Eur. J. Med. Chem. 34 (1999) 1071–1076

Short communication

Synthesis and antimycobacterial activity of some isonicotinoylhydrazones

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(Received 15 February 1999; accepted 8 June 1999)

Abstract – A series of isonicotinoylhydrazones **2** were prepared by addition of some aryloxyacetonitriles with isonicotinoylhydrazine in basic medium. These compounds have been further reacted with pyridinecarboxaldehydes to give the corresponding pyridylmethyleneamino derivatives **3**–**5**. The new synthesized hydrazones and their pyridylmethyleneamino derivatives were tested for their activity against mycobacteria, Gram-positive and Gram-negative bacteria. The cytotoxicity was also tested. Several compounds showed a good activity against *Mycobacterium tuberculosis* H37Rv and some isonycotinoylhydrazones **2** showed a moderate activity against a clinically isolated *M. tuberculosis* which was isoniazid resistant. © 1999 Éditions scientifiques et médicales Elsevier SAS

aminohydrazone derivatives / synthesis / antimycobacterial activity

1. Introduction

At present, tuberculosis is considered, by the World Health Organisation, to be the most important chronic communicable disease in the world [1, 2, 3]. Over the past decade, tuberculosis has re-emerged both in industrial and developing countries. The emergence of AIDS, decline of socioeconomic standards and a reduced emphasis on tuberculosis control programs contribute to the disease's resurgence in industrialised countries [4]. In most developing countries, although the disease has always been endemic, its severity has increased because of the global HIV endemic and extensive social restructuring due to rapid industrialisation and conflicts.

Further contributing to the increased morbidity is the emergence of new strains of *M. tuberculosis* resistant to some or all current antitubercular drugs [5, 6]. Among these, isoniazid (INH), an inexpensive and relatively safe drug, continues to be well established for the treatment of tuberculosis. The mechanism of action of INH, as well as the mechanism conferring INH resistance, are complex and not completely understood. However, several studies suggest that INH inhibits the biosynthesis of cell wall mycolic acids, thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors [7]. INH is active against the *Mycobacterium* complex (*Mycobacterium tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) at MICs ranging from 0.025–0.05 μg/mL [8], while at a higher concentration (MIC of 500 μg/mL) it inhibits the growth of other microorganisms such as the opportunists *Klebsiella*, *Serratia* and *Enterobacter*.

For these reasons the antimycobacterial pharmacophore moiety of INH is introduced in several molecules to improve their activity against Mycobacteria.

On the other hand aminohydrazone derivatives, structurally correlated to INH, have been described for their in vitro antimycobacterial activity and some of these compounds exhibited inhibitory activity toward a human strain of *M. avium* resistant to the primary drugs INH, rifampicin and ofloxacin [9, 10]. As a part of our studies on aminohydrazone derivatives [11, 12], we became interested in a new series of 2-amino-2-(isonicotinoylhydrazono)ethyl aryl ethers, assuming that the isonicotine hydrazonic moiety is an important pharmacophore to antimycobacterial activity. Here, we report the

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Table I. Chemical and spectral data of compounds **2**.

