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NOTES

Structure-Activity Relationships of Selected Phenazines against *Mycobacterium leprae* In Vitro

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Structure-activity relationships of phenazines against *Mycobacterium leprae* were investigated by using an in vitro radiorespirometric assay. In general, activity in ascending order was observed in compounds containing no chlorine atoms, a monochlorinated phenazine nucleus, and chlorines in the *para* positions of both the anilino and phenyl rings. The most active compounds contained a 2,2,6,6-tetramethylpiperidine substitution at the imino nitrogen. Most of these chlorinated phenazines were considerably more active in vitro than clofazimine (B663).

Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine; B663] has been used effectively in treating leprosy for over 25 years (2). The World Health Organization recommends its use along with rifampin and dapsone in multidrug therapy of lepromatous leprosy (8). Major advantages of B663 include low host toxicity (16); suppression of erythema nodosum leprosum (10, 11), a reactive state in leprosy; and few reports of bacterial resistance. The single most common drawback of B663 is a dose-related reddish-black skin pigmentation, making its continued use objectionable to a number of patients. The most serious and dose-limiting toxicity is gastrointestinal (16).

Recently, a series of phenazines were synthesized. They share a number of properties, including activity against clofazimine-resistant *Mycobacterium smegmatis* 607 and lack of uptake by body fat (and thus nonpigmentation) (13). We studied the relative activities of these compounds against *Mycobacterium leprae* in vitro to determine structure-activity relationships with a view to ultimately finding a less toxic substitute for clofazimine in treating leprosy.

Unlike that for in vitro-cultivable microorganisms, drug susceptibility testing of *M. leprae* has, until recently, been possible only in vivo. The established mouse footpad model is expensive and time-consuming, taking 6 to 12 months to complete, requiring gram quantities of new compounds for maximum-tolerated-dose determinations, and relying upon favorable pharmacokinetics in a nonhuman system (8). Therefore, a number of in vitro systems have been devised which involve exposure of intracellular or extracellular *M. leprae* to test compounds followed by measurement of some bacillary metabolic activity (6–9, 12, 15). Although these systems appear to be useful, most are somewhat cumbersome for large-scale screening or clinical use. We therefore recently used a simpler, semiautomated, radiorespirometric assay for rapidly assessing the metabolic status of *M. leprae* (4). This assay is based on the oxidation of palmitic acid to carbon dioxide and is similar to that used for rapid drug susceptibility testing of cultivable mycobacteria in the BACTEC method (14). We describe here its use in investigating structure-activity relationships of a number of phenazine derivatives against *M. leprae*.

Susceptibility testing was performed by a slight modification of the method of Franzblau (4). Briefly, footpads of *M. leprae*-infected, congenitally athymic nude mice were surface decontaminated by using UV irradiation, immersion in 1% iodine, and washing with 70% ethanol. Bacilli were obtained by homogenization of the footpads in Dubos-albumin medium (pH 6.5) followed by differential centrifugation. Samples were removed for contamination check on a variety of culture media (7). The remaining *M. leprae* suspension was held at 4°C for 2 days, during which time the culture medium was observed to confirm the absence of contamination. The suspension was then diluted in Dubos-albumin medium to a final density of 10^7/ml and distributed in 1-ml aliquots to 6-ml glass screw-cap vials. Phenazines were solubilized in absolute ethanol at 200 μg/ml with the aid of a sonicating bath and stored at 4°C until used. Dilutions were made in absolute ethanol, and 20 μl was added to the 1-ml cultures. Controls and heat-killed bacilli received 20 μl of ethanol. All groups consisted of quadruplicate samples,

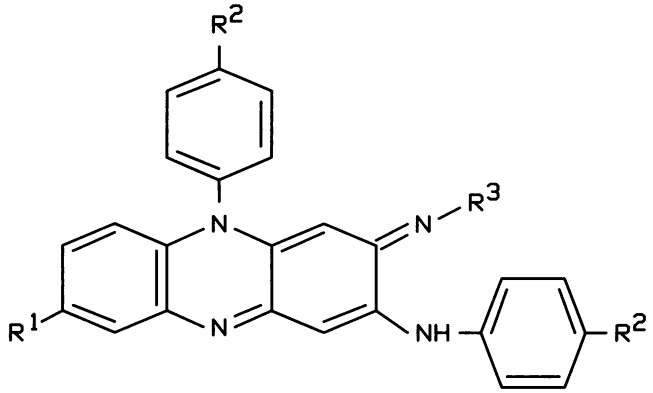

FIG. 1. Structure of parent phenazine.

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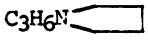
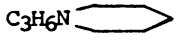
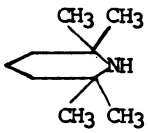
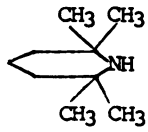
except for viable controls in which $n = 15$. Cultures were incubated for 1 week at 33°C with loose caps in chambers which were flushed on days 0, 1, 4, and 6 with 5% carbon dioxide-enriched air (Scott Medical Products, Plumsteadville, Pa.) for 5 min at 10 lb/in^2 (5). 1-^{14}C-labeled palmitic acid (1 μCi; 58 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was then added to each vial in 10 μl of ethanol, and the glass vials were placed in wide-mouth plastic scintillation vials containing a hollow cylinder of filter paper which had previously been saturated with a concentrated liquid scintillation fluor and alkalinized with 100 μl of NaOH (3). The entire assembly was then incubated at 33°C, and the evolved $^{14}CO_2$ was quantitated at 1, 3, 5, and 7 days after label addition by placing the assembly in a liquid scintillation counter, recording the counts per minute, and then reincubating at 33°C.

