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Peptide conjugates of therapeutically used antitubercular isoniazid – design, synthesis and antimycobacterial effect

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Tuberculosis (TB) is a bacterial infectious disease caused by *Mycobacterium tuberculosis*, a slow-growing, powerful human pathogen which can survive in the host macrophages. In the chemotherapy of such intracellular pathogens it is necessary to achieve relatively high level of the drug in blood to attain therapeutically effective concentration in infected cells, which presumably has several serious side effects on healthy tissues. The elimination of *M. tuberculosis* from infected phagocytes could be more efficient with target cell-directed delivery of antituberculars. A particularly promising approach is to conjugate a drug moiety to a peptide based carrier. The conjugates are chemically constructed to target release by hydrolysis (enzymatic and/or chemical) to liberate the active compound. Here we report the synthesis, characterisation and antimycobacterial evaluation of isoniazid (INH) peptide conjugates. As carrier moiety T-cell epitope of immundominant 16-kDa protein of *M. tuberculosis* and tuftsin-derived peptides were used. To conjugate INH two synthetic methods were developed, where INH was coupled directly to the peptides or through a heterobifunctional reagent. We found that all of the INH conjugates were effective against *M. tuberculosis* and the minimal inhibitory concentration (MIC) values were comparable to the free INH moiety. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Introduction

Tuberculosis (TB) is responsible for more deaths throughout the world than any other infectious disease. The disease affects both BCG-vaccinated and non-vaccinated people, and susceptibility to infection and disease is related to decreased host immunity. It is estimated that more than one-third of the world's population is infected with *Mycobacterium tuberculosis* and over three million people die each year of TB, resulting in the World Health Organisation declaring TB a global emergency [1].

M. tuberculosis is a slow-growing (replicates in 15–20 h) intracellular pathogen which has a robust complex cell wall containing mycolic acid and multimethyl-branched fatty acids, unique for pathogenic mycobacteria. The pathogenicity of *M. tuberculosis* is based on the ability (i) to arrest phagolysosome biogenesis, (ii) to neutralise the phagosomes and (iii) to block the efficient antigen processing and presentation.

In the treatment of tuberculosis long duration of a relatively high amount of antituberculars is required because the intracellular dormant mycobacteria are particularly protected against antibiotic drugs. Isoniazid (INH) is a frontline antibiotic that must be taken for a minimum of 6 months, and it inhibits the formation of mycolic acid cell wall [2,3]. INH is a prodrug activated by catalase/peroxidase activity of a KatG gene product [4,5]. Resistance to INH is based on the binding to arylamine *N*-acetyltransferase (NAT) enzyme which inactivates INH by its acetylation [6]. Although several analogues of INH have been synthesised, none of them featuring a peptide moiety where INH was covalently attached.

The development of new chemotherapy for the treatment of TB has at least four major objectives: (i) the development of faster-acting drugs (small organic compounds) to shorten the duration of treatment; (ii) the development of novel antimicrobials to counter the emergence of bacteria resistant to current therapies; (iii) the development of chemotherapeutics that specifically target dormant bacilli to treat the one-third of the world's population latently infected with tubercle bacilli [7]; and (iv) the development of synthetic delivery compounds to achieve host macrophage using specific transport for the active drug (recently used or new) moieties.

The specific transport of the drug molecules to the infected macrophages as an alternative approach would be based on receptor-mediated endocytosis through specific cell surface molecules and receptors which are expressed mainly on macrophages (i.e. tuftsin receptor, scavenger receptor) [8–10]. In the literature, conjugation of *p*-aminosalicylic acid (PAS) to maleylated bovine serum albumin was reported, and cellular uptake by macrophages through scavenger receptor was increased. The conjugate was nearly 100 times as effective as free PAS in killing intracellular mycobacteria [11]. The use of oligopeptides as targeting moiety is a new approach and has several advantages compared to protein carriers. The synthesis of covalently attached drug–peptide conjugates is absolutely reproducible, and the conjugates are chemically well characterised. In this study we have

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investigated synthetic oligopeptides described below as possible carrier molecules for receptor-mediated delivery of recently used antitubercular drug, INH.

