Detection of latent *Mycobacterium tuberculosis* infection and host cell specific targeted delivery of potential antituberculotic compounds

Background of the project

In the *Mycobacterium* genus over one hundred environmental and pathogenic species are already described and new ones are periodically reported. Species that form colonies in a week are classified as rapid growers, those requiring longer periods (up to three months) are the mostly pathogenic slow growers [1-3]. Tuberculosis (TB) remains a major public health threat; the first cause of mortality due to a single bacterial, intracellular pathogen. This is largely due to the latently infected individuals; one third of the world's population is believed to be infected with the slow-growing *Mycobacterium tuberculosis* (*MTB*) which has the unique ability to cause latent infection (LTBI) [4-7].

Nontuberculous (atypical) mycobacteria (NTM) are increasingly recognised as causative agents of various opportunistic human infections most often originating from contaminated water supplies, and their incidence (skin, soft tissue, postoperative, catheter related and disseminated infections etc., which are not limited to immunocompromised patients) is expected to rise. Their treatment involves multiple medications and it is complicated with limited efficacy, e.g., due to the high levels of natural and acquired resistance [2, 3, 8]. Among them *M. abscessus* is the most resistant rapid grower and there is an urgent need to find new agents against these strains [7, 8].

The aim of the project 104275 was **the preparation and *in vitro – in vivo* evaluation of new potential antimycobacterial agents.** Considering that mycobacteria can survive in host cell, its elimination could be more efficient with host cell directed delivery of chemoterapetic agents. **To enhance the cellular uptake, bioavailability and the host cell specificity** of the new and recently used antituberculotic compounds different approaches were developed.

The further aim of the project was to improve the immunodiagnosis of latent *Mycobacterium tuberculosis* infection (LTBI), using peptide-based synthetic antigens to make progress towards differentiating TB infection and BCG vaccination, but also between LTBI and the early stage of active TB.

Description of the result according to the research plan

I. To identify new promising antimycobacterial compounds using *in silico* docking method and chemical tailoring of antituberculotic compounds. To determine the *in vitro* activity we used extracellular and intracellular bacteria as model systems

(i) In the framework of OTKA 104275 grant **small molecular ligands** which are capable of binding to and perturbing the function of an essential dUTPase (EC 3.6.1.23; Rv2697) of *MTB* have been identified using ***in silico* docking method** to define numbers of "drug-like" compounds [9-12]. Top hit molecules were assayed *in vitro* on MTB cultures. **Our**

results are summarized in *Horvati, K., et al., Tuberculosis (Edinb), 2015. 95 Suppl 1: p. S207-211.; Ábrahám Á, et al. Colloids Surf B Biointerfaces. 2016, 147:106-115.*

(ii) Modifications of first or second line antituberculotic drugs are also widely used approach [13]. Salicylanilides (2-hydroxy-N-phenylbenzamides) are promising candidates for this purpose due to their antimycobacterial activity. Salicylanilides inhibit the bacterial two-component regulatory system, which is a signal transmission pathway. Salicylanilide derivatives are also effective on drug resistant bacteria.

Based on those above during OTKA 104275 grant **we designed and synthesised novel salicylanilide and isoniazide derivatives.** These compounds were active against *MTB*, *MTB* MDR, NTM strains. Substituted salicylanilide derivatives, 5-chloropyrazinoate-2-carboxylic acid, salicylanilide-5-chloropyrazinoates, salicylanilide-4-formylbenzoates and salicylanilide carbamates were synthesized and chemically characterized. Most of the compounds were outstandingly effective against extracellular bacteria; they possessed low inhibitory concentration against *M. abscessus*, *MTB* H_{37}Rv sensitive and *MTB* A8 MDR multidrug-resistant strains (MICs from 0.2 μM). Only few drugs have been found so far that are effective on this strain, especially not at such a low concentration. Importantly, ten salicylanilide carbamates exhibited an excellent activity against *M. abscessus*. **Our results are summarized in** *Baranyai, Z., et al., Eur J Med Chem, 2015. 101: p. 692-704. ; Krátký M, et al., Bioorg Med Chem. 2015, 15;23(4):868-75.*

