Antimycobacterial activity of peptide conjugate of pyridopyrimidine derivative against *Mycobacterium tuberculosis* in a series of *in vitro* and *in vivo* models.

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Abstract

New pyridopyrimidine derivatives were defined using a novel HTS *in silico* docking method (FRIGATE). The target protein was a dUTPase enzyme (EC 3.6.1.23; Rv2697) which plays a key role in nucleotide biosynthesis of *Mycobacterium tuberculosis* (*Mtb*). Top hit molecules were assayed *in vitro* for their antimycobacterial effect on *Mtb* H_{37}Rv culture. In order to enhance the cellular uptake rate, the TB820 compound was conjugated to a peptid-based carrier and a nanoparticle type delivery system (polylactide-co-glycolide, PLGA) was applied. The conjugate had relevant *in vitro* antitubercular activity with low *in vitro* and *in vivo* toxicity. In a *Mtb* H_{37}Rv infected guinea pig model the *in vivo* efficacy of orally administrated PLGA encapsulated compound was proved: animals maintained a constant weight gain and no external clinical signs of tuberculosis were observed. All tissue homogenates from lung, liver and kidney were found negative for *Mtb*, and diagnostic autopsy showed that no significant malformations on the tissues occurred.

keywords: *in silico* docking, pyridopyrimidines, peptide conjugate, PLGA encapsulation, guinea pig infection model

1. INTRODUCTION

In silico docking methods and structure-based drug design are useful bioinformatics tools for identifying new agents against tuberculosis. In our previous study [1], a newly developed FRIGATE docking algorithm [2] was utilized for a virtual screen of several million small molecules against the species-specific surface loop of *Mtb* DUTPase (EC 3.6.1.23; Rv2697) enzyme [3, 4]. As a result, we reported the identification of 10 new compounds, active against *Mtb* H_{37}Rv *in vitro.* The lowest minimal inhibitory concentration (MIC) was measured for TB8 compound (1 μg/mL, 3.7 μM), a pyridopyrimidine derivative. To optimize the structure of TB8 compound, more than 100 derivatives were synthesized, *in vitro* evaluated and the structure-activity relationship was analyzed. One of the derivatives, TB820 molecule (4-oxo-2-(pyrrolidin-1-yl)-pyrido[1,2-a]pyrimidine-3-carbaldehyde) was chosen in this study for further modification in order to improve its therapeutic efficacy by using advanced drug delivery system (DDS).

Targeted delivery of antitubercular drugs into macrophages, which provide the intracellular survival of the mycobacteria, has crucial pharmaceutical benefits. The phagocytic uptake process of macrophages is highly efficient due to the expression of specific receptors such as the IgG receptor and complement receptors [5]. DDS can be engineered to achieve enhanced cellular uptake and bioavailability, controlled drug release and reduced side effects. In this paper we report the design of a new type of advanced DDS, where the antitubercular agent is conjugated to a tuftsin peptide derivative and encapsulated into PLGA nanoparticles. Tuftsin is a natural phagocytosis stimulating peptide produced by enzymatic cleavage of the Fc-domain of the heavy chain of IgG. During the past decade, a new group of sequential oligopeptide carriers has been developed in our laboratory: oligotuftsin derivatives consisting of a pentapeptide unit TKPKG [6, 7]. In this study, the TKPKG peptide was elongated with palmitic acid to enhance the lipophilicity, membrane affinity and encapsulation efficacy. Nanoencapsulation into lactic and glycolic acid (PLGA) copolymers is an efficient method for sustained controlled release and intensively explored for antitubercular therapy [8]. These biodegradable and biocompatible polymer-based nanoparticles have the ability to essentially modify the biodistribution of an active compound and the release rate can be controlled by the size and composition of the particles [9]. PLGA is approved by the US Food and Drug Administration (FDA) for clinical use and it can be utilized in oral formulation [10].

