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Bioorganic & Medicinal Chemistry Letters 14 (2004) 5731–5733
Bioorganic & Medicinal Chemistry Letters

In vitro advanced antimycobacterial screening of cobalt(II) and copper(II) complexes of fluorinated isonicotinoylhydrazones

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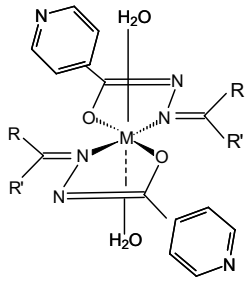
Received 21 May 2004; revised 14 September 2004; accepted 20 September 2004
Available online 7 October 2004

Abstract—The in vitro antimycobacterial activity of cobalt(II) and copper(II) complexes of some fluorinated isonicotinoylhydrazones was evaluated in a TB-infected macrophage model; all metalcomplexes exhibited excellent activity against *Mycobacterium tuberculosis* Erdman growing within macrophages, at concentrations much lower than in culture media. Moreover complexes **1b** and **2a** displayed EC_{99} values lower than that of the parent-drug, isoniazid. In addition, all tested metalchelates significantly inhibited the growth of single-drug-resistant *M. tuberculosis* strains; complexes **1a** and **2a** also possessed moderate activity against *Mycobacterium avium* complex.

In recent years the incidence of tuberculosis (TB) in both developing and industrialized countries has greatly increased. In spite of the availability of effective chemotherapy, TB is presently considered to be one of the most dangerous infective diseases throughout the world. The global resurgence of TB has been favoured by: (a) the diffusion of the human immunodeficiency virus (HIV) and the deadly synergy of TB and nontubercular mycobacterial infections with HIV;[1–4] (b) the widespread emergence of resistant strains of *Mycobacterium tuberculosis*, which are insensitive to one or more of the first-line anti-TB drugs.[5–11] Therefore the need to search for new antimycobacterial agents is critical.

In an effort to improve and extend the very selective activity spectrum of isoniazid (INH) also to resistant *M. tuberculosis* strains and nontubercular mycobacteria (NTM), in the last few years we have synthesized and assayed different series of lipophilic analogues of INH; several of them, particularly fluorinated isonicotinoylhydrazones (ISNEs) and isonicotinohydrazides, exhibited good in vitro antitubercular activity.[12–18]

In this context, we used some active fluorinated ISNEs as polydentate ligands to synthesize cobalt(II) and copper(II) complexes (**1**, **2**, Fig. 1) of stoichiometry $[M(ISNE)_2(H_2O)_2]$ that we have recently reported.[12,13,19] In the octahedral structure of metalchelates **1**, **2**, the polar 'core', consisting of the metal ion and the electronegative atoms of ISNEs involved in coordination, is surrounded by the hydrophobic moieties of the ligands, which form an outer lipophilic envelope (Fig. 1). According to our design, such an arrangement should facilitate the diffusion through biomembranes, thus enhancing the antimycobacterial effectiveness of ISNEs. In addition, we hypothesized that these metalchelates should act as repositories for the active ligands and could thus be potentially longer-acting antimycobacterial agents.[13]



1 M = CoII
2 M = CuII

a R = H, R' = 3F-C_6H_4
b R = H, R' = 3CF_3-C_6H_4
c R = H, R' = 3F,4OCH_3-C_6H_3

Figure 1.

