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Unanticipated *Mycobacterium tuberculosis* complex culture inhibition by immune modulators, immune suppressants, a growth enhancer, and vitamins A and D: clinical implications

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SUMMARY

Background: The development of novel antibiotics to treat multidrug-resistant (MDR) tuberculosis is time-consuming and expensive. Multiple immune modulators, immune suppressants, anti-inflammatories, and growth enhancers, and vitamins A and D, inhibit *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in culture. We studied the culture inhibition of *Mycobacterium tuberculosis* complex by these agents.

Methods: Biosafety level two *M. tuberculosis* complex (ATCC 19015 and ATCC 25177) was studied in radiometric Bactec or MGIT culture. Agents evaluated included clofazimine, methotrexate, 6-mercaptopurine, cyclosporine A, rapamycin, tacrolimus, monensin, and vitamins A and D.

Results: All the agents mentioned above caused dose-dependent inhibition of the *M. tuberculosis* complex. There was no inhibition by the anti-inflammatory 5-aminosalicylic acid, which causes bacteriostatic inhibition of MAP.

Conclusions: We conclude that, at a minimum, studies with virulent *M. tuberculosis* are indicated with the agents mentioned above, as well as with the thioamide 5-propothiouricil, which has previously been shown to inhibit the *M. tuberculosis* complex in culture. Our data additionally emphasize the importance of vitamins A and D in treating mycobacterial diseases.

1. Introduction

Multidrug-resistant (MDR) and total drug-resistant (TDR) tuberculosis is an increasing problem worldwide.[1–4] Amongst the multiple evolving strategies attempting to address this problem is the development of new antibiotics.[5–8] However, identifying, evaluating, obtaining regulatory approval, and marketing totally new antibiotics is time-consuming and expensive.[9–11] Existing approved pharmaceuticals that have heretofore unanticipated inhibition on *Mycobacterium tuberculosis* could more rapidly and less expensively proceed to ethically acceptable clinical evaluation.

There are increasing concerns that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be zoonotic,[12–14] and is responsible for, at a minimum, Crohn's disease.[15] We posit that the reason the pathogenesis of MAP has been missed is because, unknowingly, since 1942,[16] the medical profession has been treating MAP without understanding that was what they were doing. Multiple agents called 'immune suppressants', 'immune modulators',[17–22] and 'anti-inflammatories',[23] as well as vitamins,[24] exhibit dose-dependent inhibition of MAP in culture: they are anti-MAP antibiotics. As controls in these and other experiments,[25] we used *Mycobacterium avium* subspecies *avium* and two biosafety level 2 strains from the *M. tuberculosis* complex.

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We present herein unreported data on the dose-dependent inhibition, in culture, of the *M. tuberculosis* complex by multiple agents we have studied, and correlate these data with those from prior publications.[17–24] We compared the known anti-*M. tuberculosis* antibiotics *para*-aminosalicylic acid (PAS) and isoniazid with sulfapyridine and the anti-leprosy antibiotic clofazimine. We also evaluated the anti-inflammatory 5-aminosalicylic acid (5-ASA), the thiopurine immunomodulator 6-mercaptopurine (6-MP), and the immunosuppressants methotrexate, cyclosporine A, thalidomide, rapamycin, and tacrolimus. In addition, we studied two vitamins that inhibit mycobacteria in culture, vitamins A[24] and D.[24,26]

2. Methods

This study was approved by the Research and Development Committee at the Veterans Affairs Medical Center, Bronx NY (0720-06-038) and was conducted under the Institutional Radioactive Materials Permit (#31-00636-07).

2.1. Bacterial culture

The purpose of this study was to evaluate inhibition on the *M. tuberculosis* complex. We used two biosafety level 2 strains, bacillus Calmette–Guérin (BCG) *Mycobacterium bovis* Karlson and Lessel (ATCC 19015) and an avirulent *M. tuberculosis* strain (ATCC 25177).[27]

When indicated, comparisons of inhibition of MAP are included; the MAP was mostly that isolated from humans with Crohn's disease ('Dominic' ATCC 43545; 'Ben' ATCC 43544; 'Linda' ATCC 43015; ATCC 700535; '303' ATCC # PTA 7788[28]) and UCF-4 (gift of Saleh Naser, Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL,USA).[29] All ATCC were from ATCC Rockville, MD, USA.

All agents studied were purchased and prepared as described in previous publications.[17–19,23,24,30] The solvent in which the chemical was dissolved is identified in each table in the Results section.

Our Bactec 460 (Becton Dickinson, Franklin Lakes, NJ, USA) ^{14}C radiometric culture inhibition methods have been published in detail previously.[17–19,23,24,30] This system quantifies bacterial growth, or the lack thereof, by providing ^{14}C in palmitate, an energy source for mycobacterial growth.[31] Vials are assayed on a daily basis, quantifying the amount of ^{14}C released as $^{14}CO_2$, by the integral detector in the Bactec 460. The data are obtained as manufacturer-determined arbitrary 'growth units' (GU) of 0–999. Because the Bactec 460 is only semi-automatic and because of the onerous regulatory requirements of using radionucleotides, this exquisitely sensitive[23] system is being phased out. The Bactec 460 radiometric system has been replaced by the fully automatic, oxygen consumption detecting fluorescent probe-based MGIT 960 system (Becton Dickinson).[32,33]

In this study we performed a parallel Bactec/MGIT comparison. For this comparison, both components of the study were set up on the same day, using the same pre-culture for the bacterial inoculum. For the Bactec component, we used our previously described methods.[17–19,23,24,30] The final volume in the Bactec system was always 5 ml, and the concentration of the dissolving liquid was identical in each tube, irrespective of the concentration of the agent being tested. In this Bactec/MGIT comparison experiment, the agents were dissolved in dimethyl sulfoxide (DMSO), and the final concentration was always 3.2% DMSO. In the MGIT system the final volume was 7 ml. Accordingly, we increased the volume of the inoculum, test agent, and DMSO so that the concentration was the same for each component in the final solution.

To minimize possible confounding variables in the Bactec/MGIT comparison, mycobactin J, Oleic Acid, Albumin, Dextrose & Catalase (OADC) (Cat # BD-237510), and Tween 80 were not added to either the Bactec or the MGIT cultures. Neither OADC nor Albumin, Dextrose & Catalase (ADC) (Cat # BD-212352) nor mycobactin J is required for the growth of the *M. tuberculosis* strain.[17–19,23,24,30] MGIT computer-declared 'positivity' occurred by day 7 of the control *M. tuberculosis* inoculum. We did not use Tween 80, recommended to minimize mycobacterial clumping,[31] because we,[19] and others,[34] have found that it interferes with inhibition.

