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Anti-HIV natural product (+)-calanolide A is active against both drug-susceptible and drug-resistant strains of *Mycobacterium tuberculosis*

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Abstract—Naturally occurring anti-HIV-1 agent (+)-calanolide A was found to be active against all of the strains of *Mycobacterium tuberculosis* tested, including those resistant to the standard antitubercular drugs. Efficacy evaluations in macrophages revealed that (+)-calanolide A significantly inhibited intracellular replication of *M. tuberculosis* H37Rv at concentrations below the MIC observed in vitro. Preliminary mechanistic studies indicated that (+)-calanolide A rapidly inhibits RNA and DNA synthesis followed by an inhibition of protein synthesis. Compared with known inhibitors, this scenario is more similar to effects observed with rifampin, an inhibitor of RNA synthesis. Since (+)-calanolide A was active against a rifampin-resistant strain, it is believed that these two agents may involve different targets. (+)-Calanolide A and its related pyranocoumarins are the first class of compounds identified to possess antimycobacterial and antiretroviral activities, representing a new pharmacophore for anti-TB activity.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, a facultative intracellular bacillus, is the world's number one infectious pathogen that kills 2–3 million people annually.[1,2] One-third (about 2 billion) of the world population is estimated to be infected with *M. tuberculosis*. Worse still, the emergence of multidrug-resistant (MDR) strains of *M. tuberculosis* and the global human immunodeficiency virus (HIV) pandemic have greatly amplified the incidence of TB, reaffirming tuberculosis as a primary public health threat.[3,4]

Even though Directly Observed Therapy Short-course (DOTS), the WHO recommended program for the treatment of TB is generally successful, treating individuals infected with MDR-TB is very limited and frequently ineffectual.[5] No new classes of anti-TB drugs or drugs with new mechanisms of action have been developed in the past 30 years. Rifapentine, a derivative of rifampin, was approved in late 1998 to treat TB infection.[6] Several promising new anti-TB agents have been reported, such as oxazolidinones[7] and nitroimidazopyrans,[8] and yet the treatment benefits remain to be demonstrated in clinics. Currently, there is no standard optimal anti-TB therapy in AIDS patients and no single agent that is active against infections caused by both HIV and *M. tuberculosis*. It is clear that there is an urgent need for anti-TB drugs with improved properties such as enhanced activity against MDR strains, reduced toxicity, shortened duration of therapy, rapid mycobactericidal mechanism of action and the ability to penetrate host cells and exert antimycobacterial effects in the intracellular environment. Particularly, new agents with activity against both TB and HIV infections are in great demand.

Natural sources have been rich in providing drug leads; in fact, more than 60% of marketed anticancer and antiinfectious agents are of natural origin. In an attempt to identify novel classes of anti-TB agents, marine[9,10] and plant[11,12] natural products have been evaluated. Included among the potential natural product leads reported

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are cyclic depsipeptides, C-19 hydroxy steroids, scalarin sesquiterpenoids, tetrabromo spirocyclohexadienylisoxazolines, flavonoids and chalcones. In our continuing interest in naturally occurring pyranocoumarin compounds and in an attempt to identify agents which would be active against both HIV and TB, we embarked on the evaluation of anti-HIV active pyranocoumarins[13] for their activity against *M. tuberculosis*.

2. Results

2.1. In vitro anti-TB activity and structure–activity relationships (SARs)

Against the H37Rv strain of *M. tuberculosis* in BACTEC 12B medium using the BACTEC 460 radiometric system, (+)-calanolide A demonstrated greater than 96% inhibition at a concentration of 12.5 μg/mL. The minimum inhibitory concentration (MIC), defined as the lowest concentration inhibiting >99% of the bacterial population present at the beginning of the assay, was then determined to be 3.13 μg/mL or 8 μM (Table 1). In addition, (+)-calanolide A was weakly active against *M. avium*, exhibiting 81% inhibition at a concentration of 12.5 μg/mL.

Encouraged by the (+)-calanolide A results, eight other anti-HIV-1 active pyranocoumarin compounds (see Fig. 1 for structures) were also screened for anti-TB activity. Interestingly, compounds **2**–**6** were all determined to be active against the H37Rv strain of *M. tuberculosis*, with MIC values at the same level of (+)-calanolide A, as shown in Table 1. However, compounds **7**–**9** were weakly active, since they only exhibited 43–78% inhibition at a concentration of 12.5 μg/mL.

In order to further determine the scope of anti-TB activity and better understand the mechanism of action of this class of compounds, (+)-calanolide A was evaluated for in vitro activity, using the microdilution Alamar blue assay, against four drug-susceptible strains of *M. tuberculosis* and also strains resistant to either isoniazid, ethambutol, rifampin or streptomycin, The results are shown in Table 2. As can be seen, (+)-calanolide A was consistently active (MIC 8–16 μg/mL) against the drug-susceptible strains of *M. tuberculosis*. More importantly, (+)-calanolide A did not lose activity

Table 1. Activity of pyranocoumarin compounds against strain H37Rv of *M. tuberculosis*

Compound	% Inhibition[a]	MIC[b] (μg/mL)	IC_{50}[c] (μg/mL)	SI[d] (IC_{50}/MIC)
(+)-Calanolide A (**1**)	96	3.13	7.60	2.43
(−)-Calanolide A (**2**)	98	6.25	>10	>1.6
(−)-Calanolide B (**3**)	99	6.25	>10	>1.6
Soulattrolide (**4**)	99	3.13	>10	>3.2
(−)-7,8-Dihydrocalanolide B (**5**)	98	3.13	6.6	2.1
7,8-Dihydrosoulattrolide (**6**)	99	6.25	>10	>1.6
(+)-12-Oxocalanolide A (**7**)	78	>12.5	ND[e]	ND
(±)-**7**	59	>12.5	ND	ND
(±)-7,8-Dihydro-12-Oxocalanolide A (**8**)	43	>12.5	ND	ND
(±)-Calanolide D (**9**)	57	>12.5	ND	ND

