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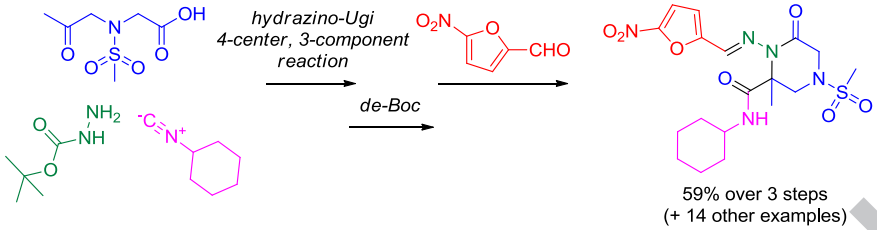

MIC *M. tuberculosis*:
$H_{37}Rv$ strain - 22 μM
"2067" strain - 11 μM
(MDR isoniazid+rifampicin+kanamycin)

Cytotoxicity:
IC_{90} (L929, KB-3-1, MCF-7) > 100 μM

New nitrofurans amenable by isocyanide multicomponent chemistry are active against multidrug-resistant and poly-resistant *Mycobacterium tuberculosis*

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ABSTRACT

A set of structurally diverse *N*-amino δ-lactams decorated with a 5-nitro-2-furyl moiety was synthesized using isocyanide-based multicomponent chemistry and evaluated for antibacterial activity. Three compounds displayed a selective and potent (MIC 22-33 μM) inhibition of *M. tuberculosis* H_{37}Rv strain growth, while other Gram-positive (MRSA and *E. faecium*) or Gram-negative (*E. coli*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*) pathogens were not affected. The compounds also displayed moderate-low cytotoxicity, as demonstrated in cell line viability assays. Several multidrug- and poly-resistant patient-derived *M. tuberculosis* strains were found to be susceptible to treatment with these compounds. The three most potent compounds share a significant structural similarity which provides a basis for further scaffold-hopping analog design.

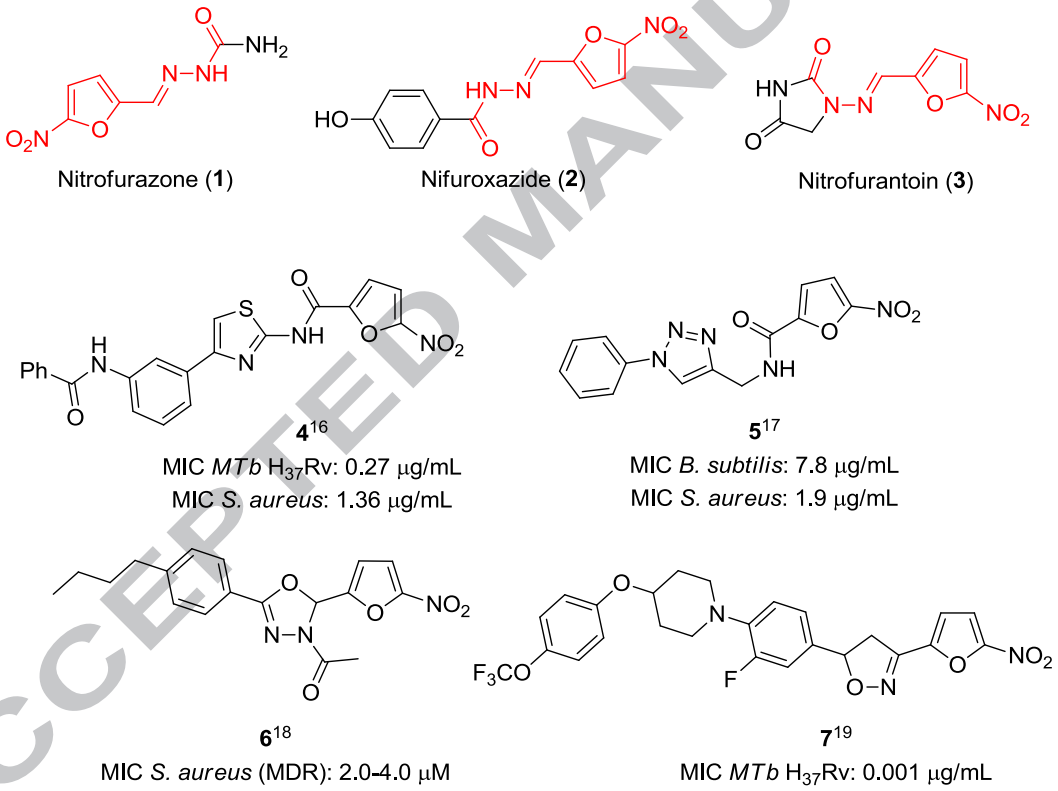
Keywords: nitrofurans, antitubercular, drug resistance, patient-derived strains, multicomponent chemistry, *N*-amino lactams

1. Introduction

There is a constant need for new lead compounds endowed with antibacterial activity due to the continued emergence of multidrug-resistant microorganisms.[1] The situation is particularly alarming for health care-associated infections of the developed world (especially due to pathogens of the so-

called ESKAPE panel), but also for non-contained infections in developing countries like tuberculosis (caused by *Mycobacterium tuberculosis*).[2] The patients acquiring or developing multidrug-resistant (MDR) or extensively drug-resistant (XDR) tuberculosis are at high risk of death from the disease, as these forms take long time to treat with first- and second-line therapies.[3] The poor economic situation in the regions where tuberculosis is a particularly pressing public health issue, and the occurrence of co-infections with HIV further exacerbate the impact of tuberculosis on life expectancy.[4] Hence, the existing anti-tubercular drug development pipeline needs to be filled with new efficacious leads, in spite of the recent approvals of Janssen's bedaquiline[5] or Otsuka's delamanid.[6]

Figure 1. Classical nitrofuran antibacterial drugs (**1**-**3**) and examples of the recently reported nitrofuran derivatives (**4**-**7**) with broad-spectrum antibacterial and anti-mycobacterial efficacy.

