ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1982, p. 981-984
0066-4804/82/120981-04$02.00/0
Vol. 22, No. 6

# In Vitro Assessment of 2-Acetylpyridine Thiosemicarbazones Against Chloroquine-Resistant *Plasmodium falciparum*

CHRIS LAMBROS,* GEORGE E. CHILDS, JOHN D. NOTSCH, JOHN P. SCOVILL, DANIEL L. KLAYMAN, AND DAVID E. DAVIDSON, JR.

*Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20012*

Received 14 May 1982/Accepted 13 September 1982

A series of 2-acylpyridine thiosemicarbazones was evaluated in vitro against a chloroquine-resistant *Plasmodium falciparum* strain. Antimalarial activity was assessed by the inhibition of uptake of [*G*-$^3$H]hypoxanthine by the parasites. Among the mono- and disubstituted derivatives tested, 13 of 17 had 50% inhibitory doses of less than 10 ng/ml. Increasing the size of the ring at $N^4$ from four to five, six, and seven members produced concomitant decreases in activity. Similarly, increasing the size of the aliphatic substituent on the azomethine carbon reduced activity. Selected compounds were also tested against a chloroquine-susceptible strain. The results suggested that the activities of these agents were not modified significantly by resistance to chloroquine. In general, in vitro activities correlate poorly with the in vivo activities in mice infected with *Plasmodium berghei*.

Klayman et al. (6, 7) have shown that 2-acetylpyridine thiosemicarbazones exhibit antimalarial activity in mice infected with *Plasmodium berghei*. In these studies, the alkylidene group attached to the 2 position of the pyridine ring was critical for antimalarial activity; all 3- and 4-pyridyl compounds were found to be inactive. Activity was also limited to those compounds in which a thiocarbonyl, rather than a carbonyl group, is present. Whereas two $N^4$-monosubstituted 2-acetylpyridine thiosemicarbazones exhibited curative action at a dose level of 160 mg/kg, the incorporation of the $N^4$ nitrogen atom into a six- or seven-membered ring produced cures at doses as low as 20 mg/kg.

The present study was undertaken to evaluate in vitro activity in a series of 2-acylpyridine thiosemicarbazones against selected strains of *Plasmodium falciparum*. Due to the emerging clinical importance of multidrug resistance, major emphasis is placed on the activity of these compounds against a chloroquine-resistant strain. To determine whether resistance to chloroquine compromised the activities of these agents, selected compounds were tested against a chloroquine-susceptible strain. An attempt has been made to correlate these in vitro data with in vivo activities of these compounds against *P. berghei* infections in mice.

## MATERIALS AND METHODS

Antimalarial activity against *P. falciparum* was determined by using the semiautomated in vitro system described by Desjardins et al. (4).

**Parasite.** The multi-drug-resistant Vietnam Smith strain (2) was obtained from a military volunteer at Ft. Detrick, Md. The chloroquine-susceptible and pyrimethamine-resistant Camp strain (3) was obtained from the Department of Immunology, Walter Reed Army Institute of Research, Washington, D.C.

Stock cultures were grown with 6% human A+ erythrocytes in RPMI 1640 medium supplemented with 10% heat-inactivated human A+ plasma in 25-ml tissue culture flasks (4); total volume was 5.0 ml. The cultures were maintained under a gas phase of 5% $O_2$–5% $CO_2$–90% $N_2$ (5) and incubated at 37°C.

**Preparation of compounds.** The thiosemicarbazones tested were synthesized in the Organic Synthesis Section of the Walter Reed Army Institute of Research. All compounds were initially dissolved in a 50/50 (vol/vol) mixture of dimethyl sulfoxide (Fisher) and ethanol to a concentration of 1 mg/ml. Subsequent dilutions were made with culture medium. The final dilution contained less than 0.02% dimethyl sulfoxide or ethanol, which we have determined to have no effect on the parasites in culture.

**Preparation of isotope.** [*G*-$^3$H]hypoxanthine (New England Nuclear Corp., Boston, Mass.; 10 Ci/mmol), supplied as a sterile aqueous solution, was diluted in culture medium to a concentration of 10 μCi/ml. Twenty-five microliters of this solution was added to each well of a 96-well microtiter plate. Incorporation of radioactivity by the parasites served as an index of parasite viability (4).