Bactec quantifies growth as the 'growth index' (GI). Sequential days of data are added together and presented as the cumulative GI (cGI). The data are then mathematically manipulated to indicate the amount of inhibition from the control as the percentage change from control cGI (inhibition as %−ΔcGI; see Greenstein et al.[23] for calculation).

MGIT data are provided by the integral MGIT computer as either growth units or as the day when the computer determines an individual inoculum has reached log phase growth and is declared 'positive.' In our Bactec/MGIT *M. tuberculosis* comparison, we present the MGIT data in both ways. Agents being tested were added at the beginning of the experiment. The calculation for MGIT 'cumulative growth units' (cGU) was made by adding the growth units from the MGIT printout until an arbitrary day post inoculation; in this particular experiment we terminated the experiment on day 16 because the controls had passed log phase growth and showed no further increase in the control growth units. The calculation for cGU was as described for cGI for Bactec data.[23] The effect (or lack thereof) of each agent in the MGIT is presented as the percentage decrease in cGU units (%−ΔcGU). The calculation of %−ΔcGI was performed in two stages (using Excel) using the following formula: step one = [(A − B)/A] = C, step two = −C × % = final result of %−ΔcGU, where A = the cGU of the control inoculum for the given diluent (in these experiments DMSO see above and in each table), B = the cGU for the particular chemical at a particular dose being tested, incubated for the same number of days as A, and C = the product of [(A − B)/A]. Days to positivity are also presented in the tables and figure (see Figure 1 legend for details).

3. Results

The inhibitory control used was PAS. There was a marked dose-dependent inhibition (>95%−ΔcGI at 1 μg/ml) of *M. tuberculosis* (Table 1). This was not as pronounced with BCG, particularly when PAS was dissolved in 7H9 (18%−ΔcGI at 1 μg/ml) or water (−86%−ΔcGI at 1 μg/ml; Table 1). Isoniazid was an additional inhibitory control. It was found to be bactericidal against *M. tuberculosis* whether dissolved in NaOH or water (99%−ΔcGI at 1 μg/ml; Table 2). BCG was best inhibited when the dissolving solution was NaOH (99%−ΔcGI at 1 μg/ml; Table 2).

Our non-inhibitory control was the intact molecule of sulfasalazine (comprising sulfapyridine coupled to 5-ASA). There was no dose-dependent inhibition of either *M. tuberculosis* complex strain studied (Table 3).

Sulfapyridine, alone or with 5-ASA (the two component molecules of our non-inhibitory control sulfasalazine), showed poor dose-dependent inhibition of *M. tuberculosis* (63%−ΔcGI at 64 μg/ml; Table 4). BCG was more susceptible to sulfapyridine (≥89%−ΔcGI at 16 μg/ml; Table 4). There was no synergy of sulfapyridine with 5-ASA on BCG (Table 4).

Alone, 5-ASA showed no dose-dependent inhibition on the *M. tuberculosis* complex (Table 5). This is in contrast to the weak, but consistent and replicable, bacteriostatic dose-dependent

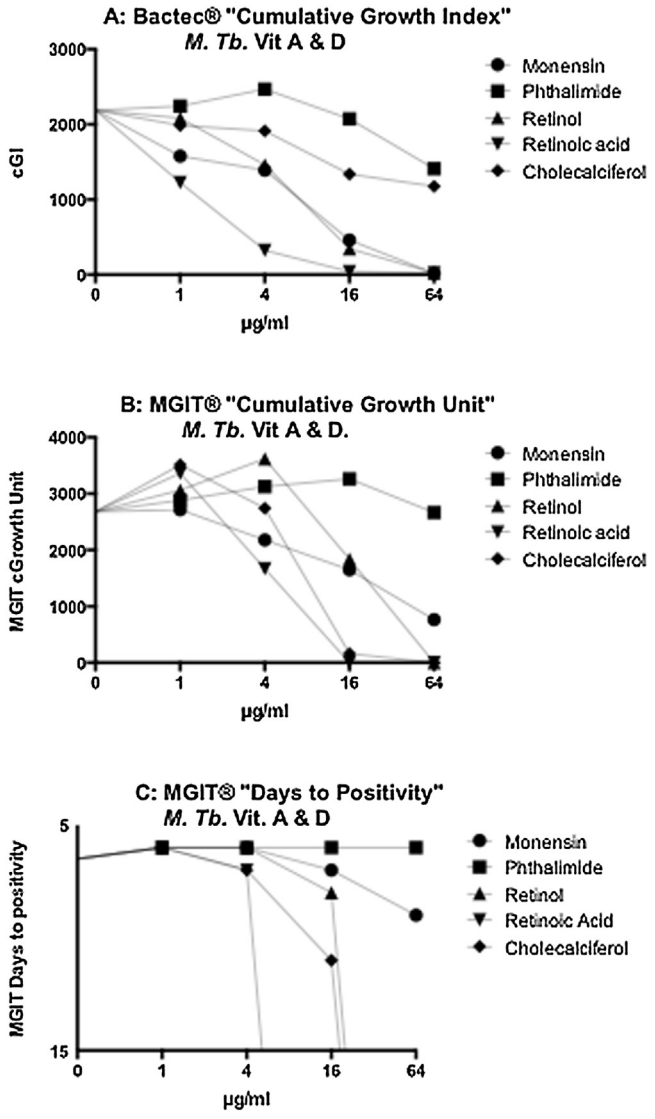

Figure 1. A simultaneous comparison of the effects of vitamins A and D was performed using both Bactec and MGIT systems. The Bactec data are presented here, as previously,[18–20,23–25,30] in graph form (cGI = cumulative growth index) (A); data in tabular form are given in Table 11. The MGIT data are presented in two ways: the manufacturer determined 'growth unit' was combined, arbitrarily, for the first 16 days of the experiment and is presented in 'cumulative growth units' (cGU) (B); alternatively, the data are presented as recommended by the manufacturer in 'days to positivity' (C). The inhibitory control was monensin and the non-inhibitory control was phthalimide.