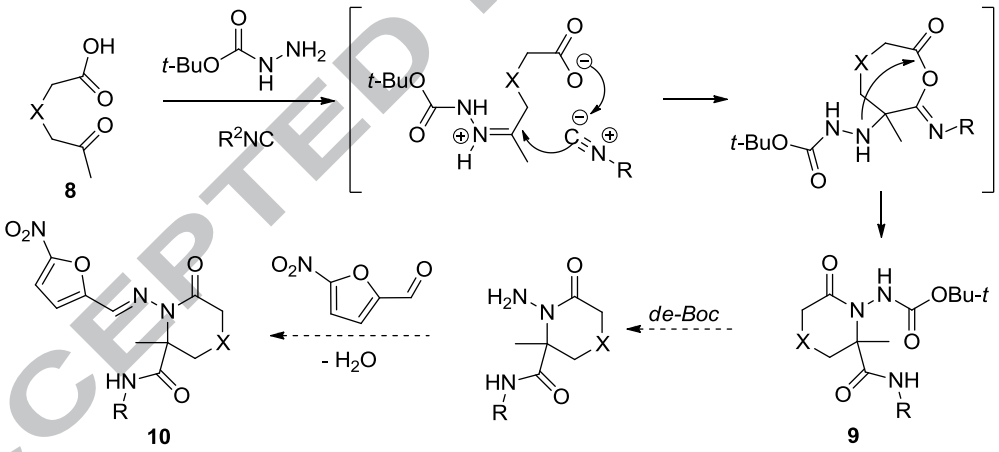


Nitrofuran-based agents have been used as antibacterials since the 1940s, starting with the introduction of nitrofurazone (**1**).[7] The compound has given rise to such next-generation nutrofurans as nifuroxazide (**2**) and nitrofurantoin (**3**).[8] Their exact mechanism of antibacterial action remains to be fully elucidated. A currently accepted view (supported by experimental data[9]) is that the nitrofuran moiety undergoes reduction at the nitro group, which leads to production of toxic free radical species. A particular challenge, however, is to avoid non-specific toxicity associated with such a mechanism of action, and it is perhaps unsurprising that the advancement of new nitrofurans into clinical setting has been limited by the observable undesired side effects.[10-11] However, the recent success of other nitroheterocyclic compounds (e. g., the antitubercular nitroimidazoles PA-

824[12] and OPC-67683[13] as well as related compounds[14]) led researchers to conclude that the carefully optimized periphery of a nitrofuran compound could help reduce toxicity and improve the therapeutic window of the newly designed chemotherapeutic agents. This is the likely reason for the recent re-emergence of new nitrofuran derivatives in the anti-infective field,[15] which is illustrated by the recently reported compounds (**4**-**7**)[16-19] having broad-spectrum antibacterial and antimycobacterial activity (Figure 1).

The presence of the *N*-acylhydazone or semicarbazone linker between the pharmacophoric 5-nitrofuryl moiety and the rest of the molecule is evident in the classical nitrofuran antibacterials **1**-**3**. This inspired us to pursue a discovery strategy based on a novel molecular scaffold containing a terminally *N′*-unsubstituted *N*-acylhydrazine or a semicarbazide moiety, that was decorated with the 5-nitro-2-furfurylidene 'warhead' via a straightforward hydrazine (semicarbazone) synthesis.[20-24] The compounds were then screened for antibacterial activity and for cellular toxicity in order to identify hits with an improved therapeutic window.

Scheme 1. Synthesis of *N*-aminolactams **9** and the intended use of them in the design of new nitrofuran antibacterials **10**.



Our research in the area of the hydrazino-Ugi reaction (i. e. a four-component isocyanide-based reaction employing hydrazines in lieu of the traditional amine component[25-29]) delivered a number of scaffolds containing a hydrazine moiety. In particular, we reported a four-center, three-component Ugi reaction of various ketoacids **8** and Boc-protected hydrazine and isocyanides that delivered *N*-aminolactams **9** with a flexible variation of the scaffold (X) and the isocyanide-derived periphery (R).[29] We reasoned that removal of the protecting group in **9** would liberate a reactive *N*-acylhydrazine nitrogen atom that can be conveniently decorated with the 5-nitro-2-furfurylidene moiety and deliver structurally diverse compounds **10** with potential antibacterial activity (Scheme 1). Herein, we report on the practical realization of this strategy and investigation of the

antibacterial, anti-mycobacterial and cytotoxic activity of the resultant compounds, as a continuation of our research program toward antibacterial lead generation.[30-31]

2. Results and discussion

The robust synthetic protocol developed earlier for the isocyanide-based *N*-aminolactam synthesis[29] permitted realization of our synthetic strategy in a three-step, single-purification format as shown in Scheme 2.

Scheme 2. Synthesis of 5-nitro-2-furfurylidene *N*-aminolactams **10a-o**.

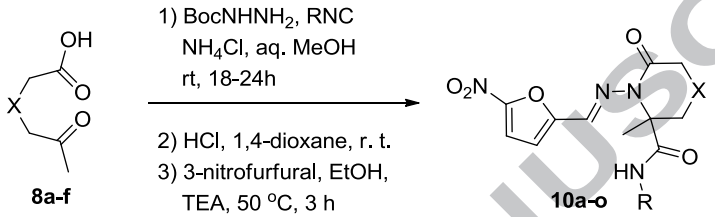


Table 1. Novel 5-nitro-2-furfurylidene N-aminolactams **10a-o** prepared in this work.

Compound	Ketoacid **8**	X	R	Isolated yield (%)
10a	**8a**	direct bond	cyclohexyl	46
10b	**8a**	direct bond	4-$ClC_6H_4CH_2$	37
10c	**8a**	direct bond	*t*-Bu	56
10d	**8a**	direct bond	Bn	42
10e	**8b**	CH_2	cyclohexyl	72
10f	**8b**	CH_2	*t*-Bu	63
10g	**8b**	CH_2	Bn	69
10h	**8b**	CH_2	4-$ClC_6H_4CH_2$	59
10i	**8c**	S	cyclohexyl	46
10j	**8c**	S	Bn	43
10k	**8d**	O	cyclohexyl	38
10l	**8d**	O	Bn	44
10m	**8d**	O	*t*-Bu	39
10n	**8e**	2-$O_2NC_6H_4SO_2N$	cyclohexyl	59
10o	**8f**	$MeSO_2N$	cyclohexyl	61

All ketocarboxylic acids **8** were either commercially available (**8a-d**, X = direct bond, CH_2, S, O) or prepared according to a literature protocol (**8e-f**, X = $ArSO_2N$, $MeSO_2N$).[32] These were reacted with equimolar amounts of Boc-hydrazine and an isocyanide in methanol containing water and ammonium chloride (as mildly acidic catalyst for the Ugi reaction).[33] In all cases, the hydrazino-Ugi reaction was complete within 24 h and the reaction was found to primarily deliver the target N-(Boc-amino)lactams **9**, according to TLC and ^{1}H NMR analysis of the crude reaction products.