Compound	R	Yield (%)	M.p. (°C) (recryst. solvent)	IR (Nujol) ν (cm^{-1})	^{1}H-NMR (DMSO-d_6/TMS) δ (ppm)
2a	H	77	174–176 (CH_3CN)	3 410, 3 280, 3 020, 1 680, 1 590, 1 570	4.50 (s, 2H, CH_2), 6.63 (s, 2H, NH_2), 6.97–7.24 (m, 5H, Ar), 7.72, 8.63 (m, 4H, Py), 10.11 (br s, 1H, NH)
2b	2-CH_3	74	178–180 (EtOH)	3 270, 3 310, 1 660, 1 605	2.17 (s, 3H, CH_3), 4.51 (s, 2H, CH_2), 6.59 (s, 2H, NH_2), 6.82–7.10 (m, 4H, Ar), 7.73, 8.63 (m, 4H, Py), 10.13 (br s, 1H, NH)
2c	3-CH_3	68	162–164 (2-PrOH)	3 170, 3 060, 1 680, 1 650	2.23 (s, 3H, CH_3), 4.49 (s, 2H, CH_2), 6.61 (s, 2H, NH_2), 6.75–7.13 (m, 4H, Ar), 7.73, 8.60 (m, 4H, Py), 10.11 (br s, 1H, NH)
2d	4-CH_3	66	208–210 (CH_3CN)	3 270, 3 080, 1 680, 1 650	2.17 (s, 3H, CH_3), 4.46 (s, 2H, CH_2), 6.60 (s, 2H, NH_2), 6.72–7.06 (m, 4H, Ar), 7.72, 8.62 (m, 4H, Py), 10.09 (br s, 1H, NH)
2e	2-OCH_3	72	175–177 (EtOH)	3 260, 3 100, 3 020, 1 670	3.73 (s, 3H, CH_3), 4.47 (s, 2H, CH_2), 6.58–6.94 (m, 6H, Ar + NH_2), 7.72, 8.63 (m, 4H, Py) 10.11 (s, 1H, NH)
2f	3-OCH_3	99	158–160 (CH_3CN)	3 270, 3 120, 1 675, 1 635	3.68 (s, 3H, CH_3), 4.49 (s, 2H, CH_2), 6.49–7.18 (m, 6H, Ar + NH_2), 7.72, 8.61 (m, 4H, Py), 10.10 (brs, 1H,NH)
2g	4-OCH_3	68	193–195 (CH_3CN)	3 360, 1 675, 1 590, 1 570	3.64 (s, 3H, CH_3), 4.44 (s, 2H, CH_2), 6.60 (s, 2H, NH_2), 6.80–6.94 (m, 4H, Ar), 7.72, 8.62 (m, 4H, Py), 10.08 (br s, 1H, NH)
2h	2-Cl	68	188–190 (EtOH)	3 410, 3 320, 1 690, 1 620	4.58 (s, 2H, CH_2), 6.62 (s, 2H, NH_2), 6.94–7.38 (m, 4H, Ar), 7.72, 8.64 (m, 4H, Py), 10.16 (br s, 1H, NH)
2i	3-Cl	64	180–182 (EtOH)	3 410, 3 080, 1 680, 1 585	4.54 (s, 2H, CH_2), 6.64 (s, 2H, NH_2), 6.95–7.31 (m, 4H, Ar), 7.72, 8.63 (m, 4H, Py), 10.12 (br s, 1H, NH)
2j	4-Cl	99	236–238 (CH_3CN)	3 240, 3 080, 1 680, 1 640	4.51 (s, 2H, CH_2), 6.64 (s, 2H, NH_2), 7.01–7.31 (m, 4H, Ar), 7.72, 8.63 (m, 4H, Py), 10.11 (br s, 1H, NH)
2k	4-NO_2	77	228–230 (EtOH)	3 360, 3 280, 3 160, 3 080, 1 680, 1 640	4.70 (s, 2H, CH_2), 6.74 (s, 2H, NH_2), 7.18–7.73 (m, 4H, Ar), 8.20, 8.63 (m, 4H, Py), 10.16 (br s, 1H, NH)
2l	4-$NHCOCH_3$	73	191–193 (EtOH)	3 220, 3 060, 1 660, 1 595	1.95 (s, 3H, CH_3), 4.46 (s, 2H, CH_2), 6.61 (s, 2H, NH_2), 6.90–7.42 (m, 4H, Ar), 7.72, 8.62 (m, 4H, Py), 9.77 (s, 1H, NH), 10.09 (br s, 1H, NH).

synthesis of these aminohydrazone derivatives and the evaluation of their activity against mycobacteria. Activity against Gram-positive and Gram-negative bacteria as well as their cytotoxicity were also evaluated.