Table 1
Inhibitory control: *para*-aminosalicylic acid (PAS)[a]

	M. tuberculosis (ATCC 25177)			BCG (ATCC 19015)					
µg/ml	NaOH	NaOH	Water	NaOH	NaOH	NaOH	7H9	NaOH	Water
1	−95%	−95%	−98%	−72%	−96%	−81%	−18%	−29%	−86%
4	−96%	−98%	−99%	−98%	−98%	−94%	−94%	−83%	−99%
16	−97%	−98%	−99%	−98%	−98%	−96%	−97%	−93%	−99%
64	−97%	−98%	−99%	−98%	−98%	−97%	−98%	−94%	−99%

BCG, Bacillus Calmette–Guérin.
[a] PAS was the first mass-produced anti-tuberculosis antibiotic and was used here as an inhibitory control. The dissolving solution is indicated for each experiment. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 2
Inhibitory control: isoniazid[a]

	M. tuberculosis		BCG		
µg/ml	NaOH	Water	NaOH	7H9	Water
1	−99%	−99%	−99%	−2%	−62%
4	−98%	−99%	−98%	32%	−25%
16	−98%	−99%	−99%	3%	−63%
64	−98%	−99%	−98%	−98%	−99%

BCG, bacillus Calmette–Guérin.
[a] Isoniazid is an acknowledged anti-tuberculosis antibiotic. Isoniazid was equally inhibitory against *M. tuberculosis* whether dissolved in NaOH or water. Efficacy against BCG was best observed when it was dissolved in NaOH. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 3
Non-inhibitory control: sulfasalazine (intact sulfasalazine: sulfapyridine/5-ASA)[a]

µg/ml	*M. tuberculosis* (ATCC 25177)		BCG (ATCC 19015)			
1	−31%	−11%	−4%	8%	−54%	46%
4	−7%	18%	4%	10%	−41%	51%
16	−8%	62%	6%	25%	−25%	100%
64	−8%	31%	55%	24%	27%	295%

5-ASA, 5-aminosalicylic acid; BCG, bacillus Calmette–Guérin.
[a] The non-inhibitory control was sulfasalazine, the intact combination of sulfapyridine and 5-ASA. NaOH was used to dissolve in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 4
Inhibition of sulfapyridine ± 5-ASA[a]

	Sulfapyridine				Sulfapyridine + 5ASA	
µg/ml	*M. tuberculosis*	BCG				
1	9%	7%	9%	7%	9%	7%
4	−6%	−13%	−6%	−13%	−6%	−13%
16	−13%	−90%	−13%	−90%	−13%	−90%
64	−63%	−99%	−63%	−99%	−63%	−99%

5-ASA, 5-aminosalicylic acid; BCG, bacillus Calmette–Guérin.
[a] Sulfapyridine was studied alone or with separate and equal weight of 5-ASA. There was dose-dependent inhibition of both *M. tuberculosis* and BCG, only by sulfapyridine. 5-ASA had no effect. NaOH was used to dissolve both agents in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 5
The effect of 5-ASA on *Mycobacterium tuberculosis*, BCG, and MAP[a]

µg/ml	M. tuberculosis		BCG				MAP
1	−42%	−24%	−16%	26%	−26%	3%	−19%
4	−38%	−13%	−7%	4%	−31%	19%	−18%
16	−56%	−10%	−9%	13%	−35%	21%	−28%
64	−24%	−4%	47%	6%	68%	149%	−45%

5-ASA, 5-aminosalicylic acid; BCG, bacillus Calmette–Guérin; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.
[a] 5-ASA was studied alone in comparing *M. tuberculosis*, BCG, and MAP (UCF-4). There was no dose-dependent inhibition observed with the *M. tuberculosis* or BCG. In contrast, the previously reported,[23] subtle, bacteriostatic, dose-dependent inhibition with MAP was reproduced here. NaOH was used to dissolve in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

inhibition of 5-ASA on MAP,[23] that we replicated here with MAP UCF-4 (Table 5, right-hand column).

The agricultural growth enhancer monensin caused dose-dependent inhibition of MAP, some strains of *M. avium*, and BCG.[30] We replicated our data with BCG[30] here and found, for the first time, profound monensin dose-dependent inhibition on *M. tuberculosis* (99%−ΔcGI starting at 4 µg/ml; Table 6).

Table 6
Monensin, a growth enhancer, inhibits both *Mycobacterium tuberculosis* and BCG[a]

μg/ml	M. tuberculosis			BCG			
1	−28%	−96%	−74%	−25%	−44%	−29%	−28%
4	−36%	−99%	−98%	−77%	−69%	−66%	−54%
16	−79%	−99%	−99%	−87%	−92%	−84%	−73%
64	−99%	−99%	−99%	−97%	−98%	−95%	−91%

BCG, bacillus Calmette–Guérin; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.

[a] The veterinarian profession calls monensin a 'growth enhancer' because cows treated with it gain weight and produce more milk. We consistently showed dose-dependent monensin inhibition with MAP, with *Mycobacterium avium* subspecies *avium* ATCC 25291, and here with the *M. tuberculosis* complex. Dimethyl sulfoxide (DMSO) was used to dissolve in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

The thiopurine immune modulator 6-MP causes profound and reproducible dose-dependent inhibition of MAP.[17,21,22] In this study we replicated our observations on MAP[17] with MAP UCF-4 that had been isolated from a patient with Crohn's disease (67%−ΔcGI at 1 μg/ml; right-hand column, Table 7) and found a profound inhibition of the *M. tuberculosis* complex, more pronounced with *M. tuberculosis* (94%−ΔcGI by 4 μg/ml; Table 7).

The immunosuppressant methotrexate causes dose-dependent inhibition of MAP.[17] We studied four additional MAP strains, two of which ('Ben' and 'Linda') had been isolated from humans with Crohn's disease (Table 8; four right-hand columns). With 'Linda', inhibition was 87%−ΔcGI at 4 μg/ml (Table 8). Methotrexate showed dose-dependent inhibition on the *M. tuberculosis* complex. This was more pronounced on BCG (≥87%−ΔcGI at 4 μg/ml) than on *M. tuberculosis* (97%−ΔcGI at 64 μg/ml; Table 8).

Table 7
6-Mercaptopurine (6-MP), an immune modulator, inhibits mycobacterial growth[a]

μg/ml	M. tuberculosis		BCG			MAP
1	−58%	−44%	−2%	−66%	−43%	−67%
4	−94%	−70%	−52%	−74%	−61%	−87%
16	−99%	−97%	−80%	−92%	−81%	−95%
64	−99%	−98%	−98%	−96%	−92%	−98%

BCG, bacillus Calmette–Guérin; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.

[a] The medical profession calls 6-MP an 'immune modulator'. We replicated 6-MP dose-dependent inhibition of MAP and *M. avium* subspecies *avium*. We now reproduced the MAP inhibition using MAP UCF-4. We additionally showed marked dose-dependent inhibition on the *M. tuberculosis* complex. Inhibition was more pronounced on *M. tuberculosis* than on BCG. NaOH was used to dissolve in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 8
Methotrexate, an 'immune suppressant', inhibits MAP and BCG more than *M. tuberculosis*[a]

	M. tuberculosis	BCG		MAP			
				Human isolates		Bovine isolates	
μg/ml				'Ben'	'Linda'	700535	303
1	−13%	−60%	18%	−4%	−41%	6%	−10%
4	−19%	−95%	−87%	−73%	−87%	−67%	−82%
16	−23%	−97%	−95%	−96%	−99%	−99%	−98%
64	−97%	−96%	−94%	−96%	−99%	−99%	−99%

BCG, bacillus Calmette–Guérin; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.