[a] Percent inhibition was obtained in BACTEC assays at a test concentration of 12.5 μg/mL.
[b] The MIC was obtained in BACTEC assays and defined as the lowest concentration inhibiting >99% of the bacterial population present at the beginning of the assay.
[c] Cytotoxicity to a VERO cell line.
[d] Selective index was defined as the ratio of the measured IC_{50} in VERO cell line to the MIC.
[e] ND, not determined.

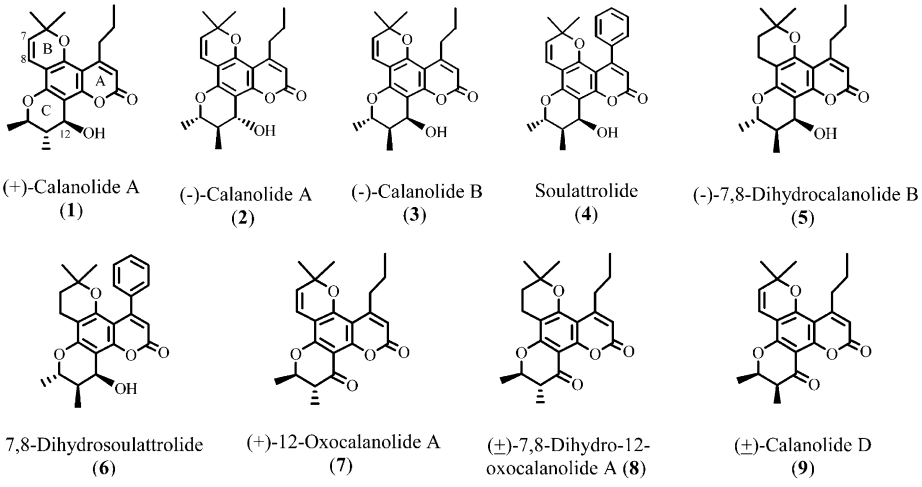

Figure 1. Structures of pyranocoumarin compounds tested against *M. tuberculosis*.

Table 2. Activity of (+)-Calanolide A against drug-susceptible and drug-resistant strains of *Mycobacterium tuberculosis*

Strains of *M. tuberculosis*	MIC (μg/mL) (fold resistance)[a]				
	(+)-Calanolide A	Isoniazid	Rifampin	Streptomycin	Ethambutol
H37Ra (ATCC[c] 25177)	16	0.063	ND[b]	ND[b]	4
H37Rv (ATCC 27294)	8	0.031	0.016	0.25	2
CSU[d] 19	16	0.031	0.016	0.25	2
CSU 33	16	0.031	0.008	0.25	4
H37Rv-INH-R (ATCC 35822)	8 (1)	>128 (>4100)	0.031 (2)	0.25 (1)	8 (4)
CSU 36	8 (1)	0.031 (1)	64 (4000)	0.25 (1)	2 (1)
CSU 38	8 (1)	0.031 (1)	0.016 (1)	>128 (>512)	2 (1)
H37Rv-EMB-R (ATCC 35837)	8 (1)	0.25 (8)	0.031 (2)	0.25 (1)	64 (32)

[a] Fold resistance was the ratio between the MIC value against individual strain and that against H37Rv strain.
[b] ND- not determined.
[c] ATCC- American Type Culture Collection.
[d] CSU- Colorado State University.

toward strains of *M. tuberculosis* which are resistant to isoniazid, rifampin, streptomycin and ethambutol, respectively.

Even though the number of compounds tested here is limited, a few key features regarding structural requirements for these pyranocoumarins to exert their anti-TB activity may be determined. First of all, the 12-OH group appears to be the single most important structural element, since compounds with such a group as in **2**–**6** are active, while those without, as in **7**–**9**, which all possess a carbonyl group at the 12-position, are inactive. In contrast to the anti-HIV activity,[13] however, the stereochemistry of the 12-OH group in these pyranocoumarins may not be critical for anti-TB activity because (−)-calanolide A (**2**), possessing the 12-OH group in the α position, was as active against TB as (+)-calanolide A (**1**) and compounds **3**–**6**, which all have a 12-OH group in the β position. Furthermore, it is apparent that the coumarin (Ring A, see the ring designation in Fig. 1) and chromene (Ring B) rings are flexible to modifications. For example, either replacement of the *n*-propyl group in **3** and **5** with a phenyl, resulting in **4** and **6**, or saturation of the 7,8-double bond in Ring B of **3** and **4**, leading to **5** and **6**, still maintains the anti-TB activity. However, the necessity of methyl groups in the chroman ring (Ring C) is not clear.

2.2. Intracellular anti-TB efficacy of (+)-calanolide A in monocytes

The J774 murine macrophage cell line, analogous to human alveolar macrophages, and the Mono Mac 6 (MM6) human acute monocytic leukemia cell line, the only human cell line to constitutively express phenotypic and functional features of mature monocytes, have been consistently used for evaluation of antimycobacterial drugs.[14,15] Consequently, the intracellular anti-TB efficacy of (+)-calanolide A was evaluated in both J774 and MM6 cell lines.

