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Antimycobacterial Activity of 2-Acetylpyridine Thiosemicarbazones in Relation to Their Antileprosy Activity[1]

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Thiacetazone has been reported to be active against both tuberculosis and leprosy ([9, 12]). The minimal inhibitory concentration (MIC) has been calculated to be between 0.2 and 0.4 μg per ml for both *M. tuberculosis* and *M. leprae in vivo* ([3, 5]). However, this drug has not been widely used in the treatment of human leprosy due to the rapid emergence of resistant strains during monotherapy ([10]). As a result, thiacetazone is only considered as a second line drug. Interest in this class of compound has been rekindled by the recent availability of a number of new 2-acetylpyridine thiosemicarbazones developed as potential antimalarials ([8]). Some of these compounds have been shown to possess considerable antibacterial activity against a number of human pathogens ([3, 4]), as well as to *M. smegmatis* ATCC #607 (Morrison, unpublished data). The latter organism has been used as a rapid primary screen in the selection of compounds with potential antileprosy activity ([13]).

The present study examines the antituberculous activity of a number of 2-acetylpyridine thiosemicarbazones compared to that seen in the foot pads of orally treated mice inoculated with *M. leprae*.

MATERIALS AND METHODS

Organisms. *M. tuberculosis* H37Rv (TMC #102), *M. kansasii* (TMC #1201 and 1204), *M. avium* (TMC #706 and 724) and *M. intracellulare* (TMC #1406 and D673) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, New York, U.S.A. Suspensions of each organism were kept at −70°C, thawed rapidly at 37°C, homogenized briefly, and diluted in sterile Middlebrook 7H10 agar just prior to inoculation into the test medium ([2]). *M. smegmatis* 607 was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A., and maintained on Löwenstein-Jensen slants ([13]).

2-acetylpyridine thiosemicarbazones. The synthesis and chemical properties of the thiosemicarbazones used in this study have been reported elsewhere ([8]). The structure of the compounds used in this study are shown in Fig. 1.

Lipophilicity (log P) determinations. The octanol/water coefficients were calculated from the additive Hansch π values ([7, 17]) for each of the thiosemicarbazone derivatives, using the tables provided by Leo, *et al.* ([9]).

Minimal inhibitory concentration (MIC) determinations. The 2-acetyl-pyridine thiosemicarbazones were dissolved in dimethylsulfoxide (DMSO) at a concentration of 4 mg per ml and diluted with 7H10 agar from 20 to 0.075 μg per ml. The final DMSO concentration was maintained at 0.5% (v/v), including the drug-free controls. The drug containing medium was incubated at 37°C overnight to check for sterility and the plates inoculated with decreasing numbers of viable bacilli (10^5, 10^4, 10^3 and 10^2 per ml). The plates were examined weekly for 3 weeks, and the number of colonies was counted. The MIC was determined as the minimum drug concentration limiting growth to 1% or less of the drug-free counts after 22 days at 37°C ([1]).

M. smegmatis 607 and its three drug-resistant mutants (DDS^R, RIF^R and $B663^R$) ([1]) were maintained in liquid Kirchner's medium containing 0.5% Tween-80. The MIC determinations were based on an end point taken as the minimum drug concentration resulting in a 95% inhibition of

