In vitro profiling of new antimycobacterial compounds and their peptide conjugates

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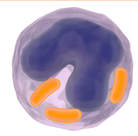
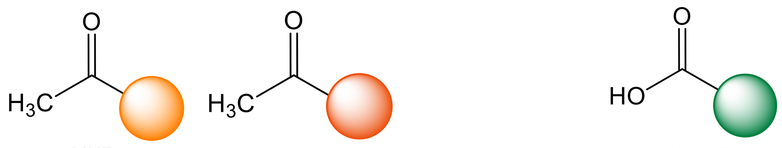
INTRODUCTION

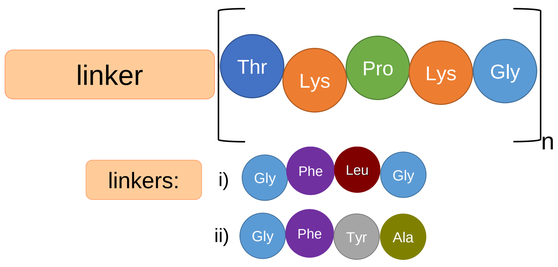
Tuberculosis is an infectious disease caused by the intracellular bacteria *Mycobacterium tuberculosis*. These pathogens live and reproduce in their host cells, mainly macrophages. Nowadays, spreading of the drug-resistant strains of *Mycobacteria* is a serious health threat and there is an urgent need for development of novel compounds with no cross-resistance to current therapeutic options. Salicylanilide derivatives have great potential as antimycobacterial compounds[1-6] with pronounced inhibitory effect against not only *M. tuberculosis* but nontuberculous (also called atypical) mycobacteria (NTM), like highly chemoresistant *M. avium* and *M. kansasii*.[7-11]



CONJUGABLE COMPOUNDS AND PEPTIDES

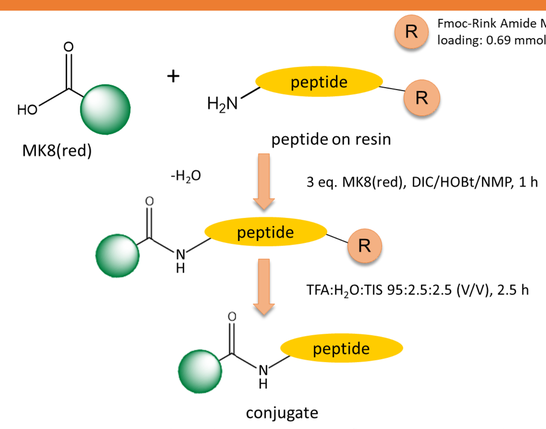


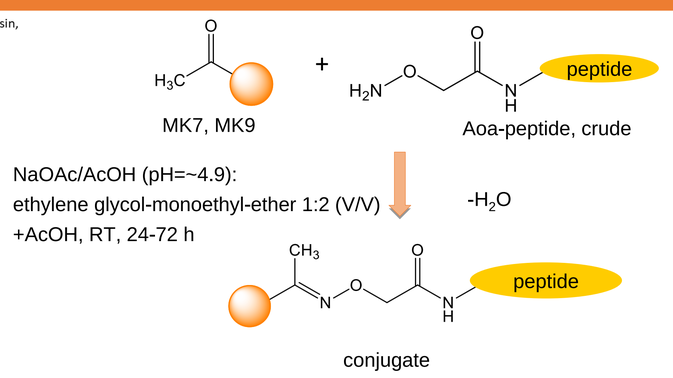
Novel hydroxybenzoic acid derivatives were designed and synthesized.



Tuftsin derivatives were used as targeting peptides.[11-13] Oligo-tuftsin peptides $[TKPKG]_n$ (n=1, 2, 3) with and without enzyme cleavable linker sequences (GFLG, GFYA)[14] were synthesized using Fmoc/^{t}Bu strategy.