Without further purification, the Boc group in **9** was removed, and the resulting hydrochloride salts were treated with 5-nitrofurfural in the presence of triethylamine to give the desired racemic hydrazones **10a-o**, which were isolated and purified chromatograhically in moderate to good yields over three steps (Table 1). Notably, the formation of *N*-amino δ-lactams was particularly high-yielding as judged from the overall yield of products **10e-h** obtained after the Boc group removal and hydrazine synthesis.

The antibacterial profile of compounds **10a-o** was evaluated for their ability to suppress the growth of Gram-positive (MRSA and *Enterococcus faecium*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*) bacteria (ESKAPE panel). As it is evident from the data presented in Table 2, the compounds were virtually inactive against the bacterial strains in the panel, except for MRSA where weak inhibition of bacterial culture growth was observed at concentrations as high as 100 μM (which was the highest testing concentration, see Experimental). To our delight, however, some of the compounds tested (in particular, **10e**, **10i** and **10n**) displayed a selective anti-mycobacterial action against *M. tuberculosis* H_{37}Rv strain. Their level of potency (MIC 22-33 μM) is moderate, but similar to that of several recently reported novel antitubercular hits.[34-36] In addition, their potency may be easily enhanced, as the scaffold is well-amenable to structural alterations.

Table 2. Growth inhibitory profile of **10a-o** against the ESKAPE panel as well as *Mycobacterium tuberculosis* H_{37}Rv strain (nt = not tested).

Compound	*MRSA*	*E. faecium*	*E. coli*	*P. aeruginosa*	*A. baumannii*	*K. pneumoniae*	*M. tuberculosis*
10a	>100	>100	(85)	(90)	nt	nt	70
10b	>100	>100	(82)	(89)	nt	nt	>100
10c	>100	>100	(86)	(92)	nt	nt	>100
10d	95	>100	(85)	(90)	nt	nt	>100
10e	100	>100	(81)	(88)	>100	>100	**33**
10f	100	>100	(83)	(85)	nt	nt	71
10g	100	>100	(87)	(85)	nt	nt	65
10h	100	>100	(84)	(98)	nt	nt	>100
10i	100	>100	(84)	(91)	>100	>100	**32**
10j	>100	>100	>100	>100	>100	>100	>100
10k	100	>100	(84)	(90)	nt	nt	66
10l	100	>100	(83)	(86)	nt	nt	65
10m	>100	>100	>100	>100	>100	>100	>100
10n	100	>100	(88)	(85)	>100	>100	**22**
10o	>100	>100	>100	>100	>100	>100	55

Data are either given as minimal inhibitory concentrations (MIC) in μM, or, if numbers are in brackets, as % inhibition at a test concentration of 100 μM. The lowest MIC values are printed in bold.

The most potent anti-mycobacterial compounds in the series (**10e**, **10i** and **10n**) were evaluated for their effects on the viability of the following immortalized cell lines: mouse fibroblasts L929, human cervix carcinoma KB-3-1, human breast cancer cell line MCF-7, and the human foreskin endothelial cell line FS4-LTM. As can be seen from the data in Table 3, all three compounds displayed some degree of cytotoxicity, although full inhibition of the cell culture growth was not achieved in the tested concentration range (up to 100 μM). Interestingly, an antiproliferative effect was not observed with the FS4-LTM cell line that reflects properties of a primary cell line.[37]

Table 3. Cytotoxicity profile (IC_{50} and IC_{90}, μM) of **10e**, **10i** and **10n** against immortalized cancer cell lines.

Compound	L929		KB-3-1		MCF-7		FS4-LTM	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
10e	50	>100	61	100	19	100	>100	>100
10i	50	>100	16	100	8.7	>100	>100	>100
10n	>100	>100	27	>100	12	>100	>100	>100

The same compounds were evaluated for the ability to suppress the growth of various patient-derived, genotyped drug-resistant mutant *M. tuberculosis* strains, which are part of the pathogenic mycobacterial strain collection of Saint Petersburg Research Institute of Phthisiopulmonology. All three compounds displayed significant activity against all poly-resistant and MDR strains and were found to be particularly active against the MDR 2067 strain (Table 4). Importantly, **10n** was found to inhibit both 2067 MDR and 5307 poly-resistant strains with an MIC of 11 μM.

Table 4. Growth inhibitory profile (MIC, in μM) of **10e**, **10i** and **10n** against MDR and poly-resistant patient-derived mutant *M. tuberculosis* strains.

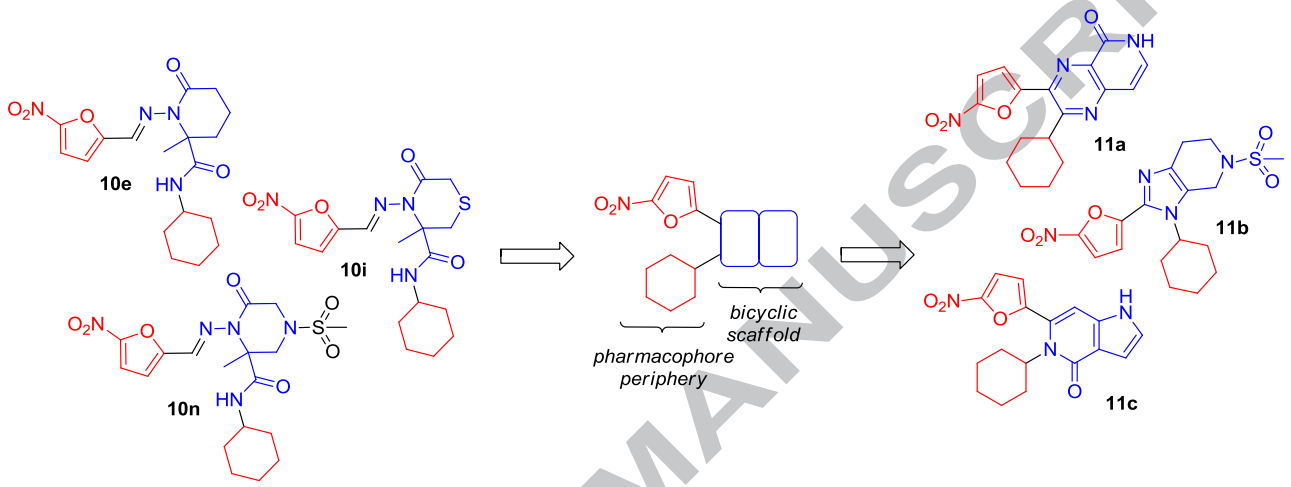
Mutant strain	Gene mutations				Drug resistance type	Drug resistance profile[a]	MIC (μM)		
	rpoB	*katG*	*inhA*	*ahpC*			**10e**	**10i**	**10n**
2067	H526R	none	none	T10	MDR	HRK	17	16	11
5227	none	S315T	none	A10	poly	SH	66	63	44
5307	none	none	T15	none	poly	SHEt	66	63	11
7106	S531L	S315T	none	none	MDR	SHREKCapAZ	66	63	44