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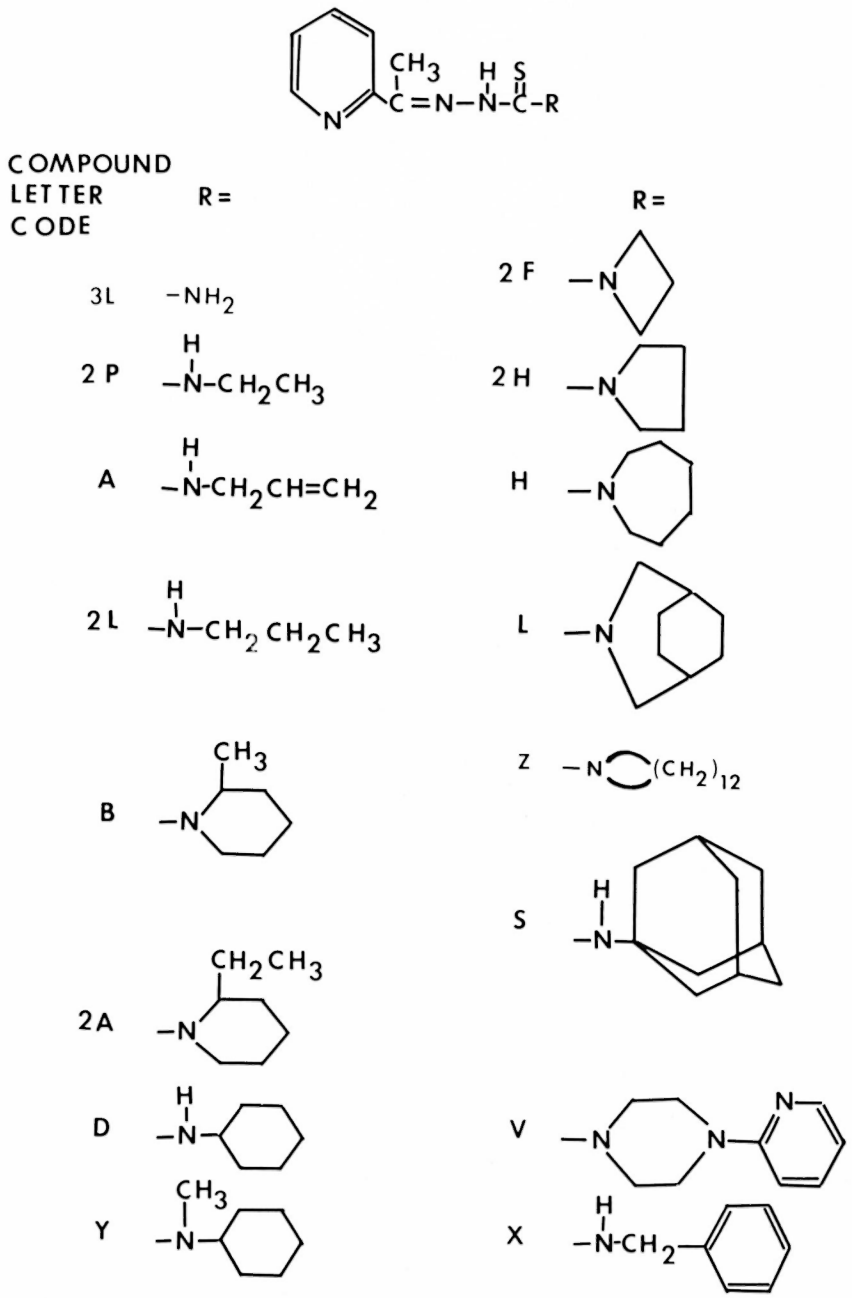

FIG. 1. Chemical structures of selected 2-acetylpyridine thiosemicarbazones.

growth compared with the drug-free controls ([13]). The MIC values were expressed as μmoles per liter.

Animals. Eight to ten week-old outbred female CD-1 mice (Charles River Farms, Massachusetts, U.S.A.) weighing 28–30 g were fed a commercially prepared, powdered diet #5002 (Ralston Purina Company, Ralston, Illinois, U.S.A.) and given sterile drinking water *ad libitum*. The thiosemicarbazones were administered in the diet at the maximum tolerated dose by mixing the drug with the chow in a liquids-solids blender (Patterson-Kelley Co., East Stroudsburg, Pennsylvania, U.S.A.). Uniform particle size distribution of the various

TABLE 1. *Minimal inhibitory concentration (MIC) determination (μmoles per liter) for 16 2-acetylpyridine thiosemicarbazones tested against* M. tuberculosis *and 5 nontuberculous mycobacteria.*

