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Original article

Biological evaluation of bisbenzaldehydes against four *Mycobacterium* species

Davie Cappoen [a,1], Delphine Forge [b,1], Frank Vercammen [a], Vanessa Mathys [c], Mehdi Kiass [c], Virginie Roupie [a], Roel Anthonissen [d], Luc Verschaeve [d,e], Jean Jacques Vanden Eynde [b], Kris Huygen [a,*]

[a] *Program Host-Pathogen Interactions, Service Immunology, O.D. Communicable & Infectious Diseases, Scientific Institute of Public Health WIV-ISP (Site Ukkel), Engelandstraat 642, B-1180 Ukkel, Belgium*
[b] *Laboratory of Organic Chemistry, University of Mons, UMONS, place du Parc 23, B-7000 Mons, Belgium*
[c] *Program Tuberculosis and Mycobacteria, Service Bacterial Diseases, O.D. Communicable & Infectious Diseases, Scientific Institute of Public Health (Site Ukkel), Engelandstraat 642, B-1180 Ukkel, Belgium*
[d] *Service Toxicology, O.D. Public Health and Surveillance, Scientific Institute of Public Health (Site Elsene), J. Wytsmanstraat 14, B-1050 Brussels, Belgium*
[e] *Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

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ABSTRACT

A series of bisbenzaldehydes and structurally related analogs, conveniently synthesized via microwave-assisted reactions, were evaluated *in vitro* against drug susceptible and multi-drug resistant *Mycobacterium tuberculosis*, against virulent *Mycobacterium bovis*, against *Mycobacterium ulcerans* and against two *Mycobacterium avium* subspecies. Among the 33 substances that were tested, compound **12**, i.e. 4,4′-[1,12-dodecanediyl(oxy)]bisbenzaldehyde, emerged as the most promising hit. Its activity was further confirmed in an intracellular growth inhibition assay of *M. tb* in murine J774 A.1 macrophages. None of the compounds showed significant cytotoxicity on human C3A hepatocytes in a neutral red dye uptake assay and no genotoxicity or mutagenicity was observed as demonstrated by a VITOTOX™ test and confirmed with a comet assay.

1. Introduction

The control of tuberculosis (TB) remains a major challenge to health care worldwide. Despite the progress made in diagnosis, treatment, improved hygiene and overall welfare, eradication of this pulmonary disease has not yet been possible. Indeed, *Mycobacterium tuberculosis* (*M. tb*), the causative agent of TB has intrinsic and adaptive resistance mechanisms that reduce the effectiveness of many drugs [1]. Firstly, the cell wall of *M. tb* is composed of mycolic acids, long carbon-chain fatty acids unique to mycobacteria, which limit penetration of most commonly used broad spectrum antibiotics into the bacterial cytoplasm [2,3]. Secondly, the bacteria can survive harsh conditions within host macrophages by shifting to a less active metabolic state. The latter induces generation of dormant bacteria ultimately leading to metabolically inactive persister cells that withstand conventional chemotherapy [4–7]. The actual first line drug regimen is still the same as it was 40 years ago: it consists of a combination of four drugs, namely isoniazid, rifampicin, ethambutol and pyrazinamide. In addition, medication must be taken for at least 6–18 months [8]. This puts a heavy burden both on patients and on public health systems, especially in less developed regions. The long duration of the treatment by itself can also lead to lack of compliance and inadequate drug dosage, resulting in the selection of multi-drug resistant (MDR) and extensively drug resistant (XDR)-strains. MDR-strains are resistant to at least isoniazid and rifampicin whereas XDR-strains show additional resistance to one fluoroquinolone and one of the three second-line injectable anti-TB drugs (amikacin, capreomycin, and kanamycin) [1,8,9]. Consequently, there is still obvious need for the identification of novel anti-*M. tuberculosis* candidates characterized by original structures, higher efficiency and good tolerability.

The activity of benzaldehydes against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* has

* Corresponding author. Tel.: +32 2 373 33 70; fax: +32 2 373 33 67.
E-mail address: kris.huygen@wiv-isp.be (K. Huygen).
[1] Both authors contributed equally.

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been reported previously by Friedman et al. In their study a 50% reduction in bacterial growth was observed at submicromolar concentrations [10]. This observation prompted us to evaluate the anti-mycobacterial effects of a library of bisbenzaldehydes and analogs available in the UMONS laboratory. Here, we report on the results of a screening of 33 such derivatives against drug susceptible *M. tb* and MDR LAM-1 *M. tb* [11]. The most promising candidates were further evaluated for their activity against *Mycobacterium bovis*, *Mycobacterium avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *Mycobacterium ulcerans*. *M. bovis*, together with *M. tb*, *Mycobacterium africanum* and *Mycobacterium microti*, is a member of the *M. tuberculosis* complex [12]. *M. avium* subsp. *avium* is pathogenic for birds and can cause opportunistic infections in AIDS patients. *M. avium* subsp. *paratuberculosis* is the etiological agent of Johne's disease or paratuberculosis (in ruminants) that has been linked to Crohn's disease in humans [13,14]. *M. ulcerans* finally causes Buruli ulcer, a necrotizing skin disease affecting mostly children in certain rural areas of West-Africa and which may lead to irreversible disabilities when left untreated [15].