CONJUGATION REACTIONS





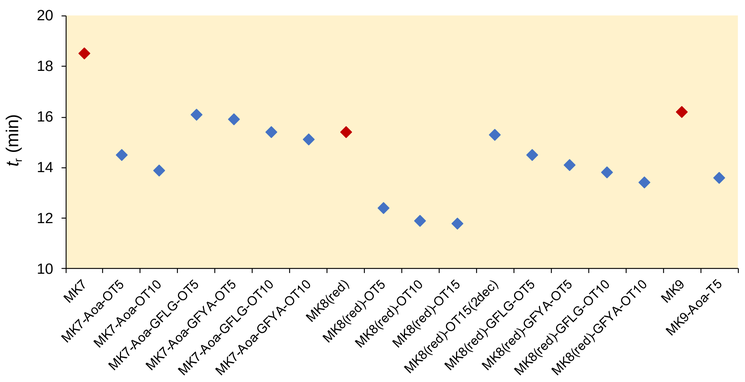
Amide bond was formed on resin. Oxime bond was formed in liquid phase under acidic conditions.[11] Crude products and reaction mixtures were purified using semi-preparative RT-HPLC.

CHEMICAL CHARACTERIZATION OF THE CONJUGATES

Name	$M_{av/mono(calc)}$	M_{meas}*	ppm	t_r** (min)
MK7-Aoa-OT5	912.0002 / 911.4501	911.4505	0.46	14.5
MK7-Aoa-GFLG-OT5	1286.4344 / 1285.6455	1285.6459	0.33	16.1
MK7-Aoa-GFYA-OT5	1350.4766 / 1349.6404	1349.6406	0.18	15.9
MK7-Aoa-OT10	1423.6152 / 1422.7619	1422.7614	-0.34	13.9
MK7-Aoa-GFLG-OT10	1798.0493 / 1796.9574	1796.9580	0.36	15.4
MK7-Aoa-GFYA-OT10	1862.0915 / 1860.9523	1860.9518	-0.25	15.1
MK9-Aoa-OT5	897.9737 / 897.4345	897.4344	-0.09	13.6

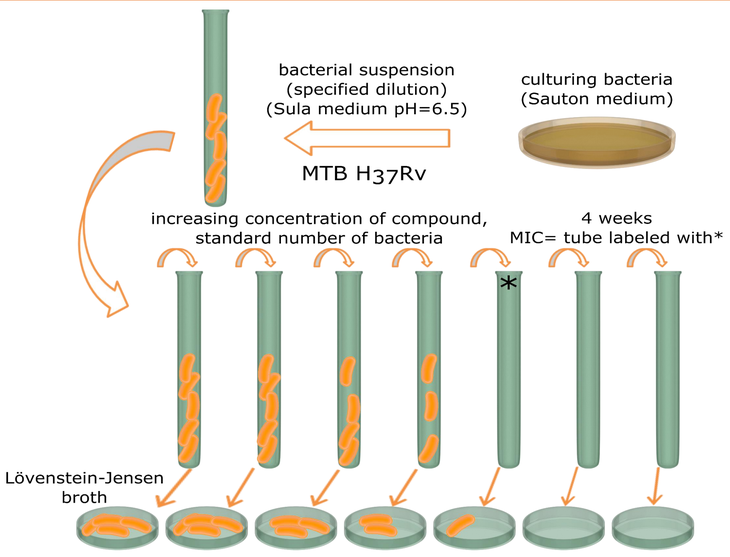
Name	$M_{av/mono(calc)}$	M_{meas}*	ppm	t_r** (min)
MK8(red)-OT5	735.8282 / 735.3915	735.3922	0.98	12.4
MK8(red)-GFLG-OT5	1110.2624 / 1109.5869	1109.5867	-0.16	14.5
MK8(red)-GFYA-OT5	1174.3046 / 1173.5819	1173.5828	0.80	14.1
MK8(red)-OT10	1247.4432 / 1246.7034	1246.7031	-0.22	11.9
MK8(red)-GFLG-OT10	1621.8873 / 1620.8988	1620.8982	-0.35	13.8
MK8(red)-GFYA-OT10	1685.9195 / 1684.8937	1684.8912	-1.46	13.4
MK8(red)-OT15	1759.0581 / 1758.0152	1758.0144	-0.43	11.8
MK8(red)-OT15(2dec)	1913.3074 / 1912.1510	1912.1512	0.13	15.3

* Thermo Scientific Q Exactive Focus, electrospray ionization (ESI), Orbitrap analyzer
**Exformma EX1600, (Waters Symmetry®, C18, 4.6x150 mm, 100 Å column), gradient: 0-60 B%, 0-10 min; 60-100 B% 10-20 min, 1 ml/min, λ = 220 nm. Eluent A: 0.1% TFA/water (V/V), eluent B: 0.1% TFA/acetonitrile/water 80:20 (V/V)



Retention times of free compounds (red) and conjugates (blue) on reverse phase column. Based on the retention times, lipophilic character of the conjugates can be estimated and compared.[11] Free compounds are more lipophilic than conjugates.