Compound	Log P	Mycobacterial culture TMC #							Mean (range)
		102	1201	1204	706	724	D673	1406	
L (50)[a]	4.33	3	3	3	1	8	8	3	4 (1–8)
Y (31)	4.26	8	4	4	4	34	34	17	15 (4–34)
2A (37)	4.37	8	4	17	4	34	34	34	19 (4–34)
H (45)	3.64	18	36	9	4	36	36	36	25 (4–36)
B (47)	3.71	9	26	36	4	36	36	36	26 (4–36)
X (14)	3.10	4	20	20	8	20	120	36	32 (4–120)
D (25)	3.71	36	74	9	36	18	36	72	40 (9–74)
S (26)	5.40	30	30	15	60	60	60	60	46 (15–60)
2L (4)	2.70	8	8	16	16	60	100	40	50 (8–100)
Z (49)	6.22	27	83	55	27	55	55	83	55 (27–83)
V (43)	1.24	88	88	88	88	29	88	88	79 (29–88)
2F (0)	2.38	85	128	128	21	42	85	85	82 (21–128)
2H (33)	2.68	80	120	20	80	120	80	120	88 (20–120)
2P (3)	2.09	135	135	135	90	90	135	135	122 (90–135)
A (5)	2.25	128	128	128	128	85	128	128	122 (85–128)
3L (9)	0.91	100	135	135	135	135	135	135	130 (100–135)

[a] Compound numbers in parenthesis according to Dobek, *et al.* (4).

thiosemicarbazones was achieved by grinding the compounds in a pestle and mortar and passing the resultant powder through a 200 mesh (75 micron) sieve.

Maximum tolerated dose determinations. These were performed by carrying out dietary feeding experiments over a 90 day period in which the thiosemicarbazones were added to the diet in increasing amounts. Diet consumption and body weight changes were measured twice weekly, and the maximum tolerated dose of each drug was taken to be the maximum dietary concentration that did not cause body weight losses over the 90 day period.

***M. leprae* inoculum.** A mouse foot pad-passaged isolate (20 transfers) of *M. leprae* (strain *B. maestre*) was received from Dr. C. C. Shepard of the Communicable Disease Center, Atlanta, Georgia, U.S.A. Both hind foot pads of a group of CD-1 mice were injected with a 0.03 ml volume containing an estimated 10^4 acid-fast bacilli (AFB). The number of organisms in the inoculum was checked by microscopic counting. The mice were placed on a diet containing each test drug given at the maximum tolerated dosage 30 days after foot pad inoculation. Each drug was continuously administered for 150 days ([15]). On day 180, the number of AFB present in the pooled hind foot pads from individual mice were counted microscopically by the method of Shepard and McRae ([16]). The hind foot pads were surgically excised, placed in a ground-glass homogenizer containing 2 ml of 0.001 M phosphate buffer (pH 7.0), and ground for 1 min at 4°C. A series of 10 μl aliquots were transferred to counting slides (Bellco Glass Co., Vineland, New Jersey, U.S.A.) and after fixation were stained by the Ziehl-Neelsen procedure and examined by light microscopy.

RESULTS

***In vitro* antimycobacterial activity.** Sixteen thiosemicarbazones were tested *in vitro* for their inhibitory activity against seven mycobacteria and the results were recorded in Table 1. Compounds L, Y, 2A, H, and B were considered to be active (average MICs of <30 μmoles per liter or 10 μg per ml) while compounds 2F, 2H, 2P, A, Z, and 3L were inactive (MIC > 50 μmoles per liter).

The average MICs for *M. smegmatis* 607 gave a completely different sensitivity profile (Table 2). Here, compounds X, 2P, A, 2F, 2L, 2H, Y, and H were active while compounds D, S, Z, and 3L were inactive.