Salicylanilide derivatives have remarkable antimycobacterial activity but most of the compounds have cytotoxic or cytostatic activity on human MonoMac6 and HepG2 cells, and also on murine bone marrow-derived macrophages. Based on these results it is an important task to increase the selectivity of the compounds. The high cytotoxic and cytostatic effects were measured on human cancer cell lines, therefore the activity of the compounds on these cells could pave the way to their application in cancer therapy as well *(Bősze, Sz., et. al.,: Contribution to the COST Action CM1106 compound library toward new collaborations: molecules and their in vitro cytotoxic/cytostatic effect on tumour cell lines with potential activity on cancer stem cells. Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells, EU COST CMST Action CM1106, 3rd Working Group Meeting, Athens 27-28 March, 2015).*

Based on the known biological activity of isoniazid-based hydrazones, hydrazones of 4-(trifluoromethyl)benzohydrazide were synthesized from various benzaldehydes and aliphatic ketones. The compounds were screened for their *in vitro* activity against *MTB* H_{37}Rv, NTM (*M. avium, M. kansasii*). The most antimicrobial potent derivatives were also investigated for their cytostatic and cytotoxic properties against three cell lines. Some derivatives avoided any cytotoxicity on two mammalian cell cultures (HepG2, BMMΦ) up to the concentration of 100 μM, but it affected the growth of MonoMac6 cells (*Krátký, M., et al., Bioorganic & Medicinal Chemistry Letters (2017), doi: https://doi.org/10.1016/j.bmcl.2017.10.050).*

(iii) To enhance cellular uptake and bioavaibility: Our goal of the conjugation was macrophage-selective delivery of compounds by endocytosis or via specific receptors [14-18]. **As a macrophage (host cell) model we have used human monocytic MonoMac6 cells.** MonoMac6 was established as a cell line which appears to have phenotypic and functional characteristic of mature blood monocytes. MonoMac6 cells are phagocytic and express the CR3 receptor, which is important for the entry of *MTB*.

Testing the compounds efficacy on intracellular bacteria *in vitro* MonoMac-6 was infected with *MTB* H_{37}Rv. A number of oligopeptides based on different cellular uptake mechanism have been developed and considered as targeting structures (**results are summarized in:** *Horváti K, et al., Amino Acids. 2017, 49(6):1053-1067.; Zsila, F; et al., RSC Adv., 2017, 7, 41091-41097*.) for the clinically used isoniazid (INH) and new candidates: covalent bond between the carriers and ligand compounds was formed (hydrazone, oxime, etc.) [14-18]. **During 104275 OTKA project receptor specific tuftsin derivatives and antimicrobial peptides** were applied as carrier moieties (*Horváti K, et el., Comparative analysis of internalisation, haemolytic, cytotoxic and antibacterial effect of membrane-active cationic peptides: aspects of experimental setup. Amino Acids. 201, 49(6):1053-1067.).*

The optimization of the cellular uptake by peptide based drug delivery systems increased the efficacy of the compounds. In order to enhance the efficacy isoniazid (INH) was covalently conjugated to a palmitoylated tuftsin derivative with a sequence of TKPKG. We observed that INH maintained its antimicrobial efficacy indicating that the applied hydrazide modification of the INH is permitted and the synthetic route can be used for modification of INH prior to conjugation. The conjugate showed relevant *in vitro* activity on *MTB* H_{37}Rv culture with low cytotoxicity and hemolytic activity on MonoMac-6 cells. The conjugate directly killed intracellular *MTB* H_{37}Rv *in vitro* and shows much greater efficacy than free INH; **results in***: Horváti K, et al., Bioconjug Chem. 2014, 25(12):2260-8.*

In the case of the salicylanilide (SAL2)-tuftsin conjugates this antimycobacterial activity was preserved with the conjugation to peptides by oxime bond. The improved intracellular efficacy of the conjugates on infected cells can be explained with the better internalization of the conjugated antitubercular drugs than the free drugs. The inhibition effect against the intracellular bacteria was increasing with the estimated intracellular uptake rate in the case of most of the conjugates. The decanoic acid containing monotuftsin conjugate, SAL2-Aoa-T5(4-dec) with moderate cytostatic activity and outstanding activity against intracellular bacteria is a favourable candidate for further investigation. Conjugates without fatty acid side chains therefore considered rather hydrophilic compounds also were able to significantly inhibit the intracellular bacteria without being cytostatic to the host cell model. **Our results are summarised in:** *Baranyai Z, et al., Eur J Med Chem. 2017, 16;133:152-173.*

