Chem Biol Drug Des 2011; **77:** 489–493

doi: 10.1111/j.1747-0285.2011.01120.x

Research Letter

Antitubercular Activity of New Coumarins

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The present article describes a series of 21 *N′*-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides 4a–4v, which were synthesized and evaluated for their cell viabilities in non-infected and *Mycobacterium bovis* Bacillus Calmette–Guerin-infected macrophages. Subsequently, the non-cytotoxic compounds 4c, 4g, 4h, 4j, 4l and 4t were assessed against *Mycobacterium tuberculosis* ATCC 27294 using the microplate Alamar Blue assay and the activity expressed as the minimum inhibitory concentration in μg/mL. These compounds exhibited a significant activity (50–100 μg/mL) when compared to the first-line drugs, such as pyrazinamide (PZA >100 μg/mL). These results could be considered a good starting point for further studies to develop new lead compounds to treat multidrug-resistant tuberculosis.

Key words: coumarins, drugs, *N′*-acylhydrazones, tuberculosis

Received 7 February 2010, revised 19 September 2010 and accepted for publication 27 February 2011

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a contagious disease which reappeared in the mid-1980s and has become a serious global public health problem. Currently, it is estimated that TB kills 2 million people per year of which 450 000 are children. Globally, the number of TB cases is rising 2% annually. It is estimated that 32% of the world population is infected by latent TB. Many factors contribute to the increase in tuberculosis cases, being AIDS pandemic and the emergence of bacterial resistance (MDR and XDR-TB) the most important (1,2).[a]

AIDS Epidemic, in the mid-1980s, has led to an outbreak of coinfection with TB in seropositive patients. As an example, the coinfection of TB and HIV has led to a fourfold increase in TB cases in several African and Asian countries. According to statistics, presently, about 10–12 million people are coinfected by these two micro-organisms, and TB is the most common opportunist infection in HIV-positive patients, being responsible for the morbidity and mortality of one in every three patients. Nowadays, there is no optimum standard anti-TB therapy for AIDS patients or any agent that is active against infection caused by both HIV and *M. tuberculosis*. Consequently, because of the high impact of TB in global society, new drugs and strategies are urgently needed to fight this disease, particularly new agents with activity against both TB and HIV infection and MDR strain. In this context, the development of potent new antitubercular agents with low-toxicity profiles, a rapid mycobactericidal mechanism of action and the ability to penetrate host cells and shortened duration of therapy are in great interest (2).

Naturally occurring coumarins are endowed with different types of biological applications. The 1,2-benzopyrone structure consists of structural units of several natural products and is widely present in pharmacologically and biologically active compounds, such as novobiocin (3), warfarin (4), 677 cumate (5) and psoralen (6). In addition to functionalized coumarins, the calanolides isolated from *Calophyllum* genus showed potent anti-HIV activity. Specifically, (+)-calanolide A was found to inhibit not only the wild type of HIV-1 but also clinically isolated resistant strains, such as A17 (7). Recently, it has been demonstrated that (+)-calanolide A was active against all of the strains of *M. 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* H_{37}Rv *in vitro* at a 3.13 μg/mL concentration (8). (+)-Calanolide A and its related coumarins are the first class of compounds identified to possess antimycobacterial and antiretroviral activities, representing a new pharmacophore for antitubercular activity.

Inspired in the antitubercular activity of (+)-calanolide A, we have proposed the synthesis of some *N′*-acylhydrazones that contained the coumarin nucleus. According to literature, many *N′*-acylhydrazones are described with a wide range of pharmacological activities (9), such as antibacterial agents. For example, hydrazone derivatives, prepared by our group, exhibit promising anti-TB activity comparable to the first-line anti-TB drugs as isoniazid, rifampicin and

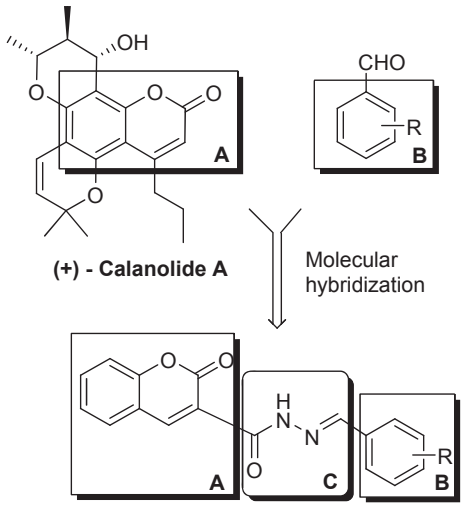
pyrazinamide (10–18). This work is part of our continuous programme in the search of new antitubercular agent candidates.

The design concept of these compounds explored the introduction of mono- and disubstituted benzaldehydes moieties (**B**) into coumarins core structure (**A**) to obtain *N*′-acylhydrazones groups (**C**) (Scheme 1). This modification aims to evaluate their selectivity against *M. tuberculosis*, their mechanism of action and the cytotoxic effects of these compounds. Furthermore, we report the synthesis (Scheme 2) and preliminary *in vitro* antitubercular and cytotoxic activities of 21 *N*′-acylhydrazones coumarins derivatives, of which fourteen are novels.