Prior to intracellular efficacy studies, the LD_{50} of (+)-calanolide A was determined to be 3.50 and 2.42 μg/mL for the J774 murine and MM6 human monocytic cell lines, respectively. It should be noted that these values are not used to evaluate toxicity of the test compound for eventual administration to mice or humans. Determination of the LD_{50} allows the assay to be set up within a concentration range that will give reliable data for that particular cell line. To conduct the evaluation properly, it is important to maintain cell viability $\geq$75% of nontreated cells. Loss of viability greater than that reduces the reliability of the results.

For the intracellular efficacy in the J774 cell line, the drug was evaluated at 0.5, 1.0, 1.5, 2.0 and 3.13 μg/mL. The results revealed that (+)-calanolide A was able to reduce intracellular replication of *M. tuberculosis* H37Rv at all the concentrations tested (Fig. 2A). This reduction was significant at the concentrations of 1.5 and 2.0 μg/mL ($P<0.001$), which were 4–5-fold below the MIC as described in Table 2. By comparison, rifampin (positive control) was able to significantly reduce intracellular replication of *M. tuberculosis* H37Rv at 0.25 and 2.0 μg/mL (Fig. 2B), which were concentrations equivalent to the MIC and 8-fold above the MIC, respectively. Rifampin was not able to significantly reduce intracellular replication of *M. tuberculosis* H37Rv at a concentration 8-fold below its MIC (i.e., 0.03 μg/mL) (Fig. 2B). The MTT cytotoxicity assay was performed on each set to determine the percent reduction in viable cells by the end of the 7-day evaluation. The percent reduction for 0.5, 1.0, 1.5 and 2.0 μg/mL concentrations of (+)-calanolide A was 22, 20, 9 and 15%, respectively. Thus, cell viability remained well above 75% at termination of the experiment. By contrast, the 3.13 μg/mL concentration of (+)-calanolide A resulted in 74% reduction in viability. Even though there was a significant reduction in *M. tuberculosis* H37Rv at that concentration, the results were not considered valid because viability was not $\geq$75% and were not included in Figure 2A for analysis.

In the MM6 cell line, the drug was evaluated at 0.5, 1.0 and 1.5 μg/mL, all of which were below the LD_{50} of 2.42 μg/mL. Results revealed that (+)-calanolide A was effective in significantly reducing intracellular replication of *M. tuberculosis* H37Rv at 1.0 μg/mL (Fig. 3A), which was 8-fold below its MIC (Table 2). By comparison, rifampin was able to significantly reduce intracellular replication of *M. tuberculosis* H37Rv at the MIC and 8-fold above MIC (Fig. 3B). As was the case with

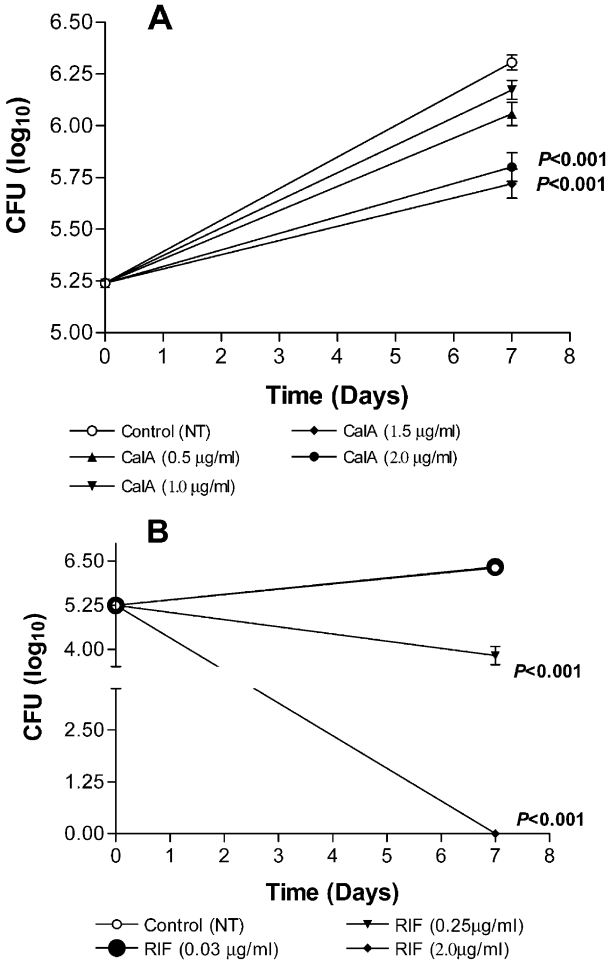

Figure 2. Intracellular efficacy of (+)-calanolide A (A) and rifampin (RIF) (B) in the J774 murine macrophage cell line, infected with *M. tuberculosis* H37Rv. CFUs were measured at time of infection (day 0) and 7 days post treatment. Cells were treated with drugs immediately following initial CFU determination at day 0 at concentrations depicted in figures. Each point represents the mean and standard error of the mean of triplicate samples. Differences in CFUs were evaluated with a one-way analysis of variance (ANOVA) to determine significant differences between the nontreated group at day 7 and each drug-treated group at day 7, as described in the Experimental. Results with p values ≥ 0.05 are considered significant.