Polymer nanoparticles (NP) occupy unique position in drug delivery technology due to their favorable properties [19-22] such as modified bioavailability, biodistribution of an active compound and biomimetic character [23-24]. **During our OTKA 104275 to improve cellular uptake and bioavaibility** *(i)* a new *in silico* identified and optimized candidate (TB515) was encapsulated into the poly(lactide-co-glycolide) (PLGA) particles. The loaded PLGA particles were internalized with significant intracellular efficacy on intracellular *MTB* model Surface modification of PLGA NPs as primary amino groups were introduced to the Pluronic surface layer: *Kiss, É., et al., Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2014. 458, 178-186.*

(ii) **INH and a palmitoylated tuftsin derivative** (pal-TKPKG, palT5) was encapsulated into PLGA NPs. For the determination of *in vivo* chemotherapeutic effect, **guinea pig infectious model was developed and employed**. Guinea pigs are highly susceptible to infection with *MTB* and develop disease that is observed in humans. The *in vivo* antitubercular activity of PLGA-palT5i was proven; the histopathological examination showed that treatment with PLGA-palT5i has resulted in considerably decreased

inflammation and minimal granulomatous involvement compared to untreated control. Furthermore, no symptoms related to conjugate toxicity were observed during the experiment and this approach was successfully repeated *in vivo* with a new pyridopyrimidine derivative (T820). **Our results are summarized in***: Horváti K, et al., Bioconjug Chem. 2014, 25(12):2260-8. Horvati, K., et al., Tuberculosis (Edinb), 2015. 95 Suppl 1: p. S207-211*

In our studies nanoparticles (NPs) were characterized in terms of size, composition, zeta potential and colloidal stability. Surface modification of PLGA NPs as primary amino groups were introduced to the Pluronic surface layer (*Kiss, É., et al., Colloid Surface A. 458: 178-186, (2014); Gyulai G. et al., Express Polymer Letters, 2016, 10 (3), 216)*

The membrane affinity of the compounds was comperatively analysed using monolayer and supported bilayer experimental systems. We successfully confimed that *in vitro* biological results with/applying penetration studies. We have assessed structural information from quartz crystal microbalance (QCM) frequency and resistance values which are also in good agreement with the data of monolayer (Langmuir), atomic force microscopy (AFM) and biological evaluations. **Our results were presented in** *Ábrahám Á, et al. Colloids Surf B Biointerfaces. 2016, 147:106-115.*

(iii) **Liposomes were investigated also as nanocarriers**, we have encapsulated of antituberculotic compounds into liposomes to enhance the efficiency on intracellular bacteria. We have prepared two types of liposomes: dipalmitoyl phosphatidylcholine (DPPC) and dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS) and pegylated distearoyl phosphatidylethanolamine (DSPE-PEG). Encapsulation efficiency was influenced by the diameter of liposome and the physico-chemical properties of antituberculotic compounds. *In vitro* liposomal formulation showed better uptake by MonoMac6 cells then free drug. The cytotoxicity of liposomal formulation was smaller then non-encapsulated antituberculotic drug candidates. **Our result are summarised in**: *Kosa, N., et al., Biophysical J., 110:3(Suppl.1) 246a (2016) DOI: 10.1016/j.bpj.2015.11.1356.*

II. To improve the immunodiagnosis of latent *MTB* infection (LTBI) using peptide-based synthetic antigens

Epitope mapping of the Rv2654c protein was evaluated during project OTKA 104275. Our data indicated that more frequently recognised peptides of Rv2654c are in the C-terminal region between amino acids 51–81. Further detailed epitope mapping resulted in the identification of peptide p51-65 which was the most dominantly recognised peptide of the Rv2654c. Computational prediction suggested that the middle region between amino acids 30–50 of the Rv2654c protein to be unrecognised by the Xhosa population and only the region near the C-terminal section of the protein was predicted to be immunogenic enough to show up in the analysis. These characteristics make peptide p51-65 that provoked significantly increased IFN-γ response in a whole blood assay. **Using peptide p51-65 in the QuantiFERON-TB Gold In-Tube assay resulted in significant boosting of the quantitative performance of the QFT test in the HIV uninfected group. Our results presented in**: *Horváti K, et. al.,Population tailored modification of tuberculosis specific interferon-gamma release assay. J Infect. 2016 Feb;72(2):179-188.*

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