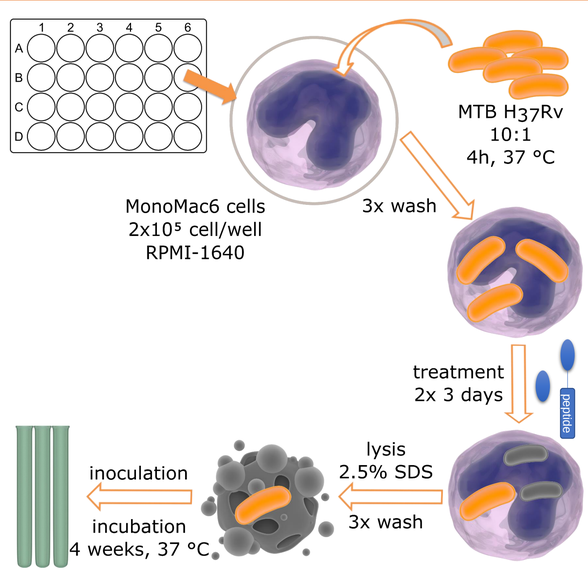
IN VITRO ANTIMYCOBACTERIAL EFFECT



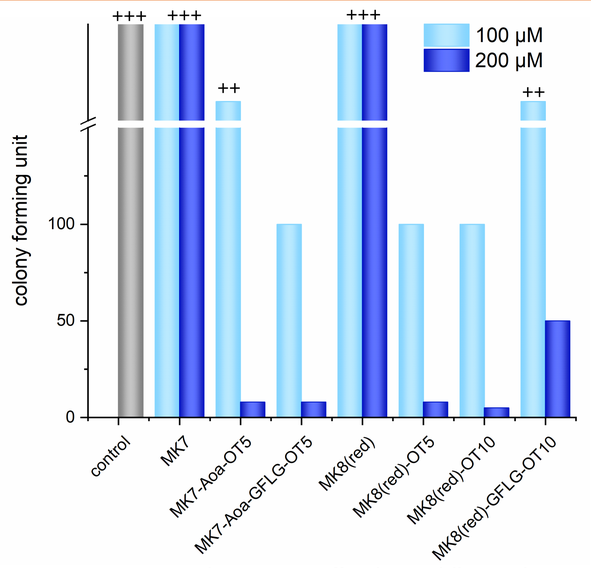
In vitro antimycobacterial activity was determined on *M. tuberculosis* $H_{37}Rv$ bacterial culture using broth dilution method.[15-19,11] Free compounds and peptide conjugates have antimycobacterial effect.

Name	MIC *M. tuberculosis* $H_{37}Rv$ (μg/ml) (μM)	CFU
MK7	40 (122)	24
MK8(red)	1 (4)	8
MK9	0.5 (1.5)	5
MK7-Aoa-OT5	20 (22)	3
MK7-Aoa-GFLG-OT5	40 (31)	14
MK7-Aoa-GFYA-OT5	60 (45)	4
MK7-Aoa-GFLG-OT10	80 (59)	8
MK7-Aoa-GFYA-OT10	80 (62)	28
MK9-Aoa-OT5	5 (6)	7
MK8(red)-OT5	20 (27)	2
MK8(red)-GFLG-OT5	20 (18)	40
MK8(red)-GFYA-OT5	40 (34)	10
MK8(red)-OT10	20 (16)	1
MK8(red)-GFLG-OT10	60 (37)	33
MK8(red)-GFYA-OT10	80 (48)	4
MK8(red)-OT15	20 (11)	0
MK8(red)-OT15(2dec)	20 (11)	6

IN VITRO INTRACELLULAR EFFECT

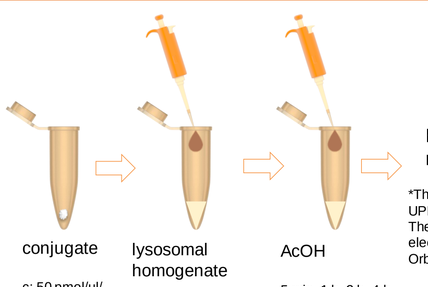


In vitro intracellular activity was determined on MonoMac6 cells infected with *M. tuberculosis* $H_{37}Rv$ bacteria.[20]