2. Results

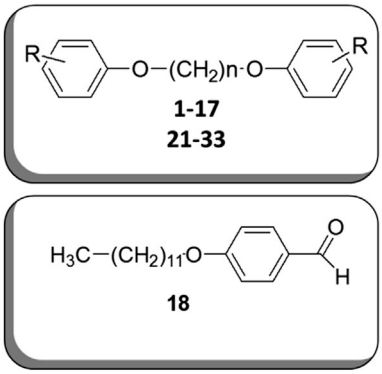
2.1. Chemistry

The structure of the 33 compounds involved in this study is described in Fig. 1. All derivatives, except **18**, **19** and **20**, are articulated on a linear chain composed of 1–14 methylene groups. Both ends of the chains are linked through an ether function to unsubstituted aromatic rings (**17**) or to aromatic rings bearing a carboxaldehyde (**1**–**16**), a nitro (**12**), or a carboxylic acid (**22**–**33**) moiety. Substance **18** is a monoaldehyde whereas **19** and **20** are bisbenzaldehydes in which the linker contains an additional hydrophilic site under the form of an ether (**19**) or an alcohol (**20**) respectively. Synthesis of the compounds has been described previously [16,17]. Briefly, the compounds were produced by a bimolecular nucleophilic substitution (SN_2) of an α,ω-dibromoalkane (or a bromoalkane for the synthesis of **18**) by the appropriate hydroxyaryl precursor in the presence of a base in boiling alcohol. Interestingly, reactions could be carried out advantageously under microwave irradiation, which enabled to reduce heating periods to a few minutes [17].

2.2. Biological activity assays

We have previously shown that luminometry is a rapid, cheap and reproducible tool to test the efficacy of novel antimycobacterial compounds [11,18,19]. In this study, we made use of the luminescent *M. tb* H37Rv laboratory strain expressing the bacterial luciferase of *Vibrio harveyi* driven by the constitutive hsp60 promoter. Addition of the luciferase substrate *n*-undecanal produces a green–blue light (490 nm) the intensity of which is directly correlated with the number of live bacteria in the culture. A first screening of compounds **1**–**33** was performed at concentrations of 10 μM, 1 μM and 0.1 μM and the results are shown in Table 1.

At 10 μM a number of the derivatives displayed a 100% growth reduction. At 1 μM significant inhibition was only detected for compounds **1**–**13**, which are bis(oxybenzaldehyde)s. Compound **18**, the (4-(dodecyloxy)benzaldehyde) (monoaldehyde) was totally ineffective at 1 μM. The anti-bacterial activity was clearly dependent on the length of the carbon chain separating the aromatic rings. Substances **1**–**5** (1–5 methylene groups in the linker) were moderately active (25–41% growth inhibition at 1 μM) whereas potency significantly increased for compounds **6**–**11** (6–11 methylene groups in the linker; 59–73% growth inhibition). 4,4′-[1,12-Dodecanediylbis(oxy)]dibenzaldehyde (**12**) was the most effective compound exhibiting a growth inhibition of 81%. Further elongation



	n	R		n	R
1	1	CHO(*para*)	**16**	12	CHO (*meta*)
2	2	CHO(*para*)	**17**	12	H
3	3	CHO(*para*)	**21**	12	NO_2 (para)
4	4	CHO(*para*)	**22**	1	COOH(*para*)
5	5	CHO(*para*)	**23**	2	COOH(*para*)
6	6	CHO(*para*)	**24**	3	COOH(*para*)
7	7	CHO(*para*)	**25**	4	COOH (*para*)
8	8	CHO(*para*)	**26**	5	COOH(*para*)
9	9	CHO(*para*)	**27**	6	COOH(*para*)
10	10	CHO(*para*)	**28**	7	COOH(*para*)
11	11	CHO(*para*)	**29**	8	COOH(*para*)
12	12	CHO(*para*)	**30**	9	COOH(*para*)
13	14	CHO(*para*)	**31**	10	COOH(*para*)
14	5	CHO (*ortho*)	**32**	11	COOH(*para*)
15	5	CHO (*meta*)	**33**	12	COOH(*para*)

Fig. 1. Structure of the 33 compounds involved in this study.

Table 1
In vitro potency of compounds **1**–**33** against *M. tb* H37Rv.

Cmpd.	G.I. (%)[a]			MiLogP[b]
	10 μM	1 μM	0.1 μM	
1	85	30	<10	3.243
2	90	41	<10	3.256
3	84	35	<10	3.526
4	87	25	<10	3.797
5	95	39	<10	4.302
6	99	65	15	4.759
7	99	62	13	5.313
8	99	64	13	5.818
9	98	59	11	6.323
10	100	71	20	6.828
11	100	73	25	7.333
12	100	**81**	**26**	7.839
13	98	57	30	8.615
14	39	15	<10	4.206
15	97	<10	<10	4.254
16	<10	<10	<10	7.791
17	<10	<10	<10	8.230
18	<10	<10	<10	7.254
19	70	12	<10	3.052
20	60	<10	<10	2.611
21	91	<10	<10	8.162
22	40	<10	<10	3.485
23	38	<10	<10	3.497
24	<10	<10	<10	3.768
25	27	26	<10	4.039
26	27	30	<10	4.544
27	39	26	<10	5.049
28	38	14	<10	5.554
29	29	29	<10	6.059
30	17	17	<10	6.565
31	40	<10	<10	7.070
32	15	<10	<10	7.575
33	<10	<10	<10	8.077
INH[c]	100	100	98	−0.969

[a] Growth inhibition (GI) of luminescent *M. tb* H37Rv measured after 6 days of culture, as compared to untreated culture.
[b] MiLogP was calculated with the property calculation logarithm at www.molinspiration.com.
[c] INH, the positive control isoniazid, a commonly used first line TB antibiotic. Inhibition values calculated on cultures tested in triplicate (Standard deviation <10).