**Preparation of microtiter plates and harvesting of parasites.** The preparation of microtiter plates and harvest of parasites were performed as previously described (4). Each drug was serially diluted 2-fold for a total of seven concentrations over a 64-fold range. The final hematocrit in each well was 1%, and parasitemia was 0.4 to 0.6%. Four wells of each microtiter plate contained chloroquine and mefloquine as controls. The plates were placed in an airtight box, flushed with a gas mixture of 5% $O_2$–5% $CO_2$–90% $N_2$ and placed in an incubator at 37°C for 24 h. After this time

TABLE 1. In vitro activity of chloroquine, mefloquine, and pyrimethamine against the Smith and Camp strains of *P. falciparum*

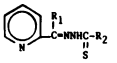
| Antimalarial drug | $ID_{50}$ (ng/ml) | |
|---|---|---|
| | Smith | Camp |
| Chloroquine ($2H_3PO_4$) | 60 ± 6.6 ($n$ = 10) | 7.6 ± 1.6 ($n$ = 5) |
| Mefloquine (HCl) | 2.5 ± 0.46 ($n$ = 10) | 3.5 ± 0.82 ($n$ = 5) |
| Pyrimethamine | >68 ($n$ = 2) | >68 ($n$ = 2) |

interval, the plates were taken out of the box, and to each well was added [$G$-$^3$H]hypoxanthine. The plates were replaced in the airtight box, gassed, and incubated for an additional 18 h. The contents from each well were then collected on filter paper with a MASH II harvester (Microbiological Associates, Bethesda, Md.). The filter paper was dried at 80°C for 60 min in a drying oven.

**Scintillation counting.** Dried filter disks were individually placed in minivials (Bio-Vial; Beckman Instruments, Inc., Irvine, Calif.) with 1.5 ml of Aquasol-2 (New England Nuclear Corp.) xylene-based scintillation fluid. Radioactivity was assayed in a Searle Delta 300 scintillation spectrometer to a counting error of less than 1%.

**Data analysis.** The resulting counts per minute were

TABLE 2. Comparative activities of 2-acetylpyridine thiosemicarbazones against *P. falciparum* in vitro and *P. berghei* infections in mice



| Compound no. | Substituent | | $ID_{50}$ (ng/ml) for *P. falciparum*[a] | | $CD_{50}$ (mg/kg) in mice infected with *P. berghei*[b] |
|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | Smith strain | Camp strain | |
| 1 | $CH_3$ | $NHC_6H_5$ | 5.9 (0.64) | ND | 724 |
| 2 | $CH_3$ | $NHCH_3$ | 3.3 (0.58) | ND | Toxicity at >40 mg/kg |
| 3 | $CH_3$ | $NHCH_2CH_3$ | 5.9 (1.1) | ND | Toxicity at >40 mg/kg |
| 4 | $CH_3$ | $NHCH_2CH_2CH_3$ | 4.6 (0.96) | ND | No cures at 160 mg/kg<br>Toxicity at 320 mg/kg |
| 5 | $CH_3$ | $NHCH_2CH{=}CH_2$ | 4.3 (1.1) | ND | >640 |
| 6 | $CH_3$ | $NHCH_2C{\equiv}CH$ | 3.2 (0.18) | ND | Toxicity at >40 mg/kg |
| 7 | $CH_3$ | NH-cyclo-$C_6H_{11}$ | 6.3 (0.91) | ND | 555 |
| 8 | $CH_3$ | $NHCH_2C_6H_4$-2-$CH_3$ | 6.4 (0.91) | ND | 539 |
| 9 | $CH_3$ | $NHC_6H_{13}$ | 7.2 (1.2) | ND | >640 |
| 10 | $CH_3$ | $NHC(CH_3)_2CH_2C(CH_3)_3$ | 17 (8.3) | ND | >640 |
| 11 | H | $N(CH_3)_2$ | 2.3 (0.65) | 1.05 (0.21) | |
| 12 | $CH_3$ | $N(CH_3)_2$ | 3.6 (0.63) | 0.65 (0.16) | No cures at 20 mg/kg<br>Toxicity at >20 mg/kg |
| 13 | $C_2H_5$ | $N(CH_3)_2$ | 4.4 (0.67) | ND | No cures at 20 mg/kg<br>Toxicity at >40 mg/kg |
| 14 | $CH_3$ | $N(C_2H_5)_2$ | 14 (4.56) | ND | One cure at 80 mg/kg<br>Toxicity at >160 mg/kg |
| 15 | $CH_3$ | $N(iso\text{-}C_4H_9)_2$ | 9.6 (1.57) | ND | >640 |
| 16 | $CH_3$ | $N(CH_3)$(cyclo-$C_6H_{11}$) | 12 (1.50) | 13 (3.36) | 326 |
| 17 | $CH_3$ |  | 1.7 (0.41) | 1.4 (0.11) | No cures at 20 mg/kg<br>Toxicity at >40 mg/kg |
| 18 | $CH_3$ |  | 4.1 (1.1) | ND | No cures at 40 mg/kg<br>Toxicity at >160 mg/kg |
| 19 | $CH_3$ |  | 8.2 (0.36) | ND | No cures at 20 mg/kg<br>Toxicity at >40 mg/kg |
| 20 | H |  | 4.3 (0.73) | 3.7 (0.26) | ND |
| 21 | $CH_3$ |  | 13 (1.26) | 15 (7.82) | 43 |
| 22 | $CH_3$ |  | 17 (6.38) | ND | 361 |