Tuftsin is a natural phagocytosis stimulating peptide produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G [12–17]. During the past decade, a new group of sequential oligopeptide carriers with discrete molecular masses and defined sequences has been developed in our laboratory: oligotuftsin derivatives consisting of tandem pentapeptide repeat unit $[TKPKG]_n$ ($n = 2, 4, 6, 8$) based on the canine tuftsin sequence TKPK [18,19]. These compounds are non-toxic, non-immunogenic and exhibit tuftsin-like biological properties, e.g. receptor binding, immunostimulatory activity and chemotactic activity on monocytes and macrophages. In this study, a tuftsin derivative (GTKPKG) and TKPKG-based oligotuftsin ($n = 4$; OT20, [18]) were used as carrier peptides [19]. The 16 kDa low-molecular weight heat shock protein (Hsp16.3) is an immundominant protein expressed by *M. tuberculosis*. This is the dominant protein accumulating in the latent stationary phase of TB infection and expressed during latency. Peptide ^{91}SEFAYGSFVRTVSL^{104} corresponding to the 91–104 sequence of the 16 kDa protein is the functional T-cell epitope, and it provokes a specific immune response [20–23]. Use of T-cell epitope peptides represents a further advantage as they can be presented directly by the surface major histocompatibility complex (MHC) molecules (human leukocyte antigen (HLA) class II molecules) of antigen presenting cells like monocytes and macrophages. Peptide ^{91}SEFAYGSFVRTVSL^{104} binds to the HLA class II molecules [24]. It is considered that naturally processed peptides enter the endosomal processing pathway in which the bulk of *de novo* synthesised HLA class II molecules are loaded [25]. By contrast, synthetic peptides are thought to have limited access to this dominant pathway and rely instead for their presentation on a small number of recirculating cytoplasmic MHC molecules or by peptide exchange occurring at the cell surface [26–30].

In this study we report for the first time the synthesis, characterisation, stability and *in vitro* antimycobacterial activity of INH–peptide conjugates. For these conjugates tuftsin derivative (GTKPKG), TKPKG-based oligotuftsin (OT20) and T-cell epitope-derived oligopeptides were used as carriers. Our primary aim was to study the effect of chemical linkage of the INH on its *in vitro* antimycobacterial activity.

Materials and Methods

Materials

INH, *N*,*N*′-diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIEA), thioanisole, 1,2-ethanedithiol (EDT), phenol and $NaIO_4$ were purchased from Fluka (Buchs, Switzerland). The amino acid derivatives were obtained from Reanal (Budapest, Hungary) and IRIS Biotech (Marktredwitz, Germany). 1-Hydroxybenzotriazole (HOBt) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were also from IRIS Biotech. Resins (MBHA and Rink-Amide MBHA) were purchased from Novabiochem (Darmstadt, Germany). Acetonitrile, TFA, *N*-methylpyrrolidone (NMP), glyoxylic acid, DMSO and $NaBH_3CN$ were from Merck (Darmstadt, Germany). Ninhydrin was from Sigma-Aldrich (Budapest, Hungary). DMF and dichloromethane (DCM) were from Reanal (Budapest, Hungary). Löwenstein-Jensen medium base and the components of the Sula medium were purchased from Sigma-Aldrich (Budapest, Hungary).

Synthesis of T-cell Epitope Based Carrier Peptides: ^{91}SEFAYGSFVRTVSLPV^{106}, S^{91}SEFAYGSFVRTVSLPV^{106}, ^{91}SEFAGAGFVRAGAL^{104}

The 16 kDa epitope peptide ^{91}SEFAYGSFVRTVSLPV^{106} and its derivatives S^{91}SEFAYGSFVRTVSLPV^{106}, ^{91}SEFAGAGFVRAGAL^{104} were produced manually by solid-phase synthesis on Rink-Amide MBHA resin (0.69 mmol/g capacity) using Fmoc/^{t}Bu strategy. The following side-chain-protected amino acids were used: Ser(^{t}Bu), Glu(O^{t}Bu), Tyr(^{t}Bu), Arg(Pbf), Thr(^{t}Bu). The protocol of the synthesis was the following: (i) Fmoc deprotection with piperidine/DBU/DMF (2 : 2 : 96 v/v) solution (four times, 2 + 2 + 5 + 10 min); (ii) washing with DMF (5 × 1 min); (iii) coupling 3 equiv. of Fmoc-amino acid derivative – DIC–HOBt dissolved in NMP (60 min); (iv) washing with DMF (5 × 1 min); (v) ninhydrin [31] or isatin [32] assay. Peptides were cleaved from the resin with TFA/H_2O/thioanisole/EDT/phenol (10 ml : 0.5 ml : 0.5 ml : 0.25 ml : 0.75 g) mixture (2 h, RT). After filtration peptides were precipitated with cold diethyl ether, centrifuged and freeze-dried in water. Crude products were purified by semi-preparative RP-HPLC as described below. Purified peptides were analysed by analytical RP-HPLC, ESI-MS and amino acid analysis.

Selective Oxidation of Peptides S^{91}SEFAYGSFVRTVSLPV^{106} and ^{91}SEFAGAGFVRAGAL^{104}

N-terminal serine containing peptides S^{91}SEFAYGSFVRTVSLPV^{106} or ^{91}SEFAGAGFVRAGAL^{104} were dissolved in 1% NH_4HCO_3 (pH 8.2, $c = 5$ mg/ml). Peptides were oxidised with 4 equiv. $NaIO_4$ in the presence of 10 equiv. of methionine (50 min, RT) [33]. After adding 10 equiv. ethylene glycol, the reaction mixture was purified by semi-preparative RP-HPLC as described below. Selectively oxidised peptides were analysed by analytical RP-HPLC, ESI-MS and amino acid analysis as described below.