of the linker (**13**) did not improve the bactericidal effect any further. At 0.1 μM only compounds **6**–**13** exerted a modest anti-mycobacterial activity. Comparing the growth inhibition by compound **12** with that of compound **17** (4,4′-[1,12-dodecanediyl(oxy)] bisbenzene) indicated that removing both aldehyde substituents significantly diminished the anti-bacterial activity. Changing the aldehyde moieties for nitro groups (**21**) decreased the antimicrobial effect as well. Furthermore, moving the aldehyde groups from the *para*-position to the *meta* or *ortho* positions with regard to the ether bonds position (**14**–**16**) also decreased the antimycobacterial activity. Introduction of an additional hydrophilic site in the linker of the bisbenzaldehydes (**19**, **20**) almost completely abolished the activity at 1 μM concentration. Finally, compounds **22**–**33** in which the aldehyde residues were replaced by carboxylic acid functions, were found to be only weakly active at the highest concentration tested.

To extend the study, antimicrobial activity against a multi-drug resistant *M. tb* isolate was determined using the radiometric BACTEC 460 TB method (Fig. 2). This standardized radiometric assay relies on the ability of mycobacteria to produce $^{14}CO_2$ from ^{14}C labeled palmitic acid. The quantity of $^{14}CO_2$ expelled by the bacteria is measured and expressed as the growth index (G.I.), a measure for the bacterial growth. The MIC_{90} and MIC_{99} values of a compound can be deduced by comparing the G.I. of the *M. tb* culture exposed to compound concentration with the G.I. of an untreated control culture that is diluted 10 times (C/10) and 100 times (C/100) respectively. As resistant *M. tb* strain, a LAM-1 clinical isolate was selected. This MDR *M. tb* strain shows resistance against isoniazid, rifampicin, rifabutin and prothionamide. The susceptibility to compounds **9**–**12** was tested at concentrations ranging from 0.4 μM to 1.2 μM. A MIC_{90} between 0.6 μM and 0.4 μM was calculated for derivative **12** and between 0.6 μM and 0.8 μM for compounds **9**, **10** and **11**. Compounds **11** and **12** also inhibited the growth by 99% inhibition at concentrations between 1 μM and 1.2 μM.

In order to examine the selectivity for *M. tuberculosis* within the genus *Mycobacterium*, the MIC_{50} (the minimal concentration at which a growth inhibition of 50% is obtained) of compounds **1**–**12** was calculated for three other mycobacterial species: *M. bovis*, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. ulcerans* (Table 2). Confirming the results of the initial screening, submicromolar MIC_{5O} values were measured for compounds **6**–**12** when tested against *M. tb* H37Rv. The most active compound, 4,4′-[1,12-dodecanediyl(oxy)]bisbenzaldehyde (**12**) showed a 50% inhibition at 0.52 μM. Compounds **6**–**12** were also very effective against *M. bovis* and could reduce the bacterial growth by 50% at submicromolar concentrations. The compounds were five to tenfold less effective against both *M. avium* subspecies and their potency against *M. ulcerans* was even lower. Only compounds **11** and **12** inhibited the bacterial growth by 50% at concentrations of 9.8 μM and 8.2 μM respectively.

M. tuberculosis being an intracellular pathogen, we wished to determine whether the compounds could reach and target *M. tb* bacilli inside macrophages. In this assay a J774A.1 murine macrophage cell line was infected with the luminescent *M. tb* H37Rv strain at a multiplicity of infection of 1. After infection, the macrophages were treated with 10, 1 and 0.1 μM dilutions of the bisbenzaldehyde derivatives **1**–**12**. After 5 days of treatment the macrophages were lysed and the intracellular *M. tb* numbers were measured by luminometry. As shown in Table 3, a reduction of the bacterial load inside the infected macrophages could be observed with all twelve compounds. As in the axenic growth experiments, the length of the carbon chain linker separating both oxybenzaldehyde groups influenced their potency: longer carbon chain linkers resulted in stronger growth inhibition. The most active compound in this assay was again 4,4′-[1,12-dodecanediyl(oxy)]bisbenzaldehyde (**12**), which showed a 74.0% inhibition at 10 μM and 53.1% inhibition at 1 μM final concentration. Under similar conditions 97.7% and 82.2% inhibitions were determined for INH.

Second line drugs currently used for treatment of tuberculosis generally cause necrosis of the liver tissue [20]. Therefore, we studied acute toxicity of compounds **1**–**12** on human liver cells and more specifically on the C3A cell line. For that purpose we used a neutral red uptake assay (Table 4). This assay is based on the property of healthy viable cells to incorporate 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red) in their lysosomes. Reduction in this dye uptake is a reflection of the toxicity of the drug [20]. Overall, the derivatives displayed a very low toxicity and some reduction in viability of the hepatocytes was only detected at concentrations above 100 μM. Substances **4**, **5**, **6** and **7** displayed the highest toxicity towards the C3A cells. Only for 4,4′-[1,5-pentanediyl(oxy)]bisbenzaldehyde (**5**), viability of the C3A cells was below 50% at the highest concentration of 180 μM. Due to the limitation of the 1 percent concentration of the solvent DMSO permitted in this assay, higher concentrations than 180 μM could not be tested. Therefore, the concentration at which there is a reduction of neutral red uptake of 50% (NI_{50}) could not be observed for the derivatives. The Selectivity Index (S.I.) was calculated for the derivatives **1**–**12** by dividing the highest concentration tested by the MIC_{50} against *M. tb*.