Synthesis and characteristics of peptide-conjugated and PLGA encapsulated TB820 is described in this work. *In vitro* antitubercular efficacy of the conjugate is compared to its cytotoxicity and hemolytic activity. Furthermore, *in vivo* chemotherapeutic effect of the orally administered PLGA-pT820 compound in a guinea pig infectious model is presented.

2. MATERIALS AND METHODS

2.1. Synthesis and characterization of PLGA encapsulated pal-T5(TB820)$_2$

The TB820 compound was conjugated to a palmitoylated tuftsin derivative. First protected tuftsin (TKPKG, T5) was produced manually by solid-phase peptide synthesis using Boc/Bzl strategy. Palmitic acid was coupled to the *N*-terminus of the peptide. The N^{ε}-Fmoc protecting groups of the side chain of Lys residues were selectively removed and free amino groups were reacted with Boc-aminooxyacetic acid. After Boc deprotection, peptide was removed from the resin with liquid HF in the presence of scavengers. Palmitoylated aminooxi-peptide was then allowed to react with the TB820 compound in solution and the precipitated conjugate was filtered and purified. Final product pal-T5(TB820)$_2$ (pT820) was characterized by analytical RP-HPLC, ESI-MS and amino acid analysis.

PLGA nanoparticles were prepared by the nanoprecipitation (solvent exchange) method, detailed previously [11]. Briefly, PLGA and pT820 conjugate were dissolved in acetone and added dropwise to an aqueous solution of Pluronic 12700. The resulting nanoparticle suspension was centrifuged, purified by dialysis and freeze-dried. Drug content of the lyophilized powder was determined by amino acid analysis.

2.2. *In vitro* tests

In vitro antitubercular activity of the compound was determined against *Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294) and multiresistant *M. tuberculosis* (MDR A8) by serial dilution method in Sula semi-synthetic medium. The minimal inhibitory concentration (MIC) was determined after incubation at 37^{o}C for 28 days. The activity of the tested compounds was confirmed using colony forming unit (CFU) enumeration by subculturing from the Sula medium onto drug-free Löwensten-Jensen solid medium (37^{o}C, 28 days).

In vitro cytotoxicity was evaluated on human peripheral blood mononuclear cells (PBMC), prepared from peripheral blood of healthy volunteers using Ficoll-Hypaque density gradient centrifugation method. The pT820 conjugate was added to the cells to give 0.5 μM – 200 μM final concentration. Cells were incubated at 37^{o}C for 4 hours, then cell viability was tested using MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

In vitro hemolytic activity of pT820 was determined on human erythrocytes. Red blood cell suspension was incubated with the compounds (0.5 μM – 200 μM final concentration) for 4 hours at 37^{o}C and after centrifugation, the absorbance of the supernatant was measured at λ=450 nm.

2.3. ***In vivo*** **evaluation**

Inbred, female guinea pigs aged 6 weeks at weight 400-500 g were housed four per cage and allowed free access to water and to standard pellet diet. All animal experiments were approved by the Hungarian Scientific Ethical Committee on Animal Experimentation (No: 22.1/3720/003/2009).

To study the *in vivo* toxicity, animals were administered orally twice a week with 50 mg/kg bw of PLGA-pT820 (n = 3 for the treatment group). To monitor the side effects, changes in body weight were determined weekly and after 7 weeks of administration, animals were euthanized and diagnostic autopsy was evaluated.

In vivo antitubercular effect of the PLGA-pT820 compound was evaluated on *Mtb* infected guinea pigs (n = 6 for treatment and 6 for control group). First, guinea pigs were infected with *Mtb* H_{37}Rv suspension by intramuscular injection. Infection was followed by sacrificing one animal each week and performing histopathological analysis from the organs. Chemotherapy was started 3 weeks after infection. PLGA-pT820 compound (50 mg/kg bw) was suspended in 1 mL sterile water and administrated orally twice a week. Control animals were administered of 1 mL sterile water with the same frequency. After 12 weeks of chemotherapy, animals were euthanized by ketamine (40 mg/kg bw) and dexmedetomidine (Dexdomitor, 0.5 mg/kg bw).