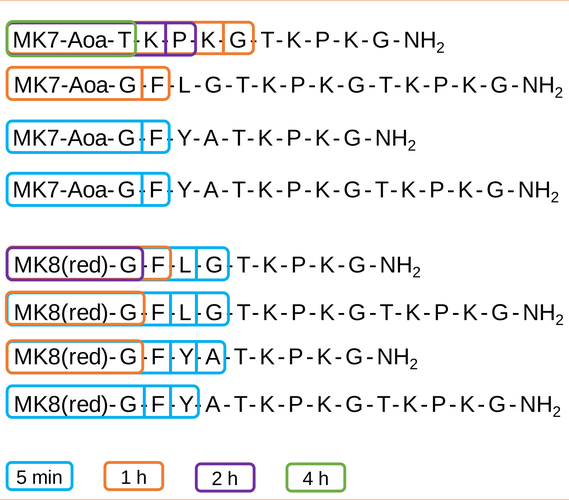


Free compounds are not effective; while conjugates inhibit the intracellular bacteria in a concentration-dependent manner.

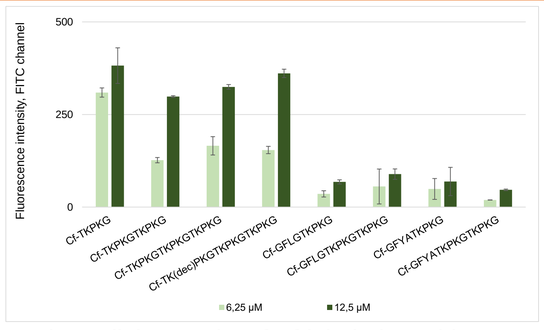
LYSOSOMAL DIGESTION PATTERN



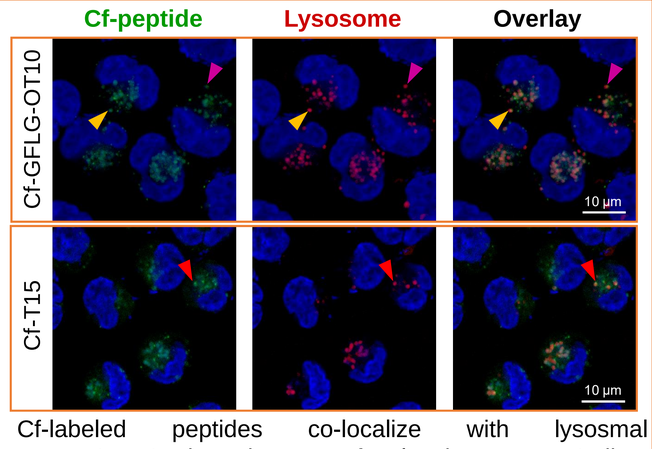
Lysosomal digestion pattern was determined in rat liver lysosomal homogenate.[11] Metabolites were identified using high resolution HPLC-MS instrument. Smallest metabolites are the conjugable compounds and the first amino acids.



IN VITRO CELLULAR UPTAKE STUDIES



In vitro cellular uptake of Cf-labeled peptides was determined using flow cytometry. Cellular uptake is concentration dependent. In comparison with non-linker containing tuftsin derivatives, peptides containing linker sequences have decreased cellular uptake.



Cf-labeled peptides co-localize with lysosmal compartments based on confocal microscopy studies. Treatment: 50 μM, 90 min. blue: nucleus.

CONCLUSION

Novel hydroxybenzoic acid derivatives have been designed and synthesized. These compounds were conjugated via oxime and amide bond to tuftsin peptide carriers. The hydroxybenzoic acid derivatives have antimycobacterial effect and this effect remains after conjugation. However, the free compounds lack intracellular activity, conjugates inhibit intracellular bacteria in a concentration-dependent manner. The lysosomal digestion pattern of the conjugates was determined. Smallest metabolites containing hydroxybenzoic acid derivatives are the compounds and the first amino acids. *In vitro* cellular uptake is concentration dependent. The Cf-labeled peptides co-localize with lysosomal compartments. The compounds have moderate *in vitro* cytostatic effect on hepatocytes and monocytes.

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