The structure of the parent phenazine is shown in Fig. 1. The effects of the various derivatives on the subsequent evolution of $^{14}CO_2$ from 1-^{14}C-labeled palmitic acid by *M. leprae* are shown in Table 1. The data presented are those

TABLE 1. Structure-activity relationships of phenazines against *M. leprae*

Phenazine	R^1	R^2	R^3	$^{14}CO_2$ [mean cpm (SD)] evolved after preincubation with phenazines at concn (ng/ml) of:				
				0	62.5	250	1,000	4,000
None				2,208 (403)				
Heat killed				38 (6)				
B663	H	Cl	$CH(CH_3)_2$		1,369 (742)	1,546 (189)	362 (59)	25 (9)
B3884	H	H	$C_3H_6N(C_2H_5)_2$		2,659 (153)	2,590 (243)	2,054 (184)	895 (145)
B4017	Cl	H	$C_3H_6N(C_2H_5)_2$		2,451 (175)	1,798 (155)	56 (18)	21 (6)
B826	H	Cl	$C_3H_6N(C_2H_5)_2$		1,598 (232)	740 (89)	32 (3)	20 (5)
B3779	H	Cl	$CH(CH_2)_3N(C_2H_5)_2$ (CH$_3$)		1,592 (218)	1,017 (179)	34 (7)	33 (5)
B3824	H	Cl	$C_3H_6N(C_2H_4OH)_2$		2,551 (248)	2,398 (64)	1,322 (106)	73 (19)
B3793	H	H	C_3H_6N 		1,514 (269)	2,871 (30)	2,707 (179)	2,748 (413)
B3849	Cl	H	C_3H_6N 		2,375 (115)	2,007 (150)	69 (17)	40 (4)
B3785	H	Cl	C_3H_6N 		1,452 (111)	1,007 (300)	47 (14)	43 (22)
B4018	Cl	H	C_3H_6N 		2,133 (355)	1,562 (260)	35 (10)	26 (6)
B3770	H	Cl	C_3H_6N 		1,235 (335)	814 (228)	29 (6)	37 (3)
B4019	Cl	H			1,193 (94)	158 (13)	27 (5)	23 (3)
B3786	H	Cl			1,277 (152)	194 (45)	42 (12)	60 (23)

obtained 24 h after addition of 1-^{14}C-labeled palmitic acid, in order to demonstrate the ability of this technique to yield significant data in as little as 8 days (7 days of preincubation with drug plus 1 day with labeled substrate). Cumulative $^{14}CO_2$ was higher for all samples at 3, 5, and 7 days post-label addition (data not shown). However, with the exception of heat-killed cells and drug-treated cells with very low $^{14}CO_2$ evolution at 1 day post-label addition, increases were proportional to the results obtained at the earlier reading.

Clofazimine (B663) demonstrated relatively potent activity, as reported previously (4). Although unhalogenated B3884 (containing a propanyldiethylamine at R^3) appeared to be inactive at ≤1,000 ng/ml, the monochloro (B4017) and dichloro (B826) analogs were active, with the latter showing the highest activity. Changing the propanyl spacer group on the dichlorinated compound to a sec-pentyl group (B3779) had little effect; however, replacing the diethylamine moiety with a diethanolamine (B3824) resulted in a marked reduction in activity, the lowest among all of the dichloro compounds in this study. Compounds containing a pyrrolidine ring (B3793, B3849, and B3785) displayed essentially the same activity as their diethylamine analogs, with activity increasing with the degree of chlorination. Expanding the ring to a piperidine had little effect, with the dichloro analog (B3770) again appearing to be more active than the corresponding monochlorinated (B4018) phenazine. Finally, compounds containing a 2,2,6,6-tetramethylpiperidine moiety at the R^3 position showed the highest activity, with no apparent difference between the monochloro (B4019) and dichloro (B3786) analogs.

This is the first in vitro drug susceptibility study with *M. leprae* which yielded structure-related activity. Both chlorine substitution at R^1 and R^2 and the nature of the R^3 group affected phenazine activity. With the exception of B3824, all of the chlorinated phenazines demonstrated activity markedly superior to that of B663 at 1,000 ng/ml. In this regard, the results obtained with dichlorinated phenazines B826 and B3785 were consistent with those obtained in a previous in vitro study which used measurement of bacillary ATP as an index of drug activity (7). The importance of the R^3 substitution was readily apparent from the relatively low activity of B3824 and the high activity of B4019 and B3786. A number of the dichlorinated compounds evaluated here have also been tested against *M. smegmatis* 607 in vitro (13). In general, they showed markedly superior activity compared with B663 against both clofazimine-susceptible and -resistant strains. In addition, consistent with the results in the present study, B3824 was less active than structural analogs B826 and B3779. Previous in vivo studies with *M. tuberculosis* revealed a potentiating effect of chlorine substitution in the peripheral anilino and phenyl rings of various phenazines (1). However, chlorine substitution alone was not sufficient to impart antituberculosis activity, and some compounds which were inactive in vivo showed in vitro activity. Indeed, compounds which do not form crystals intracellularly and do not persist in tissues (such as clofazimine does) may also have lower in vivo activity. While the efficacies of the highly active compounds in this study require in vivo confirmation, the radiorespirometric assay appears to be promising in the in vitro exploration of structure-activity relationships of antileprosy agents.

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