Doctoral (Ph.D.) thesis summary

Formulation development of a pDNA-based nanomedicine

ORSOLYA LŐRINCZ

Supervisor: Julianna Lisziewicz, Ph.D.

Genetic Immunity Kft.

DOCTORAL SCHOOL OF ENVIRONMENTAL SCIENCES

UNIVERSITY OF SZEGED

Faculty of Science and Informatics

Szeged

2013

INTRODUCTION

During the last few decades there is a great interest around the technology of nanomedicines, especially the field of gene therapy. Usually the active pharmaceutical ingredient (API) of these nanomedicines is a nucleic acid, mainly plasmid DNA (pDNA). Synthetic polymer molecules, such as polyaminoacids (e.g.: polylysine), polyethylenglicol or the understudy polyethylenimine (PEI) and its derivatives could be the solution. These have structures that are easy to modify by the coupling with different molecules to gain receptor-specific motifs which make the polymer molecule able to specifically target different cell populations. PEI is able to protect the carried nucleic acid from extra- and intracellular degradation. In addition to its targeting efficiency, one of the main advantages of most synthetic polymers is that they spontaneously form nanoparticles when mixed with nucleic acids, thus the preparation of such nucleic acid/polymer nanoparticles is very simple. pDNA/PEI nanoparticles were successful in many animal studies for various indications, like for example cancer models. Despite all the above mentioned advantages the stability of such pDNA/polymer nanoparticles is still a problem to be solved as they sediment and precipitate after a short time. This reduced stability makes the use of these nanoparticles rather difficult and often the formulations are prepared right before administration.

During my work I participated in the formulation development of an innovative nanomedicinal product-candidate, DermaVir which is a phase II clinical stage investigational medicinal product for the treatment of HIV/AIDS. Today HIV infection and the disease of AIDS is one of the most urgent unmet medical needs. There are more than 30 million HIV infected people worldwide, from whom only 5 million receive the available antiretroviral therapy. Although the antiviral drugs improved the treatment of the disease, they do not provide cure only delay the development of AIDS, therefore the development of appropriate medicament (both preventive and therapeutic) is still a problem to be solved.

The active pharmaceutical ingredient of DermaVir nanomedicine is a pDNA (polyanion) encoding 15 antigens of the HIV. This pDNA is condensed into 100-300 nm nanoparticles using a mannobiosylated linear polyethylenimine (PEIm, polycation), in 10% glucose/dextrose solution. The formed nanoparticles are pathogen-like nanoparticles as their size, shape and surface properties resemble pathogens. This is a platform technology as the

pDNA sequence can be changed to encode various antigens against other infectious diseases, cancer or allergy.

The formulation of DermaVir nanomedicine currently in the clinic is prepared freshly by a clinical pharmacist from 3 components stored at 3 different temperatures (pDNA -80°C, PEIm -20°C and 10% glucose/dextrose room temperature), and must be used within 3 hours because of the poor stability. The suspension of the nanoparticles is administered using a transdermal patch that targets the antigen presenting cells of the skin. These cells take up the particles which are recognized as pathogens and take them to the lymph nodes, where the pDNA encoded antigens are expressed, presented to the naive T cells thus generating specific immune response against the disease. After the uptake of the nanoparticles, they are closed in endosomes where pH is lowered with the use of proton pumps. In these endosomes PEIm protects the condensed pDNA thanks to the proton-sponge effect. This mechanism of protection is essential for the pDNA to reach the nucleus for potent antigen expression.

The aim is to develop a stabile formulation taking into consideration that the necessary safety and toxicology studies are already accomplished for the product thus the parameters which could be modified in the formulation are limited, to avoid the need of additional safety/toxicology studies which would delay the marketing approval. To simplify the formulation is needed so that hospital nurses could prepare the nanomedicine and to avoid the need of special storage conditions like ultra-low temperature freezer. As not only the composition is innovative but also the verification of the above mechanism of action of the nanomedicine, the structure-activity relationship is also studied.

METHODS

Preparation of the nanoparticles was performed as follows: one volume pDNA solution was diluted with three volumes of formulation solvent and one volume PEIm solution was also diluted with three volumes of formulation solvent then these diluted solutions were mixed in a 1 to 1 volume ratio by adding the polymer solution to the pDNA. Nanoparticles form spontaneously, the molar ratio of the components (nitrogen/phosphorous

or N/P) is 4.2. After preparation nanoparticle formation was allowed to proceed for 20 minutes at the temperature of the laboratory.

For the quantitative analysis of the percentage of the different topoisomers of the pDNA agarose gelelectrophoresis was used and taken gel photos were analyzed by densitometric evaluation using Image J software. For the investigation of the ratio of pDNA topoisomers inside the nanoparticle, decomplexation was performed with the addition of sodium-dodecyl-sulfate in 1000-times molar excess calculated on the pDNA concentration. After decomplexation agarose gelelectrophoresis was performed.