Conjugation of Isoniazid (INH) to *N*-glyoxylyl-^{91}SEFAYGSFVRTVSLPV^{106} and to *N*-glyoxylyl-^{92}EFAGAGFVRAGAL^{104} Through Hydrazone Linkage

Selectively oxidised peptide *N*-glyoxylyl-^{91}SEFAYGSFVRTVSLPV^{106} or *N*-glyoxylyl-^{92}EFAGAGFVRAGAL^{104} was dissolved in 0.1 M NH_4OAc (pH 4.6, $c = 10$ mg/ml). The 10 equiv. INH was dissolved in 1 ml acetonitrile and added to the peptide solution [34]. After 60 min shaking the reaction mixture was purified by semi-preparative RP-HPLC. The isolated product was lyophilised and characterised by analytical RP-HPLC and ESI-MS.

Synthesis of Isonicotinoylhydrazonoacetic Acid

The 10 mmol (1.37 g) INH was dissolved in acetonitrile/water (1 : 4 v/v) and added to the aqueous solution of glyoxylic acid monohydrate (10 mmol, 0.92 g/2 ml). After 60 min the precipitate was filtered, washed with water and dried over P_2O_5 under vacuum. The hydrazone bond containing compound was obtained as a white solid (97% yield). ESI-MS calcd 193.1 (M_{mo}) found 193.1; melting point: 204.5 °C.

Synthesis of Isonicotinoylhydrazinoacetic Acid by the Reduction of Hydrazone Linkage

An amount of 9 mmol (1.75 g) isonicotinoylhydrazonoacetic acid was suspended in 15 ml abs. methanol. Equimolar $NaBH_3CN$

(9 mmol, 0.57 g) was added and stirred for 1 h at room temperature to obtain a yellowish solution. After further 12 h of stirring the reaction mixture was evaporated to dryness under reduced pressure, then the product was recrystallised from methanol. (92% yield). ESI-MS calcd 195.1 (M_{mo}) found 195.1; melting point: 192.5 °C.

Conjugation of Isonicotinoylhydrazinoacetic Acid to ^{91}SEF-AYGSFVRTVSLPV^{106} Peptide on Solid Phase

N-terminally deprotected peptidyl-resin was treated with 5 equiv. isonicotinoylhydrazinoacetic acid, HOBt and DIC (dissolved in NMP, 5 equiv. was calculated to the capacity of the resin used). After 1 h the coupling was monitored by ninhydrin assay. The modified peptide was cleaved from the resin with TFA/H_2O/thioanisole/EDT/phenol (10 ml : 0.5 ml : 0.5 ml : 0.25 ml : 0.75 g) mixture (2 h, RT). After filtration INH–CH_2–CO–^{91}SEFAYGSFVRTVSLPV^{106} conjugate was precipitated with cold diethyl ether, centrifuged and freeze-dried in water. Crude product was purified by semi-preparative RP-HPLC and characterised by analytical RP-HPLC and ESI-MS.

Synthesis of GTKPK(INH-CH_2-CO)G Conjugate and its Tetramer [TKPK(INH-CH_2-CO)G]$_4$ Analogue

The protected tuftsin derivative (GTKPKG, T6 and [TKPKG]$_4$, OT20) peptides were produced manually by solid-phase synthesis on MBHA resin (1.1 mmol/g capacity) using Boc/Bzl strategy. The following side-chain-protected amino acids were used: Lys(ClZ), Lys(Fmoc) and Thr(Bzl). The protocol of the synthesis was the following: (i) Boc deprotection with TFA/DCM (1 : 2 v/v) mixture (2 + 20 min); (ii) washing with DCM (5 × 1 min); (iii) neutralisation with DIEA/DCM (1 : 9 v/v) (5 × 1 min); (iv) washing with DCM (5 × 1 min); (v) coupling 3 equiv. of Boc-amino acid derivative – DIC – HOBt dissolved in NMP (60 min); (vi) washing with DCM (5 × 1 min); (vii) ninhydrin [31] or isatin [32] assay.

The N^{ε}-Fmoc protecting group of the side chain of Lys residue was selectively removed with piperidine/DBU/DMF (2 : 2 : 96 v/v) solution (four times, 2 + 2 + 5 + 10 min). After DMF washing (5 × 1 min) 5 equiv. isonicotinoylhydrazinoacetic acid – DIC – HOBt (calculated to deprotected lysines) was added to the peptidyl-resin (suspended in NMP, 60 min). *N*-terminal Boc protection was removed with 33% TFA/DCM mixture (2 + 20 min). Peptide conjugates were cleaved from the resin with liquid HF in the presence of *p*-cresol (10 ml : 0.5 g) (0 °C, 1.5 h). The modified-tuftsin peptides GTKPK(INH-CH_2-CO)G and [TKPK(INH-CH_2-CO)G]$_4$ were precipitated with cold diethyl ether, dissolved in acetic acid and freeze-dried. Crude products were purified by semi-preparative RP-HPLC and characterised by analytical RP-HPLC, ESI-MS and amino acid analysis as described below.

