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Double-blind evaluation of BACTEC and Buddemeyer-type radiorespirometric assays for *in vitro* screening of antileprosy agents

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Summary Two radiorespirometric assays, the BACTEC 460 and Buddemeyer-type $^{14}CO_2$ detection systems, were evaluated in a double-blind manner for their ability to discriminate between authentic antileprosy agents and inactive compounds. Freshly harvested, nude-mouse derived *Mycobacterium leprae* were incubated in axenic media in the presence of coded test solutions prepared in a remote laboratory. Activity was assessed by comparing the rate of $^{14}CO_2$ evolution from [1-^{14}C]palmitic acid to controls. Breaking the code revealed that both systems demonstrated a dose response to ethionamide, pefloxacin and rifampicin as well as sensitivity to dapsone. Most of the water, ethanol, sucrose, dabsyl chloride and riboflavin negative-control samples failed to effect a significant reduction in radiorespirometric activity. This study confirms the ability of the radiorespirometric assays to function as a primary drug screening system in leprosy.

Introduction

Historically, the screening of compounds for activity against *Mycobacterium leprae* has had to utilize the mouse footpad model[1–3] because of the failure to cultivate the leprosy bacillus *in vitro*. The high cost, the length of time (6–12 months per experiment), the requirement for gram quantities of drug and the dependence on non-human pharmacokinetics[4] have all motivated investigators to develop rapid *in vitro* systems which measure bacterial metabolism (following exposure to antimicrobial agents) as an index of viability.[5–7]

The ability of *M. leprae* to oxidize rapidly palmitic acid to carbon dioxide[8–10] has been exploited by Franzblau *et al.*[10–17] when screening for new antileprosy drugs, using two related assay systems: a Buddemeyer-type, liquid scintillation based, two-compartment system[10] and an adaptation of the commercial BACTEC 460 system.[11] These systems are very similar in that they both quantitate the rate of $^{14}CO_2$ evolution from ^{14}C-labelled

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substrates. Both are 'automated' systems which do not require host cells, subsampling, extraction, filtration or other processing following the incubation of bacilli with drugs.

The present WHO-sponsored, double-blind study was undertaken to evaluate objectively the utility of these systems in screening for antileprosy drugs. Coded samples of compounds with known antileprosy activity, and control samples with no activity, were prepared in the London laboratory and sent to the Carville laboratory where they were tested in the two *in vitro* assays. In this way we were able to assess the reliability and reproducibility of the assays in screening for antileprosy activity.

Methods

The drug solutions were composed of stock solutions of rifampicin (RMP; 2·5 mg/ml in ethanol), ethionamide (ETH), dapsone (DDS; 5 mg/ml in ethanol), and pefloxacin (PEF; 5 mg/ml in water) and were prepared at NIMR (London). Triplicate 0·1 ml aliquots of these stock solutions, together with 2·5-fold, 5-fold and 10-fold dilutions were provided for a subsequent 100-fold dilution in the test media following filter sterilization. Negative control solutions consisted of distilled water, absolute ethanol, 1% sucrose, riboflavin (0·5 mg/ml), and dabsyl chloride (0·1 mg/ml). These last two acted as colour controls for ethionamide and rifampicin, respectively. The 70 test samples were coded, randomized, stored at −20°C and shipped on dry ice to the Carville laboratory where they were held at −20°C until used.

M. LEPRAE INOCULUM

M. leprae was harvested from the footpads of athymic nu/nu mice when the footpads had reached a bacillary load of approximately 10^{10} AFB. Footpads were surface decontaminated with iodine and ethanol, minced and homogenized in 7H12 medium (Middlebrook 7H9 broth, 0·1% casitone (Difco), 1% w/v albumin and 5 μg/ml catalase). The bulk of tissue debris was removed by slow-speed centrifugation ($108 \times g$, 5 min, 10°C) and the bacilli pelleted ($2710 \times g$, 45 min, 10°C) and resuspended in 7H12 medium to approximately 10^9/ml. Cell counts were determined by the method of Shepard & McRae.[18] The suspensions were treated with 50 μg/ml ampicillin and 2·5 μg/ml amphotericin B for 4–5 h to eliminate contaminants. These agents have repeatedly been shown to be inactive against *M. leprae*.[10,19] Aliquots of the bacillary suspension were inoculated into Middlebrook 7H11 and Lowenstein–Jensen slants, tryptic soy and thioglycollate broths and blood agar to check for contaminants.