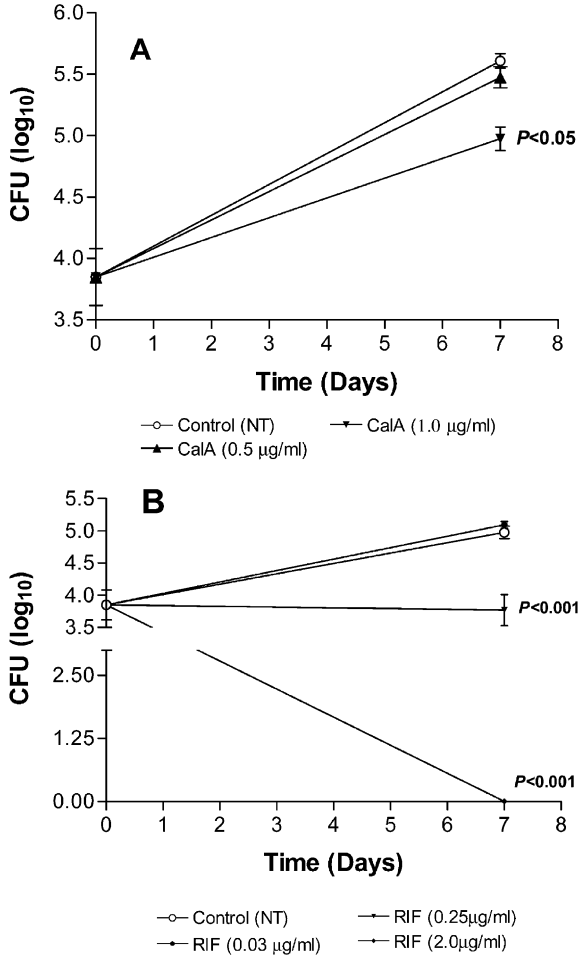

Figure 3. Intracellular efficacy of (+)-calanolide A (A) and rifampin (RIF) (B) in the Mono Mac 6 human monocytic cell line, infected with *M. tuberculosis* H37Rv. CFUs were measured at time of infection (day 0) and 7 days post treatment. Cells were treated with drugs immediately following initial CFU determination at day 0 at concentrations depicted in figures. Each point represents the mean and standard error of the mean of triplicate samples. Differences in CFUs were evaluated with a one-way analysis of variance (ANOVA) to determine significant differences between the nontreated group at day 7 and each drug-treated group at day 7, as described in the Experimental. Results with p values ≥ 0.05 are considered significant.

the J774 cell line, rifampin was not able to significantly reduce intracellular replication at eight-fold below its MIC (Fig. 3B). The MTT cytotoxicity assay revealed a 25% reduction of viability at 1.0 μg/mL of (+)-calanolide A. At 1.5 μg/mL, viability was reduced to 59% and, therefore, the results were not considered valid and were not included in Figure 3A for analysis.

It has been known that organic solvents used to dissolve test articles may have toxic effects on the whole cells. The effect of DMSO, used to dissolve (+)-calanolide A in the intracellular efficacy studies described above, was therefore evaluated on both the J774 and MM6 cell lines. The results indicate that viability of J774 cells was only reduced by 2 and 18 percent for DMSO concentrations equivalent to those used for the 1.5 and 2.0 μg/mL concentrations of (+)-calanolide A. For the MM6 cell line, treatment of DMSO at the concentration used to dissolve 1.0 μg/mL of (+)-calanolide A resulted in only 3% reduction of viability. These results demonstrate that DMSO did not affect viability of the cell lines sufficiently to alter results of intracellular efficacy evaluations.

In a separate bone marrow-derived murine macrophage model evaluated under the TAACF program, the BC99 value of (+)-calanolide A, a concentration at which 99% of the inoculum of *M. tuberculosis* is destroyed, was 1.1 μg/mL (3 μM) against strain Erdman (TMCC 107/ATCC 35801) and 1.8 μg/mL (5 μM) against strain CSU 93 (CDC-95-031551), compared to 0.7–0.8 μg/mL (5–6 μM) for the positive control drug isoniazid.

Table 3. Effects of (+)-Calanolide A on protein, RNA and DNA biosynthesis[a]

Time (h)	Nontreated	(+)-Calanolide A A 64 μg/mL (% inhibition)	Positive control	Polymyxin B At 10 μg/mL (% inhibition)
A. Protein Synthesis			Streptomycin At 10 μg/mL (% inhibition)	
0	3584	3584	3584	3584
1	6461	7449	6823	6966
2	10,180	6523 (25)	8381 (18)	8751
3	14,252	6886 (38)	12,477 (12)	12,487
5	20,382	6425 (60)	11,625 (43)	19,923
B. RNA Synthesis			Rifampin at 0.04 μg/mL (% inhibition)	
0	179	179	179	179
1	731	537 (27)	523 (28)	927
2	1483	861 (42)	709 (52)	1377
4	3715	898 (76)	1023 (72)	3117
C. DNA Synthesis			Ofloxacin at 5.0 μg/mL (% inhibition)	
0	756	756	756	756
1	1447	684 (40)	209 (93)	1428
2	4250	943 (56)	312 (92)	3730
3	7120	1658 (53)	514 (93)	6402
4	7980	1376 (47)	670 (92)	8526
5	10,559	923 (47)	716 (93)	11,188

[a] Results were determined by measuring incorporation of ^{14}C-labeled precursors into TCA-precipitable material of a log-phase culture of *M. tuberculosis* H37Ra. Each value represents the average cpm of two samples. Numbers in parentheses are the % inhibition in relation to the nontreated control set. Percent inhibition for (+)-calanolide A for protein and DNA synthesis were adjusted by subtracting the background observed in a DMSO equivalent control. Positive controls included streptomycin, rifampin and ofloxacin, for protein, RNA and DNA synthesis, respectively. Polymyxin B was used as a negative control for each parameter.