Early signs of genotoxicity of the compounds can be observed by investigating the activation of DNA repair mechanisms. The

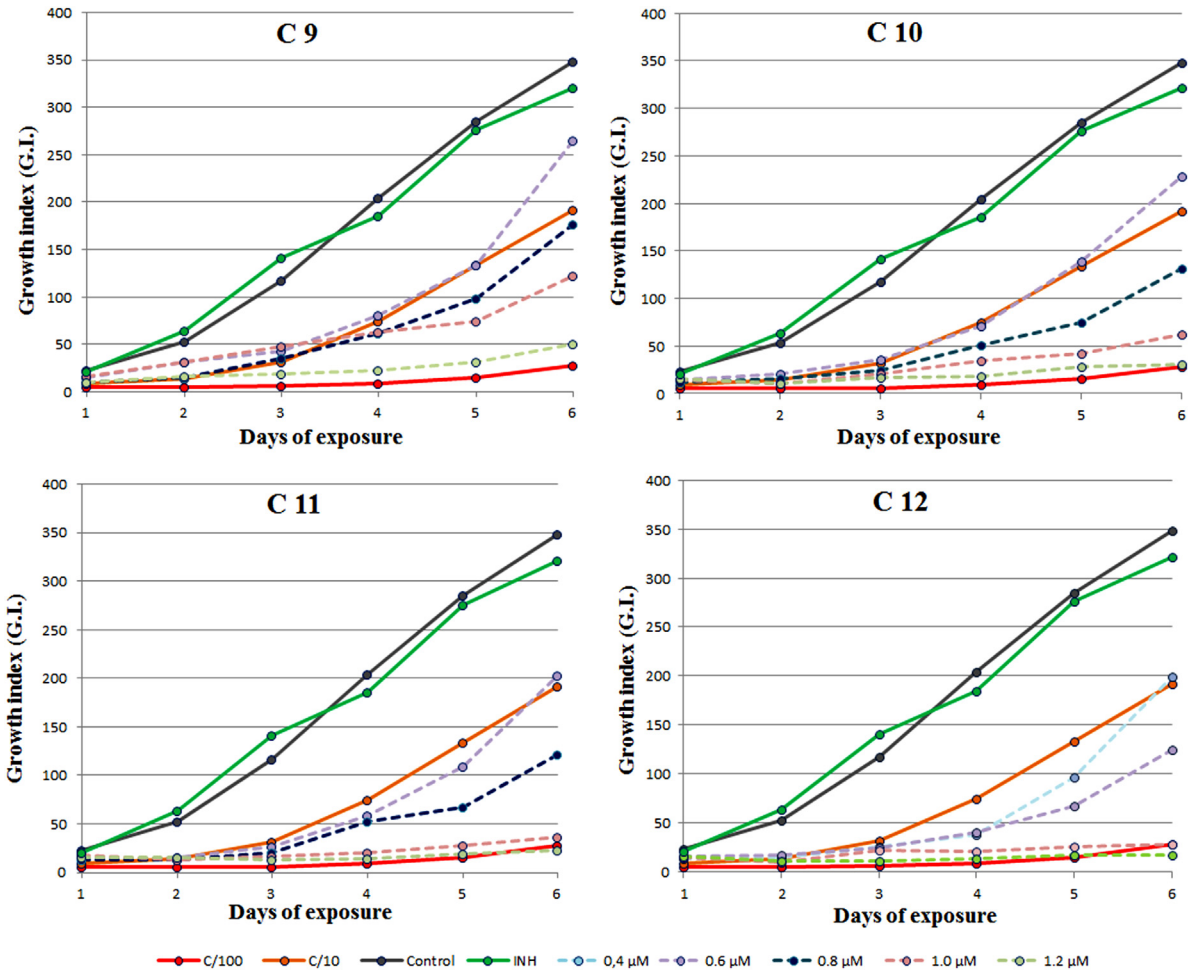


Fig. 2. Growth inhibition of a multi-drug resistant *M. tb* strain by compounds **9**–**12**. C/100 and C/10 were control samples diluted 100 times and 10 times respectively upon inoculation. INH, negative control culture treated with 0.1 μg/mL isoniazid to confirm the resistance of the *M. tb* strain.

regulatory upstream SOS operon is key to the repair of DNA damage and is activated by the binding of ssDNA fragments to the recA activator, promoting on its turn the transcription by the recN promotor and enabling the DNA repair mechanism. For the evaluation of the genotoxicity of the compound library, the VITOTOX assay was used, which employs two recombinant *Salmonella typhimurium* reporter strains and which is closely related to the AMES test [21]. The reporter strain used for the assessment of genotoxicity T104 (recN2-4) harbors a bacterial luxabcde operon originated from *Vibrio fisheri* under the transcriptional control of the recN promoter. Light produced by the bacterial luciferase increases when DNA damage occurs in the genox strain. To avoid possible false positives due to the direct action of compounds on the light production, a second cytox strain T104 (pr1) is incorporated in the assay

Table 2
MIC_{50} against *M. tb* and three other mycobacterial species.