To count the number of bacteria in the organs, a portion of lung, spleen, liver and kidney were resected and homogenized in Sula media. 1:1000 dilutions of the homogenates were plated onto Löwenstein-Jensen solid media and after 8 weeks of incubation CFU was enumerated. For histopathological analysis, lung, spleen, liver, kidney, inguinal lymph nodes and heart muscle of each animal were removed and fixed in 8% neutral buffered formalin for 24 hours at room temperature. Tissue specimens were dehydrated in a series of ethanol and xylene baths and embedded in paraffin wax. Sections (3-4 μm) were stained with haematoxylin and eosin (HE.). For *in situ* visualization of the acid-fast bacilli Ziehl–Neelsen (ZN) staining method was applied on similarly pre-treated sections.

3. RESULTS AND DISCUSSION

Palmitoylated tuftsin derivative was used as a carrier for the TB820 compound. Chemical structure and homogeneity of the conjugate was proved by mass spectrometry (M_{mo}(calculated) = 1362.8; M_{mo}(found) = 1362.8), analytical RP-HPLC and amino acid analysis. Structure and mass spectra of the pT820 conjugate is given in FIGURE 1.

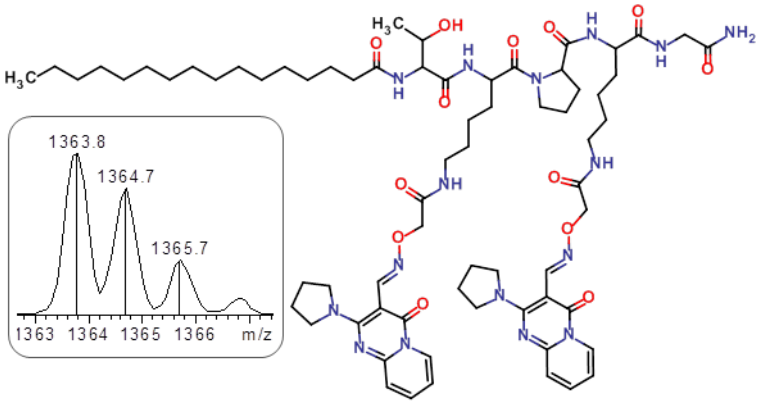


FIGURE 1. Chemical structure of the pT820 conjugate. The *N*-terminal of TKPKG pentapeptide is palmitoylated, the *C*-terminus is amidated. The TB820 compound is coupled to the side chains of the two lysine amino acids through oxime bond. High resolution mass spectrum shows the isotopic distribution of the pT820 molecule.

The pT820 conjugate was encapsulated into PLGA nanoparticles by nanoprecipitation method. The encapsulation efficacy was up to 83%. The drug content of the PLGA-pT820 nanoparticles was 20.2%.

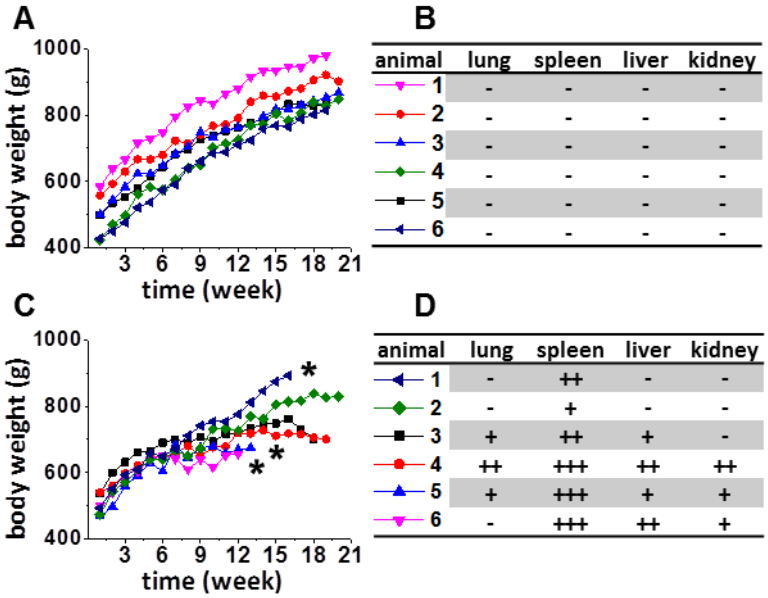
3.1. *In Vitro* Antitubercular effect, cytotoxicity and hemolytic activity