Relationship of log P to antimycobacterial activity. When the log MIC values for the

TABLE 2. *Minimal inhibitory concentration (MIC) determinations (μmoles per liter) for 16 2-acetylpyridine thiosemicarbazones tested against four strains of* M. smegmatis *607.*

Compound	Log P	*M. smegmatis* #				Mean (range)
		607	607 DDS^R	607 RIF^R	607 B663^R	
X (14)[a]	3.10	1	1	1	3	2 (1–3)
2P (3)	2.09	4	4	9	9	6 (4–9)
A (5)	2.25	8	4	8	8	7 (4–8)
2F (0)	2.38	8	8	8	12	9 (8–12)
2L (4)	2.70	8	8	8	16	10 (8–16)
2H (33)	2.68	8	12	8	16	11 (8–16)
Y (31)	4.37	17	13	34	34	24 (13–30)
H (45)	3.64	14	36	18	36	26 (14–36)
2A (37)	4.37	17	34	34	34	30 (17–34)
V (43)	1.24	23	17	29	58	32 (17–58)
L (50)	4.33	16	82	16	24	35 (16–85)
B (47)	3.71	21	36	36	72	41 (21–72)
D (25)	3.71	18	—	31	181	77 (18–181)
Z (49)	6.22	277	277	277	277	277
3L (1)	0.91	309	309	309	309	309
S (26)	5.40	457	457	457	457	457

[a] Compound numbers in parenthesis according to Dobek, *et al.* (4).

thiosemicarbazones were plotted against the corresponding lipophilicity (log P) values, parabolic regression curves were obtained using both sets of test organisms (Fig. 2). The regression coefficients (R^2) for both curves were significant ($p < 0.05$). The log P_{max} for the slow-growing mycobacteria was approximately 4.0. On the other hand, when the MIC values for these same compounds were determined using the 4 *M. smegmatis* #607 strains, the log P_{max} shifted to 3.0. Compounds H, Y, and 2A exhibited activity against both groups of organisms while the parent compound 3L was inactive in both tests (Tables 1 and 2).

Antileprosy activity of 2-acetylpyridine thiosemicarbazones. Compounds 2P, A, 2L, X, Y, 2A, H, L, and 3L were mixed into the diet at their respective maximum tolerated doses (0.1%, except for 2P and 2L where the dosage had to be reduced to 0.05% for reasons of toxicity). Thiacetazone (0.1%) was included as a positive drug control. The activity of each compound was assessed after 180 days of infection. The compounds included in this test were selected on the basis of their MIC values for *M. smegmatis* 607 (Table 2) and covered a log P range from 1.0 to 4.5 (Table 3). Of the 10 compounds tested, 2P, A, and 2L were highly active against *M. leprae in vivo*

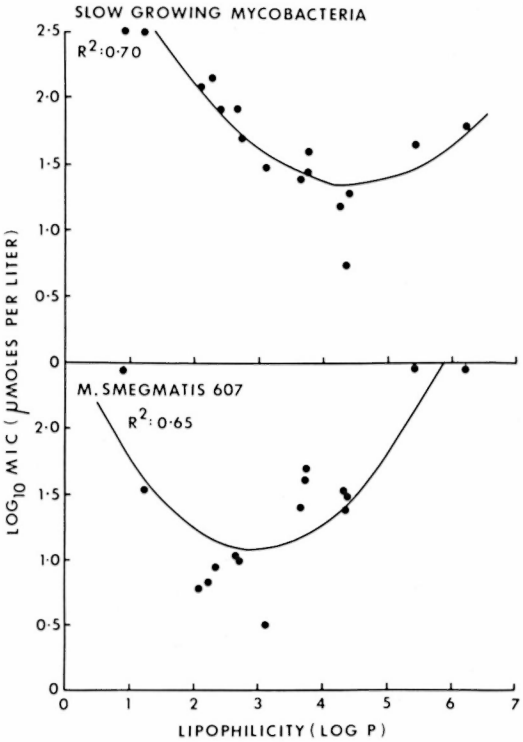

FIG. 2. Regression curves obtained when log MIC values for 2-acetylpyridine thiosemicarbazones were plotted against the corresponding lipophilicity (log P) values using *M. smegmatis* 607 (bottom) or slow-growing mycobacteria (top). The regression coefficients (R^2) were significant at the 5% level.