	MIC_{50} [a] (μM)				
	M. tuberculosis H37Rv ATCC27294	*M. bovis* AN5	*M. avium* subsp. *avium* ATCC15769	*M. avium* subspp. *paratuberculosis* ATCC19698	*M. ulcerans* 1615
1	3	2.93	>10	>10	>10
2	2.23	2.41	>10	>10	>10
3	2.65	2.65	>10	>10	>10
4	3.21	2.51	>10	>10	>10
5	1.63	1.69	7.32	8.69	>10
6	0.83	0.94	6.18	6.56	>10
7	0.85	0.97	5.12	5.05	>10
8	0.85	0.95	5.36	5.83	>10
9	0.95	1.01	5.32	5.95	>10
10	0.77	0.81	5.61	5.45	>10
11	0.64	0.71	4.96	4.23	9.82
12	0.52	0.64	5.01	4.75	8.21
INH	<0.1	<0.1	<0.1	<0.1	0.39

[a] Minimal concentration at which 50% of the bacterial growth is inhibited.

Table 3
Inhibition of intracellular *M. tb* H37Rv replication by compounds **1**–**12**.

Cmpd.	Inhibition of intracellular *M. tb* (%)[a]		
	10 μM	1 μM	0.1 μM
1	41.3 ± 1.5	35.2 ± 6.3	1.6 ± 8.6
2	39.2 ± 3.3	32.6 ± 7.2	0.3 ± 6.5
3	42.0 ± 3.6	29.9 ± 1.3	−1.2 ± 1.6[b]
4	41.3 ± 9.2	39.0 ± 0.9	−0.9 ± 0.9
5	46.8 ± 4.0	38.1 ± 8.3	0.2 ± 0.2
6	52.6 ± 3.2	46.6 ± 9.2	3.2 ± 6.9
7	55.0 ± 5.6	43.3 ± 5.2	−1.2 ± 7.3
8	58.7 ± 4.8	42.9 ± 4.3	0.6 ± 0.3
9	62.1 ± 7.8	45.7 ± 0.2	−0.5 ± 1.6
10	60.9 ± 8.9	39.6 ± 3.6	11.1 ± 7.3
11	69.4 ± 5.3	48.2 ± 6.2	5.2 ± 8.2
12	74.0 ± 0.8	53.1 ± 0.3	13.5 ± 3.9
INH	97.7 ± 5.1	82.3 ± 1.5	37.0 ± 6.5

[a] Inhibition of intracellular *M. tb* replication by compounds tested at 10, 1 and 0.1 μM concentration. Results represent mean ± SD % inhibition of samples tested in triplicate.
[b] Negative values result from higher intracellular growth in presence than in absence of the molecules.

expressing the luxabcde genes under the transcriptional control of a strong constitutive pr1 promoter. With this cytox strain, an increase in light signal is not related to toxicity but is the result of bacterial proliferation. A decrease in signal, on the other hand, can be interpreted as reduced viability of the strain and provides important information on the toxicity of the compounds towards the model organism. Both recombinant strains (genox and cytox) lack the oxidative machinery necessary to metabolize the compounds. To study the possible genotoxic effects of the metabolites of the compounds, enzymes lacking in the model are provided by the addition of S9 liver extract from aroclor treated rats.

Light emission in this study was recorded real time, every 5 min after addition of compounds during a 4 h period. Compounds were tested both in the presence and absence of the metabolizing S9 fraction. Genotoxicity is measured as a function of the signal to noise ratio (S/N), this being the light produced by the bacterial suspension exposed to the compounds divided by the light produced by a non-exposed bacterial suspension. As shown in Table 5, no significant activation of the SOS operon by the compounds was detected for concentrations as high as 10 μM. This was observed both for compounds and their respective metabolites. No significant toxicity was observed with the cytox strain.

The potential genotoxic properties of the most promising compound **12** were further assessed with a COMET assay [22]. With this assay further downstream genotoxic effects such as fragmentation of the genomic DNA can be detected. Briefly, C3A cells are exposed to a compound concentration for 24 h and immobilized onto agarose. The immobilized cells are lysed and the remaining nuclei are submitted to electrophoresis. Through pore exclusion of the agarose, fragmented DNA will migrate further with applied current. After staining with gel red, genomic DNA of an undamaged nucleus maintains its spherical organization (HEAD) whereas fragmented DNA migrates further across the gel (TAIL). The ratio DNA in the TAIL/DNA in the COMET is a measure of DNA fragmentation and reflects the genotoxic properties of the compounds for the C3A cells.

As shown in Fig. 3, no significant increase in DNA fragmentation was detected in the C3A cells exposed to compound **12** at concentrations of 1 μM, 3 μM and 10 μM.

Table 4
Acute toxicity of compounds **1**–**12** for C3A hepatocytes.

Cmpd.	Neutral red uptake assay			S.I.[b]
	Viability C3A cells (%)[a]			
	60 μM	120 μM	180 μM	
1	96.8	91.6	78.6	>60.0
2	93.6	85.0	70.5	>80.7
3	94.9	94.8	77.4	>67.9
4	86.1	73.2	55.7	>56.1
5	90.4	72.6	45.6	>110.4
6	93.4	85.9	59.3	>216.9
7	100.4	78.9	53.4	>211.8
8	97.9	95.3	69.0	>211.8
9	100.2	93.0	75.7	>189.5
10	97.7	96.6	72.8	>233.8
11	93.3	94.1	75.4	>281.3
12	101.3	89.9	68.1	>346.2

[a] The degree at which treated cells were able to bind and incorporate neutral red dye as compared to untreated control cells is expressed in % viability.
[b] S.I., selectivity index calculated as the NI_{50} divided by the MIC_{50} against *M. tb*.