BACTEC

The *M. leprae* suspension was diluted to 10^8/ml in 7H12 medium and 0·1 ml aliquots (10^7 AFB) delivered via tuberculin syringe to BACTEC 12B media (4 ml in 20 ml serum vials) supplemented with 50 μg/ml ampicillin and 2·5 μg/ml amphotericin B. The controls were 16 cultures that had received 0·1 ml 7H9 broth. Other cultures received 0·1 ml of coded test solutions in quadruplicate. All vials were flushed with 2·5% oxygen, 10% carbon dioxide, balance nitrogen using the BACTEC 460 instrument and were then incubated at 33°C. The growth index (GI, $^{14}CO_2$ evolution) was determined at weekly intervals for 3 weeks in

the BACTEC 460 by flushing with the above gas mixture. Readings from week 2 were used in evaluating activity.

BUDDEMEYER

The *M. leprae* suspension was diluted to 10^6/ml in 7H12 medium and 1 ml aliquots (10^6 AFB) dispensed to 6 ml screw-capped vials (Wheaton, Millville, NJ, USA). The controls were 16 cultures who received 10 μl 7H9 broth. Other cultures received 10 μl of coded test solutions in quadruplicate. Vials, with loose caps, were then incubated for 2 weeks at 33°C under an atmosphere of 2·5% oxygen, 10% carbon dioxide in an incubator with automatic oxygen and carbon dioxide control capability, and 1 microCurie of [1-^{14}C] palmitic acid (58 mCi/mmole; New England Nuclear, Boston, MA, USA) was added to each vial in a volume of 10 μl ethanol. The glass vials, with loose caps, were then placed within wide-mouth scintillation vials (Poly-Q; Beckman, Brea, CA) containing a 2 × 4 cm strip of Whatman No. 42 filter paper which had previously been dipped into a mixture of 20 ml Liquifluor concentrate (New England Nuclear), 15 g PPO, 5 ml Triton-X 100 and 5 ml 2N NaOH (in MeOH). The entire assembly was incubated at 33°C and $^{14}CO_2$ evolution determined at daily intervals for 1 week by placing the double vial assemblies in a liquid scintillation counter. Cumulative counts per minute from the day 7 reading were used in evaluating activity.

STATISTICAL EVALUATION

Statistical significance was determined by the Student's *t*-test.

Results

BACTEC

Using a *p* value of 0·05 as a cut-off for active substances, there were 6 false positives out of 22 non-antileprosy substances (Table 1). While 11 of 12 DDS samples were active, there was little, if any, dose-response over the entire range of 0·1–5·0 μg/ml. Both ETH and PEF displayed no activity at 0·1 μg/ml; 2 of 3 samples were active at 0·5 μg/ml and all 2·0 and 5·0 μg/ml samples effected significant reductions compared to controls. The dose response was more apparent with PEF than with ETH. All RMP concentrations demonstrated highly significant activity and effected a clear dose-response.

BUDDEMEYER

Only 1 of the 22 non-antileprosy substances appeared active, possibly due to the large SD obtained with this sample (A20) (Table 2). Results with DDS were variable, although 2 of 3 samples were positive at 0·5, 2·0 and 5·0 μg/ml. Both ETH and PEF gave inconclusive results at 0·1 μg/ml and significant dose-responsive activity at the 3 higher concentrations. All concentrations of RMP again demonstrated highly significant, dose-dependent activity.