TABLE 3. *Growth inhibitory effects of ten thiosemicarbazones against* M. leprae *multiplying in the mouse foot pad.*

Compound	Log P	% control count[a]
2P (3)	2.09	3.5
A (5)	2.25	6.5
2L (4)	2.70	17.5
X (14)	3.10	26.0
Y (31)	4.37	37.0
2A (37)	4.37	47.5
H (45)	3.64	50.0
3L (1)	0.91	53.0
L (50)	4.26	100.0
Thiacetazone	0.90	25.0

[a] Control counts = $1.2 (\pm 0.26) \times 10^6$ acid-fast bacilli per foot pad.

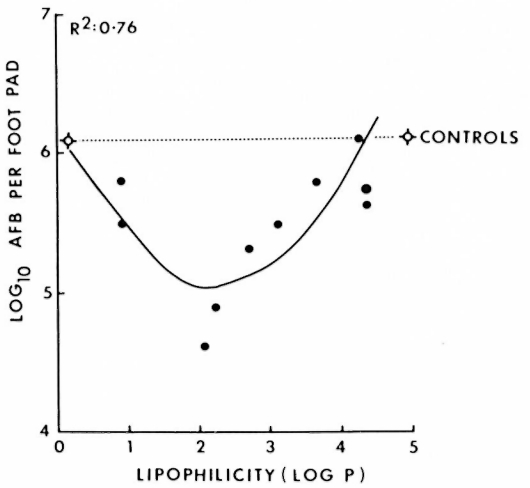

FIG. 3. Regression curve obtained when the log of the number of acid fast bacilli (AFB) per mouse foot pad was plotted against the lipophilicity (log P) values for 10 thiosemicarbazones tested in mice infected with 10^4 *M. leprae*. The control counts are represented by the dotted line.

while compounds 2A, H, L, and 3L were essentially inactive (Fig. 3). Thiacetazone and compounds Y and X fell into an intermediate position. When these counts were plotted against the log P values for the 10 compounds, a parabolic distribution was observed with a significant regression coefficient, $R^2 = 0.76$. The curve showed a log $P_{max} = 2.0$ for *M. leprae* (Fig. 3).

DISCUSSION

The present study indicates that a progressive shift occurred in the log P_{max} values as the test organisms changed from the slow-growing nontuberculous mycobacteria to *M. leprae* (Figs. 2, 3). These data indicate that a number of compounds (L, Y, 2A, and H) which appeared to be highly active against the non-tuberculous mycobacteria *in vitro* were ineffectual when tested against *M. leprae in vivo*. This disparity was predictable on the basis of the *M. smegmatis* 607 data (Table 2) in which the six most active compounds out of the 16 tested had log P values in the 2–3 range. Four of these were also active against *M. leprae* (Fig. 3). This suggests that *M. smegmatis* 607 serves as a useful primary screen for the potential antileprosy activity of the class of compound.

The log P_{max} value for the thiosemicarbazones when tested against *M. leprae* would appear to be consistent with the log P range for most clinically active antileprosy drugs (Table 4). Clofazimine is an apparent exception to this rule, having a calculated log P value of 7.48. Presumably, other factors are important in determining the activity of this highly insoluble drug *in vivo* ([14]). The log P_{max} of 2.0 for the active thiosemicarbazones against *M. leprae* (Fig. 3) suggests a positive contribution by tissue diffusional factors within the foot pad in determining the level of drug activity *in vivo*. Significantly, it has been calculated that the optimum log P value for hypnotic and depressant drugs known to effectively penetrate human nerve tissue is approximately 2.0 ([6]). This finding takes on an