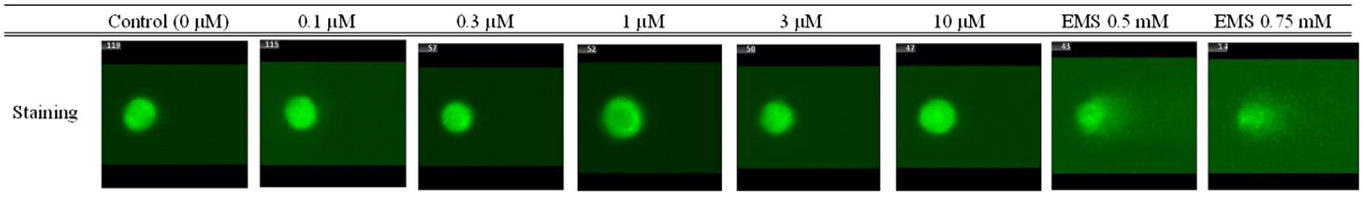
Table 5
Genotoxicity observed with the VITOTOX™ model.

Cmpd.	VITOTOX			
	Cytotox[a]		Genotox[b]	
	−S9[c]	+S9[d]	−S9	+S9
1	>10 μM[e]	>10 μM	>10 μM	>10 μM
2	>10 μM	>10 μM	>10 μM	>10 μM
3	>10 μM	>10 μM	>10 μM	>10 μM
4	>10 μM	>10 μM	>10 μM	>10 μM
5	>10 μM	>10 μM	>10 μM	>10 μM
6	>10 μM	>10 μM	>10 μM	>10 μM
7	>10 μM	>10 μM	>10 μM	>10 μM
8	>10 μM	>10 μM	>10 μM	>10 μM
9	>10 μM	>10 μM	>10 μM	>10 μM
10	>10 μM	>10 μM	>10 μM	>10 μM
11	>10 μM	>10 μM	>10 μM	>10 μM
12	>10 μM	>10 μM	>10 μM	>10 μM

[a] Cytotoxicity observation made with the TA104 pr1 strain.
[b] Genotoxicity observations made with the TA104 RecN2-4 strain.
[c] Toxic effects caused by the compounds in absence of metabolic enzymes.
[d] Toxic effects caused by the compounds in presence of metabolic enzymes.
[e] 10 μM was the highest concentration tested.

3. Discussion and conclusions

A library of thirty-three bisbenzaldehydes and structurally related analogs has been synthesized and tested for antimycobacterial activity against *M. tuberculosis* H37Rv. Structure–activity analysis revealed the importance of the aldehyde functions to observed growth inhibition of *M. tuberculosis*. Indeed, derivatives bearing no substituent on the aryl moieties or bearing carboxylic acid or nitro groups (instead of aldehydes) were less potent. We also noticed that the most efficient candidates were bisbenzaldehydes in which the aryl groups were linked to an aliphatic chain through ether bonds located in *para* with regard to the aldehyde functions. Activity increased as the number of carbon atoms in the linker increased up to an optimal value of 12. Thus, 4,4′-[1,12-dodecanediyl(oxy)]bisbenzaldehyde (**12**) emerged as the most promising hit not only to inhibit growth of *M. tb* but also to inhibit growth of *M. bovis*. A multi-drug resistant *M. tb* strain was highly susceptible to **12** and a 99% growth inhibition could be obtained at a concentration between 1.2 μM and 1.0 μM. Growth inhibition of 90% was detected at a concentration between 0.6 μM and 0.4 μM. Substances **1**–**12** showed activity against intracellular *M. tb* and compound **12** could reduce the intracellular bacterial load by 74.0% ± 0.8 at 10 μM and 53.1% ± 4.3 at 1 μM. In addition, compounds **1**–**12** displayed very low toxicity on C3A hepatocytes and their genotoxicity as well as that of possible metabolites could be excluded at antimycobacterially effective concentrations. Finally, compound **12** scored negative in a COMET assay, excluding DNA fragmentation.



	Control (0 μM)	0.1 μM	0.3 μM	1 μM	3 μM	10 μM	EMS 0.5 mM	EMS 0.75 mM
% DNA	6.65	5.72	7.64	7.62	7.44	6.97	63.44	65.15
# cells	50	50	50	50	50	50	50	50
SD	6.58	6.14	8.62	6.88	8.26	7.39	6.80	6.42
Median	3.82	3.14	4.78	5.95	5.43	3.39	63.60	65.15
P-Level		0.426	0.868	0.619	0.804	0.634		

Fig. 3. DNA fragmentation detection by the COMET assay. % DNA: the percentage of DNA measured in the COMET tail. *p*-level; significance obtained with Mann–Whitney *U* test. EMS, ethylmethanesulphonate at 0.5 mM and 0.75 mM as the positive control.

Therefore compound **12** can be considered as a novel drug candidate to fight *M. tb* and it deserves further *in vivo* studies. It is important to note that this derivative is characterized by a very simple chemical structure and that it can be prepared readily in a one-step reaction from commercially available precursors.

4. Experimental protocols

4.1. Biological data

Materials – 7H9 growth medium was purchased as a powder from BD Science (Franklin Lakes, NJ, USA). DMEM, glutamax, non essential amino acids, sodium pyruvate, gentamicin, β-mercaptoethanol, PBS were from GIBCO Invitrogen (Carlsbad, CA, USA). OADC, mycobactin J, penicillin, fungizone and hygromicin were from Roche (Basel, CH) and Triton X-100, glycerol, Tween 80 and *n*-decanal substrate were purchased from Sigma Aldrich (St. Louis, MO, USA).