Amino Acid Analysis

Amino acid analysis was performed on Sykam S433H Amino Acid analyser (Eresing, Germany) equipped with an ion exchange separation column and post-column derivatisation. Prior to analysis, samples were hydrolysed with 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h. For post-column derivatisation the ninhydrin method was used.

High-performance Liquid Chromatography

Peptides, amino acid derivatives and the reaction mixtures were analysed by RP-HPLC using a laboratory-assembled Knauer HPLC system (Bad Homburg, Germany) with an Eurospher-100 C-18 column (250 mm × 4 mm, 5 µm particle size, 300 Å pore size) (Knauer, Bad Homburg, Germany). The gradient elution system consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80/20 (v/v) (eluent B). The eluent B content was 5% for 5 min, then varied from 5 to 60% in 35 min with 1 ml/min flow rate at room temperature. 20 µl of sample was injected, and peaks were detected at λ = 214 nm.

Crude peptides and peptide derivatives were purified on semi-preparative C-18 Phenomenex Jupiter column (250 mm × 10 mm) packed with 10 µm silica (300 Å pore size) (Torrance, CA, USA). The same eluents were used as described above with linear gradient elution of 10–80% B in 40 min. An amount of 5–10 mg sample in 2 ml A eluent was injected to the column with 4 ml/min flow rate.

Mass Spectrometry

Mass spectrometric analyses were performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany) equipped with ESI source. Spray voltage was set to 4.0 kV, and 40.0 V orifice voltage was applied. Samples were dissolved in a mixture of acetonitrile/water = 1/1 (v/v) containing 0.1% acetic acid and introduced by a syringe pump with a flow rate of 10 µl/min. The instrument was used in positive mode in the range of 50–3000 *m/z* with 13.000 *m/z/s* scan resolution.

Evaluation of Antimycobacterial Activity

In vitro antimycobacterial activity of the compounds was determined on *M. tuberculosis* H_{37}Rv (ATCC 27294) in Sula semi-synthetic medium, which was prepared in-house [35–37] at pH 6.5 by serial dilution. The test compounds were added to medium as aqueous solutions. The following final concentrations of the tested compounds were used: 0.05, 0.10, 0.14, 0.16, 0.18, 0.20, 0.22, 0.24, 0.32 and 0.40 µg/ml. Minimal inhibitory concentration (MIC) was determined after incubation at 37 °C for 28 days. MIC was the lowest concentration of a compound at which the visible inhibition of the growth of *M. tuberculosis* H_{37}Rv occurred. The activities of the tested compounds were confirmed using a colony forming unit (CFU) determination by subculturing from the Sula medium onto drug-free Löwensten-Jensen solid medium. The samples were incubated for further 28 days [36,38,39]. The experiments were repeated at least two times with similar results.

Stability of Isoniazid Conjugates

The stability of hydrazone and hydrazide bond containing INH conjugates was analysed by RP-HPLC sampling over 24 h. Compounds were dissolved in Sula semi-synthetic liquid medium (pH 6.5) in 0.5 mg/ml concentration. Hourly 20 µl of the samples was injected to Eurospher-100 C-18 column (250 mm × 4 mm, 5 µm particle size, 300 Å pore size) (Knauer, Bad Homburg, Germany). Linear gradient elution was used (5%B, 5 min; 5–60% B, 35 min) with 1 ml/min flow rate. The amount of the intact conjugate was determined from the peak area on the chromatogram.

Results

INH was conjugated to immunodominant T-cell epitope peptides and tuftsin derivatives. INH was coupled to peptides in two

different ways: (i) in solution, where first the peptides were modified, then reacted with INH to obtain a hydrazone derivative. Alternatively, (ii) glyoxylic acid as a heterobifunctional reagent was used to obtain an INH derivative which can be coupled to peptides (to *N*-terminal and to Lys side chains) on solid phase.

Synthesis of Carrier Peptides

To achieve antitubercular drug–peptide conjugates, carrier peptides were synthesised using standard Fmoc/^{t}Bu or Boc/Bzl strategies. All of the peptides were obtained with amide *C*-terminal after final cleavage. These peptides were also used as control compounds in the further experiments. Data concerning analytical characterisation of peptides are presented in Table 1. All purified peptides demonstrated a single peak on analytical RP-HPLC with a mass coinciding with the theoretically calculated molecular mass. The amino acid composition of peptides were proved by amino acid analysis where the accuracy was less than 5%.

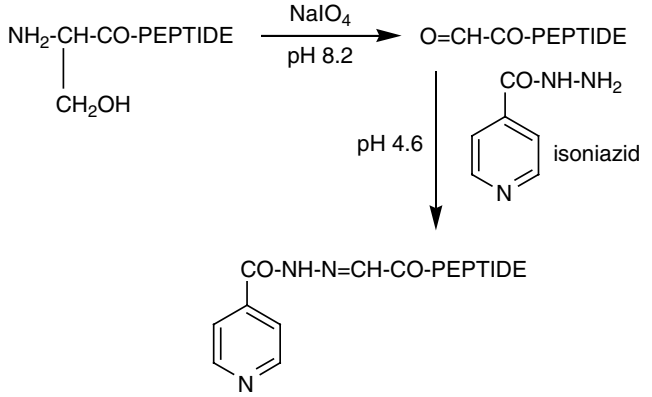

Figure 1. Outline of the conjugation of isoniazid (INH) to a peptide aldehyde. Peptide elongated with *N*-terminal Ser residue was first selectively oxidised with sodium periodate. After purification the peptide aldehyde was reacted with the excess of INH to result a hydrazone.