2. Chemistry

Isonicotinoylhydrazones **2** and their pyridylmethyleneamino derivatives **3**, **4** and **5** described in this study are shown in *tables I* and *II*, and a reaction sequence for their preparation is outlined in *figure 1*. The starting aryloxyacetonitriles **1** were prepared according to known procedures [13]. Aryloxyacetonitriles **1** were converted to hydrazones **2** by addition of isonicotinoylhydrazine in the presence of catalytic amounts of sodium ethoxide in anhydrous ethanolic solution.

Heating compounds **2** with pyridinecarboxaldehyde in ethanolic solution in the presence of piperidine afforded pyridylmethyleneamino derivatives **3**–**5**.

The structures for all new obtained compounds **2–5** were determined by examining their IR and ^{1}H-NMR spectra as well as by elemental analyses (*tables I* and *II*).

3. Microbiology

Antimycobacterial activity was investigated against *M. tuberculosis* H37Rv ATCC 25584, *M. avium* ATCC 19421 and *M. Fortuitum* ATCC 9820, and *M. tuberculosis* resistant to isoniazid (INH-R) isolated from a patient with an active clinical infection treated at the Università di Cagliari. The antimicrobial activity of compounds **2**–**5** was also evaluated against Gram-positive and Gram-negative bacteria isolated from clinical specimens and *Candida albicans* ATCC E10931. At the same time, cell cytotoxicity of all compounds was tested in vitro on Vero cells.

4. Results and discussion

With the exception of compound **2f** which showed toxicity at concentrations higher than 62.5 μg/mL, the tested compounds exhibited high values of maximum non-toxic dose (MNTD) on Vero cells, ranging from 250 μg/mL for **4j**, to 500–1 000 μg/mL for the other members of the series.

Table II. Chemical and spectral data of compounds **3**, **4** and **5**.