Table 4. Effects of (+)-calanolide A on lipid synthesis in *M. tuberculosis* H37Ra[a]

Time (h)	Nontreated control	Cerulenin (% inhibition, *p*)	(+)-Calanolide A (% inhibition, *p*)		
			1.0 μg/mL	8.0 μg/mL	64 μg/mL
12	44,110±5661	12,423±1146 (72%, <0.001)	50,102±37.14 (0%, NS[b])	17,467±1656 (60%, <0.001)	262±19 (99%, <0.001)
24	20,954±1535	6764±866 (68%, <0.001)	18,785±1337 (10%, NS[b])	9140±1038 (56%, <0.001)	267±11 (99%, <0.001)

[a] Inhibitors were added at time 0, followed by addition of [1,2-^{14}C]-acetate. Cerulenin (10 μg/mL) was used as a positive control. Values are reported as the mean±standard error of the mean counts per min per 50 μg lipid ($n=3$). Percent inhibition is given in parentheses following each cpm data entry, along with the *p* value as determined by a one-way analysis of variance (ANOVA) and a Tukey–Kramer Multiple Comparisons Test (InStat/GraphPad Software).
[b] NS, not significant.

2.3. Effects of (+)-calanolide A on macromolecular synthesis

(+)-Calanolide A was evaluated for its effect on the syntheses of protein, RNA, DNA and lipids in *M. tuberculosis* H37Ra. The H37Ra strain was used to facilitate these labor-intensive experiments by precluding the use of Biosafety Level 3 facilities. These studies were designed to examine the immediate effect of (+)-calanolide A on the major macromolecular syntheses.

For these experiments, (+)-calanolide A was evaluated at three concentrations, 1.0, 8.0 and 64 μg/mL. For protein, RNA and DNA synthesis, (+)-calanolide A at a concentration of 1.0 and 8 μg/mL had no effect on incorporation of [^{14}C]-phenylalanine (protein) or [^{14}C]-uracil (RNA and DNA) throughout a 5-h exposure period, indicating no immediate effect on these parameters (data not shown). At a concentration of 64 μg/mL, (+)-calanolide A did reduce uptake of [^{14}C]-phenylalanine by 25%, but inhibition was not observed until after 2 h exposure (Table 3A). This inhibition persisted up to 5 h, where reduction increased to 60%. The most immediate effect of (+)-calanolide A at 64 μg/mL was on RNA and DNA synthesis, which was observed after 1 h treatment, resulting in 27 and 40% inhibition, respectively (Table 3B and C). This inhibition persisted throughout 5 h, with the highest level of inhibition being observed for RNA synthesis at 76%. Compared with known inhibitors, this overall effect is more closely associated with inhibitory effects observed with rifampin.[16]

The effect of (+)-calanolide A on lipid synthesis was evaluated at concentrations of 1.0, 8.0 and 64 μg/mL. The results (Table 4) show that (+)-calanolide A affected incorporation of [1,2-^{14}C]-acetate in *M. tuberculosis* in a dose-dependent manner. At the highest concentration tested (64 μg/mL), treatment with (+)-calano-

lide A resulted in 99% inhibition, as compared to cerulenin, the positive control (Table 4). The negative control, Polymyxin B (10 μg/mL), resulted in 34 and 39% inhibition by 12 and 24 h, respectively. It should be noted that treatment with DMSO at a concentration equivalent to that used to dissolve the 64 μg/mL dose, resulted in 51 and 56% inhibition, respectively, at 12 and 24 h.

3. Discussion

M. tuberculosis is one of the leading opportunistic infections associated with AIDS. HIV increases a person's susceptibility to TB infection due to the decline of CD4+ lymphocytes in number and function, resulting in a weakened immune system incapable of preventing the growth and local spread of *M. tuberculosis*. An individual co-infected with HIV and *M. tuberculosis* has a 10 times greater risk of developing TB, compared to an individual who is not infected with HIV. On the other hand, TB infection in an HIV-infected person may allow HIV to multiply more quickly and lead to a more rapid disease progression of AIDS.

Challenges in the management of TB in patients with AIDS are significantly higher than in patients without AIDS. The first challenge is the pill burden. A DOTS program for TB requires a patient to take 10–12 pills a day and the recommended highly active antiretroviral therapy (HAART) for HIV infection easily adds another 20 pills. All the medications have to be taken daily, around the clock, with or without food restrictions, creating a tremendous difficulty for the patient to adhere to the drug regimen. The second challenge is the interactions between drugs for TB and HIV infections, which may lead to regimen intolerance and/or contraindication and add more difficulties in the treatment design. For example, rifampin has been recommended not to be used concurrently with almost all the anti-HIV NNRTIs and protease inhibitors, due to their contraindicated interactions.[17] The third challenge is that HIV-infected TB patients may be exposed only to functional monotherapy even while such a patient is under treatment with the recommended combination therapy, which may exacerbate the spread of MDR-TB in HIV-infected TB patients. Resistance to rifampin in HIV-infected TB patients, as well as linkage between HIV and MDR-TB in small-scale outbreaks, has been documented.[18] Clearly, agents with dual activities against both TB and HIV would be advantageous to patients co-infected with these pathogens.