[a] S – strepromycin, H – isoniazid, R – rifampicin, E – ethambutol, K – kanamycin, Et – ethionamide, Cap – capreomycin, A – amikacin, Z – pyrazinamide.

Interestingly, the three compounds which showed the highest anti-mycobacterial potency (**10e**, **10i** and **10n**) all shared a cyclohexyl substitution at the exocyclic amide bond, the only variation being

the linker X. We intend to take this as a basis for the next-generation analog design (using a scaffold-hopping approach[38]), where the pharmacophoric 5-nitrofur-2-yl and cyclohexyl substituents could be affixed onto a novel bicyclic scaffold (as in **11a-c**). Such scaffolds are devoid of the potentially labile hydrazone linkage, a potential source of toxic metabolites[39] (Figure 2).

Figure 2. Molecular similarity among **10e**, **10i** and **10n** inspires the scaffold-hopping design of next-generation analog series **11a-c**.



3. Conclusion

Using isocyanide-based multicomponent chemistry, we synthesized a series of skeletally diverse *N*-amino δ-lactams. Their 5-nitro-2-furyl periphery was attached to endow the compounds with antibacterial potency, while the scaffold was expected to alleviate the cytotoxicity which may have arisen from the nitrofuran moiety itself. The compounds displayed almost no activity against several pathogenic bacterial strains, but turned out to be anti-mycobacterial agents devoid of significant cytotoxicity as determined in immortalized cancer cell viability tests. Moreover, several multidrug-resistant (MDR) and poly-resistant patient-derived mutant strains of *M. tuberculosis* were found to be susceptible to the most potent hits (**10e**, **10i** and **10n**), which share a significant structural similarity. This observation will be used in designing next-generation analogs of the series without the hydrazone linker. These efforts are currently underway in our laboratories; the results thereof will be reported in due course.

4. Experimental section

4.1. General experimental

All reactions were conducted in oven-dried glassware in atmosphere of nitrogen. All reagents and solvents were obtained from commercial sources and used without purification. NMR spectra were

recorded on Bruker 400 and 300 spectrometers (^{1}H: 400 and 300 MHz; ^{13}C: 100 and 75 MHz, respectively; chemical shifts are reported as parts per million (δ, ppm); the residual solvent peaks were used as internal standards: 7.28 and 2.50 ppm for ^{1}H in $CDCl_3$ and DMSO-d_6 respectively, 40.01 and 77.02 ppm for ^{13}C in DMSO-d_6 and $CDCl_3$ respectively; multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad; coupling constants, *J*, are reported in Hz. Mass spectra were recorded on a Bruker micrOTOF spectrometer (ESI ionization). Melting points were determined in open capillary tubes on Stuart SMP30 Melting Point Apparatus. Elemental analysis was obtained at Research Institute for Chemical Crop Protection (Moscow, Russia) using Carlo ErbaStrumentazione 1106 analyzer.

4.2. Synthetic organic chemistry

4.2.1. General procedure for preparation of ketocarboxylic acids 8.[32]

Triethylamine (0.5 mol) was added to a 15 ºC suspension of glycine ethyl ester hydrochloride (0.2 mol) and a respective sulfonyl chloride (0.2 mol) in THF (250 mL). The mixture was heated at 50 ºC for 3 h, cooled to rt, filtered and the filter cake was washed with more THF (100 mL). The combined filtrate and washings were concentrated under reduced pressure and the residue crystallized from ethanol. This material (which represented ethyl *N*-sulfonylglycinate of at least 90% purity by ^{1}H NMR) was dissolved in acetonitrile (100 mL). Chloroacetone (0.17 mol), anhydrous potassium carbonate (0.2 mol) and 18-crown-6 (100 mg) were added and the mixture was heated at reflux for 3 h. It was cooled to rt, concentrated to a volume of about 25 mL, poured into 5% aqueous KCl solution (250 mL) and the resulting mixture was stirred for 30 min. It was poured into a separatory funnel and extracted with $CHCl_3$ (2 x 150 mL). The combined extracts were washed with brine and water, dried over anhydrous $MgSO_4$, Filtered and concentrated *in vacuo*. The residue was suspended in 2% solution of KOH in 50% aqueous ethanol (100 mL) and heated at 50 ºC for 3 h (until the solid residue completely dissolved). The reaction mixture was cooled down, filtered, concentrated to a volume of 50 mL. The pH of this solution was adjusted to ~3 with 2% HCl and the resulting precipitate was separated by filtration. The filter cake was washed with water, air dried and then dried *in vacuo* for 24 h to provide analytically pure title compound.

4.2.1.1. 2-(*N*-(2-oxopropyl)methylsulfonamido)acetic acid (8e, X = $MeSO_2N$).[32]

Yield 21.3 g (51%). Grey solid, mp = 78-80 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.63 (s, 2H), 4.25 (s, 2H), 3.02 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.4, 171.9, 57.4, 43.4, 35.9, 26.9.

4.2.1.1. 2-(2-Nitro-*N*-(2-oxopropyl)phenylsulfonamido)acetic acid (8f, X = 2-$O_2NC_6H_4SO_2N$).