Table 1. BACTEC system

Coded sample	De-coded sample and concentration (μg/ml)	Mean	SD	p	Rating
Control		746	113		
Heat killed		2	1	$<0{\cdot}001$	
A13	Water	670	39	0·212	Inactive
A18	Water	684	63	0·315	Inactive
A22	Water	574	108	0·014	Active*†
A25	Water	613	48	0·036	Active*†
A32	Water	643	37	0·094	Inactive
A65	Water	576	27	0·010	Active*†
A10	Sucrose	639	50	0·088	Inactive
A37	Sucrose	654	35	0·192	Inactive
A08	Ethanol	633	31	0·068	Inactive
A12	Ethanol	732	44	0·808	Inactive
A23	Ethanol	626	49	0·057	Inactive
A36	Ethanol	648	45	0·113	Inactive
A39	Ethanol	642	25	0·100	Inactive
A59	Ethanol	752	51	0·926	Inactive
A04	Dabsyl Cl	725	9	0·724	Inactive
A52	Dabsyl Cl	807	32	0·308	Inactive
A61	Dabsyl Cl	736	38	0·864	Inactive
A69	Dabsyl Cl	614	21	0·035	Active*†
A20	Riboflavin	698	77	0·437	Inactive*
A26	Riboflavin	591	37	0·017	Active*†
A38	Riboflavin	703	117	0·513	Inactive
A70	Riboflavin	528	76	0·002	Active*†
A03	DDS 0·1	564	12	0·006	Active
A34	DDS 0·1	516	12	0·001	Active*
A57	DDS 0·1	611	18	0·032	Active*
A02	DDS 0·5	501	48	0·001	Active
A48	DDS 0·5	669	41	0·204	Inactive*
A56	DDS 0·5	599	3	0·021	Active
A01	DDS 2·0	512	26	0·001	Active
A31	DDS 2·0	462	24	$<0{\cdot}001$	Active*
A67	DDS 2·0	480	15	$<0{\cdot}001$	Active
A27	DDS 5·0	476	13	$<0{\cdot}001$	Active
A50	DDS 5·0	588	2	0·014	Active*
A68	DDS 5·0	425	32	$<0{\cdot}001$	Active
A16	ETH 0·1	651	43	0·123	Inactive*
A44	ETH 0·1	775	47	0·630	Inactive
A55	ETH 0·1	759	19	0·824	Inactive
A43	ETH 0·5	635	165	0·129	Inactive*
A62	ETH 0·5	570	22	0·007	Active
A64	ETH 0·5	570	17	0·007	Active
A07	ETH 2·0	537	46	0·002	Active
A40	ETH 2·0	469	56	$<0{\cdot}001$	Active
A41	ETH 2·0	551	70	0·005	Active
A14	ETH 5·0	449	36	$<0{\cdot}001$	Active
A30	ETH 5·0	395	50	$<0{\cdot}001$	Active
A47	ETH 5·0	503	43	0·001	Active
A19	PEF 0·1	728	48	0·758	Inactive*
A42	PEF 0·1	816	49	0·316	Inactive
A49	PEF 0·1	ND			

Table 1. (*Contd.*)

Coded sample	De-coded sample and concentration (μg/ml)	Mean	SD	p	Rating
A11	PEF 0·5	643	61	0·100	Inactive*
A15	PEF 0·5	616	79	0·047	Active
A29	PEF 0·5	604	22	0·025	Active
A17	PEF 2·0	536	50	0·002	Active
A28	PEF 2·0	457	35	<0·001	Active
A45	PEF 2·0	561	64	0·006	Active
A09	PEF 5·0	362	29	<0·001	Active
A53	PEF 5·0	418	5	<0·001	Active
A66	PEF 5·0	318	46	<0·001	Active
A24	RMP 0·05	458	32	<0·001	Active
A33	RMP 0·05	440	41	<0·001	Active
A63	RMP 0·05	ND			Active
A06	RMP 0·25	394	26	<0·001	Active
A35	RMP 0·25	290	37	<0·001	Active
A54	RMP 0·25	395	28	<0·001	Active
A21	RMP 1·0	204	35	<0·001	Active
A58	RMP 1·0	262	27	<0·001	Active
A60	RMP 1·0	258	14	<0·001	Active
A05	RMP 2·5	226	10	<0·001	Active
A46	RMP 2·5	222	37	<0·001	Active
A51	RMP 2·5	212	8	<0·001	Active

For controls, $n = 16$; for test samples, $n = 4$.
Rating criteria: active = $p < 0{\cdot}05$; inactive = $p > 0{\cdot}05$ *vs.* controls.
*, Apparent disagreement with results of Buddemeyer assay.
ND, not determined; †, false positive result.

Discussion

This double-blind, 2-laboratory evaluation of these *in vitro* radiorespirometric systems confirms previous reports on the ability of these assays to detect activity of established antileprosy drugs at concentrations below peak obtainable plasma levels.[10–12] All of the known antileprosy compounds were found to be active at the concentrations which they would be predicted to be active and there was good concordance between replicate samples. Results from earlier studies and this report suggest a correlation between degree of drug activity in the mouse footpad[19–24] and radiorespirometric systems.[11,12,14,16] In general, highly active, potent drugs such as RMP produced lower levels of $^{14}CO_2$ than bacteriostatic agents such as DDS. Since all concentrations of RMP were active in both Buddemeyer and the BACTEC systems, we were unable to determine an MIC. The MIC of ethionamide, as measured by the *in vitro* systems, appeared to be between 0·1 and 0·5 μg/ml (compared to 0·05 μg/ml as estimated by the mouse footpad system). All concentrations of DDS were active in the BACTEC system, whereas in the Buddemeyer system occasional samples at all concentrations were found to be inactive, making it difficult to compare the MIC *in vitro* with that estimated using the mouse footpad technique (approximately 0.003 μg/ml). Pefloxacin appears to have an MIC of approximately 0·5 μg/ml *in vitro*.