Antitubercular effect of the compounds was measured against *Mtb* H_{37}Rv strain. The MIC value of the TB820 molecule was 1 μg/mL (4.1 μM), while the MIC value of the pT820 conjugate was 5 μg/mL (3.7 μM). From this result we can conclude that the chemical modification of the TB820 molecule did not influence its antimicrobial efficacy. The antitubercular effect of the compounds was also tested on multiresistant *Mtb* (MDR A8) strain. The MIC value was 1 μg/mL (4.1 μM) for the TB820 molecule and 10 μg/mL (7.4 μM) for the pT820 conjugate. Before *in vivo* experiments, we assayed the cytotoxicity and hemolytic activity *in vitro*. We found that the pT820 conjugate was not cytotoxic to human PBMC and to human erythrocytes even at the highest concentration (IC_{50} > 200 μM; HC_{50} > 200 μM).

3.2. *In Vivo* toxicology and chemotherapeutic effect of PLGA-pT820

To monitor the toxicity of PLGA-pT820, 3 guinea pigs were administered orally twice a week with a dose of 50 mg/kg bw. During the experiment animals maintained a constant weight gain and no symptoms, related to compound toxicity were observed. After 7 weeks of administration of PLGA-pT820, animals were euthanized and diagnostic autopsy proved that no significant malformations on the tissues occurred.

To study the chemotherapeutic effect, guinea pigs were infected intramuscularly with *Mtb* $H_{37}Rv$ suspension. After 3 weeks of incubation, the animals were randomly divided into two groups. Six guinea pigs were treated orally with PLGA-pT820 (50 mg/kg bw, twice a week for a period of 12 weeks). Six animals receiving sterile water with the same frequency were employed as controls. As illustrated in FIGURE 2/A, all PLGA-pT820 treated guinea pigs steadily gained weight throughout the whole experiment. No death occurred and no external clinical signs of tuberculosis were observed. Furthermore, the plated tissue homogenates were bacteria free after 8 weeks of incubation (FIGURE 2/B). In comparison, 3 infected animals died at the 13rd, 14th and 19th week of the experiment (FIGURE 2/C). Other signs, related to tuberculosis, such as weight loss, scuffed fur or lethargy, were also observed in three other cases. These external clinical signs were proved by mycobacterial CFU, detected in spleen, lung, liver and kidney homogenates as presented in FIGURE 2/D.



animal	lung	spleen	liver	kidney
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-

animal	lung	spleen	liver	kidney
1	-	++	-	-
2	-	+	-	-
3	+	++	+	-
4	++	+++	++	++
5	+	+++	+	+
6	-	+++	++	+

FIGURE 2. Chemotherapy of *Mtb* infected guinea pigs. PLGA-pT820 treated animals gained weight steadily (**A**) and no mycobacterial colonies were observed in the tissue homogenates (**B**). In panel **C**, the weight gain of infected but untreated control animals is presented (* indicates the death of an animal). To prove TB infection, CFU was determined from the plated tissue homogenates (**D**), (+++ : confluent colonies; ++ : innumerable colonies, but not confluent; + : 50-100 colonies; - : no colonies were observed).