[a] The medical profession calls methotrexate an 'immune suppressant'. Methotrexate inhibits MAP. Here we reproduced that observation in four species of MAP, two of which were isolated from patients with Crohn's disease. Dose-dependent inhibition was most pronounced with BCG and MAP, less so with *M. tuberculosis*. NaOH was used to dissolve in all experiments. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Clofazimine is an acknowledged anti-mycobacterial antibiotic used routinely to treat leprosy,[35] which inhibits MAP in culture[18,19,36] and has been used in trials on putative MAP gastrointestinal infections.[37–39] Clofazimine was profoundly inhibitory against the *M. tuberculosis* complex (>99%−ΔcGI at 1 μg/ml for both *M. tuberculosis* and BCG, the lowest dose we tested; Table 9). The immune suppressants cyclosporine A, rapamycin, and tacrolimus inhibit MAP in culture.[18] Cyclosporine A was more inhibitory against *M. tuberculosis* (96%−ΔcGI at 16 μg/ml; Table 9) than BCG (85%−ΔcGI at 64 μg/ml; Table 9). Rapamycin inhibited *M. tuberculosis* (85%−ΔcGI at 16 μg/ml; Table 9) but not BCG (no dose-dependent inhibition; Table 9). Tacrolimus was the least effective macrolide antibiotic immune suppressant we studied. It inhibited *M. tuberculosis* (99%−ΔcGI at 64 μg/ml; Table 9) but had no inhibition on BCG (Table 9).

Vitamins A[24] and D[24,26] inhibit mycobacteria in culture. We replicated our data[24] using *M. tuberculosis*. Both retinoic acid (85%−ΔcGI at 4 μg/ml; Table 10) and retinol (84%−ΔcGI at 16 μg/ml; Table 10) were more inhibitory than vitamin D_3 (46%−ΔcGI at 64 μg/ml; Table 10).

A comparison of the Bactec and MGIT systems using *M. tuberculosis* was performed with vitamin A, its metabolite retinoic acid, and vitamin D. The previously reported inhibition[24] was replicated and was found to be more pronounced with vitamin A and retinoic acid than with vitamin D and was more elegantly seen using the Bactec system (Figure 1 and Tables 11 and 12).

4. Discussion

In this study we clearly showed the dose-dependent inhibition of the *M. tuberculosis* complex by agents conventionally called immune suppressants, immune modulators, and growth enhancers, and vitamins A and D, in part reproducing previous publications by ourselves and others. The most obvious concern about any inferences that could be drawn from this study is that pathogenic *M. tuberculosis* strains were not evaluated. Our unfunded laboratory is approved for biosafety level 2 experiments; hence our study was limited to two biosafety level 2 representatives of the *M. tuberculosis* complex. The results presented cannot be ascribed to a simple pH effect for two reasons. First the experimental control always contained the same exact concentration of the dissolving solution as did each vial, irrespective of the amount of agent being tested. Second, because of buffering, in the final incubation vial, the pH was always within the manufacturer's recommended range of 6.6 ± 0.2 (data not presented).

The experimental inhibitory controls used in this study were PAS[40] and isoniazid,[41] both acknowledged anti-tuberculosis antibiotics. The non-inhibitory control was the intact molecule of sulfasalazine comprising two molecules, sulfapyridine and 5-ASA.[16] Neither intact sulfasalazine nor 5-ASA inhibited the *M. tuberculosis* complex, although we reproduced our subtle bacteriostatic dose-dependent 5-ASA inhibition of MAP (Tables 3 and 5 and Greenstein et al.[23]). In contrast, sulfapyridine showed dose-dependent inhibition, more pronounced with BCG than *M. tuberculosis*. From our inhibition curves, we conclude that sulfapyridine is not likely to be clinically useful in the therapy of MDR tuberculosis and 5-ASA has no potential role.

Clofazimine is known to inhibit *M. tuberculosis* in culture.[42–47] These data are corroborated in this study where it was found to cause dose-dependent inhibition of both of our *M. tuberculosis* complex strains. Clofazimine is used to treat leprosy[35] and putative MAP infections of the gastrointestinal tract.[37,38] Clofazimine is already used in combination to treat MDR tuberculosis[48–50] (see Dooley et al.[1], Gopal et al.[51], Dey et al.[52] and Cholo et al.[53] for reviews). We conclude that the preexisting clinical use of clofazimine, coupled with our culture inhibition data, justify

Table 9
Clofazimine (an anti-leprosy antibiotic) and the immune suppressants cyclosporine A, rapamycin, and tacrolimus inhibit mycobacterial growth[a]

	Clofazimine		Cyclosporine A		Rapamycin		Tacrolimus	
μg/ml	*M. tuberculosis*	BCG	*M. tuberculosis*	BCG	*M. tuberculosis*	BCG	*M. tuberculosis*	BCG
1	−99%	−99%	8%	30%	3%	11%	11%	3%
4	−99%	−98%	−11%	4%	−28%	15%	−6%	12%
16	−99%	−98%	−96%	−12%	−85%	0%	−13%	−5%
64	−99%	−99%	−99%	−85%	−99%	−28%	−99%	−11%

BCG, bacillus Calmette–Guérin; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.
[a] Comparison of clofazimine and the immune suppressants. Clofazimine is an anti-leprosy antibiotic and is used to treat human MAP infections. Clofazimine shows bactericidal inhibition of MAP in culture[42–47] and is used to treat tuberculosis.[51,52] Here we found pronounced inhibition of the *M. tuberculosis* complex. The immune suppressants cyclosporine A, rapamycin, and tacrolimus are used at high dose to prevent transplanted organ rejection, and at lower doses in inflammatory diseases. Cyclosporine A was more active against *M. tuberculosis* than against MAP. Rapamycin and tacrolimus are both from the macrolide family of antibiotics. Rapamycin showed greater inhibition against *M. tuberculosis* than did tacrolimus. All agents were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 3.2%. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 10
Mycobacterium tuberculosis is inhibited by vitamins A and D[a]

μg/ml	Monensin	Phthalimide	Retinol	Retinoic acid	Cholecalciferol
1	−28%	2%	−5%	−44%	−9%
4	−36%	13%	−33%	−85%	−13%
16	−79%	−5%	−84%	−98%	−39%
64	−99%	−36%	−99%	−99%	−46%

[a] We have previously shown dose-dependent inhibition of MAP and *Mycobacterium avium* by vitamins A and D. We here replicated that inhibition with vitamin A, retinoic acid, and vitamin D. The metabolite retinoic acid was more inhibitory than vitamin A; both were consistently more inhibitory than vitamin D. Inhibition data are presented as %−ΔcGI (see Methods for calculation).

