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Triazine Inhibits *Toxoplasma gondii* Tachyzoites In Vitro and In Vivo

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The triazine WR99210 [4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichlorophenoxypropyloxy)-1,3,5 triazine] inhibits *Toxoplasma gondii* in vitro at nanomolar levels ($P < 0.05$). The 50% inhibitory concentration (IC_{50}) was approximately 50 nM. It is a potent inhibitor in vitro and is also effective in vivo. Administration of WR99210 parenterally (i.e., intraperitoneally) reduced the mean number of RH strain tachyzoites present in peritoneal fluid substantially 4 days after intraperitoneal infection of mice. There was a mean of approximately 35 million parasites in control mice as contrasted with approximately 2 million parasites in mice treated with 1.25 mg WR99210/kg of body weight in a representative experiment ($P < 0.05$). In addition the prodrug PS-15 N′-[3-(2,4, 5-trichlorophenoxy)propyloxy]-N9-(1-methylethyl) imidocarbonimidicdiamide is converted to 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichlorophenoxypropyloxy)-1,3,5 triazine in vivo when the prodrug is administered orally. PS-15 administered by gavage also reduced intraperitoneal RH strain *T. gondii* tachyzoite numbers. WR99210 has high efficacy and relatively low toxicity because of its substantial effect on *T. gondii* dihydrofolate reductase (DHFR) but not the mammalian host DHFR. Amino acid sequences of *T. gondii*, *Plasmodium falciparum*, and *Homo sapiens* DHFRs were compared. It is of interest that of the DHFR amino acids considered to be interacting with WR99210 in *P. falciparum* within interatomic distances within 3 to 5 Å, four of eight were shared with *T. gondii* DHFR. *H. sapiens* also shared four amino acids thought to be interacting with WR99210. Efficacy of intraperitoneal administration of WR99210 and peroral administration of PS-15 demonstrate the potential usefulness of this class of compounds in treatment of toxoplasmosis administered either parenterally or perorally. The recent development program for this class of antimicrobials as antimalarials makes our proof of principle of improved efficacy of triazines (compared with the gold standard treatment, pyrimethamine) against *T. gondii* especially promising.

The dihydrofolate reductase (DHFR) domain of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) molecule is one of the molecular targets for gold standard treatments for diseases caused by *Plasmodium falciparum* and *Toxoplasma gondii*. Those compounds that have demonstrated inhibition of *P. falciparum* and/or *T. gondii* DHFR-TS in vitro and in vivo include pyrimethamine, cycloguanil, and trimethoprim. Rapid development of resistance of malaria parasites to pyrimethamine or cycloguanil led to the development of WR99210, which demonstrated no cross-resistance to other antifolates. The triazine WR99210 [4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichlorophenoxypropyloxy)-1,3,5-triazine] (Fig. 1A) has been found to be a tight-binding and potent inhibitor of *P. falciparum* DHFR-TS (2–4, 9–11), with activity 2 logs greater than that of pyrimethamine. It is also active against *P. vivax* (5, 6, and S. Y. Hunt, M. D. Hastings, H.-M. Shieh, J. Terpinski, D. P. Jacobus, G. A. Schiehser, and C. H. Sibley, Abstr. 53rd Annu. Meet. Am. Soc. Trop. Med. Hyg., abstr. 302, 2004, and M. D. Hastings and C. H. Hopkins, Abstr. 53rd Annu. Meet. Am. Soc. Trop. Med. Hyg., abstr. 524, 2004). No significant resistance to the triazines has been demonstrated for plasmodia. In addition to the potency of triazines, the very high avidity of this compound for the *P. falciparum* DHFR differs from its avidity to mammalian DHFR (13–15), increasing the therapeutic/toxic ratio.

The current use of pyrimethamine for treatment of *T. gondii* infection is associated with suppression of bone marrow and can result in neutropenia even when accompanied with leucovorin supplements. Furthermore, this treatment is not used to treat congenital toxoplasmosis in the first trimester of gestation when folate depletion can have additional detrimental consequences for early fetal development. Moreover, pyrimethamine is given in a synergistic combination with sulfadiazine to treat toxoplasmosis; this combination can give rise to further concern due to allergy, kidney stones, or hepatic or renal complications. We (8) had previously evaluated the active triazine metabolite of proguanil (cycloguanil) against *T. gondii* tachyzoites, and thus it was of particular interest to determine whether WR99210, a highly active triazine, would be active against *T. gondii* at low-nanomolar levels. Given its therapeutic index (7, 13), WR99210 offers the promise of avoiding the aforementioned inadequacies of pyrimethamine and potentially eliminating the requirement for simultaneous administration of a sulfonamide. Structures of WR99210 and its prodrug PS-15 [N-3-(2,4,5-trichlorophenoxypropyloxy)-N9-

