Interaction of bioactive substances with cell membrane model systems

Ph. D. Theses

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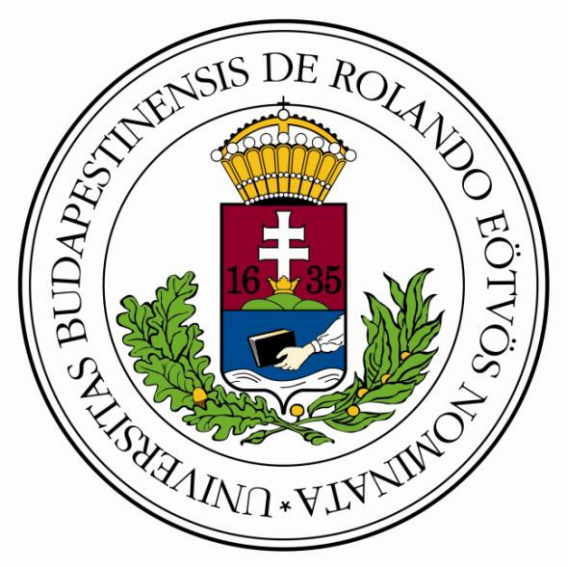

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Introduction and Aims

Tuberculosis (TB), as a fatal chronic bacterial disease is one of the leading causes of death in the world. The pathogen *Mycobacterium tuberculosis* (*Mtb*) infects 8 million people and nearly two million people die from TB every year. The World Health Organization **(**WHO) reported in 2012 that there were an estimated 8.7 million new cases of TB (13% co-infected with HIV) and 1.4 million people died from TB in 2011, including almost one million deaths among HIV-negative individuals and 430 000 among people who were HIV-positive.

Mbt is an obligate parasite that is fairly resistant against many drugs. *Mtb* is a large rod-shaped bacterium with 2-4 μm length and 0.2-0.5 μm in width. The bacterium is a facultative intracellular parasite, usually of macrophages, with a slow generation time of 15-20 hours, having significant virulence. *Mbt* is an aerobe bacillus, so in the classic case of tuberculosis, *Mtb* complexes are always found in the well-aerated upper lobes of the lungs. The difficulties of the therapy for tuberculosis are: the long duration of treatment, the drug toxicity which is increasing with the dose, and heavy side effects like the damage of liver and kidney, furthermore the emergence of drug resistance. The aim is to shorten the duration of treatment hence reducing harmful side effects. In this case there are researching on developing new drugs against *Mtb*. To reach his goal there are efforts in reach and develop of new therapeutic agents.

Conjugation of drug molecules with peptide carrier or other moieties is a promising approach to increase the efficacy of widely used drugs for example izoniazid (INH) by enhancing their cell uptake and targeting. Peptide conjugates improve the interaction with cell membrane enhanced transport by specific receptor mediated endocytosis. Hereby fewer doses are enough to reach the desired effect and the sight effects are remarkably reduced as a consequence.

Another method to find effective drug candidates is the „*in silico*" identification of chemical species able to block vital bacterial enzymes. Knowing the crystal structure of the enzyme among the molecules matching of that's active centrum drug candidates were chosen with further selections. One of the most important of those is the determination of the minimum inhibitor concentration (MIC).

The aspecific mechanism of a large group of antibacterial drugs is based upon disturbing the structure of cell membrane and destructing the lipid bilayer. Cationic molecules, peptides (penetratin) and polymers are applicable for this membrane destruction and membrane leakage. There are also synthetic polymers developed to this aim systematically changing their structure, polarity, charging and hydrophobic character to find the most effective substance.

In this work affinity of drugs to cell membrane models was investigated with Langmuir and Langmuir-Blodgett techniques.

The simplest biologic organisations are also very complex systems. Their items create complex connection networks in different organisation levels that are in continuous dynamic alteration. Investigation of these complex systems is fairly difficult, especially when a given process would be determined. In this case it is reasonable to create a model system which is the most simple as far as possible but the investigated process is can be studied in details.

Drugs have to be transported through various membranes to reach the infected target cell. The quality of the interface influences which kind of drug candidates are able to interact with it: to absorb or penetrate. Lipid Langmuir monolayer is one of the simplest model of cell membranes. It is much less complex than any biological membrane but a well defined system allowing many parameters such as packing, density, nature of lipids just as the subphase composition and temperature to be varied. The molecular interactions between those membrane models and different types of bioactive molecules were investigated. I prepared monolayers of various ordered amphiphylic lipid molecules for that and their properties at 24 and 36^{o}C were characterized. The membrane affinity of bioactive substances was determined from their penetration abilities. The structural changes of model membranes due to the penetration of drug molecules were also studied.