Table 11
Comparison of Bactec and MGIT for monensin (inhibitory control) and phthalimide (non-inhibitory control)[a]

	Bactec	MGIT	
Measurement	%−ΔcGI	%−ΔcGU	Days to positivity
Monensin			
1 μg/ml	−28%	6%	6
4 μg/ml	−36%	−27%	6
16 μg/ml	−79%	−51%	7
64 μg/ml	−99%	−70%	9
Phthalimide			
1 μg/ml	2%	7%	6
4 μg/ml	13%	16%	6
16 μg/ml	−5%	22%	6
64 μg/ml	−36%	−1%	6

[a] Comparison of Bactec and MGIT for inhibitory and non-inhibitory controls: we compared the manner in which Bactec and MGIT data are presented. The experiment was set up on the same day, from the same inoculum culture. Bactec inhibition data are presented as %−ΔcGI (see Methods for calculation) and the MGIT data are expressed as %−ΔcGU, calculated in the same manner as the Bactec data (see Methods). The conventional manner in which MGIT data are expressed is 'days to positivity' (right hand column).

Table 12
Comparison of Bactec and MGIT: retinol (vitamin A), retinoic acid (vitamin A metabolite), and cholecalciferol (Vitamin D_3)[a]

	Bactec	MGIT	
Measurement	%−ΔcGI	%−ΔcGU	Days to positivity
Retinol			
1 μg/ml	−5%	14%	6
4 μg/ml	−33%	35%	6
16 μg/ml	−84%	−31%	8
64 μg/ml	−99%	−100%	No growth
Retinoic acid			
1 μg/ml	−44%	25%	6
4 μg/ml	−85%	−38%	7
16 μg/ml	−98%	−100%	No growth
64 μg/ml	−99%	−100%	No growth
Cholecalciferol			
1 μg/ml	−9%	31%	6
4 μg/ml	−13%	2%	7
16 μg/ml	−39%	−94%	11
64 μg/ml	−46%	−100%	No growth

[a] Comparison of Bactec and MGIT for vitamins A and D. We compared the manner in which Bactec and MGIT data are presented. The experiment was set up on the same day, from the same inoculum culture. Bactec inhibition data are presented as %−ΔcGI (see Methods for calculation) and the MGIT data are expressed as %−ΔcGU, calculated in the same manner as the Bactec data (see Methods). The conventional manner in which MGIT data are expressed is 'days to positivity' (right hand column). 'No growth' is to day 61, when the experiment was terminated. See also Figure 1.

additional consideration of other inhibitory agents discussed in this manuscript.

Others, using a less sensitive growth detection method, have failed to show inhibition of virulent *M. tuberculosis* by methotrexate.[9] Against BCG, we found consistent inhibition at doses comparable to doses used in clinically significant putative human MAP infections (Table 8 and Greenstein et al.[17]). However, against our avirulent *M. tuberculosis* strain, higher doses of methotrexate were necessary to inhibit growth in culture. We conclude that our data do not justify considering methotrexate clinical application in humans; merely further culture inhibition studies on virulent *M. tuberculosis*.

In contrast to methotrexate, the thioguanine immune modulator 6-MP was found to be more inhibitory against *M. tuberculosis* than BCG. Furthermore the inhibitory potency found in culture was equal to 6-MP dosages used clinically in putative human MAP infections (Table 7 and Greenstein et al.,[17] Shin and Collins,[21] and Krishnan et al.[22]). We conclude that 6-MP is more likely than methotrexate to be of use in treating virulent *M. tuberculosis*.

The immune suppressants cyclosporine A[54] (a cyclic undecapeptide), rapamycin[55] and tacrolimus[56] (both from the macrolide family of antibiotics), are used most conventionally in the prevention of organ transplant rejection.[57] In addition, they are used, always at lower doses (see Table 7 in Greenstein et al.[18]), in the therapy of several autoimmune diseases, including those we suggest may be caused by MAP.[58] All three of these immune suppressants cause dose-dependent inhibition of MAP in culture.[18] In the current study we found that these immune suppressant agents also inhibited *M. tuberculosis* in culture. From our data (Table 9), we conclude that of the four agents tested, clofazimine is the most potent and potentially clinically useful agent and that cyclosporine A and rapamycin are potentially of more use as anti-tuberculosis agents than tacrolimus.

Monensin[59] is called a growth enhancer by the agricultural community, because cows fed it gain weight and produce more milk.[60,61] In addition to being a cocciomycotic in poultry,[62] monensin decreases fecal MAP shedding in infected cows.[63] Of interest to this manuscript, monensin is an inhibitor of MAP,[30,64] *M. avium*, and BCG in culture.[30] We have now demonstrated its inhibition of *M. tuberculosis* (Table 6) with greater potency than observed with BCG.[30]

To our knowledge there is no accepted safety profile of monensin for human use. Safety concerns mainly address whether or not residual monensin could be detected in food from animals exposed to monensin,[65–68] or the effect of monensin on human cells in culture.[69–73] In a solitary case report, a young agricultural employee, naively conceptualizing that the 'growth enhancer' acted as an anabolic steroid, took three times the pro-rated lethal dose for cattle, developed fatal rhabdomyolysis, renal and cardiac failure.[74] Monensin is used extensively and safely in ruminants[60,75] and poultry.[76] In the current study we found it to be a potent anti-tuberculosis agent that may be of use in humans. We conclude that studies of monensin for human safety and efficacy in MDR tuberculosis may be indicated.

Vitamin D utility in treating human tuberculosis[77–79] and animals infected with MAP[80,81] is conventionally ascribed to vitamin D enhancing the immune response of the infected host.[82] Direct vitamin D inhibition of *M. tuberculosis* was first documented in 1948.[26] In 2012 we observed,[24] and replicated herein, vitamin A as well as D_3 inhibition of MAP, *M. avium*, and the *M. tuberculosis* complex. We infer there is a direct vitamin A and D mycobacterial prokaryotic inhibition that is synergistic with eukaryotic immune system enhancement. We conclude adequate nutrition should be emphasized when treating mycobacterial diseases.