Conjugation of Isoniazid

In order to prepare INH-bearing peptide conjugates two strategies were developed. In the first case as outlined in Figure 1, peptide elongated with *N*-terminal Ser residue was selectively oxidised with sodium periodate in 1% NH_4HCO_3 (pH 8.2). Met was used in the reaction as scavenger. After stopping the reaction with excess of ethylene glycol, *N*-glyoxylyl-peptide derivatives were lyophilised and dissolved in 0.1 M NH_4OAc (pH 4.6) and allowed to react with INH. The INH conjugates were purified and carefully characterised (Table 1).

In the other strategy as summarised in Figure 2, INH was reacted with glyoxylic acid to produce isonicotinoylhydrazonoacetic acid in a good yield (97%). To synthesise a chemically more stable hydrazide analogue the compound was mildly reduced with $NaBH_3CN$ in methanol. Isonicotinoylhydrazinoacetic acid was obtained in a good yield (92%). The reaction of isonicotinoyl-hydrazinoacetic acid with Fmoc-deprotected peptides on solid phase was used for the introduction of INH. Using standard coupling reagents (DIC, HOBt) INH was conjugated to *N*-terminus or Lys side chains of the carrier peptide. In the case of tuftsin tetramer analogue four copies of INH were coupled. The produced hydrazides remain stable during TFA and HF final cleavage. The purified conjugates were characterised by RP-HPLC and ESI-MS (Table 1).

Antitubercular Effect of Conjugates

The *in vitro* antimycobacterial activity of the new conjugates was characterised by the determination of the MIC using *M. tuberculosis* $H_{37}Rv$ strain with 4-week exposure period. The data obtained are summarised in Table 2. The MIC value of the free INH was close to the values mentioned in the literature (INH: 0.02–0.2 γ) [40]. All of the INH conjugates were effective against the bacteria and exhibited almost the same MIC value (0.16–0.40 γ) as free INH.

Table 1. Analytical characterisation of peptide–drug conjugates and free carrier peptides

Compound	M_{mo}[a] calcd/found	R_t[b] (min)	amino acid composition[c] found (calcd)
^{91}SEFAGAGFVRAGAL^{104}	1350.7/1350.7	28.3	S 0.92 [1]; E 1.14 [1]; G 2.86 [3]; A 4.03 [4]; V 1.00 [1]; L 1.03 [1]; F 1.92 [2]; R 0.97 [1]
N-glyoxylyl-^{92}EFAGAGFVRAGAL^{104}	1319.7/1319.8	29.2	E 1.18 [1]; G 2.77 [3]; A 4.05 [4]; V 1.05 [1]; L 1.02 [1]; F 2.07 [2]; R 0.89 [1]
INH=CH-CO-^{92}EFAGAGFVRAGAL^{104}	1438.8/1438.8	29.8	
^{91}SEFAYGSFVRTVSLPV^{106}	1756.9/1756.9	32.3	T 0.94 [1]; S 2.78 [3]; E 1.08 [1]; P 1.11 [1]; G 1.08 [1]; A 1.11 [1]; V 2.97 [3]; L 1.05 [1]; Y 0.90 [1]; F 2.03 [2]; R 1.05
N-glyoxylyl-^{91}SEFAYGSFVRTVSLPV^{106}	1812.9/1813.0	33.0	
INH=CH-CO-^{91}SEFAYGSFVRTVSLPV^{106}	1932.0/1932.0	31.4	
INH-CH_2-CO-^{91}SEFAYGSFVRTVSLPV^{106}	1934.0/1934.0	30.7	
GTKPKG	585.3/585.3	9.5	T 0.96 [1]; P 1.05 [1]; G 1.98 [2]; K 1.98 [2]
GTKPK(INH-CH_2-CO)G	762.4/762.4	15.1	
$[TKPKG]_4$	2063.5/2063.5*	14.8	T 3.94 [4]; P 4.12 [4]; G 3.88 [4]; K 7.65 [8]
$[TKPK(INH\text{-}CH_2\text{-}CO)G]_4$	2771.9/2772.0*	18.8	

C-terminus of the peptides and peptide conjugates were in amidated form.
[a] Measured monoisotopic molecular mass by Bruker Esquire 3000+ ESI-MS.
[b] RP-HPLC, Knauer, Eurospher-100, C-18, 5 µm, 250 mm × 4 mm column; 1 ml/min flow rate; detection at λ = 214 nm gradient: 5% B, 5 min; 5–60% B, 35 min. A eluent: 0.1% TFA/H_2O, B eluent: 0.1% TFA/acetonitrile : H_2O = 80 : 20 (v/v).
[c] Amino acid analysis after hydrolysis (6 M HCl, 110 °C, 24 h). *Average molecular mass.