Yield 29.1 g (46%). Beige solid, mp = 148-151 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (m, 2H), 8.02 (m, 2H), 4.45 (s, 2H), 4.15 (s, 2H), 2.23 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.4, 171.9, 140.5, 128.6, 127.7, 122.2, 119.2, 117.3, 57.4, 43.4, 27.0. Anal. calcd for $C_{13}H_9ClN_2O_5S$: C, 41.77; H, 3.82; N, 8.86; found: C, 41.79; H, 3.77; N, 8.91.

4.2.2. General procedure for preparation of compound 10a-o.

Ketoacid **8** (1 mmol) and *tert*-butoxycarbonylhydrazine (1 mmol) were dissolved in MeOH (5 mL). Solid NH4Cl (22 mg, 0.5 mmol) was added followed by water (1 mL). Once a clear solution formed, isocyanide (1 mmol) was added and the reaction mixture was stirred at rt for 24 h. It was partitioned between ethyl acetate (25 mL) and water (25 mL). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was dissolved in 1,4-dioxane (5 mL) and 4M solution of HCl in 1,4-dioxane (300 μL) was added. The reaction mixture was stirred at rt for 3 h (or until the removal of Boc group was complete according to TLC analysis) and concentrated *in vacuo*. The resulting gooey residue was dissolved in ethanol (5 mL), 5-nitrofurfural (1 mmol) was added followed by trimethylamine (1.2 mmol). The reaction mixture was heated at 50 ºC for 3 h and cooled down to rt. Silica gel (10 g) was added and the volatiles were removed from the resulting slurry *in vacuo*. The residue (bright-yellow crude product absorbed on silica gel) was loaded on top of a silica gel chromatography column and the title compound was isolated by elution with a 0→5% gradient of methanol in CH_2Cl_2.

4.2.2.1. *N*-Cyclohexyl-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-5-oxopyrrolidine-2-carboxamide (10a)

Yield 166 mg (46%). Yellow solid, mp 134-135 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.30 (s, 1H, N=CH), 7.75 (d, *J* = 2.9 Hz, 1H, HetArH), 7.60 (d, *J* = 7.9 Hz, 1H, NH), 7.26 (d, *J* = 3.0 Hz, 1H, HetArH), 3.53 (bs, 1H, N-CH), 2.65 – 2.35 (m, 2H), 2.28 – 2.08 (m, 1H), 2.07 – 1.90 (m, 1H), 1.82 – 1.59 (m, 5H, AlkH), 1.55 (s, 3H, CH_3), 1.37 – 0.95 (m, 5H, AlkH).^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 171.0, 152.1, 151.9, 138.8, 115.8, 114.5, 67.8, 48.2, 32.1, 32.0, 29.6, 28.9, 25.2, 24.8, 24.7, 21.6. HRMS (ESI), *m/z* calcd for $C_{17}H_{22}N_4O_5$ $[M+Na]^+$ 385.1482, found 385.1484.

4.2.2.2. *N*-(4-Chlorobenzyl)-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-5-oxopyrrolidine-2-carboxamide (10b)

Yield 150 mg (37%). Yellow solid, mp 149-150 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.38 (s, 1H, N=CH), 8.53 (bs, 1H, NH), 7.79 (d, *J* = 2.5 Hz, 1H, HetArH), 7.45 – 7.05 (m, 5H, HetArH+ArH), 4.50 – 4.05 (m, 2H, N-CH_2), 2.70 – 2.35 (m, 2H), 2.30 – 2.10 (m, 1H), 2.10 – 1.95 (m, 1H), 1.58 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 172.4, 152.1, 151.8, 139.5, 138.5, 131.2, 128.8,

128.1, 116.2, 114.5, 67.9, 41.8, 29.4, 29.0, 21.8. HRMS (ESI), *m/z* calcd for $C_{18}H_{17}ClN_4O_5$ $[M+Na]^+$ 427.0780, found 427.0796.

4.2.2.3. *N*-(*tert*-Butyl)-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-5-oxopyrrolidine-2-carboxamide (10c)

Yield 188 mg (56%). Yellow solid, mp 121-122 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.27 (s, 1H, N=CH), 7.74 (d, *J* = 3.7 Hz, 1H, HetArH), 7.25 (d, *J* = 3.7 Hz, 1H, HetArH), 7.12 (bs, 1H, NH), 2.62 – 2.34 (m, 2H), 2.34 – 2.18 (m, 1H), 2.04 – 1.86 (m, 1H), 1.53 (s, 3H, CH_3), 1.25 (s, 9H, $3CH_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.4, 171.3, 152.1, 151.9, 138.4, 115.8, 114.5, 68.2, 50.73, 29.4, 29.0, 28.4, 21.7. HRMS (ESI), *m/z* calcd for $C_{15}H_{20}N_4O_5$ $[M+Na]^+$ 359.1328, found 359.1326.

4.2.2.4. *N*-Benzyl-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-5-oxopyrrolidine-2-carboxamide (10d)

Yield 155 mg (42%). Yellow solid, mp 132-133 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.65 (s, 1H, N=CH), 7.40 – 7.18 (m, 6H, HetArH+ArH), 6.84 (bs, 1H, NH), 6.76 (d, *J* = 3.8 Hz, 1H, HetArH), 4.62 – 4.35 (m, 2H, N-CH_2), 2.89 – 2.78 (m, 1H), 2.69 – 2.46 (m, 2H), 2.04 – 1.91 (m, 1H), 1.69 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 172.3, 152.1, 151.9, 139.4, 128.1 (2C), 126.8, 126.6, 116.0, 114.5, 68.0, 42.4, 29.5, 29.0, 21.8. HRMS (ESI), *m/z* calcd for $C_{18}H_{18}N_4O_5$ $[M+Na]^+$ 393.1169, found 393.1163.

4.2.2.5. *N*-Cyclohexyl-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-6-oxopiperidine-2-carboxamide (10e)

Yield 270 mg (72%). Yellow solid, mp 163-165 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.23 (s, 1H, N=CH), 7.75 (d, *J* = 2.9 Hz, 1H, HetArH), 7.48 (d, *J* = 8.0 Hz, 1H, NH), 7.20 (d, *J* = 3.1 Hz, 1H, HetArH), 3.54 (bs, 1H, N-CH), 2.65 – 2.35 (m, 2H), 2.23 – 2.02 (m, 1H), 2.00 – 1.82 (m, 1H), 1.81 – 1.40 (m, 10H, CH_3+AlkH), 1.36 – 0.94 (m, 5H, AlkH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.4, 169.4, 152.1, 151.9, 143.4, 115.4, 114.4, 69.0, 48.3, 34.6, 33.8, 32.3, 32.1, 25.3, 24.9, 24.8, 23.9, 16.8. HRMS (ESI), *m/z* calcd for $C_{18}H_{24}N_4O_5$ $[M+Na]^+$ 399.1639, found 399.1655.