Strains and growth conditions – M. tuberculosis H37Rv (American Type Culture Collection 27294) is known to be sensitive to the five first line anti-tuberculosis drugs (streptomycin, isoniazid, rifampin, ethambutol and pyrazinamide). The LAM-1 strain is a clinical isolate from a patient diagnosed with TB. This strain has been spoligotyped for identification and characterized for antibiotic resistance by the national TB reference lab of the Institute of Public Health of Belgium. With resistance to isoniazid, rifampicin, rifabutin and prothionamide this strain was classified as a Multi-Drug Resistant (MDR) strain [11]. Four other strains used for screening were *M. bovis* strain AN5, *M. avium* subsp. *avium* ATCC 15769, *M. avium* subsp. *paratuberculosis* ATCC19698 and *M. ulcerans* 1615. Bacteria were cultured as a surface pellicle on synthetic Sauton medium for 14–28 days, bacteria were harvested and stored at −80° as frozen stock solutions in 20% glycerol. For the drug screening, bacteria were cultivated in 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.2% glycerol. For *M. bovis*, the glycerol was replaced with 0.05% Tween 80. The growth medium of *M. avium* subsp. *paratuberculosis* was further supplemented with mycobactin J (2 μg/mL) required for optimal *in vitro* growth. Both *M. avium* strains and the *M. bovis* strain were grown at 39 °C, *M. ulcerans* was grown at 32 °C and *M. tb* was grown at 37 °C.

Monitoring mycobacterial growth by luminometry – The minimal inhibitory concentration (MIC) against mycobacteria of all synthesized compounds was evaluated by testing serial dilutions. The *in vitro* assay was based on a method in which luminescent mycobacteria transformed with pSMT1 luciferase reporter plasmid were used. The compounds were solubilized in DMSO (Sigma–Aldrich) at stock concentrations of 10 mM. Serial dilutions of each compound were made in liquid 7H9 medium [Middlebrook 7H9 broth based (Difco)] + 10% FCS (Gibco). Volumes of 20 μL of the serial dilutions were added in triplicate to 96 well, flat-bottomed microwell plates. The bacterial suspension was made by thawing and dissolving a frozen mycobacteria pellet in 7H9-10% FCS. The dissolved pellet was passed through a 5.0 μM filter (Millipore) to eliminate clumps and left for 1 h to recover at 37 °C, 5% CO_2. Next, the bacterial suspension was diluted in 7H9-10% FCS to obtain 50 000 Relative Light Units (RLU)/mL and a volume of 180 μL of bacteria was added to each well. Bacterial replication was analyzed by luminometry after 6 days of culture. The bacterial suspension from each well was collected, and brought in a 2.5 mL Eppendorf tube. Each well was washed four times with 200 μL PBS (Difco). To measure the luminescence, 100 μL of 1% *n*-decanal in ethanol (Sigma–Aldrich) was added to the Eppendorf tube and light emission was measured over 10 s using a Turner Modulus Single Tube Luminometer from Biosystems.

Activity against multi-drug resistant M. tuberculosis – Antimicrobial activity of the bisbenzaldehydes was tested using the LAM-1 strain using the BACTEC 460-TB detection system [11]. The BACTEC System relies on the unique capability of mycobacteria to metabolize palmitic acid expelling CO_2. By radioactively labeling the palmitic acid, the $^{14}CO_2$ expelled in the gaseous phase can be measured in a Beta counter and interpreted as a measurement of bacillary growth inside the BACTEC tubes [23]. The compounds were solubilized in DMSO (Sigma) at stock concentrations of 10 mM. Serial dilutions of each compound were made in 7H9 containing 10% OADC, at 40-fold the final concentrations. *M. tb LAM-1* was pre-cultured in a 4 mL BACTEC vial to a growth index (GI) of 300. Then 100 μL of this pre-culture was inoculated into a new 4 mL BACTEC vial together with 100 μL of the serial dilutions of the compounds. As a positive control for resistance, the MDR LAM-1 culture was inoculated with 0.1 μg/mL isoniazid. The GI was measured each day. To determine the MIC_{90}, the cultures treated with the compounds were compared with an untreated culture.

Inhibition of intracellular M. tb growth – The compounds were tested on the murine J774.A1 macrophage cell line infected with *M. tb* H37Rv^{lux}. The J774 macrophages were grown at 37 °C, 5% CO_2 in complete DMEM medium until a semi confluent layer was formed. The macrophages were washed in fresh complete DMEM medium and seeded in a flat-bottomed 96-well microtiter plate at a cell density of 40,000 cells per well. The cells were left to recover overnight and were washed three times in complete DMEM medium. *M. tb* H37Rv^{lux} was grown at 37 °C in 7H9 containing 10% FCS and 0.2% hygromycin to an OD_{580} of 0.6–1.0. The fully-grown bacterial suspension was measured and brought into complete DMEM-Pen/

Fung [DMEM medium containing 0.1% penicillin and 0.8% Fungizone but without gentamycin]. Compounds were solubilized in DMSO at stock concentrations of 10 mM. Serial dilutions of the compounds were made in DMEM-Pen/Fung at 2 times the concentration of each compound to be tested. A volume of 100 μL of the bacterial suspension in DMEM-Pen/Fung containing 4000 RLU of bacteria (multiplicity of infection of 0.1) and 100 μL of the serial compound dilutions were added to the macrophage cultures. To measure the effects of the compounds on intracellular growth of *M. tb*, the infected macrophages were washed three times on day 5 to remove all extracellular bacteria, incubated 1 h with 1% gentamycin to kill residual extracellular bacteria, lysed with 200 μL 1% Triton X-100 (Sigma) and the wells washed four times with 200 μL PBS. The lysate was transferred in a 2.5 mL tube together with the 4 PBS washings. One hundred μL of 1% *n*-decanal in ethanol were added to the tube and luminescence measured. RLU values shown were obtained from six replicate cultures. Cell viability of the J774 A1 macrophages was visually observed by Trypan blue dye exclusion.