recorded on paper punch tape and processed with a Tektronix 4051 computer. The data were analyzed by nonlinear regression analysis (4) to obtain the 50% inhibitory dose ($ID_{50}$), the inhibitory drug concentration corresponding to 50% inhibition of the uptake of radiolabeled hypoxanthine by the parasites. The $ID_{50}$ values of chloroquine, mefloquine, and pyrimethamine tested against both strains are presented in Table 1. The 50% curative dose ($CD_{50}$) values were determined by using a computerized log-probit analysis.

## RESULTS

**In vitro activity.** Generally, the alkyl compounds 1 to 16 (Table 2) with various substituents at $R_2$ exhibited comparable activity against the Smith strain. $ID_{50}$ values ranged from 2.3 ng/ml (compound 12) to 17 ng/ml (compound 10).

Among the azacycloalkyl-substituted derivatives where $R_1 = CH_3$, increasing the ring size from four (compound 17) to five, six, and seven members (compounds 18, 19, and 31, respectively) produced a parallel increase in $ID_{50}$ values, ranging from 1.7 to 15 ng/ml.

Of the piperazinyl-substituted compounds 33 to 36, the carbethoxypiperazinyl derivative was the most active, with an $ID_{50}$ of 4.8 ng/ml (compound 33).

The effect of structural changes at $R_1$ was observed in compounds 20 ($R_1$ = H) and 21 ($R_1 = CH_3$); 30 ($R_1$ = H), 31 ($R_1 = CH_3$), and 32 ($R_1 = C_2H_5$); and 37 ($R_1$ = H) and 38 ($R_1 = CH_3$). In each instance, increasing the size of the aliphatic

TABLE 2—*Continued*

| Compound no. | Substituent | | $ID_{50}$ (ng/ml) for *P. falciparum*[a] | | $CD_{50}$ (mg/kg) in mice infected with *P. berghei*[b] |
|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | Smith strain | Camp strain | |
| 23 | $CH_3$ |  | 12 (3.43) | ND | 35 |
| 24 | $CH_3$ |  | 29 (20.05) | ND | 281 |
| 25 | $CH_3$ |  | 22 (5.72) | ND | 43 |
| 26 | $CH_3$ |  | 19 (6.04) | 16 (7.13) | 113 |
| 27 | $CH_3$ |  | 14 (1.53) | ND | >640 mg/kg |
| 28 | $CH_3$ |  | 37 (20.18) | ND | 128 |
| 29 | $CH_3$ |  | >68 (−) | ND | 198 |
| 30 | H |  | 3.1 (0.67) | 2.9 (0.46) | No cures at 20 mg/kg Toxicity at >40 mg/kg |
| 31 | $CH_3$ |  | 15 (2.49) | 17 (1.68) | 162 |
| 32 | $C_2H_5$ |  | 31 (7.41) | 29 (7.12) | No cures at 40 mg/kg Toxicity at >160 mg/kg |
| 33 | $CH_3$ |  | 4.8 (0.95) | 5.4 (0.81) | 40 |
| 34 | $CH_3$ |  | 17 (9.6) | ND | 121 |
| 35 | $CH_3$ |  | 18 (3.7) | 10 (1.1) | 21 |
| 36 | $CH_3$ |  | 29 (8.21) | ND | 369 |
| 37 | H |  | 3.6 (0.64) | 1.8 (0.49) | >160 mg/kg |
| 38 | $CH_3$ |  | 10 (1.91) | 13 (2.22) | 33 |

[a] Numbers in parentheses are the standard error of the nonlinear regression analysis of single determinations for the corresponding $ID_{50}$ (8). A minus sign indicates that the value is out of the test range. ND, Not determined.

[b] Compounds were tested at the Leo Rane Laboratory, University of Miami, Miami, Fla.

substituent produced a concomitant reduction in activity.

Activities of selected compounds against the chloroquine-susceptible Camp strain produced a dichotomy of results as compared to the Smith strain. A striking difference in the activities of compound 12 was noted, whereas modest differences were observed with compounds 11 and 37. Comparable activities were evident among the remaining compounds tested.

**In vivo activity.** Six compounds exhibited antimalarial activity, with $CD_{50}$ values ranging from 21 to 43 mg/kg: 21, 23, 25, 33, 35, and 38. Only compound 33 showed high antimalarial activity both in vivo ($CD_{50}$ = 40 mg/kg) and in vitro ($ID_{50}$ = 4.8 ng/ml). Generally, those compounds showing high antimalarial activities in vitro did not have corresponding activities in vivo.