Compound	R	Pyr	Yield (%)	M.p. (°C) (recryst. solvent)	IR (Nujol) ν (cm^{-1})	^{1}H-NMR (DMSO-d_6 /TMS) δ (ppm)
3a	H	2-pyridyl	42	168–169 (Benzene)	3 050, 1 610, 1 590, 1 540	4.74 (s, 2H, CH_2), 6.64 (s, 1H, CH), 6.93–7.81 (m, 10H, Ar), 8.54 (s, 1H, NH), 8.63 (m, 3H, Py)
4a	H	3-pyridyl	55	164–165 (2-PrOH)	3 180, 3 030, 1 615, 1 600, 1 550	4.81 (s, 2H, CH_2), 6.75 (s, 1H, CH), 6.93–8.62 (m, 13H, Ar), 8.68 (s, 1H, NH)
5a	H	4-pyridyl	63	190–191 (Benzene)	3 070, 1 620, 1 595	4.80 (s, 2H, CH_2), 6.69 (s, 1H, CH), 6.93–8.64 (m, 13H, Ar), 8.71 (s, 1H, NH)
5b	2-CH_3	4-pyridyl	48	162–163 (Toluene)	3 360, 3 020, 1 620, 1 590	2.13 (s, 3H, CH_3), 4.81 (s, 2H, CH_2), 6.70 (s, 1H, CH), 6.83–8.64 (m, 13H, Ar + NH)
4c	3-CH_3	3-pyridyl	57	124–125 (Toluene)	3 060, 1 610, 1 540	2.24 (s, 3H, CH_3), 4.81 (s, 2H, CH_2), 6.78 (s, 1H, CH), 6.80–8.68 (m, 13H, Ar + NH)
5c	3-CH_3	4-pyridyl	59	174–175 (Toluene)	3 060, 1 620, 1 540	2.23 (s, 3H, CH_3), 4.79 (s, 2H, CH_2), 6.70 (s, 1H, CH), 6.78–8.65 (m, 12H, Ar), 8.71 (s, 1H, NH)
4d	4-CH_3	3-pyridyl	64	152–153 (Toluene)	3 020, 1 620, 1 600, 1 540	2.19 (s, 3H, CH_3), 4.77 (s, 2H, CH_2), 6.74 (s, 1H, CH), 6.86–8.74 (m, 13H, Ar + NH)
5d	4-CH_3	4-pyridyl	56	159–160 (Toluene)	3 060, 1 620, 1 560	2.22 (s, 3H, CH_3), 4.78 (s, 2H, CH_2), 6.70 (s, 1H, CH), 6.88–8.66 (m, 12H, Ar), 8.72 (s, 1H, NH)
4e	2-OCH_3	3-pyridyl	68	134–135 (Toluene)	3 080, 3 020, 1 630, 1 590	3.74 (s, 3H, CH_3), 4.80 (s, 2H, CH_2), 6.79 (s, 1H, CH), 6.87–8.65 (m, 13H, Ar + NH)
5e	2-OCH_3	4-pyridyl	67	129–130 (Toluene)	1 630, 1 600	3.72 (s, 3H, CH_3), 4.76 (s, 2H, CH_2), 6.71 (s, 1H, CH), 6.83–8.64 (m, 13H, Ar + NH)
4f	3-OCH_3	3-pyridyl	50	137–138 (Toluene)	3 160, 3 060, 1 640, 1 590	3.66 (s, 3H, CH_3), 4.79 (s, 2H, CH_2), 6.51–9.13 (m, 14H, Ar + NH + CH)
5f	3-OCH_3	4-pyridyl	52	157–158 (Toluene)	3 140, 3 080, 1 620, 1 600	3.66 (s, 3H, CH_3), 4.79 (s, 2H, CH_2), 6.70–8.63 (m, 13H, Ar + CH), 8.71 (s, 1H, NH)
4g	4-OCH_3	3-pyridyl	72	123–124 (Toluene)	3 150, 3 020, 1 620, 1 580	3.67 (s, 3H, CH_3), 4.75 (s, 2H, CH_2), 6.75 (s, 1H, CH), 6.83–8.64 (m, 12H, Ar), 8.67 (s, 1H, NH)
5g	4-OCH_3	4-pyridyl	60	129–130 (Toluene)	3 100, 1 630, 1 620, 1 595	3.65 (s, 3H, CH_3), 4.73 (s, 2H, CH_2), 6.68 (s, 1H, CH), 6.81–8.64 (m, 12H, Ar), 8.69 (s, 1H, NH)
4h	2-Cl	3-pyridyl	60	173–174 (Toluene)	3 350, 1 630, 1 610, 1 550	4.92 (s, 2H, CH_2), 6.78 (s, 1H, CH), 6.95–8.65 (m, 13H, Ar + NH)
5h	2-Cl	4-pyridyl	50	184–185 (Toluene)	3 340, 3 020, 1 620, 1 590, 1 540	4.90 (s, 2H, CH_2), 6.71 (s, 1H, CH), 6.95–8.63 (m, 12H, Ar), 8.68 (s, 1H, NH)
4i	3-Cl	3-pyridyl	51	139–140 (Toluene)	3 060, 1 620, 1 590, 1 550	4.88 (s, 2H, CH_2), 6.76 (s, 1H, CH), 6.96–8.73 (m, 13H, Ar + NH)
5i	3-Cl	4-pyridyl	67	139–140 (Toluene)	3 060, 1 620, 1 590, 1 540	4.88 (s, 2H, CH_2), 6.72 (s, 1H, CH), 6.97–8.66 (m, 12H, Ar), 8.74 (s, 1H, NH)
4j	4-Cl	3-pyridyl	20	164–165 (Ethyl acetate)	3 100, 3 020, 1 630, 1 610, 1 550	4.84 (s, 2H, CH_2), 6.75 (s, 1H, CH), 7.00–8.62 (m, 12H, Ar), 8.67 (s, 1H, NH)
5j	4-Cl	4-pyridyl	51	159–160 (Toluene)	1 600, 1 550	4.82 (s, 2H, CH_2), 6.69 (s, 1H, CH), 6.99–8.64 (m, 12H, Ar), 8.71 (s, 1H, NH)
4k	4-NO_2	3-pyridyl	65	189–190 (2-PrOH)	1 620, 1 580, 1 500	5.04 (s, 2H, CH_2), 6.79 (s, 1H, CH), 7.20–8.63 (m, 12H, Ar), 8.74 (s, 1H, NH)
5k	4-NO_2	4-pyridyl	62	184–185 (2-PrOH)	1 600, 1 580, 1 540	5.02 (s, 2H, CH_2), 6.73 (s, 1H, CH), 7.19–8.64 (m, 12H, Ar), 8.78 (s, 1H, NH)