The data from the present study showed that (+)-calanolide A and its related pyranocoumarin compounds are active against *M. tuberculosis*. To the best of our knowledge, they are the first compounds in this class identified to possess activities against both TB and HIV. The anti-TB activity (i.e., MIC values) of these naturally occurring and semi-synthetic compounds may be considered to be marginal when compared with the first-line antimycobacterial agents. However, there are a few unique features that render these molecules attractive for the discovery and development of anti-TB drugs. First, being structurally different from all the clinically used anti-TB drugs, they are expected to be active against drug-resistant strains of *M. tuberculosis*. As demonstrated (Table 2), (+)-calanolide A did not lose activity toward strains that are resistant to isoniazid, rifampin, streptomycin and ethambutol.

Second, the pyranocoumarins, representing a new pharmacophore for anti-TB activity, may possess a novel mechanism of action against *M. tuberculosis*. The most immediate effect of (+)-calanolide A on macromolecular synthesis was observed for RNA and DNA synthesis using a concentration of 64 μg/mL, or a concentration only 4-fold above that obtained in vitro for the H37Ra strain. This occurred after 1 h treatment and persisted throughout 5 h. Although (+)-calanolide A also affected protein synthesis, it did so to a lesser degree and inhibition was not observed until after 2 h treatment.

Rifampin is a known inhibitor of DNA-dependent RNA polymerase with little activity against the equivalent mammalian enzymes,[19] while (+)-calanolide A is a potent inhibitor of HIV-1 reverse transcriptase, an enzyme responsible for RNA-dependent DNA polymerase, DNA-dependent RNA polymerase and ribonuclease hybrid. Taken together, the preliminary mechanistic studies imply that the primary target in *M. tuberculosis* upon which (+)-calanolide A acts is involved in the RNA synthesis, similar to rifampin.[16] However, (+)-calanolide A is active against a rifampin-resistant strain, suggesting that both compounds may affect the same biological event (RNA synthesis) but exert their inhibitory effects in different ways. For instance, they may act on different targets involved in different stages of RNA synthesis or on the same target but with different modes of action (e.g., different binding sites).

Our results indicate that after 12–24 h exposure to (+)-calanolide A, lipid biosynthesis is also affected. However, this effect is probably secondary because polymyxin B, the negative control, also had some effect on lipid synthesis in this time frame. This secondary effect might be expected by a potential inhibitor of key events that precede the synthesis of lipid precursors, that is RNA and DNA synthesis. However, it is reassuring to know that (+)-calanolide A does affect a key component that is essential in the macromolecular composition of the mycobacterial cell.

Third, the tubercle bacillus is a facultative intracellular parasite; therefore, drugs should also be able to penetrate host cells. Thus, an ideal method for treating TB infection would be one that not only is able to safely deliver drugs systemically for the long term, but also would be able to target drugs to the intracellular environment in which the tubercle bacilli are found (i.e., macrophages). Previously, (+)-calanolide A has been demonstrated to possess anti-HIV activity in established and fresh human cells such as T-cells CEM-SS, H9 and MT2, the B-cell line AA5, the monocytic line U937 and the T-cell/B-cell hybrid line 174×CEM, as well as fresh human peripheral blood leukocytes and monocyte-

macrophages.[13] The present study showed significant effects on intracelluarly replicating *M. tuberculosis* at 1.5 and 2.0 μg/mL in the J774 cell line, at 1.0 μg/mL in the case of the MM6 cell line and at 1–2 μg/mL in bone marrow-derived murine macrophages.

These results are significant because they not only demonstrate that (+)-calanolide A has intracellular efficacy against *M. tuberculosis*, but that the effect is observed at concentrations below the MIC determined in vitro. It is important to note that these observations were made from two independent studies, involving three different macrophage assays. The enhanced intracellular anti-TB activity observed suggest that (+)-calanolide A may stimulate the macrophage to be more bactericidal, perhaps as a result of cytokine induction. This hypothesis remains to be investigated.

Finally, (+)-calanolide A, a natural product originally isolated from the rainforest tree *Calophyllum lanigerum*,[20] is being investigated in clinical trials for HIV infection.[21] In clinical trials involved with normal healthy and HIV-negative volunteers, (+)-calanolide A, after oral administration in either single[22] or 5-day multiple[23] doses, gives rise to only mild to moderate adverse events which were all transient. The pharmacokinetic data indicate that the (+)-calanolide A concentrations in plasma may be in comparable to the observed in vitro MICs against *M. tuberculosis*. Therefore, the dual activities may render (+)-calanolide A a possible chemoprophylactic agent for TB in HIV-infected people or vice versa if clinical benefits can be demonstrated.

4. Conclusion

A novel anti-TB pharmacophore, pyranocoumarin, has been identified. The structure–activity analysis has led to the determination of several key structural requirements for the pyranocoumarin derivatives to exert their anti-TB activity, which provide insights into further structural modifications. The preliminary investigation of effects conferred by (+)-calanolide A on major macromolecular biosyntheses has laid a solid foundation to pursue subsequent studies in an attempt to identify the specific target(s) for the drug.

5. Experimental

5.1. Chemistry

The structures of the compounds studied in this report are presented in Figure 1. All the known pyranocoumarins were either isolated from plant materials (**3** and **4**)[24] or synthesized according to the published procedures (**1**, **2** and **9**,[25] **5**,[26] as well as **7**[27]). All the compounds obtained for testing were at least 96% pure and were characterized by physical, chromatographic and spectroscopic data that was in full agreement with structural assignments and identical to those reported in the literature. Two new pyranocoumarins **6** and **8** were prepared by adapting the literature methods.