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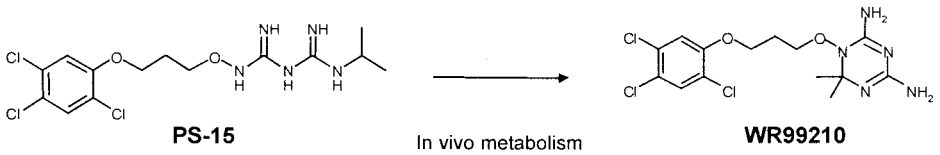
A.



B.

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B. bovis        MSTVYEGCGGLTIYVAIALN------------------RVIGHQNQIPWPHIIHDFRFLRN
H. sapiens      MVGS------LNCIVAVSQN------------------MGIGKNGDLPWPPLRNEFRYFQR
T. gondii       MQKP------VCLVVAMTPK------------------RGIGINNGLPWPHLTTDFKHFSR
P. falciparum   MMEQVCDVFDIYAICACCKVESKNEGKKNEVFNNYTFRGLGNKGVLPWKCNSLDMKYFCA
                *                *                   *     **

B. bovis        GTTYIPPEVLSKN-----------------------PNIQNVVIFGRKTYESIPKASLPLK
H. sapiens      MTTTS-------------------------------SVEGKQNLVIMGKKTWFSIPEKNRPLK
T. gondii       VTKTTPEEASRLNGWLPRKFA--KTGDSGLPSPSVGKRFNAVVMGRKTWESMPRKFRPLV
P. falciparum   VTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKLQNVVVMGRTSWESIPKKFKPLS
                 *                                   *  *     * *    **

B. bovis        NRINVIISRTVKEV------------PGCLVYEDLSTAIRDLR-ANVPH--NKIFILGGS
H. sapiens      GRINLVLSRELKEPP-----------QGAHFLSRSLDDALKLTEQPELANKVDMVWIVGGS
T. gondii       DRLNIVVSSSLKEEDIAAEKPQAEGQQRVRVCASLPAALSLLE-EEYKDSVDQIFVVGGA
P. falciparum   NRINVILSRTLKKED----------FDEDVYIINKVEDLIVLLG----KLNYYKCFITGGS
                 * *   *   *                                          **

B. bovis        YLYKEVLDNGLCDKIYLTRINKEYPGDTFFPDIP--------------------------
H. sapiens      SVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID--------------------------
T. gondii       GLYEAALSLGVASHLYITRVAREFPCDVFFPAFPGDDILSNKSTAAQAAAPAESVFVPFC
P. falciparum   VVYQEFLEKKLIKKIYFTRINSTYECDVFFPEIN--------------------------
                  *               **       * ***

B. bovis        ----------DTFEITAISPTFSTDFVSYDFVIYERK----DCKTVFPDPPFDQLLLK--
H. sapiens      ------------------------------------------------------------
T. gondii       PELGREKDNEATYRPIFISKTFSDNGVPYDFVVLEKRRKTDDAATAEPSNAMSSLTSTRE
P. falciparum   ------------------------------------------------------------

B. bovis        -----------GVDIIVP---------------KPKYVACPGVRIRNHEEFQYLDILADV
H. sapiens      ------------------------------------------------LEKYKLLPEYPGV
T. gondii       TTPVHGLQAPSSAAAIAPVLAWMDEEDRKKREQKELIRAVPHVHFRGHEEFQYLDLIADI
P. falciparum   ------------------------------------------------ENEYQIISVS---

B. bovis        LSHGVLKPNRTGTDAYSKFGYQMRFD
H. sapiens      LS-----DVQEEKGIKYKFEVYEKND
T. gondii       INNGRTMDDRTGVGVISKFGCTMRYS
P. falciparum   -------DVYTSNNTTLDFIIYKKTNN
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FIG. 1. Diagram of structures of WR99210 and PS-15 and multisequence alignment of DHFR. A. Structures of WR99210 and PS-15. B. Clustal X multiple sequence alignment of DHFR from *T. gondii* with *B. bovis*, *H. sapiens*, and *P. falciparum.* Amino acids considered to be interacting with WR99210 (interatomic distances within 3.5 Å) in *P. falciparum* (Ile14, Cys15, Asp54, Met55, Phe58, Leu119, Ile164, and Tyr170) are shown in reverse type (10). Identical amino acids in other species are also shown in reverse type. Amino acids identical in all four species are marked by asterisks.