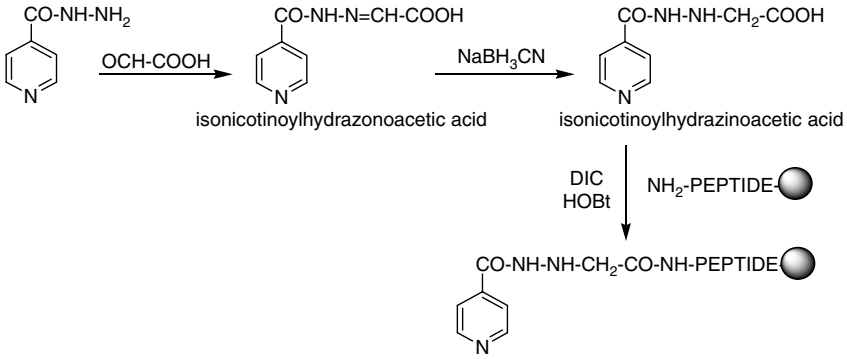

Figure 2. Summary of the solid-phase synthesis of INH–peptide conjugates, where glyoxylic acid was used as a heterobifunctional reagent. The resulted isonicotinoylhydrazonoacetic acid was reduced with sodium cyanoborohydride. Isonicotinoylhydrazinoacetic acid was coupled to the *N*-terminus or ε-amino group of Lys of the carrier peptide on the resin.

Table 2. *In vitro* antitubercular activity of peptide–drug conjugates, INH and free carriers

Compound	MIC[a] γ (μg/ml) calculated to INH content	MIC[a] γ (μg/ml) calculated to compound	CFU[b]
Isoniazid (INH)	0.16	0.16	12
Isonicotinoylhydrazonoacetic acid	0.40	0.56	60
Isonicotinoylhydrazinoacetic acid	0.40	0.57	6
INH=CH-CO-^{92}EFAGAGFVRAGAL^{104}	0.24	2.52	40
INH=CH-CO-^{91}SEFAYGSFVRTVSLPV^{106}	0.18	2.54	30
INH-CH_2-CO-^{91}SEFAYGSFVRTVSLPV^{106}	0.16	2.26	2
GTKPK(INH-CH_2-CO)G	0.18	1.00	20
[TKPK(INH-CH_2-CO)G]$_4$	0.32	6.47	n.d.
^{91}SEFAGAGFVRAGAL^{104}	No effect	–	–
^{91}SEFAYGSFVRTVSLPV^{106}	No effect	–	–
GTKPKG	No effect	–	–
[TKPKG]$_4$	No effect	–	–

[a] Minimal inhibitory concentration determined on *M. tuberculosis* H_{37}Rv in Sula medium [35–37].
[b] Colony forming unit on Löwenstein-Jensen solid medium [36,38,39].

Stability of the Hydrazone and Hydrazide INH Conjugates

The stability of the *in vitro* effective INH conjugates was analysed by RP-HPLC sampling over 24 h. The results are summarised in Figure 3. Interestingly the hydrazone remained intact during a longer period of time (approx. 15 h) than the hydrazide conjugate. During the hydrolysis of the hydrazone conjugate free INH and *N*-glyoxylyl-peptide are generated. At pH 6.5 the association of these moieties could be expected. Both types of conjugates were >90% intact in 6 h. After this period of time the release of INH was observed which is responsible for the antimycobacterial activity. This could be very important for further development of selective delivery into infected macrophages.

Discussion

Problems and issues surrounding the treatment of TB infection and disease are more complex than ever. Tubercle bacilli can remain in viable form for many years in the host cells. As intracellular pathogen the *M. tuberculosis* can be attacked through specific surface molecules and receptors of their host macrophages. We developed synthetic oligopeptides as future candidates for specific delivery of antituberculars to the infected host macrophages [19,24,29,30,41]. As carrier molecules we used a tuftsin derivatives GTKPKG and $(TKPKG)_4$ oligotuftsin [18,19]) and peptide ^{91}SEFAYGSFVRTVSLPV^{106} corresponding to the 91–110 T-cell epitope region of the 16 kDa protein of *M. tuberculosis* [20–24].

The goal of this study was to investigate the influence of the chemical linkage between INH moiety and oligopeptides. INH is an important drug in the management of TB with bactericidal activity against intracellular organisms. Except in resistant cases it is a highly potent drug which is selective to mycobacteria, low in cost and readily available. The mechanism of action of INH is to inhibit a cell-wall biosynthetic pathway. Using bioactive conjugates controlled location and concentration of antituberculars can be reached in the infected macrophages, furthermore side effects and therapeutical doses can be reduced.