4.2.2.6. *N*-(*tert*-Butyl)-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-6-oxopiperidine-2-carboxamide (10f)

Yield 220 mg (63%). Yellow solid, mp 143-144 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.22 (s, 1H, N=CH), 7.75 (d, *J* = 3.9 Hz, 1H, HetArH), 7.20 (d, *J* = 4.0 Hz, 1H, HetArH), 6.99 (bs, 1H, NH), 2.61 – 2.39 (m, 2H), 2.29 – 2.12 (m, 1H), 1.94 – 1.62 (m, 3H), 1.55 (s, 3H, CH_3), 1.23 (s, 9H, $3CH_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.8, 169.5, 152.1, 152.0, 143.3, 115.3, 114.4, 69.4,

50.7, 34.2, 33.7, 28.5, 23.8, 16.9. HRMS (ESI), *m/z* calcd for $C_{16}H_{22}N_4O_5$ $[M+Na]^+$ 373.1482, found 373.1482.

4.2.2.7. *N*-Benzyl-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-6-oxopiperidine-2-carboxamide (10g)

Yield 265 mg (69%). Light yellow solid, mp 105-106 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.28 (s, 1H, N=CH), 7.43 – 7.18 (m, 6H, HetArH+ArH), 6.76 (d, *J* = 3.9 Hz, 1H, HetArH), 6.65 (bs, 1H, NH), 4.73 – 4.56 (m, 1H), 4.47 – 4.27 (m, 1H), 2.78 – 2.52 (m, 3H, AlkH), 2.00 – 1.78 (m, 3H, AlkH), 1.69 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.6, 169.5, 152.2, 151.9, 143.4, 139.6, 128.0, 126.9, 126.5, 115.7, 114.5, 69.2, 42.5, 34.7, 34.0, 24.1, 16.8. HRMS (ESI), *m/z* calcd for $C_{19}H_{20}N_4O_5$ $[M+Na]^+$ 407.1326, found 407.1327.

4.2.2.8. *N*-(4-Chlorobenzyl)-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-6-oxopiperidine-2-carboxamide (10h)

Yield 247 mg (59%). Yellow solid, mp 165-166 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.24 (s, 1H, N=CH), 8.43 (m, 1H, NH), 7.78 (d, *J* = 3.8 Hz, 1H, HetArH), 7.35 – 7.06 (m, 5H, HetArH+ArH), 4.50 – 4.31 (m, 1H), 4.23 – 4.05 (m, 1H), 2.65 – 2.37 (m, 2H), 2.23 – 2.05 (m, 1H), 2.04 – 1.87 (m, 1H), 1.85 – 1.66 (m, 2H), 1.62 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.7, 169.4, 152.2, 151.8, 143.7, 138.7, 131.1, 128.9, 127.9, 115.8, 114.5, 69.1, 41.9, 34.7, 33.9, 24.0, 16.8. HRMS (ESI), *m/z* calcd for $C_{19}H_{19}ClN_4O_5$ $[M+Na]^+$ 441.0936, found 441.0945.

4.2.2.9. *N*-Cyclohexyl-3-methyl-4-(((5-nitrofuran-2-yl)methylene)amino)-5-oxothiomorpholine-3-carboxamide (10i)

Yield 181 mg (46%). Yellow solid, mp 105-107 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.24 (s, 1H, N=CH), 7.38 (d, *J* = 3.8 Hz, 1H, HetArH), 6.93 (d, *J* = 3.6 Hz, 1H, HetArH), 6.25 (d, *J* = 8.0 Hz, 1H, NH), 3.90 – 3.76 (m, 1H, N-CH), 3.61 – 3.43 (m, 3H), 2.91 (d, 1H, *J* = 13.6 Hz), 1.98 – 1.84 (m, 2H, AlkH), 1.74 (s, 3H, CH_3), 1.72 – 1.64 (m, 2H, AlkH), 1.46 – 1.31 (m, 2H, AlkH), 1.30 – 1.12 (m, 4H, AlkH).^{13}C NMR (75 MHz, DMSO-d_6) δ 169.6, 164.8, 152.3, 151.2, 145.9, 116.3, 114.3, 71.6, 48.3, 36.3, 32.6, 32.2, 32.1, 25.2, 24.9, 24.8, 23.0. HRMS (ESI), *m/z* calcd for $C_{17}H_{22}N_4O_5S$ $[M+Na]^+$ 417.1203, found 417.1212.

4.2.2.10. *N*-Benzyl-3-methyl-4-(((5-nitrofuran-2-yl)methylene)amino)-5-oxothiomorpholine-3-carboxamide (10j)

Yield 173 mg (43%). Yellow solid, 141-143 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.19 (s, 1H, N=CH), 7.42 – 7.15 (m, 6H, HetArH+ArH), 6.79 (d, *J* = 3.6 Hz, 1H, HetArH), 6.68 (bs, 1H, NH), 4.74 – 4.56 (m, 1H), 4.49 – 4.33 (m, 1H), 3.65 – 3.42 (m, 3H), 2.96 (d, *J* = 14.2 Hz, 1H), 1.79 (s,

3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 164.9, 152.3, 150.6, 146.0, 137.4, 128.3, 127.2, 127.1, 113.9, 112.2, 72.0, 58.0, 43.6, 37.1, 18.0. HRMS (ESI), *m/z* calcd for $C_{18}H_{18}N_4O_5S$ $[M+Na]^+$ 425.0890, found 425.0880.