(1-methyl-ethyl)imido-carbonimidicdiamide] (Jacobus Pharmaceutical Company, Princeton, NJ) are shown in Fig. 1A. The biguanide prodrug is converted in vivo to the biologically active dihydrotriazine through P450 metabolism. As such, in vitro experiments are always conducted with the dihydrotriazine. When administered orally to Aotus monkeys infected with resistant *Plasmodium falciparum*, PS-15 (prodrug) was more active and less toxic than WR99210 (dihydrotriazine) (2). Intolerance to dihydrotriazines was earlier demonstrated in a 1973 clinical study where WR99210 exhibited gastrointestinal intolerance as well as limited bioavailability (2).

MATERIALS AND METHODS

***Toxoplasma gondii*.** Tachyzoites of the RH strain were passaged in human foreskin fibroblasts.

In vitro assays. *T. gondii* tachyzoites also were used to infect fibroblasts to determine antimicrobial effects of candidate compounds. Outcome was assessed with microscopy and uracil uptake after 4 days in culture as described previously (12) with minor modifications.

Assays to assess inhibition of *T. gondii* tachyzoite growth in vitro. Human foreskin fibroblasts were cultured in 96-well plates (Corning) at a concentration 1×10^4 in 100 μl in IMDM-C until the monolayer reached 90 to 100% confluence. Confluent cultures were then infected with 2×10^3 *T. gondii* parasites per well for 1 h prior to the addition of antimicrobial agents in a 25-μl volume. After