Two synthetic approaches were developed to achieve INH conjugates: (i) reacting selectively oxidised serine elongated peptides with INH to get a hydrazone compound, (ii) coupling modified INH with glyoxylic acid to peptides on solid phase. All INH conjugates showed relevant antimycobacterial activity at the same concentration range as the free INH moiety.

This synthetic approach gives us a powerful tool to develop new promising drug conjugates against *M. tuberculosis*.

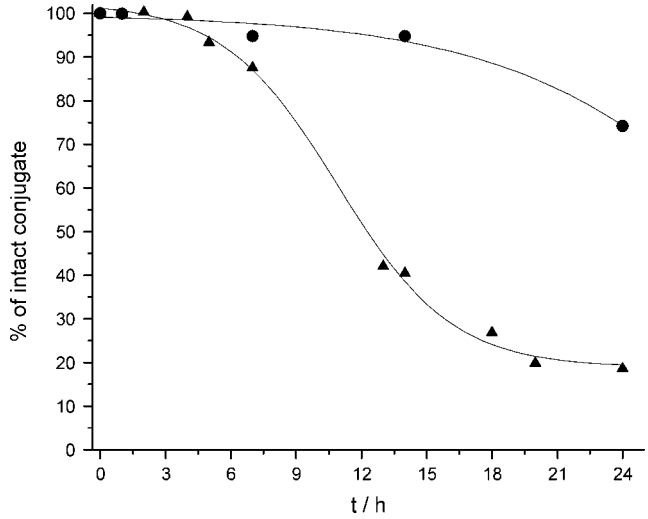

Figure 3. Stability of the INH conjugates as function of time in Sula semisynthetic medium (pH 6.5). Symbols represent the percentage of the intact hydrazone (●) and hydrazide (▲) bond containing conjugate according to the area under the peak on analytical RP-HPLC chromatogram. Knauer RP-HPLC, Eurospher-100, C-18, 5 µm, 250 mm × 4 mm column; 1 ml/min flow rate; detection at $\lambda = 214$ nm, gradient: 5–60% B in 35 min. A eluent: 0.1% TFA/H_2O; B eluent: 0.1% TFA/acetonitrile:H_2O = 80:20 (v/v).

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References

1. N°104 Fs. WHO, 2008; 1–304.
2. Slayden RA, Barry CE 3rd. The genetics and biochemistry of isoniazid resistance in *Mycobacterium tuberculosis*. *Microbes Infect.* 2000; **2**: 659–669.
3. Slayden RA, Lee RE, Barry CE 3rd. Isoniazid affects multiple components of the type II fatty acid synthase system of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 2000; **38**: 514–525.
4. Bodiguel J, Nagy JM, Brown KA, Jamart-Gregoire B. Oxidation of isoniazid by manganese and *Mycobacterium tuberculosis* catalase-peroxidase yields a new mechanism of activation. *J. Am. Chem. Soc.* 2001; **123**: 3832–3833.
5. Heym B, Zhang Y, Poulet S, Young D, Cole ST. Characterization of the katG gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. *J. Bacteriol.* 1993; **175**: 4255–4259.
6. Sandy J, Holton S, Fullam E, Sim E, Noble M. Binding of the anti-tubercular drug isoniazid to the arylamine N-acetyltransferase protein from *Mycobacterium smegmatis*. *Protein Sci.* 2005; **14**: 775–782.
7. Kumari S, Ram VJ. Advances in molecular targets and chemotherapy of tuberculosis. *Drugs Today (Barc).* 2004; **40**: 487–500.
8. Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* 1994; **63**: 601–637.
9. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. Macrophage receptors and immune recognition. *Annu. Rev. Immunol.* 2005; **23**: 901–944.
10. Taylor PR, Gordon S, Martinez-Pomares L. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol.* 2005; **26**: 104–110.
11. Majumdar S, Basu SK. Killing of intracellular *Mycobacterium tuberculosis* by receptor-mediated drug delivery. *Antimicrob. Agents Chemother.* 1991; **35**: 135–140.
12. Nishioka K, Sato PS, Constantopoulos A, Najjar VA. The chemical synthesis of the phagocytosis-stimulating tetrapeptide tuftsin (Thr-Lys-Pro-Arg) and its biological properties. *Biochim. Biophys. Acta* 1973; **310**: 230–237.
13. Nishioka K, Constantopoulos A, Sato PS, Mitchell WM, Najjar VA. Characteristics and isolation of the phagocytosis-stimulating peptide, tuftsin. *Biochim. Biophys. Acta* 1973; **310**: 217–229.
14. Nishioka K, Constantopoulos A, Satoh PS, Najjar VA. The characteristics, isolation and synthesis of the phagocytosis stimulating peptide tuftsin. *Biochem. Biophys. Res. Commun.* 1972; **47**: 172–179.
15. Siemion IZ, Kluczyk A. Tuftsin: on the 30-year anniversary of Victor Najjar's discovery. *Peptides* 1999; **20**: 645–674.
16. Najjar VA. Tuftsin, a natural activator of phagocyte cells: an overview. *Ann. N.Y. Acad. Sci.* 1983; **419**: 1–11.
17. Amoscato AA, Davies PJ, Babcock GF, Nishioka K. Receptor-mediated internalization of tuftsin. *Ann. N.Y. Acad. Sci.* 1983; **419**: 114–134.
18. Manea M, Przybylski M, Hudecz F, Mező G. Design, structural, and immuno-analytical properties of antigenic bioconjugates comprising a beta-amyloid-plaque specific epitope. *Biopolymers* 2008; **90**: 94–104.
19. Mező G, Kalászi A, Reményi J, Majer Z, Hilbert A, Láng O, Kőhidai L, Barna K, Gaál D, Hudecz F. Synthesis, conformation, and immunoreactivity of new carrier molecules based on repeated tuftsin-like sequence. *Biopolymers* 2004; **73**: 645–656.
20. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, Voskuil MI, Weldingh K, Andersen P, Schoolnik GK, Arend SM, Ottenhoff TH, Klein MR. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect.* 2006; **8**: 2052–2060.
21. Valdez MM, Clark JI, Wu GJ, Muchowski PJ. Functional similarities between the small heat shock proteins *Mycobacterium tuberculosis* HSP 16.3 and human alphaB-crystallin. *Eur. J. Biochem.* 2002; **269**: 1806–1813.
22. Yuan Y, Crane DD, Barry CE 3rd. Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J. Bacteriol.* 1996; **178**: 4484–4492.
23. Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR, Barry CE 3rd. The 16 kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 1998; **95**: 9578–9583.
24. Wilkinson KA, Hudecz F, Vordermeier HM, Ivanyi J, Wilkinson RJ. Enhancement of the T cell response to a mycobacterial peptide by conjugation to synthetic branched polypeptide. *Eur. J. Immunol.* 1999; **29**: 2788–2796.
25. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol.* 1997; **15**: 821–850.
26. Watts C. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat. Immunol.* 2004; **5**: 685–692.
27. Lanzavecchia A, Watts C. Peptide partners call the tune. *Nature* 1994; **371**: 198–199.
28. O'Sullivan D, Sidney J, Appella F. Characterisation of the specificity of peptide binding to four DR haplotypes. *J. Immunol.* 1990; **145**: 1799–1808.
29. Monji T, McCormack AL, Yates JR 3rd, Pious D. Invariant-cognate peptide exchange restores class II dimer stability in HLA-DM mutants. *J. Immunol.* 1994; **153**: 4468–4477.
30. Ferrante A, Anderson MW, Klug CS, Gorski J. HLA-DM mediates epitope selection by a "compare-exchange" mechanism when a potential peptide pool is available. *PLoS ONE* 2008; **3**: e3722.
31. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
32. Kaiser E, Colescott RL, Bossinger CD, Olser DB. Color test for terminal prolyl residues in the solid-phase synthesis of peptides. *Anal. Chem. Acta* 1980; **118**: 149–151.
33. Geoghegan KF, Stroh JG. Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a