Keywords: Antimycobacterial activity; Metalcomplexes; Isoniazid derivatives.
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doi:10.1016/j.bmcl.2004.09.052

The in vitro antimycobacterial activity of our compounds was assayed by the Tuberculosis Antimicrobial Acquisition Coordinating Facility (TAACF) antituberculosis drug discovery programme,[9] coordinated by the Southern Research Institute (Birmingham, AL, USA) under the direction of the National Institute of Allergy and Infectious Diseases. In the TAACF primary screening metalcomplexes **1**, **2** had exhibited very good in vitro activity against *M. tuberculosis* H37Rv with MIC values ranging between <0.1 and 0.2 μg/mL. Their in vitro cytotoxicity in VERO cells was low, with very favourable selectivity indexes (SI = IC_{50}/MIC > 50).[12,13]

According to the TAACF protocols, compounds with MICs ⩽ 6.25 μg/mL and SIs ⩾ 10 were submitted to further assays in order to investigate the antimycobacterial properties of the new agents more extensively.[9] Herein we report on the results of this advanced screening of metalchelates **1**, **2**, consisting in the in vitro evaluation of antimycobacterial activity in a TB-infected macrophage model and against single-drug-resistant (SDR) *M. tuberculosis* strains as well as against *M. avium* complex (MAC).

Metalchelates **1a**–**c**, **2a** and **2c** (Fig. 1) were tested for killing of *M. tuberculosis* Erdman (ATCC 35801) in monolayers of mouse bone marrow macrophages, as previously described,[20] at fourfold concentrations equivalent to 0.25, 1, 4 and 16 times the MIC. EC_{90} and EC_{99} are the lowest concentrations effecting 90% and 99% reductions in colony forming units (CFU), respectively, at seven days compared to drug-free controls. Since *M. tuberculosis* is an intracellular parasite living and multiplying inside macrophages, this assay is an useful method for determining the capacity of new antimycobacterial agents to inhibit the growth of mycobacteria within this relevant intracellular environment. In fact, many factors can be considered that influence the activity of a drug within the infected host cell but are not reflected in a broth culture assay (e.g., transport mechanisms, chemical modification in the macrophage cytoplasm, interference with macrophage functions, potential cytotoxic side effects).[20–22]

In this assay all tested chelates **1**, **2** exhibited excellent activity, expressed by EC_{90} values ranging between 0.045 and 0.28 μg/mL and EC_{99} values ranging between 0.206 and 0.72 μg/mL, whereas in broth culture their MICs against the same *M. tuberculosis* strain ranged between 0.2 and 6.25 μg/mL (Table 1). In particular, **1b** and **2a** were 140- and at least 50-fold more effective in macrophages than in culture media, respectively, and, interestingly, their EC_{99}s were lower than those of INH and rifampin, used as reference drugs. Complexes **1c** and **2c** also exhibited EC_{99}s similar to that of INH and lower than that of rifampin (Table 1).

MICs against a panel of SDR strains (i.e., each strain being resistant to a single anti-TB drug), typically an *M. tuberculosis* strain resistant to isoniazid (ATCC 35822), a strain resistant to rifampin (ATCC 35838) and one or more additional SDR strains, were then determined using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA).[23,24] In this assay all tested complexes **1**, **2** showed high activity levels against rifampin-, ethambutol-, kanamycin- and ciprofloxacin-resistant *M. tuberculosis* strains (Table 1). Instead, they revealed cross-resistance with INH, as expected since they are INH derivatives.

In addition, compounds **1a**–**c** and **2a** exhibited bactericidal activity against *M. tuberculosis* H37Rv and rifampin-resistant *M. tuberculosis*[25] with MBCs rather low and close to MICs (Table 1); the bactericidal potency of **1b** against H37Rv (MBC = 0.39 μg/mL) was at least 8-fold greater than that of the corresponding free ligand (MBC > 3.2 μg/mL).[15]

The TAACF antimycobacterial investigation includes the evaluation of new agents against *M. avium* complex, which is an emergent dangerous NTM responsible for frequent lethal infections in terminal AIDS patients.[4,9] In the primary screening, MICs were determined in the MABA against a strain of *Mycobacterium avium* (ATCC 25291) at the single concentration of 6.25 μg/mL. At this concentration, compounds **2a** and **1a** produced 98% and 68% inhibition, respectively, whereas the other analogues showed MICs higher than 6.25 μg/mL. It is worth noting that INH displayed MIC > 32 μg/mL against the same strain.[23] Complexes **1a** and **2a** were moved forward for evaluation in additional *M. avium* assays; they were tested at a range between 0.25 and 32 μg/mL