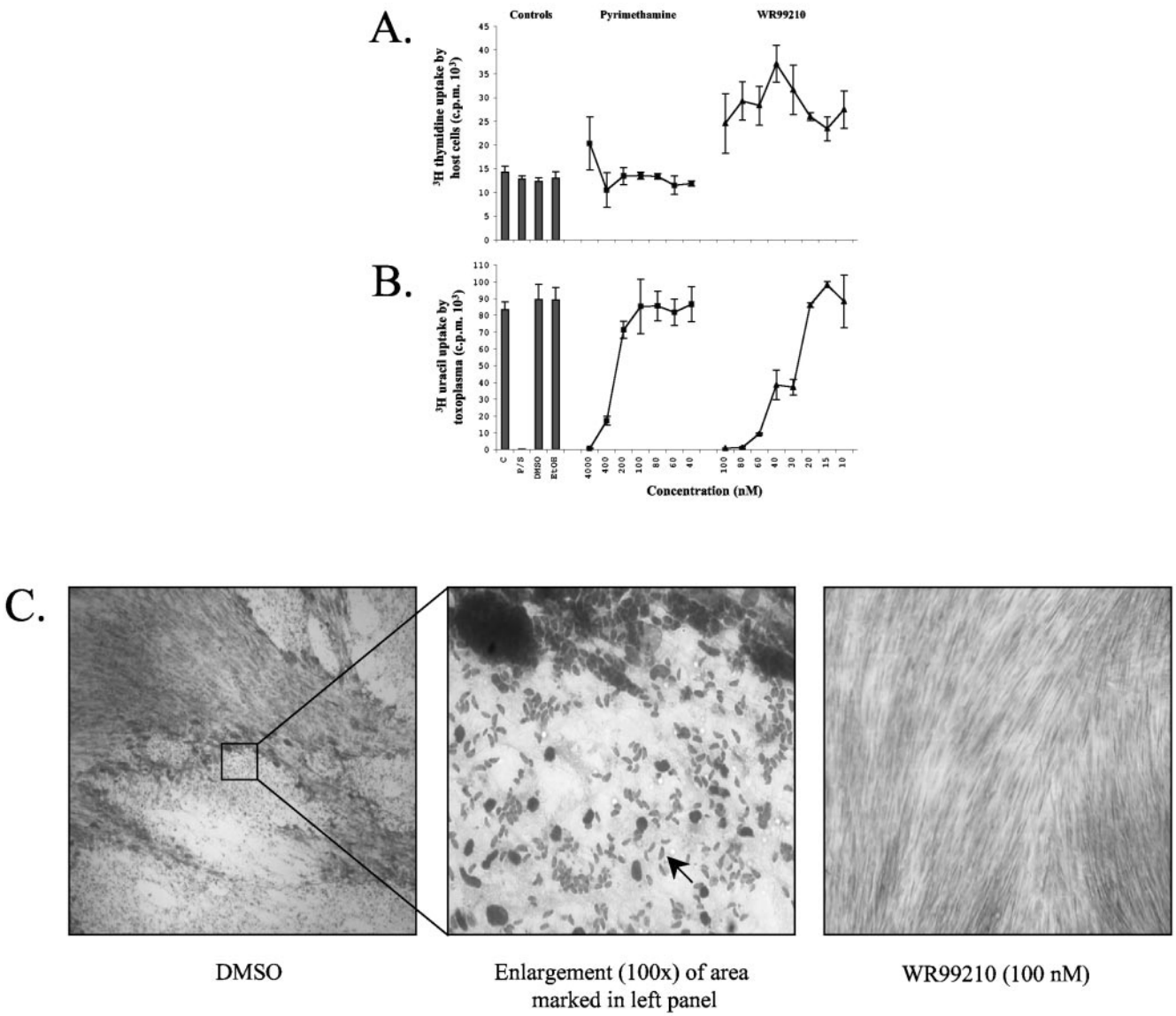

FIG. 2. Effect of WR99210 on *T. gondii* in human foreskin fibroblasts. A. Thymidine uptake assay demonstrates no toxic effect on host cells. B. Uracil uptake assay demonstrates that triazines are effective against *T. gondii* at low-nanomolar concentrations. C. Micrographs showing marked inhibition of *T. gondii* by WR99210. Note absence of plaques and parasites in treated cultures. Concentrations prepared are shown. Actual concentrations of soluble compound measured were fourfold less. EtOH, ethanol; P/S, pyrimethamine/sulfadiazine; C, control.

72 h, 25 μl IMDM containing 2.5 μCi of [5,6-^{3}H]uracil (Moravek Biochemical) was added to each well, and cultures were incubated for an additional 20 h (12, 13). Cells were dislodged and harvested onto a 96-well filter plate using a Filtermate 196 harvester (Packard) and was counted with a Topcount liquid scintillation spectrophotometer (Packard). Triplicate cultures were untreated or treated with either pyrimethamine or WR99210. For a positive control in these studies, a combination of pyrimethamine (0.1 μg/ml) and sulfadiazine (25 μg/ml) was used. Labtek slides with parallel experimentally treated cultures also were fixed in aminoacridine, stained with Giemsa, and examined microscopically.

Effect of antimicrobial agents on host cells in vitro. Human foreskin fibroblasts were cultured, collected, and processed as described above in the *T. gondii* growth inhibition assay, except that the fibroblasts were cultured to about 10% confluence, allowing for their growth to be measured, and no parasites were added. After 72 h, 25 μl of IMDM containing 1.25 μCi of [5,6-^{3}H] thymidine (Amersham) was added to each well and cultures were incubated for an additional 20 h. Thymidine uptake was measured as described above for uptake of uracil into parasites. Effect on host cell monolayers was also evaluated microscopically.

Infection of mice. Tachyzoites also were used to infect mice. Outbred Swiss Webster mice were bred in our specific-pathogen-free colony. When they were approximately 30 g, they received 10,000 tachyzoites intraperitoneally; numbers of parasites present in peritoneal fluid were quantitated 4 days later as described previously (12).

Triazine. A stock solution of WR99210, utilized in vitro, was initially dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in complete tissue culture medium (IMDM-C) (Iscove's modified Dulbecco's medium with $NaHCO_3$ and 25 mM HEPES [Cambrex Bio Science, Walkersville, MD], 10% fetal bovine serum [Gibco, Grand Island, NY], 1× antibiotic-antimycotic solution [Cellgro; Mediatech], and 2 mM L-glutamine [Gibco]). Working concentrations of WR99210 were made using IMDM-C. Concentrations measured ranged from 10 to 100 nM. WR99210 utilized in vivo was initially dissolved in 100% DMSO and then diluted 100-fold in 1× phosphate-buffered saline (PBS) without calcium or magnesium (Cellgro). When PS-15 was used for gavage, this was administered daily beginning within 1 h of intraperitoneal injection of parasites. The concentration used was 26 mM, giving a total amount of 387 mg/kg of body weight/day.