With the withdrawal of support for the Bactec 460 system by its manufacturer (Becton Dickinson), mycobacterial investigators require other sensitive methods of documenting mycobacterial bacteriostatic, in addition to bactericidal, effects. We compared the radiometric Bactec 460 with the fluorometric MGIT 960 system (Tables 11 and 12 and Figure 1). We have presented our method of analyzing the MGIT data using the computer-generated growth unit provided by the MGIT system. Our analyses (Tables 11 and 12 and Figure 1) indicate alternative ways of analyzing MGIT data that may be of use to investigators when studying mycobacteria in culture.

This manuscript contains only unpublished data. We have shown that the thioamides, thiourea, and methimazole inhibit MAP and *M. avium*, but not the *M. tuberculosis* complex in culture.[20] It is of considerable interest that in those experiments a control, 5-propothiouricil, markedly inhibited *M. tuberculosis* in culture (97%−ΔcGI at 16 μg/ml; Table 9 in Greenstein et al.[20]). We conclude that 5-propothiouricil should be included if the thioamide family is evaluated for potential therapy of tuberculosis.

In summary, we have presented data justifying the evaluation of the inhibition of virulent *M. tuberculosis* by multiple pharmaceuticals, known as immune modulators and immune suppressants, a ruminant growth enhancer, and vitamins A and D, as potential novel therapeutic agents for tuberculosis in humans.

Conflict of interest: Dr Greenstein has submitted patents based on his published work in this field. Patents issued: (1) US Patent # 7,846,420: issue date December 7, 2010. *Mycobacterium Avium* Subspecies *Paratuberculosis* Vaccines and Methods for Using the Same. US Continuation-in-part Application entitled "Combination Vaccines Against Mycobacterium Species and Methods of Using Same." Serial # 12/956,064: Filing Date: November 30, 2010. Issued: June 18, 2013. (2) US Patent # 7,902,350: issue date March 8, 2011. Method for Monitoring the Efficacy of a *Mycobacterium Avium* Subspecies *Paratuberculosis* Therapy. (3) US Patent # 8,507,251: issue date August 13, 2013 "Medium and Method for Culturing *Mycobacterium Avium* Subspecies *Paratuberculosis*" Serial # 12/892,039: Filing Date September 28, 2010. Patent submitted: Serial # 12/119,657: Filing Date: May 13, 2008: Methods for Diagnosing and Treating a *Mycobacterium avium* Subspecies *paratuberculosis* Infection. Dr Sheldon T. Brown has the following potential conflict of interest: STB was a member of the National Academy of Sciences of the USA panel that issued the report "Diagnosis and control of Johne's disease." None of the other authors reports any potential conflict of interest.

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References

1. Dooley KE, Obuku EA, Durakovic N, Belitsky V, Mitnick C, Nuermberger EL. World Health Organization group 5 drugs for the treatment of drug-resistant tuberculosis: unclear efficacy or untapped potential? *J Infect Dis* 2013;**207**:1352–8.
2. Klopper M, Warren RM, Hayes C, Gey van Pittius NC, Streicher EM, Muller B, et al. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. *Emerg Infect Dis* 2013;**19**:449–55.
3. Keshavjee S, Farmer PE. Tuberculosis, drug resistance, and the history of modern medicine. *N Engl J Med* 2012;**367**:931–6.
4. Chang KC, Yew WW, Tam CM, Leung CC. WHO Group 5 drugs and difficult multidrug-resistant tuberculosis: a systematic review with cohort analysis and meta-analysis. *Antimicrob Agents Chemother* 2013;**57**:4097–104.
5. Rex JH, Eisenstein BI, Alder J, Goldberger M, Meyer R, Dane A, et al. A comprehensive regulatory framework to address the unmet need for new antibacterial treatments. *Lancet Infect Dis* 2013;**13**:269–75.
6. Shlaes DM, Sahm D, Opiela C, Spellberg B, Commentary:. The FDA reboot of antibiotic development. *Antimicrob Agents Chemother* 2013;**57**:4605–7.
7. Spellberg B, Bartlett JG, Gilbert DN. The future of antibiotics and resistance. *NEngl J Med* 2013;**368**:299–302.
8. Cooper MA, Shlaes D. Fix the antibiotics pipeline. *Nature* 2011;**472**:32.
9. Rouse D. Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF). Research Triangle Park, NC: RTI International; 2006. Available at: http://www.taacf.org (accessed January 2007).
10. Goldman RC, Laughon BE, Reynolds RC, Secrist 3rd JA, Maddry JA, Guie MA, et al. Programs to facilitate tuberculosis drug discovery: the tuberculosis antimicrobial acquisition and coordinating facility. *Infect Disord Drug Targets* 2007;**7**:92–104.
11. Goldman RC, Plumley KV, Laughon BE. The evolution of extensively drug resistant tuberculosis (XDR-TB): history, status and issues for global control. *Infect Disord Drug Targets* 2007;**7**:73–91.
12. Greenstein RJ, Collins MT. Emerging pathogens: is *Mycobacterium avium* subspecies *paratuberculosis* zoonotic? *Lancet* 2004;**364**:396–7.
13. Greenstein RJ, Gillis TP, Scollard DS, Brown ST, Mycobacteria:. Leprosy, a battle turned; tuberculosis, a battle raging; paratuberculosis, a battle ignored. In: Fratamico P, Smith J, Brogden K, editors. *Sequelae and long-term consequences of infectious diseases*.. First ed., Washington DC: ASM Press; 2009. p. 135–68.
14. Greenstein RJ, Brown ST. Genomewide association study of leprosy. *N Engl J Med* 2010;**362**:1447. author reply 1447-8.
15. Greenstein RJ. Is Crohn's disease caused by a Mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis* 2003;**3**:507–14.
16. Svartz N. Salazopyrin, a new sulfanilamide preparation. A. Therapeutic results in rheumatic polyarthritis. B. Therapeutic results in ulcerative colitis. C. Toxic manifestations in treatment with sulfanilamide preparations. *Acta Med Scand* 1942;**110**:577–98.
17. Greenstein RJ, Su L, Haroutunian V, Shahidi A, Brown ST. On the action of methotrexate and 6-mercaptopurine on *M. avium* subspecies *paratuberculosis*. *PLoS One* 2007;**2**:e161.
18. Greenstein RJ, Su L, Juste RA, Brown ST. On the action of cyclosporine A, rapamycin and tacrolimus on *M. avium* including subspecies *paratuberculosis*. *PLoS One* 2008;**3**:e2496.
19. Greenstein RJ, Su L, Brown ST. On the effect of thalidomide on *Mycobacterium avium* subspecies *paratuberculosis* in culture. *Int J Infect Dis* 2009;**13**:e254–63.
20. Greenstein RJ, Su L, Brown ST. The thioamides methimazole and thiourea inhibit growth of *M. avium* subspecies *paratuberculosis* in culture. *PLoS One* 2010;**5**:e11099.
21. Shin SJ, Collins MT. Thiopurine drugs (azathioprine and 6-mercaptopurine) inhibit *Mycobacterium paratuberculosis* growth in vitro. *Antimicrob Agents Chemother* 2008;**52**:418–26.
22. Krishnan MY, Manning EJ, Collins MT. Effects of interactions of antibacterial drugs with each other and with 6-mercaptopurine on in vitro growth of *Mycobacterium avium* subspecies *paratuberculosis*. *J Antimicrob Chemother* 2009;**64**:1018–23.
23. Greenstein RJ, Su L, Shahidi A, Brown ST. On the action of 5-amino-salicylic acid and sulfapyridine on *M. avium* including subspecies *paratuberculosis*. *PLoS One* 2007;**2**:e516.
24. Greenstein RJ, Su L, Brown ST, Vitamins A. and D inhibit the growth of mycobacteria in radiometric culture. *PLoS One* 2012;**7**:e29631.
25. Greenstein RJ, Su L, Brown SL. Growth of *M. avium* subspecies *paratuberculosis* in culture is enhanced by nicotinic acid, nicotinamide, and α and β nicotinamide adenine dinucleotide. *Dig Dis Sci* 2010;**56**:368–75.
26. Raab W, Vitamin D. its bactericidal action. *Diseases of the Chest* 1946;**12**:409–15.