Table 1. In vitro antimycobacterial activity of compounds **1**, **2**

Compound	MIC[a]	EC_{90}[b]	EC_{99}[c]	EC_{90}/MIC		MIC[a]					MBC[a]		
	Erdman			Erdman[d]	H37Rv[d]	INH-R	RIF-R	EMB-R	KAN-R	CIP-R	H37Rv	INH-R	RIF-R
1a	1.6	0.28	0.72	0.175	2.8	>3.25	0.2	0.1	0.2	0.8	0.8	>3.25	0.2
1b	6.25	0.045	0.26	0.0072	0.23	>6.25	0.2	0.1	0.2	0.78	0.39	>6.25	0.1
1c	0.78	0.053	0.4	0.068	0.27	>6.25	0.39	0.39	0.39	1.56	0.39	>6.25	1.56
2a	>3.25	0.069	0.206	<0.021	0.69	>3.25	0.1	0.1	0.2	0.8	1.6	>3.25	0.05
2c	0.2	0.066	0.323	0.33	0.66	>3.25	0.2	0.1	0.1	0.2	—	—	—
INH	—	0.03	0.42	—	0.6–1.2	>0.2	—	—	—	—	0.1	>1.6	—
RMP	—	0.04–0.1	0.5–1.5	—	0.16–1.67	—	—	—	—	—	—	—	—

INH-R = isoniazid-resistant; RIF-R = rifampin-resistant; EMB-R = ethambutol-resistant; KAN-R = kanamycin-resistant; CIP-R = ciprofloxacin-resistant.

[a] MIC and MBC values are expressed in μg/mL.

[b,c] EC_{90} and EC_{99} are defined as the concentrations (μg/mL) effecting 90% and 99% reduction in CFU, respectively, at 7 days compared to drug-free controls.

[d] Compounds with EC_{90}/MIC < 16 are considered active.

against five *M. avium* clinical isolates (labelled as 100, 101, 108, 109, 116) in Middlebrook 7H9 broth using the MABA and BACTEC 460 systems.[23,24] Strains 100, 101, 109 represent serotypes frequently encountered in AIDS patients.[9] However, in this latter test **1a** and **2a** showed less in vitro activity, with MICs ranging between 16 and >32 μg/mL.

In conclusion, taken together the results of this advanced TAACF screening allowed us to define the interesting in vitro antimycobacterial profiles of cobalt(II) and copper(II) complexes of fluorinated ISNEs. The cobalt(II) complex of 3-trifluoromethylbenzaldehyde isonicotinoylhydrazone (**1b**) was shown to possess the most promising antitubercular profile since: (a) it was 140 times more potent against bacilli growing within macrophages than in broth culture, with an EC_{99} value lower than that of parent-drug INH; (b) it exhibited bactericidal properties against H37Rv with MBC at least 8 times lower than that of the free ligand.

Chelate **2a** was also shown to be at least 50-fold more active in the macrophage model than in culture media, its EC_{99} value being lower than that of INH; in addition it showed moderate inhibitory activity against MAC.

Although the corresponding free ligand 3-trifluoromethyl- and 3-fluorobenzaldehyde isonicotinoylhydrazones also proved to be more active against bacilli growing within macrophages than in broth culture (4 and 36 times, respectively),[15] the increase in effectiveness was much more significant for metalchelates **1b** and **2a**. These findings corroborated our initial hypothesis that the coordination of active ISNEs to a metal ion could ameliorate their antimycobacterial profile, particularly favouring the uptake within cells and enhancing ability to kill intracellular mycobacteria, which live and multiply inside macrophages.

Acknowledgements

The authors gratefully acknowledge the staff of the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF), coordinated by the Southern Research Institute (Birmingham, AL) under the direction of the US National Institute of Allergy and Infectious Diseases.

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