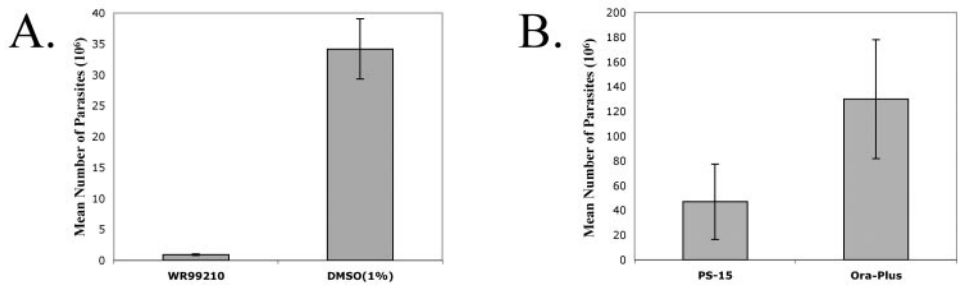

FIG. 3. Reduction of numbers of parasites in peritoneal fluid by treatment with WR99210 parenterally (A) and PS-15 administered by gavage (B). Number of mice was as follows: (A) 1% DMSO in PBS (control), 4; WR99210 in 1% DMSO in PBS, 4; (B) Ora-Plus, 4; treated with PS-15, 3.

Quantitation of inhibitory compounds. WR99210 and PS-15 levels were quantitated using a high-performance liquid chromatography (HPLC) system comprised of a Spectra System P4000 pump, AS300 autosampler, UV2000 detector, and ChromJet integrator. The column is a Phenomenex Synergi (4 m) MAX-RP 80A (150 by 4.6 mm), serial number 219259. Elution was effected with a gradient of Mobile Phase A (0.05% aqueous trifluoroacetic acid) and Mobile Phase B (0.025% trifluoroacetic acid in acetonitrile). The flow rate was 0.5 ml/min, the injection volume was 20 ml, and the detector was set to 290 nm. Observed retention times for WR99210 and PS-15 were 9.5 and 15.7 min, respectively.

Analysis of DHFR sequences. Amino acid sequences of DHFR from *T. gondii* (accession no. Q07422) with *B. bovis* (accession no. AAP57962), *H. sapiens* (accession no. RDHUD), and *P. falciparum* (accession no. AAA96491) obtained from GenBank were aligned using Clustal X and manually adjusted.

Statistics. Significance of differences were determined using a Mann Whitney U test or Student's *t* test. All experiments were performed at least twice, and representative experiments are shown.

RESULTS

In vitro experiments. WR99210 was highly effective against *T. gondii* tachyzoites in tissue culture. A representative experiment of at least two trials is shown in Fig. 2. The 50% inhibitory concentration (IC_{50}) and IC_{90} for WR99210 were approximately 50 nM and 70 nM, respectively. The IC_{50} and IC_{90} for pyrimethamine were approximately 0.3 mM and 4 mM, respectively. Differences between control and treated groups at these and higher concentrations were statistically significant ($P < 0.05$). We observed some precipitation of the compounds in the stock solutions, so the supernatant was analyzed by HPLC. We found that the actual concentrations measured in the supernatants were approximately fourfold less than those shown in Fig. 2 as calculated initially.

Data are shown both as uptake of [^{3}H]uracil into nucleic acid of the parasites and with photomicrographs of Giemsa-stained microscopic preparations, with efficacy confirmed by both methods. Lack of toxicity for fibroblasts also is shown in Fig. 2.

In vivo experiments. WR99210 was also highly effective against *T. gondii* tachyzoites in a mouse model. A representative experiment (one of two replicate studies) with 4 mice per group is shown in Fig. 3A. In these experiments, mice were infected intraperitoneally (i.p.) with 10,000 tachyzoites of the RH strain of *T. gondii* for 15 min prior to initial treatment with WR99210. Treated mice received a dose of 1.25 mg/kg/day of WR99210, administered i.p., for the next 3 days. Male mice were used for studies with WR99210. Control mice received an equivalent amount of DMSO (1%) in 1× PBS. In a separate experiment, DMSO was shown not to modify subsequent parasite numbers when compared to intraperitoneal inoculation of PBS. Intraperitoneal parasite numbers were 2 logs less in the WR99210-treated mice on the fifth day (Fig. 3A). Mice treated with WR99210 appeared sleek and active. In contrast, infected control mice appeared ill, with ruffled fur and hunched posture. The differences between control and treated mice were statistically significant ($P < 0.05$). Similar trends in reduction of parasite numbers also occurred for mice that received PS-15 by gavage (Fig. 3B).