2-amino alcohol. Application to modification at *N*-terminal serine. *Bioconjugate Chem.* 1992; **3**: 138–146.
34. Gaertner HF, Rose K, Cotton R, Timms D, Camble R, Offord RE. Construction of protein analogues by site-specific condensation of unprotected fragments. *Bioconjugate Chem.* 1992; **3**: 262–268.
35. Sula L. WHO Cooperative studies on a simple culture technique for the isolation of mycobacteria. 1. Preparation, lyophilization and reconstitution of a simple semi-synthetic concentrated liquid medium; culture technique; growth pattern of different mycobacteria. *Bull. World Health Organ.* 1963; **29**: 589–606.
36. Sula L. WHO Cooperative studies on a simple culture techniques for the isolation of mycobacteria: 2. Comparison of the efficacy of lyophilized liquid medium with that of Lowenstein-Jensen (L-J) medium. *Bull. World Health Organ.* 1963; **29**: 607–625.
37. Vinsova J, Cermakova K, Tomeckova A, Ceckova M, Jampilek J, Cermak P, Kunes J, Dolezal M, Staud F. Synthesis and antimicrobial evaluation of new 2-substituted 5,7-di-tert-butylbenzoxazoles. *Bioorg. Med. Chem.* 2006; **14**: 5850–5865.
38. Jensen KA. Reinzüchtung und Typenbestimmung von Tuberkelbazillenstämmen. Eine Vereinfachung der Methoden für die Praxis. *Zentralb. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig.* 1932; **125**: 222–239.
39. Löwenstein E. Die Züchtung der Tuberkelbazillen aus dem strömenden Blute. *Zentralb. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig.* 1931; **120**: 127–129.
40. Zhang Y, Young D. Molecular genetics of drug resistance in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* 1994; **34**: 313–319.
41. Bai KB, Láng O, Orbán E, Szabó R, Kőhidai L, Hudecz F, Mező G. Design, synthesis, and *In Vitro* activity of novel drug delivery systems containing tuftsin derivatives and methotrexate. *Bioconjugate Chem.* 2008; **19**: 2260–2269.