4.2.2.11. ***N*-Cyclohexyl-3-methyl-4-(((5-nitrofuran-2-yl)methylene)amino)-5-oxomorpholine-3-carboxamide (10k)**

Yield 144 mg (38%). Yellow solid, mp 127-128 °C. ^1H NMR (300 MHz, DMSO-d_6) δ 9.40 (s, 1H, N=CH), 7.76 (d, *J* = 3.9 Hz, 1H, HetArH), 7.67 (d, *J* = 8.1 Hz, 1H, NH), 7.29 (d, *J* = 3.8 Hz, 1H, HetArH), 4.27 (s, 2H), 4.07 (d, *J* = 11.9 Hz, 1H), 3.83 (d, *J* = 11.9 Hz, 1H), 3.64 – 3.44 (m, 1H, N-CH), 1.79 – 1.43 (m, 8H, CH_3+AlkH), 1.32 – 0.96 (m, 5H, AlkH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.7, 166.2, 152.3, 151.3, 143.6, 116.3, 114.3, 71.9, 68.5, 67.9, 48.5, 32.2, 25.2, 24.8, 24.7, 19.6. HRMS (ESI), *m/z* calcd for $C_{17}H_{22}N_4O_6$ $[M+Na]^+$ 401.1432, found 401.1450.

4.2.2.12. *N*-Benzyl-3-methyl-4-(((5-nitrofuran-2-yl)methylene)amino)-5-oxomorpholine-3-carboxamide (10l)

Yield 170 mg (44%). White solid, mp 105-106 °C. ^1H NMR (300 MHz, DMSO-d_6) δ 9.48 (s, 1H, N=CH), 8.67 – 8.53 (m, 1H, NH), 7.80 (d, *J* = 3.8 Hz, 1H, HetArH), 7.31 (d, *J* = 3.8 Hz, 1H, HetArH), 7.26 – 7.09 (m, 5H, ArH), 4.53 – 4.40 (m, 1H), 4.30 (s, 2H), 4.25 – 4.14 (m, 1H), 4.11 (d, *J* = 12.1 Hz, 1H), 3.90 (d, *J* = 12.0 Hz, 1H), 1.59 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.2, 166.4, 152.3, 151.4, 143.6, 139.3, 128.1, 126.7, 126.6, 116.6, 114.4, 72.1, 68.7, 68.1, 42.5, 19.8. HRMS (ESI), *m/z* calcd for $C_{18}H_{18}N_4O_6$ $[M+Na]^+$ 409.1119, found 409.1138.

4.2.2.13. *N*-(*tert*-Butyl)-3-methyl-4-(((5-nitrofuran-2-yl)methylene)amino)-5-oxomorpholine-3-carboxamide (10m)

Yield 137 mg (39%). Orange solid, mp 110-111 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 9.57 (s, 1H, N=CH), 7.39 (bs, 1H, HetArH), 6.95 (bs, 1H, HetArH), 6.39 (bs, 1H, NH), 4.48 (d, *J* = 11.2 Hz, 1H), 4.43 – 4.26 (m, 2H), 3.70 (d, *J* = 11.9 Hz, 1H), 1.59 (s, 3H, CH_3), 1.38 (s, 9H, $3CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.9, 166.1, 152.3, 150.7, 144.0, 114.2, 112.2, 72.1, 68.8, 67.8, 51.3, 28.2, 20.8. HRMS (ESI), *m/z* calcd for $C_{15}H_{20}N_4O_6$ $[M+Na]^+$ 375.1275, found 375.1292.

4.2.2.14. *N*-Cyclohexyl-2-methyl-1-(((5-nitrofuran-2-yl)methylene)amino)-4-((2-nitrophenyl)sulfonyl)-6-oxopiperazine-2-carboxamide (10n)

Yield 332 mg (59%). Yellow solid, mp 102-104 °C. ^1H NMR (300 MHz, DMSO-d_6) δ 9.23 (s, 1H, N=CH), 8.18 – 7.85 (m, 4H, ArH), 7.84 – 7.71 (m, 2H, NH+HetArH), 7.32 (d, *J* = 3.8 Hz, 1H, HetArH), 4.19 – 3.91 (m, 3H), 3.62 – 3.45 (m, 2H), 1.79 – 1.47 (m, 8H, CH_3+AlkH), 1.32 – 0.97 (m, 5H, AlkH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.2, 163.0, 152.4, 150.9, 147.9, 145.1, 135.3,

132.7, 130.9, 128.7, 124.5, 117.0, 114.2, 68.1, 51.8, 49.3, 48.7, 32.1, 25.3, 24.9, 24.8, 20.6. HRMS (ESI), *m/z* calcd for $C_{23}H_{26}N_6O_9S$ $[M+H]^+$ 563.1555, found 563.1566.

4.2.2.15. *N*-Cyclohexyl-2-methyl-4-(methylsulfonyl)-1-(((5-nitrofuran-2-yl)methylene)amino)-6-oxopiperazine-2-carboxamide (10o)

Yield 277 mg (61%). Light tan solid, mp 179-180 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.27 (s, 1H, N=CH), 7.77 (d, *J* = 3.8 Hz, 1H, HetArH), 7.73 (d, *J* = 7.9 Hz, 1H, NH), 7.32 (d, *J* = 3.8 Hz, 1H, HetArH), 4.18 – 3.91 (m, 2H), 3.83 (d, 1H, *J* = 12.4 Hz), 3.64 – 3.37 (m, 2H), 3.01 (s, 3H, CH_3), 1.89 – 1.43 (m, 8H, CH_3+AlkH), 1.36 – 0.92 (m, 5H, AlkH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.5, 163.7, 152.4, 151.0, 144.8, 116.8, 114.2, 68.1, 51.7, 49.7, 48.6, 35.2, 32.2, 25.3, 24.9, 24.8, 20.8. HRMS (ESI), *m/z* calcd for $C_{18}H_{25}N_5O_7S$ $[M+K]^+$ 494.1106, found 494.1107.

