USA **90:**5489–5483.

25a. **Nash, T. E., and T. Miller.** Personal communication.

26. **Rice, W. G., D. C. Baker, C. A. Schaeffer, L. Graham, M. Bu, S. Terpening, D. Clanton, R. Schulz, J. P. Bader, R. W. Buckheit, Jr., L. Field, P. K. Singh, and J. A. Turpin.** 1997. Inhibition of multiple phases of human immunodeficiency virus type 1 replication by a dithiane compound that attacks the conserved zinc fingers of retroviral nucleocapsid proteins. Antimicrob. Agents Chemother. **41:**419–426.
27. **Rice, W. G., et al.** 1993. Inhibition of HIV infectivity by zinc-ejecting aromatic C-nitroso compounds. Nature **361:**473–475.
28. **Rice, W. G., et al.** 1995. Novel inhibitors of HIV-1 p7NC zinc fingers as candidates for the treatment of AIDS. Science **270:**1194–1197.
29. **Rice, W. G., et al.** 1996. Evaluation of selected chemotypes in coupled cellular and molecular target-based screens identifies novel HIV-1 zinc finger inhibitors. J. Med. Chem. **39:**3606–3616.
30. **Rosenthal, B., Z. Mai, D. Caplivski, S. Ghosh, H. de la Vega, T. Graf, and J. Samuelson.** 1997. Evidence for the bacterial origin of genes encoding fermentation enzymes of the amitochondriate protozoan parasite *Entamoeba histolytica*. J. Bacteriol. **179:**3736–3745.
31. **Scheibel, L. W., A. Adler, and W. Trager.** 1979. Tetraethylthiuram disulfide (Antabuse) inhibits the human malaria parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. USA **76:**5303–5307.
32. **Smith, P. D., F. D. Gillin, W. M. Spira, and T. E. Nash.** 1982. Chronic giardiasis: studies on drug sensitivity, toxin production, and host immune response. Gastroenterology **83:**797–803.
33. **Sogin, M. L., J. H. Gunderson, H. J. Elwood, R. A. Alonso, and D. A. Peattie.** 1989. Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. Science **243:**75–77.
34. **Walker, E. J., D. J. Cannon, M. E. Reifsteck, K. A. Hobbs, H. F. Hardin, M. M. Jones, and J. K. Skeeles.** 1987. Effects of diethyldithiocarbamate and structural analogs in mice with systemic candidal infections. Res. Commun. Chem. Pathol. Pharmacol. **56:**253–263.
35. **Zhang, Y. Y., S. B. Aley, S. L. Stanley, and F. D. Gillin.** 1993. Cysteine-dependent zinc binding by membrane proteins of *Giardia lamblia*. Infect. Immun. **61:**520–524.