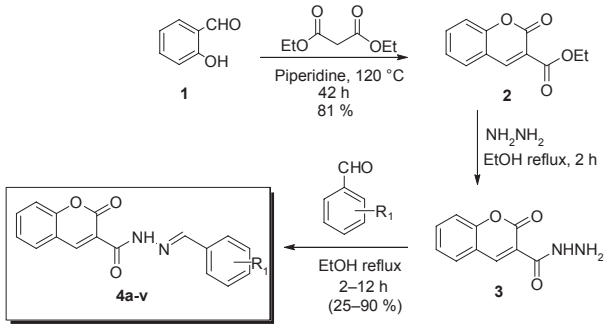
General procedure for the synthesis of *N*′-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides derivatives

The strategy for the synthesis of *N*′-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides derivatives **4a–4v** from salicylaldehyde **1** is described in Scheme 2. All target compounds **4a–4v** were obtained, with moderate to good yields (Table 1), by the nucleophilic attack of the most basic nitrogen of hydrazine **3** to the carbonyl group of the aldehyde derivative. The synthetic route employed for the preparation of **4a–4v** is outlined in Scheme 2. Primarily, salicylaldehyde **1,** diethyl malonate and piperidine catalyst were refluxed at 120 °C for 42 h to produce ethyl 2-oxo-2*H*-chromene-3-carboxylate derivatives **2** under *Knoevenagel* reaction conditions (19). In the second step, 2-oxo-2*H*-chromene-3-carbohydrazide derivative **3** was synthesized by refluxing the coumarin-3-carboxylate ethyl ester with 80% hydrazine solution in ethanol. Finally, reflux with aldehyde in ethanol leads to the target compounds **4a–4v**. The properties of *N*′-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides derivatives are summarized in Table 1.

Compounds **4a–4v** were identified by spectral data. In general, IR spectra showed the strong bands of aromatic and heteroaromatic



Scheme 1: Design concept of *N*-acylhydrazones coumarin derivatives.



Scheme 2: Synthetic route for the preparation of *N*′-benzylidene-coumarins-3-carbohydrazides derivatives.

cores in the 1570–1320 cm^{-1} region and in the 900–686 cm^{-1} region, originated in the angular deformation outside the rings plane and C-H. The stretching vibrations of N–H binding were observed in the 3588–3331 cm^{-1} region while stretching vibrations of C=O (lactone and amide) in the 1720–1620 cm^{-1} region. In the 1275–1271 cm^{-1} region, stretching vibrations of C–O binding were observed.

The analysis of ^{1}H NMR spectrum showed the signal of imine proton (H–C=N–) as singlet at 9.14–8.70 ppm, the hydrazide proton (N–H) as a singlet at 11.96–11.30 ppm and five signals corresponding to the protons of 1,2-benzopyrone moiety at 9.00–7.44 ppm. The ^{13}C NMR spectrum showed the nine coumarin carbon signals at the region of 168.0–108.9 ppm and the hydrazide carbon (–N=CH–R) signals at 168–162 ppm.

In the case of each of the **4b** (2-Cl), **4f** (4-Br), **4n** (2-NO_2), **4o** (3-NO_2), **4q** (2,3-Cl), **4s** (2,6-Cl) derivatives, the occurrence of stereoisomerism at the N=CH bond results in the formation of a pair of (*E*) and (*Z*)-diastereomers. The presence of these diastereomers was confirmed by the ^{1}H NMR spectra results, which in each case displays two separate sets of signals. The assignment of the relative configuration of (*E*) and (*Z*)-diastereomers of these *N*′-acylhydrazones derivatives was made in agreement with previous results obtained by Karabatsos *et al.* (20–22), which describes that imine-attached hydrogen signal of the (*E*)-diastereomer is downfielded by 0.2–0.3 ppm from the corresponding hydrogen atom signal in the (*Z*)-diastereomer. Therefore, after careful analysis of the ^{1}H NMR spectra of the mixture of diastereomers, we were able to evidence that the main one presents *(E)* configuration similar to that found for other *N*′-acylhydrazones in literature (23,24).

The antimycobacterial activities of these compounds were assessed against *M. tuberculosis* ATCC 27294 (29) using the microplate Alamar Blue assay made in triplicate (30) (Table 1). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric methods (31, 32). These results showed that compounds **4c**, **4h**, **4j**, **4l**, **4p** and **4t** (Entry 3, 8, 10, 11, 15 and 19, respectively) exhibit an antimycobacterial activity of 100 μg/mL, while the compound **4g** (Entry 7) presented an activity of 50 μg/mL. These compounds were more active than the first-line drug reference, pyrazinamide (Entry 23).