My aim was to support the design of more effective constructions and the selection between numerous drug candidates with the characterization of the membrane affinity of drug candidates, drug conjugates and antibacterial substances.

Applied Methods

Membrane model systems were formed by Langmuir technique. One and two component lipid systems were selected. One component lipid monolayers were prepared from Phospholipon® 100 H and DPPC. Mixture of DPPC-DPPG, DPPC-cardiolipin and DPPC-mycolic acid with various compositions were applied as two component systems.

Penetration ability of bioactive molecules was determined to characterize their membrane affinity quantitatively by tensiometric experiments performed in Langmuir balance at various surface pressures and temperatures (24 and 36^{o}C). Penetration threshold pressure was deduced from the results as a single parameter expressing the membrane affinity. Threshold pressure is the highest surface pressure of the lipid layer above which the drug no longer penetrates the monolayer.

Atomic force microscopy was used to study the structure of lipid and penetrated lipid films. Lipid monolayer was transferred to mica or glass substrates by Langmuir-Blodgett method. Structural changes caused by penetration were evaluated by comparing the roughness and structural details of pure lipid and drug containing lipid layers.

New Scientific Results

(Serial number of publication where the results are discussed in more detailed is given at each point.)

1. Surface pressure-area isotherms and stability of lipid model systems used for penetration experiments were determined by Langmuir-balance technique at various surface pressures and temperatures (24 and 36^{o}C). It was found that the stability of DPPC and Phospholipon® 100 H model membranes is similar, while the stability of mycolic acid containing lipid mixture sharply decreases with increasing mycolic content at both temperatures studied. The one component lipid film is more expanded and fluid at 36^{o}C than at 24^{o}C. On the contrary to that, the DPPC-mycolic acid films present composition dependent compactness according to the additivity analysis. (2, 4)

2. Penetration experiments demonstrated that among the *in silico* identified drug candidates blocking the vital enzyme of *Mycobacterium tuberculosis* the TB501 and TB502 exhibited significant and similar membrane affinity, while the TB505 did not penetrate the lipid layer. Degree of penetration for TB502 was higher than for TB501 at 36^{o}C, and it has outstanding threshold pressure above 50mN/m. (2)

3. It was verified that the polar and water soluble isoniazide (INH) has no affinity to lipid monolayers. The peptide and lipopeptide conjugates of INH (INH-red-Ser, pal-T_5-$(INH)_2$) however, possess substantial penetration ability. Conjugation with peptides resulted in enhanced penetration ability for other small molecular drug candidates as TB803 and TB852. (1, 3)

4. It was shown by atomic force microscopy investigations that the surface of monolayer of Phospholipon® 100 H monolayer at 24 ºC is smooth and

homogeneous with a thickness corresponding to the length of the lipid molecule. The surface of lipid layer is similar at 36 ºC but with smaller thickness indicating the less oriented arrangement of lipid molecules at higher temperature. Opposing to that the DPPC-mycolic acid film is heterogeneous containing aggregates of mycolic acid with different shape and size in the range of micrometer.

The introduction of bioactive molecules into the lipid monolayer during penetration was proven and visualized by AFM images revealing the increased surface roughness of lipid monolayer.

(2, 4, 5)

5. Comparing the membrane affinity of drugs and drug conjugates at 24 and 36^{o}C it was concluded that the penetration in general is more favoured to Phospholipon and DPPC monolayers at 36^{o}C due to the less compact structure of the monolayer. The interaction of pal-T_5-$(INH)_2$ with mixed layer of DPPC and mycolic acid at 3:1 mass ratio was found to be different from that expectation presenting significantly higher interaction at 24^{o}C. This phenomenon might be related to the appearance of hydrophobic mycolic acid domains.

(2, 4)

6. Membrane affinity of antibacterial amphiphilic polyelectrolytes was evaluated using neutral and negatively charged lipid models for the penetration experiments. Comparing to the unmodified PEI the degree of penetration of the polymers modified with hydrophobic chains was multiplied. The great membrane affinity exceeding the corresponding surface activity is found to be governed by hydrophobic interaction while the electrostatic interaction is negligible under the given conditions. The unexpected dependence of penetration on the alkyl chain length is explained by conformational changes.

(5)

Publications and Presentations

Publications

1. K. Hill, Cs. B. Pénzes, **D. Schnöller**, K. Horváti, Sz. Bősze, F. Hudecz, T. Keszthelyi, É. Kiss:
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2. K. Hill, Cs. Pénzes, **D. Schnöller**, K. Horváti, Sz. Bősze, T. Keszthelyi, É. Kiss:
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5. É. Kiss, K. Pribranská, **D. Schnöller**, Cs. B. Pénzes, K. Hill, K. Horváti, Sz. Bősze:
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