Analyses of DHFR sequences and potential interaction with triazines. Alignment of the DHFR amino acid sequences from *T. gondii*, *Babesia bovis*, *Homo sapiens*, and *Plasmodium falciparum* allowed comparison of amino acids in the active site known to interact with WR99210 (Fig. 1B). Notably, of the seven amino acids (Ile14, Cys15, Asp54, Met55, Phe58, Leu119, Ile164, and Tyr170) identified in the *P. falciparum* DHFR, which are within an interatomic distance of 3.5 Å and therefore assumed to interact with WR99210, four are identical in the *T. gondii* enzyme (Asp54, Phe58, Leu119, and Tyr170).

DISCUSSION

WR99210 is highly active against *T. gondii* in vitro at low nanomolar amounts and also is effective in vivo by intraperitoneal injection or oral administration. Pyrimethamine, which is currently used to treat humans, is substantially less active (1.5 logs) than WR99210.

The structure of the *P. falciparum* DHFR has been solved, and the key residues are identified as Ile14, Ala16, Trp48, Asp54, Phe58, Ser108, Ile164, and Thr185 (14). These have been demonstrated to interact with dihydrofolate and the NADH cofactor. Folate inhibitors, including pyrimethamine and WR99210, also bind within this active site. Thus, the amino acids that have interatomic distances within 3.5 Å and therefore considered to be interacting with WR99210 are Ile14, Cys15, Asp54, Met55, Phe58, Leu119, Ile164, and Tyr170 (14). It has been noted that mutation of a number of these residues or those adjacent (Ala16, Cys50, Asn41, Cys59, Ser108, and Ile164) have been linked to resistance to antifolates (14). Comparison of the *T. gondii* and *P. falciparum* DHFR domains reveals that four out of the seven residues directly involved in the interaction with WR99210 are identical (Asp54, Phe58, Leu119, and Tyr170). Either differences in assay conditions and times or the differences between the *P.*

falciparum and *T. gondii* DHFRs, as shown in Fig. 1B, may contribute to the greater sensitivity of *P. falciparum* to WR99210. There is a clinically very promising related compound, JPC-2056, being progressed and currently under development with IC_{50}s ranging from 0.01 to 0.02 ng/ml (G. A. Schiehser et al., Abstr. 53rd Annu. Meet. Am. Soc. Trop. Med. Hyg., abstr. 727, 2004).

We identified one other published data set which suggested that a triazine might be effective against *T. gondii*. This was an interesting and complicated rat model relevant to toxoplasmosis in patients with AIDS and patients with other conjoint infections (1). There were many confounding variables (e.g., steroid treatment and suppressed immune response and conjoint infections) (1) in addition to the triazine treatment per se, which could have influenced the results. In this study, PS-15 (the prodrug of WR99210) administered in vivo to rats alone or in combination with dapsone, in a model of conjoint infection with *Pneumocystis carinii* followed by immunosuppression and then intraperitoneal infection with the RH strain of *T. gondii*, partially protected the rats and reduced tissue parasite burdens (1). PS-15 was administered 5 days a week for 5 weeks until death of the rats due to *T. gondii* infection.

We attempted to place the prodrug PS-15 in drinking water as well as administering it subcutaneously in peanut oil, but difficulties with maintaining a suspension made it impossible to administer consistent amounts of the compound in these experiments. The compound administered by gavage was protective.

This class of DHFR-inhibiting compounds has considerable promise as an improved, less toxic means to treat toxoplasmosis. For example, Winstanley et al. (13) illustrate (their Fig. 1) the closeness of the therapeutic index between *P. falciparum* and human DHFR for pyrimethamine, which contrasts with a much better therapeutic index for WR99210. The potential of these compounds to act in the absence of sulfadiazine may increase tolerance and decrease detrimental side effects, including allergy. Modeling of these compounds in the *T. gondii* DHFR active site or empirical cocrystallization studies might inform further optimization of these inhibitors for the *T. gondii* enzyme.