Table 1: Antimycobacterial activities, melting points, clogP measurements and yields of *N'*-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides derivatives

		Substituents					
Entry	Compound	R	R_1	Yield (%)	cLogP[a]	mp (°C)	MIC[b] (μg/mL)
1	**4a**	H	H	30	3.10	133–137 (25)	Res.
2	**4b**	H	2-Cl	82	3.74	86–88 (26)	Res.
3	**4c**	H	3-Cl	88	3.76	70–71	100.0
4	**4d**	H	4-Cl	80	3.78	121–122 (27)	Res.
5	**4e**	H	3-Br	73	3.89	140–141	Res.
6	**4f**	H	4-Br	76	3.91	143–144	Res.
7	**4g**	H	3-OH	45	2.60	111–113 (25)	50.0
8	**4h**	H	4-OH	25	2.62	173–176	100.0
9	**4i**	H	2-OMe	48	3.11	133–136	Res.
10	**4j**	H	3-OMe	53	3.14	84–85	100.0
11	**4l**	H	4-OMe	40	3.16	85–86	100.0
12	**4m**	H	4-$N(CH_3)_2$	62	3.21	155–156	Res.
13	**4n**	H	2-NO_2	65	3.01	87–88	Res.
14	**4o**	H	3-NO_2	68	3.04	123–125 (26)	Res.
15	**4p**	H	4-NO_2	58	3.06	135–138 (26)	100.0
16	**4q**	H	2,3-Cl	57	4.36	111–112	Res.
17	**4r**	H	2,4-Cl	68	4.39	124–125	Res.
18	**4s**	H	2,6-Cl	73	4.36	83–85	Res.
19	**4t**	H	2,4-OH	35	2.54	131–134	100.0
20	**4u**	H	2,5-OH	45	2.54	121–124	Res.
21	**4v**	H	3,4-OMe	55	2.75	123–124 (28)	Res.
22	**PZA**	–	–	–	–0.71	–	>100.

PZA, pyrazinamide; MIC, minimum inhibitory concentration.
[a]Calculated using http://www.molinspiration.com.
[b]Res. indicates that the strain is resistant to the tested substance.

Furthermore, these derivatives that showed antitubercular activity were submitted to the cellular viability in non-infected or *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG)-infected macrophages. The test was determined by Mosman's MTT (3-(4,5-dimethylthylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay (33, 34). The results were represented as cell viability percentage (Table 2). This table shows that only the compound **4p** (4-NO_2) was cytotoxic (70% cell viability) in its respective minimum inhibitory concentration (100 μg/mL); therefore, the derivatives **4c**, **4g**, **4h**, **4j**, **4l** and **4t** (Entries 1, 2, 3, 4, 5 and 7) were selected to be tested on macrophages infected with *M. bovis* BCG (Table 3).

Table 2: Cellular viability data for a macrophage cell line J774 (ATTC TIB-67™) by Mosmans's assay

		% Cell viability/dose (μg/mL)		
Entry	Compound	50	100	150
1	**4c**	100	100	98
2	**4g**	100	100	75
3	**4h**	95	80	100
4	**4j**	100	95	98
5	**4l**	100	89	100
6	**4p**	69	62	57
7	**4t**	100	100	100
8	**PZA**	100	100	100

The purpose was to evaluate the action of these compounds against macrophages that show metabolism change after infection. Nevertheless, if we analyse Tables 2 and 3, it can be verified that the derivatives **4c**, **4h**, **4j**, **4l** and **4t** were not cytotoxic, because <5% of the cells were killed at the minimum concentration tested.

The synthesis of 21 *N'*-benzylidene-2-oxo-2*H*-chromene-3-carbohydrazides derivatives **4a–4v** was performed with moderate to good yields (25–90%), among which fourteen are new compounds (**4c**, **4e**, **4f**, **4h**, **4i**, **4j**, **4l**, **4m**, **4n**, **4q**, **4r**, **4s**, **4t**, **4u**). Only the derivatives **4c**, **4g, 4h**, **4j**, **4l**, **4p** and **4t** that showed antitubercular activity were submitted to the cellular viability in non-infected

Table 3: Cellular viability data for a macrophage cell line J774 infected (ATTC TIB-67™) with BCG by Mosmans's assay

		% Cell viability/dose (μg/mL)		
Entry	Compound	50	100	150
1	**4c**	100	100	93
2	**4g**	60	74	84
3	**4h**	99	80	85
4	**4j**	100	89	100
5	**4l**	100	100	100
6	**4t**	100	100	100

BCG, Bacillus Calmette–Guerin.

or *M. bovis* BCG-infected macrophages. In relation to the antimycobacterial activity, it was found that the compounds **4c**, **4g**, **4h**, **4j**, **4l**, **4p** and **4t** (50–100 μg/mL) exhibited better activities than PZA (>100 mg/mL), when Alamar Blue assay was used and that the derivatives **4c**, **4h**, **4j**, **4l**, **4p** and **4t** were not cytotoxic. These results suggest promising perspectives for MDR/XDR-TB. However, it is necessary to examine the action mechanism in detail to ascertain the reason for the increased activity of the *N*′-acylhydrazones.

Acknowledgments

We gratefully acknowledge the financial support of Fiocruz, UFRJ, CNPq.

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Note

[a]World Health Organization. Tuberculosis: A World Free of TB. http://www.who.int/tb/en/ (accessed April 10, 2009).