Table 2. Buddemeyer system

Coded sample	De-coded sample and concentration (μg/ml)	Mean	SD	p	Rating
Control		7213	992		
Heat killed		148	10	<0.001	
A13	Water	6704	331	0·333	Inactive
A18	Water	6671	552	0·313	Inactive
A22	Water	6731	982	0·395	Inactive*
A25	Water	6833	243	0·465	Inactive*
A32	Water	7325	958	0·842	Inactive
A65	Water	6963	987	0·656	Inactive*
A10	Sucrose	7257	605	0·935	Inactive
A37	Sucrose	6501	533	0·188	Inactive
A08	Ethanol	6693	758	0·343	Inactive
A12	Ethanol	7384	435	0·745	Inactive
A23	Ethanol	6361	603	0·174	Inactive
A36	Ethanol	7316	1300	0·876	Inactive
A39	Ethanol	7444	1251	0·696	Inactive
A59	Ethanol	7879	306	0·210	Inactive
A04	Dabsyl Cl	6272	1496	0·141	Inactive
A52	Dabsyl Cl	6548	466	0·215	Inactive
A61	Dabsyl Cl	6896	1003	0·575	Inactive
A69	Dabsyl Cl	8143	1165	0·122	Inactive*
A20	Riboflavin	5399	1971	0·023	Active*†
A26	Riboflavin	7092	268	0·814	Inactive*
A38	Riboflavin	6760	681	0·403	Inactive
A70	Riboflavin	7252	544	0·941	Inactive*
A03	DDS 0·1	5623	648	0·007	Active
A34	DDS 0·1	6258	330	0·078	Inactive*
A57	DDS 0·1	6310	213	0·093	Inactive*
A02	DDS 0·5	5543	100	0·004	Active
A48	DDS 0·5	5392	804	0·003	Active*
A56	DDS 0·5	5746	781	0·014	Active
A01	DDS 2·0	5439	828	0·004	Active
A31	DDS 2·0	6621	414	0·265	Inactive*
A67	DDS 2·0	6080	814	0·050	Active
A27	DDS 5·0	5805	1204	0·025	Active
A50	DDS 5·0	6268	1076	0·110	Inactive*
A68	DDS 5·0	5193	290	0·001	Active
A16	ETH 0·1	5884	1235	0·034	Active*
A44	ETH 0·1	6501	335	0·181	Inactive
A55	ETH 0·1	7035	854	0·746	Inactive
A43	ETH 0·5	5038	1131	0·001	Active*
A62	ETH 0·5	5791	456	0·013	Active
A64	ETH 0·5	5296	1626	0·007	Active
A07	ETH 2·0	1699	460	<0.001	Active
A40	ETH 2·0	1953	570	<0.001	Active
A41	ETH 2·0	ND			Active
A14	ETH 5·0	1086	39	<0.001	Active
A30	ETH 5·0	1163	237	<0.001	Active
A47	ETH 5·0	1270	85	<0.001	Active
A19	PEF 0·1	5789	1252	0·025	Active*
A42	PEF 0·1	ND			
A49	PEF 0·1	8480	550	0·026	Inactive

Table 2. (*Contd.*)

Coded sample	De-coded sample and concentration (μg/ml)	Mean	SD	*p*	Rating
A11	PEF 0·5	5206	644	0·001	Active*
A15	PEF 0·5	4732	469	<0·001	Active
A29	PEF 0·5	5527	961	0·007	Active
A17	PEF 2·0	3319	430	<0·001	Active
A28	PEF 2·0	3438	264	<0·001	Active
A45	PEF 2·0	3136	717	<0·001	Active
A09	PEF 5·0	2506	187	<0·001	Active
A53	PEF 5·0	2458	19	<0·001	Active
A66	PEF 5·0	2608	307	<0·001	Active
A24	RMP 0·05	4179	557	<0·001	Active
A33	RMP 0·05	4423	922	<0·001	Active
A63	RMP 0·05	5346	389	0·002	Active
A06	RMP 0·25	2174	388	<0·001	Active
A35	RMP 0·25	2266	348	<0·001	Active
A54	RMP 0·25	2082	57	<0·001	Active
A21	RMP 1·0	453	139	<0·001	Active
A58	RMP 1·0	832	122	<0·001	Active
A60	RMP 1·0	802	168	<0·001	Active
A05	RMP 2·5	633	77	<0·001	Active
A46	RMP 2·5	696	47	<0·001	Active
A51	RMP 2·5	631	87	<0·001	Active

For controls, $n = 16$; for test samples, $n = 4$.
Rating criteria: active = $p < 0{\cdot}05$; inactive = $p > 0{\cdot}05$ *vs.* controls.
*, Apparent disagreement with results of BACTEC assay.
ND, not determined; † false positive result.