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REFERENCES

1. **Brun-Pascaud, M., F. Chau, L. Garry, D. Jacobus, F. Derouin, and P.-M. Girard.** 1996. Combination of PS-15, epiroprim, or pyrimethamine with dapsone in prophylaxis of *Toxoplasma gondii* and *Pneumocystis carinii* dual infection in a rat model. Antimicrob. Agents Chemother. **40:**2067–2070.
2. **Canfield, C. J., W. K. Milhous, A. L. Ager, R. N. Rossan, T. R. Sweeney, N. J. Lewis, and D. Jacobus.** 1993. PS-15: a potent orally active antimalarial from a new class of folic acid antagonists. Am. J. Trop. Med. Hyg. **49:**121–126.
3. **Childs, G. E., and C. Lambros.** 1986. Analogues of N-benzyloxydihydro triazines: in vitro anti-malarial activity against *Plasmodium falciparum.* Ann. Trop. Med. Parasitol. **80:**177–181.
4. **Fiddock, D. A., and T. E. Wellems.** 1997. Transformation of *Plasmodium falciparum* with human DHFR confers resistance to WR99210. Proc. Natl. Acad. Sci. USA **94:**10931–10936.
5. **Hastings, M. D., and C. H. Sibley.** 2002. Pyrimethamine and WR 99210 exert opposing selection on dihydrofolate reductase from *Plasmodium vivax.* Proc. Natl. Acad. Sci. USA **99:**13137–13141.
6. **Hastings, M. D., K. M. Porter, J. D. Maguire, L. Susanti, W. Kania, M. J. Bangs, C. H. Sibley, and J. K. Baird.** 2004. Dihydrofolate reductase mutations in *Plasmodium vivax* from Indonesia and therapeutic response to sulfodoxine plus pyrimethamine. J. Infect. Dis. **189:**744–750.
7. **Hekmat-Nejad, M., and P. K. Rathod.** 1997. *Plasmodium falciparum*: kinetic interactions of WR99210 with pyrimethamine-sensitive and pyrimethamine-resistant dihydrofolate reductase. Exp. Parasitol. **87:**222–228.
8. **Holfels, E., J. McAuley, D. Mack, W. K. Milhous, and R. McLeod.** 1994. In vitro effects of artemisinin ether, cycloguanil hydrochloride (alone and in combination with sulfadiazine), quinine sulfate, mefloquine, primaquine phosphate, trifluoperazine hydrochloride, and verapamil on *Toxoplasma gondii.* Antimicrob. Agents Chemother. **38:**1392–1396.
9. **Peterson, D. S., W. K. Milhous, and T. E. Wellems.** 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA **87:**3018–3022.
10. **Rastelli, G., W. Sirawaraporn, P. Sompornpisut, T. Vilaivan, S. Kamchonwongpaisan, R. Quarrell, G. Lowe, Y. Thebtaranonth, and Y. Yuthavong.** 2000. Interaction of pyrimethamine, cycloguanil, WR99210 and their analogues with *Plasmodium falciparum* dihydrofolate reductase: structural basis of antifolate resistance. Bioorg. Med. Chem. **8:**1117–1128.
11. **Rathod, P. K., and M. A. Phillips.** 2003. Prized malaria drug target nailed. Nat. Struct. Biol. **10:**316–318.
12. **Samuel, B. U., B. Hearn, D. Mack, P. Wender, J. Rothbard, M. J. Kirisits, E. Mui, S. Wernimont, C. W. Roberts, S. P. Muench, D. W. Rice, S. T. Prigge, A. B. Law, and R. McLeod.** 2003. Delivery of antimicrobials into parasites. Proc. Natl. Acad. Sci. USA **100:**14281–14286.
13. **Winstanley, P. A., E. K. Mberu, I. S. F. Szwandt, A. M. Breckenridge, and A. M. Watkins.** 1995. In vitro activities of novel antifolate drug combinations against *Plasmodium falciparum* and human granulocyte CFUs. Antimicrob. Agents Chemother. **39:**948–952.
14. **Yuvaniyama, J., P. Chitnumsub, S. Kamchonwongpaisan, J. Vanichtanankul, W. Sirawaraporn, P. Taylor, M. D. Walkinshaw, and Y. Yuthavong.** 2003. Insights into antifolate resistance from malarial DHFR-TS structures. Nat. Struct. Biol. **10:**357–365.
15. **Zhang, K., and P. K. Rathod.** 2002. Divergent regulation of dihydrofolate reductase between malaria parasite and human host. Science **290:**545–547.