The tested compounds showed activity against mycobacteria with MIC values ranging from 6.25 μg/mL to over 100 μg/mL. MIC values as well as the results of cytotoxicity assays are reported in *table III*. The most active compounds against *M. tuberculosis* H37Rv were **2d**, **2j** and **2k** (MIC 6.25 μg/ml). However, a significant activity was also exhibited by compounds **2c**, **2f**, **2g** and **2i** (MIC 12.5 μg/mL) and **2b**, **2h** and **4a** (MIC 25 μg/mL). The most effective compounds in the inhibition of growth of *M. tuberculosis* INH-R were **2g**, **2j** and **2k** (MIC 25 μg/mL). All the tested compounds showed a higher activity against *M. tuberculosis* H37Rv than *M.*

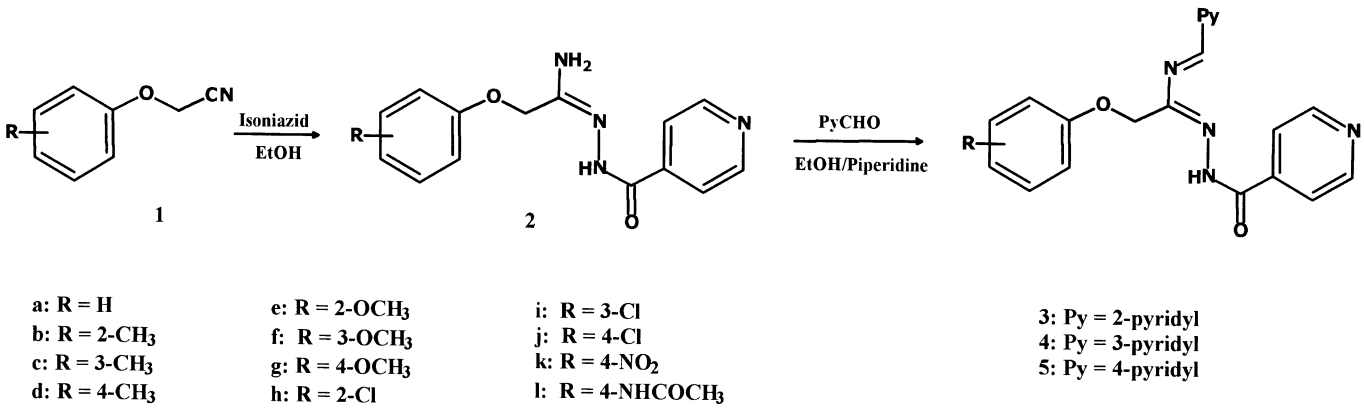

Figure 1. Synthetic pathways to compounds **2**, **3**, **4** and **5**.

tuberculosis INH-R, with the exception of compounds **4j**, **5g** and **5j** that were inactive against *M. tuberculosis* H37Rv (MIC > 100 μg/mL) but showed a weak activity against *M. tuberculosis* INH-R (MIC 50μg/mL). Isonycotinoylhydrazones **2g**, **2j** and **2k** were the most active compounds in the whole series, with potencies superior to INH against *M. tuberculosis* INH-R. Although none of the tested compounds exhibited a significant activity against *M. fortuitum*, we observed a poor activity by **2g** and **2i** (MIC 100 μg/mL) against *M. avium*.