5.1.1. (10,11-*trans*)-12-Hydroxy-6,6,10,11-tetramethyl-4-phenyl-7,8,11,12-tetrahydro-6*H*,10*H*-dipyrano[2,3-*f*;2′,3′-*h*]chromen-2-one (6). Soulattrolide (**4**) (4.04 g, 10.0 mmol) was hydrogenated in 200 mL of EtOH/CH_2Cl_2 (1:1) under 2 atm. H_2 pressure and in the presence 212.8 mg (0.2 mmol) of 10% Pd/C. After shaking on a Parr shaker at rt for 2 h, there was no more H_2 consumption. The catalyst was removed through filtration and washed with EtOH (5 mL×3). The solvents were evaporated and the crude product obtained was purified by column chromatography on silica gel eluenting with hexane and EtOAc (8:2). The pure compound **6** was obtained as white crystalline solid (2.3 g, 57% yield), along with recovery of the starting material (1.5 g, 37%). Compound **6**: mp 165–167 °C; $[\alpha]_D$ −36.9° ($CHCl_3$, *c* 0.8); ^{1}H NMR ($CDCl_3$) δ 0.82 (s, 3H), 0.84 (s, 3H), 1. 17 (d, *J* = 7.2 Hz, 3H), 1.43 (d, *J* = 6.0 Hz, 3H), 1.57 (t, *J* = 6.6 Hz, 2H), 1.77 (ddq, *J* = 3.5, 10.5, 7.0 Hz, 1H), 2.44 (broad-s, 1H), 2.55 (dt, *J* = 2.1, 6.8 Hz, 2H), 4.28 (dq, *J* = 11.2, 6.2 Hz, 1H), 5.07 (broad-s, 1H), 5.94 (s, 1H), 7.19–7.22 (m, 2H), 7.33–7.37 (m, 3H); FTIR (KBr) 3460, 3062, 2973, 1700, 1594, 1558, 1442, 1377, 1148, 1120 cm^{-1}; EIMS *m*/*e* 407 (100, M + 1), 389 (60, M−OH); elem. anal. calcd. for $C_{25}H_{26}O_5$: C, 73.87; H, 6.45. Found: C, 73.65; H, 6.49.

5.1.2. (10,11-*trans*)-6,6,10,11-Tetramethyl-4-propyl-7,8,10,11-tetrahydro-6*H*-dipyrano[2,3-*f*; 2′,3′-*h*]chromen-2,12-dione (8). (±)-**7** was reduced in the same manner as described above. From 534 mg (1.45 mmol) of (±)-**7**, 441 mg (82% yield) of pure compound **8** was obtained as white plates after purification by passing through a short silica gel plug with an eluent of CH_2Cl_2/MeOH (97:3), followed by recrystallization from EtOAc. Compound **8**: mp 165 °C; ^{1}H NMR ($CDCl_3$) δ 1.01 (t, *J* = 7.3 Hz, 3H), 1.21 (d, *J* = 6.8 Hz, 3H), 1.42 (s, 3H), 1.44 (s, 3H), 1.53 (d, *J* = 6.2 Hz, 3H), 1.61 (m, 2H), 1.84 (dt, *J* = 2.4, 6.7 Hz, 2H), 2.53 (dq, *J* = 11.2, 6.8 Hz, 1H), 2.69 (dt, *J* = 3.4, 6.7 Hz, 2H), 2.88 (t, *J* = 7.5 Hz, 2H), 4.28 (dq, *J* = 11.2, 6.2 Hz, 1H), 6.02 (s, 1H); FTIR (KBr) 2986, 2876, 1740, 1557, 1109 cm^{-1}; EIMS *m*/*e* 370 (100, M^+), 314 (99, M-C_4H_8), 286 (65, M-C_5H_8O); elem. anal. calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.00; H, 7.22.

5.2. Measurement of MICs by microdilution Alamar Blue assay

MICs for *M. tuberculosis* strains for the results shown in Table 2 were determined using a microdilution broth assay that incorporates the REDOX indicator Alamar blue.[28] Briefly, the assay involved serially diluting each stock solution of drug in broth medium (7H9 + ADC enrichment + 0.2% glycerol) at twice the desired concentration, and then adding 0.05 mL to each well in duplicate. The inoculum for each well consisted of 0.05 mL of culture standardized to about 1×10^5 CFU/mL. Each plate also contained uninoculated drug controls, a viability control and an uninoculated medium control. The plates were then covered with a lid, placed in polyethylene bags and incubated at 37 °C for either 6 days or 13 days, depending upon the strain. The REDOX dye Alamar blue, diluted in Tween 80, was then added to

each well, the plates incubated for an additional 16–20 h and read visually. Growth and viability were indicated by the metabolic reduction of the dye from blue to red. The MIC was recorded as the lowest concentration of drug yielding no visible growth as indicated by the lack of change in color of the dye. Each drug was tested initially in duplicate at 0.128, 1.28, 12.8 and 128 μg/mL to determine the range of concentrations within which the MIC fell. The assay was then repeated using two-fold serial dilutions of drug. A stock solution of (+)-calanolide A was prepared by dissolving the drug in DMSO at a concentration of 10.24 mg/mL and stored at refrigeration before use. Ethambutol, isoniazid, rifampin and streptomycin were used as positive drug controls on each day of assay. The diluent, DMSO, was diluted in the same manner as (+)-calanolide A and tested on each day of assay.

MICs of the test compounds, as shown in Table 1, were also obtained for H37Rv strain of *M. tuberculosis* with the radiometric BACTEC assay through the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) (NIH, NIAID contract No. NO1-AI45246).[29] The MIC of each compound was defined as the lowest concentration which inhibits >99% of the bacterial population present at the beginning of the assay.