The histopathological analysis of tissues of *Mtb* infected guinea pigs clearly demonstrates the formation of typical tuberculotic lesions, e.g. formation of multiple epitheloid granulomas in the lymph nodes, lungs, liver and spleen of the infected animals, as a result of type IV, T_H1 mediated hypersensitivity reaction. Ziehl-Neelsen staining demonstrated the acid-fast bacteria in the cytoplasm of the epitheloid cells, or in the multinucleated giant cells (FIGURE 3).

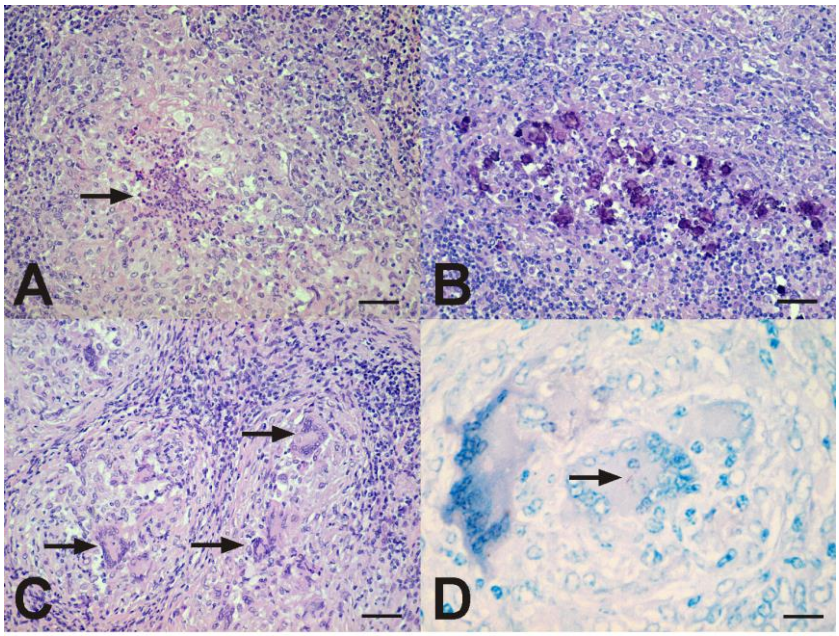


FIGURE 3. Representative histological spectrum of TB using intramuscular infection model of guinea pigs. A typical tubercle is observed (panel **A**, spleen section), where the arrow indicates the central necrosis, which is surrounded by epitheloid macrophages. In panel **B** the deep purple precipitations represent multifocal calcification in the necrotic areas, whereas in panel **C** the arrows point at multinucleated Langhans type giant cells as a result of epitheloid macrophage fusion. In panel **D**, rod-shaped Ziehl-Neelsen stained *Mycobacterium* is shown. Panel **B**, **C** and **D** are lymph node sections. Panel **A**, **B**, **C**: Sections were stained with HE. 200× Bar, 50μm; **D**: ZN staining, 1000× Bar, 10μm.

As a summary of this study we can conclude that a new type of peptide based drug delivery system was applied for the TB820 molecule with relevant chemotherapeutic efficacy against experimental tuberculosis. Palmitoylated tuftsin peptide conjugate of TB820 found to be effective against susceptible and multiresistant *Mtb* strains with a relatively high *in vitro* selectivity (IC_{50} and HC_{50} was more than 50-times higher than MIC). The pT820 conjugate was entrapped into PLGA nanoparticles with high encapsulation efficacy. The PLGA-pT820 compound was not toxic to guinea pigs at the therapeutic concentration. Guinea pigs were infected with virulent *Mtb* H_{37}Rv and treated with PLGA-pT820. Untreated control animals show numerous severe lesions with extensive

parenchymal involvement. These signs of infection were characterized by the evidence of necrosis with intralesional mineralization, thus reflecting progression of the disease. In contrast, PLGA-pT5i treated animals had considerably decreased inflammation and minimal granulomatous involvement.

4. ACKNOWLEDGEMENTS

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