Assessment of Cytotoxicity. Inhibitory effect on C3A human hepatocytic cells was determined for the derivatives using a neutral red uptake assay as described before. The C3A cells were grown in DMEM + 10% FCS until a semi confluent monolayer of cells was obtained. The cells were trypsinized, washed and 40 000 cells were seeded per well of a 96 well plate and left for recovery at 37 °C, 5% CO_2. The following days, the compounds were solubilized in DMSO (Sigma–Aldrich) to stock concentrations of 18 mM. A serial dilution of each compound was made in DMEM + 10% FCS. The C3A cells were washed and exposed to the derivatives by adding the serial dilutions of the compounds to the wells. The plates were left for incubation at 37 °C, 5% CO_2 for 24 h. After exposure, the cells were washed with 200 μL PBS and 200 μL neutral red working solution (Sigma) was added per well. Subsequently the plates were incubated for 3 h at 37 °C, 5% CO_2. The wells were washed with 200 μL PBS and 200 μL of an ethanol/acetic acid (50%/1%) mixture was added. The plates were left on the shaker until the color became homogenous purple and the optical density was measured at 540 nm (NR max) and 620 nM (reference wavelength) with the Paradigm detection platform. OD of treated cells was compared to OD of untreated cells (equals 100% viability).

Vitotox assay. Possible genotoxicity of the compounds was analyzed using the Gentaur Vitotox™ kit and the included protocol was followed. In brief, TA104 RecN2-4 (genox) and TA104 (cytox) *Salmonella typhimurium* bacteria were cultivated shaking at 36 °C for 16 h in poor LB BROTH medium (20 g LB broth − 1 g glucose − 0.345 g $CaCl_2 \cdot 2H_2O$ + antibiotics/liter) (1% LB broth, 0.5% glucose, 0.172% $CaClH_2O$, 2.25% NaCl). The bacterial culture was diluted 125 times with poor LB broth MEDIUM (2 g LB BROTH, 1 g glucose, 0.375 g $CaCl_2 \cdot 2H_2O$, 4.5 g NaCl/liter) times incubated shaking for 1 h at 36 °C. Cultures were diluted 10 fold with poor LB medium, S9 (S9 liver fraction from aroclor treated rats) was added to the designated +S9 cultures to test the genotoxic/cytotoxic effects of the metabolites of the compounds. The bacterial suspensions were then incubated at 30 °C and the luminescent signal was measured for 4 h with a 5 min interval.

Comet assay. Possible DNA breakage effects of the derivatives on C3A cells were examined in the alkaline comet assay. The C3A cells were grown in DMEM + 10% FCS until a semi confluent layer of cells was obtained. The cells were trypsinized, washed and seeded at 40,000 cells per well of a 24 well plate and left for recovery at 37 °C, 5% CO_2. The following day, compound **12** was dissolved in DMSO as a stock concentration of 1 mM. Serial dilutions of each compound were made in DMEM + 10% FCS to obtain the final concentrations (10 μM, 3 μM, 1 μM, 0.3 μM, 0.1 μM). The C3A cells were washed and exposed to the derivatives by adding 1 mL of the serial dilutions to each well. The plates were incubated at 37 °C, 5% CO_2 for 24 h. After incubation, the cells were trypsinized, washed and resuspended with PBS, and 10 μL of cell suspension was mixed with 300 μL low melting point agarose. The agarose/cell suspension mix dissolved cell suspension was then placed onto a frosted microscope slide and left on ice for 5 min. After removal of cover slip, the slide was subsequently placed in a jar containing lysis buffer (10% DMSO, 89% lysing stock solution, 1% Triton x-100) for 1 h. After lysis, the agarose cell suspension was subjected to denaturation for 40 min (denaturation buffer pH > 13) electrophoresis (same buffer) for 20 min at 300 mA (temperature +/−17 °C for both denaturation/electrophoresis). The slides were washed 3 times with neutralization buffer for 5 min and dried in ice cold ethanol for 10 min. Staining of the DNA was done with gel red (Biotum-VWR). For the quantification of the DNA migration a fluorescence microscope was used and the percentage of DNA in the comet tail of the cells' nuclei core was calculated in proportion to the total DNA present in the nuclei (comet head + tail) by imager software from Metasystems Altlussheim Germany.

4.2. Chemistry

All compounds were prepared according to previously described procedures [16,17]. Briefly, a phenol derivative (0.015 mol) and a dibromoalkane (0.0075 mol) were dissolved in a mixture of ethanol and water (9/1) containing an alkaline hydroxide (0.015 mol). The mixture was heated under microwave irradiation in an Initiator® Biotage oven for 20 min at 120 °C. After cooling, the precipitate was filtered and thoroughly washed with water, ethanol and ether.

All compounds have been described in the literature [16,24]. The synthesized products were identified by comparison of the spectral data. The purity of the compounds was estimated at more than 98% based on NMR spectra. An example of a complete characterization (FTIR, HPLC, mass spectrometry, ^1H and ^{13}C NMR spectra) of two samples is shown in the Supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.023.

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