Up to now the radiorespirometric systems evaluated in this report have been used to screen 6 macrolides,[14] 17 clofazimine derivatives,[15,17] 20 fluoroquinolones,[16] 1 tetracycline,[11] 5 aminoglycosides, 3 rifamycins and 2 lincosamides[12] for activity against *M. leprae*. Thus, 2 agents, clarithromycin[14] and sparfloxacin,[16] were first recognized as being the most active clinically relevant agents in their respective classes and are currently in clinical trials in leprosy. Another potential antileprotic identified by radiorespirometry is fusidic acid,[13] an established antibiotic which has poor pharmacokinetics in mice (in contrast to humans) and is inactive against *M. leprae* in the mouse footpad model (Colston, unpublished results).

There was fairly good agreement (79%) between the BACTEC and Buddemeyer systems, especially considering that the two assays were evaluated in separate experiments at different times using different nude mouse-derived inocula. The BACTEC system requires approximately 10 times more bacilli than the Buddemeyer-type system to obtain usable readings. This relatively lower sensitivity also results in smaller differences observed between control and drug-treated samples than that observed in the Buddemeyer system. The BACTEC system, however, appears to be more sensitive to weakly-active agents such as DDS and results in lower variance among replicate samples. This may be due to the ability to produce efficiently and maintain the desired (microaerophilic) environment using the BACTEC 460 instrument, which appears optimal for maintenance of *M. leprae* viability *in vitro*.[25] With either test (consisting of approximately 300 vials—70

samples in quadruplicate plus 4 heat-killed and 16 drug-free controls), the numbers evaluated in the present study can be read within a normal 8 hour working day.

Conclusion

Radiorespirometry, using either the BACTEC 460 or Buddemeyer $^{14}CO_2$ detection systems, is capable of differentiating between antileprosy agents and inactive substances following a 2-week incubation of freshly harvested, viable leprosy bacilli under appropriate incubation conditions.

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Évaluation en double-aveugle des tests radiorespirométriques BACTEC et type Buddemeyer pour le triage *in vitro* des agents antilépreux

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Résumé Nous avons comparé deux essais radiorespirométriques, le système BACTEC 460 et le système type Buddemeyer de détection de $^{14}CO_2$, en double-aveugle, pour évaluer leur aptitude à différencier entre les agents antilépreux authentiques et les composés inactifs. *Mycobacterium leprae* provenant de souris "nude", fraîchement récolté, a été incubé en milieu axénique en présence des solutions à tester identifiées par code et préparées dans un laboratoire éloigné. L'activité a été déterminée en comparant le contôle du taux d'évolution de $^{14}CO_2$ partir de l'acide (1-^{14}C) palmitique. En décodant, nous avons observé que les deux systèmes présentaient une résponse proportionnée à la dose à l'éthionamide, la pefloxacine et la rifampicine, de même qu'une sensibilité à la dapsone. La plupart des échantillons témoins-négatifs eau, éthanol, sucrose, chlorure de dabsyl et riboflavine n'ont produit aucune réduction significative de l'activité radiorespirométrique. Cette étude confirme l'aptitude des essais radiorespirométriques à fonctionner comme un système de triage primaire des agents antilépreux.

La evaluación doble-ciego de las pruebas radiorespirométricos BACTEC y de tipo Buddemeyer en los estudios *in vitro* de los agentes contra la lepra

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Resumen Dos pruebas radiorespirométricas, los sistemas de detección de $^{14}CO_2$ BACTEC 460 y el tipo Buddemeyer, fueron evaluados de un modo doble-ciego para su habilidad de discriminar entre agentes verdaderamente antileprosos y los compuestos inactivos. *Mycobacterium lepræ* recién obtenido de ratones desnudos fue incubado in medios axénicos, en la presencia de soluciones de prueba codificadas, preparadas en un laboratorio remoto. Se evaluó la actividad comparando la velocidad de evolución de $^{14}CO_2$ del ácido [1-^{14}C]palmítico. Después de revelar el código, ambos sistemas mostraron una respuesta de dósis a la etionamida, pefloxacina y rifampicina, además de sensibilidad a la dapsona. La mayoría de muestras de control negativo, de agua, etanol, sucrosa, cloruro de dabsilo y riboflavina no lograron una reducción significativa de actividad radiorespirométrica. El estudio confirma la manera en que las pruebas radiorespirométricas pueden funcionar como un sistema de examen primario para drogas contra la lepra.