Compounds **2–5** were inactive at inhibiting the growth of the Gram-negative species tested. A weak activity against *Staphyloccocus aureus* was shown by compounds **2h**, **2i** and **2j** (MIC 100 μg/mL) and by compound **3a** against *C. albicans* (MIC 100 μg/mL).

From a first examination of these results, it appears that compounds **2**, containing the NH_2 group, show better activity against *M. tuberculosis* H37Rv and *M. tuberculosis* INH-R with respect to derivatives **3**, **4** and **5**. Further studies to acquire more information about structure-activity relationships are in progress in our laboratories.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler hot stage and are uncorrected. IR spectra were recorded on Nujol mulls between salt plates in a Perkin-Elmer 398 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian Unity 300 spectrometer. Elemental analyses were carried out with a Carlo Erba Model 1106 Elemental Analyzer.

5.1.1. 2-Amino-2-(isonicotinoylhydrazono)ethyl aryl ethers **2**

General Procedure: to a stirred solution of NaOEt (0.005 mol) in dry EtOH (5 mL) the appropriate aryloxyacetonitrile **1** (0.05 mol) was added. The mixture was stirred at room temperature for 1 h and after cooling to 0 °C, an ethanolic solution of INH (6.8 g, 0.05 mol) was added dropwise. The resulting solution was stirred at the

Table III. Cytotoxicity and antimycobacterial activity of compounds **2**–**5**.

Compound	MNTD (μg/mL)	MIC (μg/mL)		
	Vero cells	*M. tuberculosis* H37Rv ATCC 25584	*M. tuberculosis* INH-R	*M. avium* ATCC 19421
2b	1 000	25	100	> 100
2c	1 000	12.5	50	> 100
2d	500	6.25	50	> 100
2f	62.5	12.5	100	> 100
2g	500	12.5	25	100
2h	500	25	100	> 100
2i	1 000	12.5	50	100
2j	500	6.25	25	> 100
2k	1 000	6.25	25	> 100
2l	1 000	50	> 100	> 100
4a	1 000	25	50	> 100
4j	250	> 100	50	> 100
4k	1 000	50	100	> 100
5c	1 000	50	> 100	> 100
5g	500	> 100	50	> 100
5i	500	50	50	> 100
5j	500	> 100	50	> 100
INH	1 000	0.06	50	100

same temperature for 2 h and then allowed to reach room temperature and to stand overnight. The formed precipitate was collected by filtration and recrystallised from the solvent shown in *table I*.

5.1.2. 2-(Pyridylmethyleneamino)-2-(isonicotinoylhydrazono)ethyl aryl ethers **3**, **4** *and* **5**

General procedure: to a solution of compounds **2** (1.85 mmol) and 2-, 3- or 4-pyridinecarboxaldehyde (1.85 mmol) in EtOH (15 mL), a few drops of piperidine were added. After heating at reflux for 6 h the solvent was removed in vacuo and the residue treated with H_2O (20 mL) and extracted with $CHCl_3$ (3 × 10 mL). The organic layers were dried (Na_2SO_4) and the solvent evaporated to give the crude **3**, **4** and **5** that were purified by crystallisation from the solvent indicated in *table II*.

5.2. Microbiology

For the antimicrobial and cytotoxicity studies, compounds were dissolved in DMSO at 10 mg/mL and kept at –20 °C. The working solutions were prepared in the same medium used for the tests. To avoid interference by the solvent [14], the highest DMSO concentration was 1%. The MICs of various compounds against Gram-positive, Gram-negative and *C. albicans* were determined by a standard broth serial dilution method [15, 16].