27. Zheng H, Lu L, Wang B, Pu S, Zhang X, Zhu G, et al. Genetic basis of virulence attenuation revealed by comparative genomic analysis of *Mycobacterium tuberculosis* strain H37Ra versus H37Rv. *PLoS One* 2008;**3**:e2375.
28. Chiodini RJ, Van Kruiningen HJ, Thayer WR, Coutu JA. Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J Clin Microbiol* 1986;**24**:357–63.
29. Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 2004;**364**:1039–44.
30. Greenstein RJ, Su L, Whitlock RH, Brown ST. Monensin causes dose dependent inhibition of *Mycobacterium avium* subspecies *paratuberculosis* in radiometric culture. *Gut Pathog* 2009;**1**:4.
31. Siddiqi SH, Libonati JP, Middlebrook G. Evaluation of rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1981;**13**:908–12.
32. Rusch-Gerdes S, Pfyffer GE, Casal M, Chadwick M, Siddiqi S. Multicenter laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of *Mycobacterium tuberculosis* to classical second-line drugs and newer antimicrobials. *J Clin Microbiol* 2006;**44**:688–92.
33. Krishnan MY, Manning EJ, Collins MT. Comparison of three methods for susceptibility testing of *Mycobacterium avium* subsp. *paratuberculosis* to 11 antimicrobial drugs. *J Antimicrob Chemother* 2009;**64**:310–6.
34. Damato JJ, Collins MT. Growth of *Mycobacterium paratuberculosis* in radiometric, Middlebrook and egg-based media. *Vet Microbiol* 1990;**22**:31–42.
35. Britton WJ, Lockwood DN. Leprosy. *Lancet* 2004;**363**:1209–19.
36. Rastogi N, Goh KS, Labrousse V. Activity of clarithromycin compared with those of other drugs against *Mycobacterium paratuberculosis* and further enhancement of its extracellular and intracellular activities by ethambutol. *Antimicrob Agents Chemother* 1992;**36**:2843–6.
37. Borody TJ, Bilkey S, Wettstein AR, Leis S, Pang G, Tye S. Anti-mycobacterial therapy in Crohn's disease heals mucosa with longitudinal scars. *Dig Liver Dis* 2007;**39**:438–44.
38. Borody TJ, Leis S, Warren EF, Surace R. Treatment of severe Crohn's disease using antimycobacterial triple therapy—approaching a cure? *Dig Liver Dis* 2002;**34**:29–38.
39. Selby W, Pavli P, Crotty B, Florin T, Radford-Smith G, Gibson P, et al. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology* 2007;**132**:2313–9.
40. Lehmann J. Para-aminosalicylic acid in the treatment of tuberculosis. *Lancet* 1946;**1**:15–6.
41. Noufflard H, Deslandes M. [Bactericidal action of isoniazid (isonicotinic acid hydrazide) on *Mycobacterium tuberculosis*]. *Ann Inst Pasteur (Paris)* 1952;**83**:769–73.
42. Abate G, Miorner H, Ahmed O, Hoffner SE. Drug resistance in *Mycobacterium tuberculosis* strains isolated from re-treatment cases of pulmonary tuberculosis in Ethiopia: susceptibility to first-line and alternative drugs. *Int J Tuberc Lung Dis* 1998;**2**:580–4.
43. Peloquin CA. *Mycobacterium avium* complex infection. Pharmacokinetic and pharmacodynamic considerations that may improve clinical outcomes. *Clin Pharmacokinet* 1997;**32**:132–44.
44. Pfyffer GE, Bonato DA, Ebrahimzadeh A, Gross W, Hotaling J, Kornblum J, et al. Multicenter laboratory validation of susceptibility testing of *Mycobacterium tuberculosis* against classical second-line and newer antimicrobial drugs by using the radiometric BACTEC 460 technique and the proportion method with solid media. *J Clin Microbiol* 1999;**37**:3179–86.
45. Rastogi N, Goh KS, Bryskier A, Devallois A. In vitro activities of levofloxacin used alone and in combination with first- and second-line antituberculous drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1996;**40**:1610–6.
46. Rastogi N, Labrousse V, Goh KS. In vitro activities of fourteen antimicrobial agents against drug susceptible and resistant clinical isolates of *Mycobacterium tuberculosis* and comparative intracellular activities against the virulent H37Rv strain in human macrophages. *Curr Microbiol* 1996;**33**:167–75.
47. Satana D, Coban AY, Uzun M. Testing susceptibility of multidrug-resistant *Mycobacterium tuberculosis* to second-line drugs by use of blood agar. *J Clin Microbiol* 2010;**48**:4291–3.
48. Xu HB, Jiang RH, Xiao HP. Clofazimine in the treatment of multidrug-resistant tuberculosis. *Clin Microbiol Infect* 2012;**18**:1104–10.
49. Fortun J, Martin-Davila P, Navas E, Perez-Elias MJ, Cobo J, Tato M, et al. Linezolid for the treatment of multidrug-resistant tuberculosis. *J Antimicrob Chemother* 2005;**56**:180–5.
50. Schecter GF, Scott C, True L, Raftery A, Flood J, Mase S. Linezolid in the treatment of multidrug-resistant tuberculosis. *Clin Infect Dis* 2010;**50**:49–55.
51. Gopal M, Padayatchi N, Metcalfe JZ, O'Donnell MR. Systematic review of clofazimine for the treatment of drug-resistant tuberculosis. *Int J Tuberc Lung Dis* 2013;**17**:1001–7.
52. Dey T, Brigden G, Cox H, Shubber Z, Cooke G, Ford N. Outcomes of clofazimine for the treatment of drug-resistant tuberculosis: a systematic review and meta-analysis. *J Antimicrob Chemother* 2013;**68**:284–93.
53. Cholo MC, Steel HC, Fourie PB, Germishuizen WA, Anderson R, Clofazimine:. current status and future prospects. *J Antimicrob Chemother* 2012;**67**:290–8.
54. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* 1976;**6**:468–75.