TABLE 4. *Lipophilicity of antileprosy drugs.*

Drug	Formula	Molecular weight	Log P
Dapsone	$C_{12}H_{12}N_2O_2S$	248	0.97[a]
Rifampin	$C_{43}H_{58}N_4O_{12}$	823	1.29[a]
Prothionamide	$C_9H_{12}N_2S$	180	1.75[c]
Thiacetazone	$C_{10}H_{12}N_4OS$	236	0.90[b]
Compound 2P	$C_{10}H_{15}N_4S$	222	2.09[c]
			Range 0.90–2.09

[a] Reference 7.
[b] Reference 17.
[c] Calculated from ([7,9]).

added significance when it is considered that any effective thiosemicarbazone must penetrate *M. leprae*-infected nerve tissue. The most active compounds (2P, A, and 2L) all have log P values relatively close to this tissue diffusional optimum ([5, 17]). In addition, other thiosemicarbazones having log P values in the 1–3 range could be examined more thoroughly for their potential activity against *M. leprae*. The log P value of these new compounds relative to the tissue diffusional factor is likely to be critically important in determining the level of drug activity expressed by these lipophilic compounds. This parameter should also be considered during the synthesis of new thiosemicarbazones with enhanced antileprosy activity ([11]).

SUMMARY

Antimycobacterial assays were carried out on sixteen 2-acetylpyridine thiosemicarbazones using a number of culturable mycobacteria *in vitro*. The resulting MIC determinations were plotted against the lipophilicity (log P) values for the various test compounds. Plots of log MIC vs log P values conformed to a parabolic regression curve having a log P_{max} of 4.0 for the slow-growing mycobacteria and 3.0 for the rapid grower, *M. smegmatis*. Ten thiosemicarbazone compounds covering a log P range of 1.0 to 4.5 were tested for their antileprosy activity in *M. leprae*-inoculated mouse foot pads. The resulting activity curve had a log P_{max} of 2.0. The significance of these findings is discussed in terms of the role played by limiting diffusional factors within the tissue so far as the penetration of these thiosemicarbazones into the intracellular environment is concerned.

RESUMEN

Se probó *in vitro* el efecto antibacteriano de dieciseis 2-acetil-piridino tiosemicarbazonas sobre diversas micobacterias cultivables. Los valores de las concentraciones mínimas inhibitorias (CMI) resultantes se expresaron graficamente en función de los valores de liofilicidad (log P) para cada compuesto probado. Las gráficas de los valores de log CMI contra log P, tuvieron la forma de curvas parabólicas, con un log P_{max} de 4.0 para las micobacterias de lento crecimiento y de 3.0 para el *M. smegmatis* de rápido crecimiento. Se probaron 10 tiosemicarbazonas con valores de P entre 1.0 y 4.5 en cuanto a su actividad antileprosa usando el modelo de la inoculación del *M. leprae* en el cojinete plantar del ratón. La curva de actividad resultante tuvo un log P_{max} de 2.0. Se discute el papel que juegan los factores de difusibilidad en relación a la penetración de estas tiosemicarbazonas en el medio intracelular.

RÉSUMÉ

Des épreuves antimycobactériennes ont été menées sur seize 2-acetyl-pyridine thiosemicarbazones, en utilisant un certain nombre de mycobactéries cultivables *in vitro*. Des déterminations subséquentes du MIC ont été mises en regard avec les valeurs de la lipophilicité (log P) pour les divers produits étudiés. Les graphiques de log MIC en relation avec les valeurs de log P obéissaient à une courbe de régression parabolique présentant un log P_{max} de 4,0 pour les mycobactéries à croissance lente, et de 3,0 pour *M. smegmatis* et les mycobactéries à croissance rapide. Dix composés à base de thiosemicarbazone, couvrant une gamme de log P allant de 1,0 à 4,5, ont été étudiés quant à leur activité antilépreuse dans les coussinets de souris inoculées avec *M. leprae*. La courbe d'activité résultant de ces essais présentait un log P_{max} de 2,0. La signification de ces observations est discutée en ce qui regarde le rôle joué par les facteurs limitant la diffusion à l'intérieur des tissus, pour autant que la pénétration de ces thiosemicarbazones dans l'environnement intracellulaire soit en cause.

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