4.3 Antibacterial testing

4.3.1. Investigation of activity against Gram-positive and Gram-negative bacteria

Cultures of the bacteria were grown aerobically at 37 °C overnight in either Müller-Hinton broth with added 1% glucose at pH 7.2 (for Gram-negative strains) or in 30 g/L trypticase soy broth containing 3 g/L yeast extract medium at pH 7.2 (for Gram-positive strains). The cultures were adjusted to 1_106 cfu/mL (which resulted in 5_105 cfu/mL in the test). 25 µL of test culture was added to 25µL of a serial dilution of the test compounds in the appropriate medium for the different strains in accordance with standardized procedures.[40] Test compounds from stock solutions in DMSO were used at final concentrations of 100, 50, 25, 12.5, 6,25, 3.125, 1.56, 0.78, 0.39, 0.2 µM. The highest DMSO concentration in the assay was 1%, which had no apparent effect on the growth of the bacteria. After an incubation time of 18 h at 37 °C under moist conditions, the optical density at 600 nm was measured with a Fusion Universal Microplate Analyser (Perkin–Elmer, Waltham, USA). The lowest concentration that completely suppressed growth defined the MIC values. The following bacterial strains were used. Gram-negative: *Acinetobacter baumannii* (DSM 30007), *Escherichia coli* (DSM 1116), *Klebsiella pneumoniae* (DSM 11678) and *Pseudomonas aeruginosa* PA7 (DSM 24068). Gram-positive: *Enterococcus faecium* (DSM 20477), *Staphylococcus aureus* MRSA (DSM 11822), *Staphylococcus aureus* MRSA (clinical isolate, RKI 11-02670)

4.3.2. Determination of anti-mycobacterial activity

Mycobacterium tuberculosis H_{37}Rv strain (originated from the Institute of Hygiene and Epidemiology in Prague, 1976) was obtained on August 7th, 2013 from the Federal Scientific Center for Expertise of Medical Products (RF Ministry of Health Care). The lyophilized strain was seeded on Löwenstein–Jensen growth medium. The 3-weeks culture was suspended in physiological

solution containing glycerol (15%) was transferred into cryotubes and kept at -80°C. Three weeks in advance of the experiment, the culture was brought to ambient temperature and re-seeded into Löwenstein–Jensen growth medium. Thus, the 2nd generation of the original *M. tuberculosis* culture was used in present study.

The minimal inhibitory concentration (MIC) of the compounds was determined using the REMA (resazurin microtitre plate assay).[41] A 3-week *M. tuberculosis* culture was transferred into a dry, sterile tube containing 8-9 3-mm glass beads. The tube was placed on a Vortex shaker for 30-40 s and then 5 mL Middlebrook 7H9 Broth (Becton Dickinson, catalogue No. 271310) was introduced. The turbidity of bacterial suspension was adjusted to 1.0 McFarland units (corresponding to approximately $3x10^8$ bacteria/mL) and diluted 20-fold with Middlebrook 7H9 Broth containing OADC enrichment (Becton Dickinson, catalogue No. 245116). The same culture medium was used to prepare the 1:100 *M. tuberculosis* (1% population) control. The stock solutions of the compounds in DMSO (10 mg/mL) were diluted with Middlebrook 7H9 Broth (containing OADC enrichment) to a concentration of 800 μg/mL. 200 μL of the solution thus obtained was introduced into the 2nd row of a 96-well microtitre plate. This raw was used to perform 2-fold serial dilutions using and 8-channel pipette to obtain final concentrations of 1.6, 3.1, 6.2, 12.5, 25, 50, 100, 200 and 400 μg/mL concentrations of the compound in rows 2-9 (accounting for 100 μL of bacterial suspension introduced for testing). Row 10 – MTb suspension control, row 11 – same culture diluted 10-fold (the 1% control). Row 12 was used as a blank control for optical density reading (200 μL of the grown medium). The bacterial suspension (100 μL) was introduced into each well except rows 11 (1% population control) and 12 (blank culture medium), to the total volume of 200 μL in each well. The plates were incubated at 35 °C for 7 days. At that point, 0.01% aqueous solution (30 μL) of resazurin (Sigma, product No. R7017) was introduced in each well and the incubation continued for 18 h at 35 °C. The fluorescence reading was performed using FLUOstar Optima plate reader operating at λ_{ex} = 520 nm and λ_{em} = 590 nm. The bacterial viability was determined by comparing the mean values (± SD at p = 0.05) of fluorescence in the control wells (row 12, blank and row 11, 1% control) and the wells containing the compound tested. The MIC was determined as the compound concentration at which the fluorescence reached a plateau or was statistically (t criterion) similar to that of 1% control.

The mutant, patient-derived multidrug- and poly-resistant strains of *M. tuberculosis* were obtained from the pathogenic mycobacterial strain collection of Saint Petersburg Research Institute of Phthisiopulmonology. These strains were genotyped using microchip technology and were confirmed to contain various combinations of mutations in genes *rpoB* (resistance to rifampicin), *katG*, *inhA* and *ahpC* (resistance to isoniazid) as shown in Table 4.

4.3.3. Cell viability assay

The effect of compounds on cell viability was probed with WST-1 tests using the procedure of Ishiyama *et al.*[42] as modified by Sasse *et al.*[43] The following immortalized cell lines were used: mouse fibroblast cell line L929 (DSM ACC 2), human cervix carcinoma cell line KB-3-1 (DSM ACC 158) and human breast cancer cell line MCF-7 (DSM ACC 115). In addition, the conditionally immortalized human fibroblast cell line FS4-LTM was used without doxycyclin to induce primary cell-like behavior (Pub. No.: US2011/0189142 A2). Briefly, the subconfluent cells were washed with Earle's Balanced Salt Solution, Gibco, without Ca and Mg, trypsinized and re-suspended in Dulbecco's modified eagle's medium that contained 5% fetal bovine serum (FBS; L929, KB-31, FS4-LTM) or Roswell Park Memorial Institute medium that contained 5% FBS, 0.5% Minimum Essential Medium Non-Essential Amino Acids, Gibco (MEM NEAA), 0.5% GlutaMAX (Gibco) and insulin at 5 μg/mL (MCF-7). 25μl μL of serial dilutions of the test compounds were added to 25μl μL aliquots of a cell suspension (1500 cells for KB3-1 and L929, 3000 cells for MCF-7) in384 well microtiter plates. Blank and solvent controls were incubated under identical conditions. The compounds were incubated for 5 days with cell lines L929, KB-3-1, and MCF-7, and for 24 h with cell line FS4-LTM. After the incubation period 3 μL WST-1 (ready to use solution by Roche) was added. The microsomal triglyceride transfer proteins were briefly shaken and then centrifuged at 1800 g for 3 min. The incubation time of the plates at 37 °C varied between the cell lines from 20 min for KB-3-1 to 2 h for MCF-7 before measuring at 450 nm (reference 600 nm) at the Infinite 200 PRO plate reader (Tecan, Männedrof, Switzerland). The percentage of viable cells was calculated as the mean with respect to the controls set to 100%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/xxx.

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