5.3. Vero cell cytotoxicity

The cytotoxicity for Vero cells was determined using the Promeaga CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega Corp., Madison, WI, USA) through the TAACF and reported as IC_{50}, the concentration of a compound which inhibited cell growth by 50% of the control. The selectivity index (SI) was determined as the ratio of the IC_{50} to the MIC (BACTEC assay).

5.4. Monocyte cell lines

The J774 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC [TIB 67], Rockville, MD, USA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (J774 medium). The MM6 cells were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany and were originally established by Ziegler-Heitbrock et al.[14] MM6 cells were maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvic acid, and 9 μg of bovine insulin (Sigma Chemical Co., St. Louis, MO, USA) per mL of MM6 medium. Cell lines were routinely assayed to verify the absence of mycoplasma contamination by using the Gen-Probe Mycoplasma Rapid Detection system (Gen-Probe, San Diego, CA, USA).

5.5. LD_{50} Determination of (+)-calanolide A for monocytic cell lines

The LD_{50} determination was performed by means of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [thiazolyl blue]) (Sigma) cytotoxicity assay as previously described by Barrow et al. for use with intracellular efficacy testing.[30] Viability is reported as the percentage of the nontreated cells. In some of the assays, DMSO was used as a solvent control at concentrations equivalent to those used to dissolve (+)-calanolide A.

5.6. Treatment of monocytes with (+)-calanolide A

The J774 and MM6 monocytic cell lines were infected with *M. tuberculosis* strain H37Rv as previously reported[31] and treated with placebo, positive control drug or (+)-calanolide A. The number of mycobacterial colony forming units (CFU) was determined at 0 h and at 7 days post-infection. To obtain CFUs, cells were lysed with 0.25% (wt/vol) sodium dodecyl sulfate (SDS) in PBS, serially diluted and plated onto 7H10 agar plates. To facilitate serial dilution, it was necessary to decrease viscosity of cell lysate by adding 5 μL (5 U of activity) of RQ Dnase (Promega Corp., Madison, WI, USA) with $MgSO_4$ (5 mM) to each well after addition of SDS. This procedure was repeated at 7 days post-infection and CFUs enumerated after three weeks. Each evaluation included an infected set of macrophages without treatments of drugs or compounds and was performed in triplicate. A statistically significant increase in CFUs at the end of 7 days for the nontreated infected group served as an internal control. Also included in each evaluation was a positive control, in which *M. tuberculosis*-infected macrophages were treated with rifampin at three concentrations: MIC, 8×'s MIC (8MIC) and 8×'s less MIC (−8MIC). Final concentrations used for (+)-calanolide A were determined after evaluation of the cytotoxicity profile. Equivalent amounts of DMSO, used to dissolve each concentration of (+)-calanolide A, were also incorporated in the evaluation as controls. Results are reported as the mean CFU's ± standard error of the mean (SEM) at day 0, immediately following infection, and day 7, for each infected group (treated and nontreated). Differences in CFUs were evaluated with a one-way analysis of variance (ANOVA) to determine significant differences between the nontreated group at day 7 and each drug-treated group at day 7 (InStat). A correction for multiple comparisons (posttest) was performed with the Tukey–Kramer multiple comparisons test. Results with p values ≤ 0.05 are considered significant. A set of viability controls was included in order to verify that handling, in combination with drug dosing, did not affect cell viability.

5.7. Inhibition of biosyntheses of *M. tuberculosis* by (+)-calanolide A

M. tuberculosis H37Ra was used to evaluate the effect of (+)-calanolide A on protein, RNA, DNA and lipid synthesis. Each mode of synthesis was assessed by measuring incorporation of appropriate radiolabeled precursors into mycobacterial macromolecular components. [2-^{14}C]-L-Phenylalanine, [1,2-^{14}C]-acetate and [2-^{14}C]-uracil were used for assessment of protein, lipid, RNA and DNA synthesis, respectively. Trichloracetic acid (TCA) precipitates were collected on glass filters and counted in a scintillation counter to assess radiolabel

incorporation. For DNA synthesis, RNA was hydrolyzed with sodium hydroxide (NaOH) prior to DNA precipitation with trichloracetic acid (TCA, 10%). It is important to note that radiolabeled uracil was used instead of thymine or thymidine because of the presence of thymidine phosphorylase that hydrolyses the substrate and results in misleading incorporation data.[32] For lipid synthesis, mycobacterial cultures, in exponential growth, were treated for 6 h prior to addition of [1,2-^{14}C]-acetate (1.0 μCi/mL), and mycobacteria were harvested 6 h and 18 h after addition of [1,2-^{14}C]-acetate. Lipid was then extracted and assayed for radiolabel incorporation. Thus, incorporation of [1,2-^{14}C]-acetate was determined at 12 and 24 h following addition of inhibitor.

Known inhibitors of each biosynthetic pathway were used as positive controls, including streptomycin sulfate for protein synthesis, rifampin for RNA synthesis, ofloxacin for DNA synthesis and cerulenin for fatty acid synthesis. Polymyxin B was used as a negative control. (+)-Calanolide A was evaluated at three concentrations encompassing its MIC. Those concentrations were 1, 8 and 64 μg/mL. Data is presented as the mean counts per min (cpm) of two samples with the percent of inhibition given in parenthesis. Percent inhibition was determined by the following formula: [1−(cpm [^{14}C] incorporation in treated samples/cpm [^{14}C] incorporation in non-treated samples)]×100 = % inhibition.

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