55. Vezina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot (Tokyo)* 1975;**28**:721–6.
56. Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, et al. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. *J Antibiot (Tokyo)* 1987;**40**:1249–55.
57. Allison AC. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology* 2000;**47**:63–83.
58. Lichtiger S, Present DH, Kornbluth A, Gelernt I, Bauer J, Galler G, et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 1994;**330**:1841–5.
59. Haney Jr ME, Hoehn MM. Monensin, a new biologically active compound. I. Discovery and isolation. *Antimicrob Agents Chemother (Bethesda)* 1967;**7**:349–52.
60. Melendez P, Goff JP, Risco CA, Archbald LF, Littell R, Donovan GA. Pre-partum monensin supplementation improves body reserves at calving and milk yield in Holstein cows dried-off with low body condition score. *Res Vet Sci* 2007;**82**:349–57.
61. Zahra LC, Duffield TF, Leslie KE, Overton TR, Putnam D, LeBlanc SJ. Effects of rumen-protected choline and monensin on milk production and metabolism of periparturient dairy cows. *J Dairy Sci* 2006;**89**:4808–18.
62. Shumard RF, Callender ME. Monensin, a new biologically active compound. VI. Anticoccidial activity. *Antimicrob Agents Chemother (Bethesda)* 1967;**7**:369–77.
63. Hendrick SH, Kelton DF, Leslie KE, Lissemore KD, Archambault M, Bagg R, et al. Efficacy of monensin sodium for the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle. *Prev Vet Med* 2006;**75**:206–20.
64. Brumbaugh GW, Simpson RB, Edwards JF, Anders DR, Thomson TD. Susceptibility of *Mycobacterium avium* sbsp *paratuberculosis* to monensin sodium or tilmicosin phosphate in vitro and resulting infectivity in a murine model. *Can J Vet Res* 2004;**68**:175–81.
65. World Health Organization. Evaluation of certain veterinary drug residues in food. World Health Organ Tech Rep Ser 2012;(969):i–vii, 1-101.
66. Bagg R, Vessie GH, Dick CP, Duffield T, Wilson JB, Aramini JJ. Milk residues and performance of lactating dairy cows administered high doses of monensin. *Can J Vet Res* 2005;**69**:180–5.
67. Callaway TR, Edrington TS, Rychlik JL, Genovese KJ, Poole TL, Jung YS, et al. Ionophores: their use as ruminant growth promotants and impact on food safety. *Curr Issues Intest Microbiol* 2003;**4**:43–51.
68. Dorne JL, Fernandez-Cruz ML, Bertelsen U, Renshaw DW, Peltonen K, Anadon A, et al. Risk assessment of coccidostatics during feed cross-contamination: animal and human health aspects. *Toxicol Appl Pharmacol* 2013;**270**:196–208.
69. Biagini C, Bender V, Borde F, Boissel E, Bonnet MC, Masson MT, et al. Cytochrome P450 expression–induction profile and chemically mediated alterations of the WIF-B9 cell line. *Biol Cell* 2006;**98**:23–32.
70. Chatzistavraki M, Kyratzi E, Fotinopoulou A, Papazafiri P, Efthimiopoulos S. Downregulation of AbetaPP enhances both calcium content of endoplasmic reticulum and acidic stores and the dynamics of store operated calcium channel activity. *J Alzheimers Dis* 2013;**34**:407–15.
71. Kazmi F, Hensley T, Pope C, Funk RS, Loewen GJ, Buckley DB, et al. Lysosomal sequestration (trapping) of lipophilic amine (cationic amphiphilic) drugs in immortalized human hepatocytes (Fa2N-4 cells). *Drug Metab Dispos* 2013;**41**:897–905.
72. Parente R, Trifiro E, Cuozzo F, Valia S, Cirone M, Di Renzo L. Cyclooxygenase-2 is induced by p38 MAPK and promotes cell survival. *Oncol Rep* 2013;**29**:1999–2004.
73. Puckeridge M, Chapman BE, Conigrave AD, Grieve SM, Figtree GA, Kuchel PW. Stoichiometric relationship between Na(+) ions transported and glucose consumed in human erythrocytes: Bayesian analysis of (23)Na and (13)C NMR time course data. *Biophys J* 2013;**104**:1676–84.
74. Caldeira C, Neves WS, Cury PM, Serrano P, Baptista MA, Burdmann EA. Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am J Kidney Dis* 2001;**38**:1108–12.
75. Tedeschi LO, Fox DG, Tylutki TP. Potential environmental benefits of ionophores in ruminant diets. *J Environ Qual* 2003;**32**:1591–602.
76. McDougald LR. Anticoccidial action of monensin in turkey poults. *Poult Sci* 1976;**55**:2442–7.
77. Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, et al. High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 2011;**377**:242–50.
78. Wilkinson RJ, Llewelyn M, Toossi Z, Patel P, Pasvol G, Lalvani A, et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case–control study. *Lancet* 2000;**355**:618–21.
79. Coussens AK, Wilkinson RJ, Hanifa Y, Nikolayevskyy V, Elkington PT, Islam K, et al. Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc Natl Acad Sci U S A* 2012;**109**:15449–54.
80. Sorge US, Molitor T, Linn J, Gallaher D, Wells SW. Cow-level association between serum 25-hydroxyvitamin D concentration and *Mycobacterium avium* subspecies *paratuberculosis* antibody seropositivity: a pilot study. *J Dairy Sci* 2013;**96**:1030–7.
81. Stabel JR, Goff JP, Whipple DL, Ackermann MR, Reinhardt TA. Low calcium diet and 1,25-dihydroxyvitamin D(3) infusion modulate immune responses during *Mycobacterium paratuberculosis* infection in beige mice. *Vet Immunol Immunopathol* 1996;**50**:127–43.
82. Bikle DD, Vitamin D. regulation of immune function. *Vitam Horm* 2011;**86**:1–21.