5.2.1. Antibacterial assay

The antimicrobial activity of compounds **2**–**5** was evaluated against six Gram-positive and five Gram-negative species isolated at the Sezione di microbiologia e virologia, Dipartimento di Scienze Chirurgiche e Trapianti d'Organo, from clinical specimens obtained from patients treated at the Università di Cagliari. In particular, compounds **2**–**5** were tested against *Staphylococcus aureus* (isolated from urine), *S. epidermidis* (isolated from urine by suprapubic aspirate), *Streptococcus agalactiae* (isolated from vaginal swab), *S. faecalis* (isolated from urine), *Bacillus licheniformis* (isolated from blood), *B. subtilis* (isolated from eye swab), *Escherichia coli* (isolated from urine), *Pseudomonas aeruginosa* (isolated from urine), *Salmonella typhi* (isolated from faeces), *Proteus mirabilis* (isolated from urine) and *Klebsiella pneumoniae* (isolated from urine). Tests with Gram-positive and Gram-negative bacteria were carried out in Mueller Hinton broth (Difco). The compounds were diluted in the test medium to obtain final concentrations ranging from 100–0.19 μg/mL. Tubes were inoculated with 1×10^5 cells/mL and were incubated at 37 °C for 18 or 24 h. The effects on the growth of mycobacteria was investigated against *M. tuberculosis* H37Rv ATCC 25584, *M. avium* ATCC 19421, *M. fortuitum* ATCC 9820 and *M. tuberculosis* resistant to isoniazid (INH-R) isolated from a patient with an active clinical infection. The determination of MICs against mycobacteria were carried out by the two-fold agar dilution method [17] using 7H11 agar (Difco Laboratories) containing compounds **2**–**5** at concentrations that ranged between 100–0.19 μg/mL, on which 100 μL of the test bacterial suspension were spotted. Suspensions to be used for drug susceptibility testing were prepared from 7H9 broth cultures supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) enrichment (Difco Laboratories) and 0.05% (v/v) Tween 80 to avoid clumping. Cells were then washed, suspended in saline, shaken and sonicated in a bath type ultrasonicator (output power 80 W) until visibile clumps were disrupted (usually from 15–30 s). Suspensions were then diluted in saline to a turbidity of no. 1 McFarland (*M. tuberculosis*) or no. 0.5 McFarland (*M. avium* and *M. fortuitum*) and then diluted to obtain inocula of 3×10^5 cells per well of *M. tuberculosis* and 1.5×10^4 cells per well of *M. avium* and *M. fortuitum*. The MICs of the compounds were determined after 7 (*M. fortuitum*) or 21 (slow growers) days of cultivation at 37 °C in a CO_2 (5% CO_2/95% humidified air) incubator.

5.2.2. Antifungal assay

For the evaluation of the antifungal activity, *Candida albicans* ATCC E10931 was employed. Antifungal activity against *C. Albicans* ATCC E10231 was evaluated in yeast extract peptone dextrose medium (Difco) [18].

5.2.3. In vitro cytotoxicity assay

Cell cytotoxicity of compounds **2**–**5** was tested in vitro by two methods. In the first method, RPMI 1640 medium (Gibco) with 2% foetal calf serum (FCS, Gibco) alone, or RPMI 1640 with 2% FCS containing compounds at concentrations ranging from 1 000–62.5 μg/mL, were inoculated onto cultures of Vero cells in 6 well tissue culture plates. The cells were observed daily for 6 days for any sign of cell cytotoxicity compared with the controls. In the second method, a cell viability assay previously reported [19, 20] was used. Monolayers of Vero cells in 96 multiwell plates were incubated with the testing compounds at concentrations of 1 000–62.5 μg/mL in RPMI 1640 with 5% FCS for 48 h and the medium replaced with 50 μL of 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in RPMI without phenol red. Cells were incubated at 37 °C for 3 h, the untransformed MTT removed and 0.04 N HCl isopropanolic solution (50 μL) was added to each well. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were

read using an automatic plate reader with a 650 nm test wavelength and a 690 nm reference wavelength.

Acknowledgements

Financial support from the Ministero della Università e Ricerca Scientifica e Tecnologica is gratefully acknowledged.

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