## DISCUSSION

Variation of substituents of 2-acetylpyridine thiosemicarbazones provided compounds with high antimalarial activity against the chloroquine-resistant Smith strain of *P. falciparum*.

Thirteen of seventeen compounds among the mono- and disubstituted (noncyclic) derivatives had $ID_{50}$s of less than 10 ng/ml against *P. falciparum*, but against *P. berghei* the majority of these compounds were either toxic at low levels or inactive.

The most active among the cyclic-substituted 2-acetylpyridine thiosemicarbazones against the Smith strain was the four-member compound 17; a progressive increase in ring size substitutions was paralleled by decreasing activity (see compounds 18, 19, and 31). On the other hand, activity against *P. berghei* was found in the six-member ring substitution (compounds 21 to 29). Within this group, the parent compound 19 was inactive (yet active against *P. falciparum*), but placement of methyl groups about the ring imparted activity (compounds 21, 23, and 25). The presence of 2-ethyl (compound 26), 4-phenyl (compound 28), or 4-benzyl (compound 29) maintained antimalarial activity but at higher doses. The hydroxylated compound 27 was inactive. Against *P. falciparum*, none of these molecular modifications approached the level of activity of compound 19 ($ID_{50}$ = 8.2 ng/ml).

Increasing the size of the substituent attached to the azomethine carbon ($R_1$ substitution) resulted in a diminution of activity against *P. falciparum*. This was evident in compounds 30, 31, and 32, in which $N^4$ is incorporated into a seven-member ring. As $R_1$ was changed from a hydrogen to methyl and then to ethyl, the respective $ID_{50}$s increased from 3.1 to 31 ng/ml. This was also observed for compounds with $N^4$ incorporated either into a six-member ring with a methyl group in the 2 position (compounds 20 and 21) or into an azabicyclononyl moiety (compounds 37 and 38). The compound with $R_1$ = H was considerably more active than that in which $R_1$ = $CH_3$. The reverse is true for *P. berghei*, against which compounds having $R_1$ = $CH_3$ were most active.

Based on our findings on the in vitro antimalarial action of a series of 2-acylpyridine thiosemicarbazones against chloroquine-resistant *P. falciparum*, these compounds warrant further definitive investigations. Perhaps a select few could be introduced into the *Aotus trivirgatus-P. falciparum* in vivo model (1) to fully ascertain their potential as possible antimalarial agents. Although many of these compounds exhibited toxicity for mice at low doses, some had activities against *P. berghei* greater than that of mefloquine (calculated $CD_{50}$, 120 mg/kg).

These analyses of structure-activity relationships should serve as a guide for synthesis and investigations of additional analogs. Newer and more effective drugs are required because of the relentless emergence of strains of human plasmodia (especially *P. falciparum*) resistant to currently available antimalaria drugs.

### ACKNOWLEDGMENTS

We thank the staff of the Walter Reed Blood Donor Center for providing plasma.

This paper has been designated as contribution no. 1557 to the Army Research Program on Antiparasitic Drugs.

### LITERATURE CITED

1. **Canfield, C. J., and M. H. Heiffer.** 1978. The U.S. Army Drug Development Program. Adv. Pharmacol. Ther. **10**:99–108.
2. **Canfield, C. J., E. G. Whiting, W. H. Hall, and B. MacDonald.** 1971. Treatment of acute falciparum malaria from Vietnam with trimethoprim and sulfalene. Am. J. Trop. Med. Hyg. **20**:524–526.
3. **Degowin, R. L., and R. D. Powell.** 1965. Drug resistance to a strain of *P. falciparum* from Malaya. Am. J. Trop. Med. Hyg. **14**:519–528.
4. **Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay.** 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Chemother. **16**:710–718.
5. **Haynes, J. D., C. A. Diggs, F. A. Hines, and R. E. Desjardins.** 1976. Culture of human malaria parasites *Plasmodium falciparum*. Nature (London) **263**:767–769.
6. **Klayman, D. L., J. F. Bartosevich, T. S. Griffin, C. J. Mason, and J. P. Scovill.** 1979. 2-Acetylpyridine thiosemicarbazones. 1. A new class of potential antimalarial agents. J. Med. Chem. **22**:855–862.
7. **Klayman, D. L., J. P. Scovill, J. F. Bartosevich, and C. J. Mason.** 1979. 2-Acetylpyridine thiosemicarbazones. 2. $N^4$,$N^4$-Disubstituted derivatives as potential antimalarial agents. J. Med. Chem. **22**:1367–1373.
8. **Rodbard, D., V. B. Faden, D. M. Hutt, and J. C. Huston, Jr.** 1980. Sigmoid, four parameter logistic curve-fitting program. Biomedical Computing Technology Information Center, Nashville, Tenn.