With UV-spectrophotometry component and nanoparticle spectra were collected in the range of 190-1100 nm with 5 nm band width. As pDNA has absorbance maximum at 260 nm in most of the cases this is the subject of investigation/evaluation. The observed hyperchromicity of the nanoparticles is calculated as follows: the increase of absorbance of the nanoparticle at 260 nm compared to the sum of absorbance of the components at 260 nm expressed in percentages.

Particle size measurement was performed by the principle of dynamic light scattering using Brookhaven Instruments equipment. The measured values are the hydrodynamic diameters of the nanoparticles calculated by the software of the equipment based using the Stokes-Einstein equation. The same samples were used for zeta potential analysis using the Smoluchowski model on the same equipment.

Atomic force microscopy (AFM) measurements were performed by drying 7 μl nanoparticle suspension on fresh mica in argon stream. Images were recorded in tapping mode and analysis was performed using Nanoscope Imaging and Image J software.

The protonation state of PEIm polymer was investigated by pH-potentiometric titration and the pK was estimated using the PSEQUAD software.

To quantify the biological activity of the formulations, they were tested in an *in vitro* cell culture. DermaVir formulations were added to the cells and after 21 hours supernatants were collected and the expressed antigens were quantified by HIV-1 ELISA (enzyme-linked immunosorbent assay).

For the quantification of the inorganic ions present in pDNA- and PEIm solutions inductively coupled plasma mass spectrometry (ICP-MS) was used. Measurements were performed according to EPA 6020 European Standard using three parallel samples. For the quantification of PEIm carbon-content total organic carbon (TOC) measurement was performed according to the international Standard MSZ EN 1484:1998 using three parallel samples. For the quantification of the chlorine content of PEIm solution adsorbable organic halogens (AOX) method was used according to the International Standard MSZ EN ISO 9562:2005 using three parallel samples.

RESULTS

1. Optimization of DermaVir components and their specification

We observed that although they all confirmed their specification in all parameters, the different lots of pDNA and PEIm showed different biological activity. When performing detailed elemental analysis it was revealed, that it is the ionic environment that differs; the ionic strength in case of the pDNA (mainly the NaCl content of the solution) and the cationic degree of the PEIm (the amount of bound hydrochloride). To maintain potent gene expression and achieve stability the components were investigated one by one as well as the nanoparticle itself; we found the critical parameters, explored their optimum and expanded the specifications respectively. To achieve stability, the glucose solution as formulation solvent was changed because the side reaction that occurred between the reductive glucose molecule and PEIm. From numerous tested buffers/solutions as stabilizers for both pDNA itself and the nanoparticle, the most commonly used Tris, phosphate buffer, ETDA or physiological saline was not suitable, as none of them was able to increase the shelf life of the nanoparticle and to maintain biological activity even for short periods. We found a buffer system, which has the suitable buffer capacity range between pH 7.3-8.3. This buffer, the triethanolamine-hydrochloride buffer is a compendial material. During developmental tests it became clear that sugar-like molecule is need for efficacy. To avoid the side reaction caused by glucose, we investigated different sugar-alcohols which do not contain reducing groups. These were mannitol, sucrose and sorbitol, from which the first two containing formulations showed high biological activity. Finally, by optimizing the pH of the buffer, we chose a

mannitol-containing triethanolamine-hydrochloride buffer (TEAM) with pH 7.5 (instead of the former pH ~3 for glucose/dextrose solution), and without reducing group containing glucose. TEAM buffer is indifferent in terms of the nanoparticle and its components and also improves the biological activity.

2. Stabile DermaVir formulations

After optimizing the components and choosing a new formulation solvent we started long-term stability tests and developed a stabile liquid formulation which was able to maintain its stability; after storage for 3 weeks at 37°C and 8 weeks at 4°C biological activity was the same as the freshly prepared control, first time in literature. Parallel the one-vial liquid formulation we also tested for longer period a two-vial frozen formulation stored at -20°C. This is comprised of the pDNA solution diluted with the TEAM buffer in a vial and the PEIm in another. After thawing the nanomedicine is prepared by simply mixing the two solutions. This formulation has 1 year stability data and unlike the present clinical formulation it does not need special storage conditions or the clinical pharmacist to prepare, it can simply be used by a nurse.

3. Hyperchromicity as the measure of structure-activity relationship and influencing parameters

During the development we explored the structure-activity relationship of DermaVir nanomedicine. We investigated the present clinical formulation in glucose and the new TEAM containing, where the pDNA and PEIm components are both the optimized ones. Atomic force microscopy images revealed that the nanoparticles of the present formulation are not compact, the pDNA strands are perturbed from below the PEIm envelop, although the nanoparticles of the new formulation have coherent PEIm shell on the outside this way providing more efficient protection for the pDNA in the core. This result explains the difference between the biological activity of the two formulations, as the new formulation shows ~30-50% more potency. By investigating more physico-chemical properties we found that it is only the hyperchromicity of the nanoparticles – measured by UV-spectrophotometry – that is able to show the difference between the biological activities. Hyperchromicity occurs when the nanoparticle is formed and shows different values for the present clinical and the new formulations, although the particle size and the size distribution are very similar

for the two formulations. The hyperchromicity of the new formulation is consequently higher than the present clinical formulation's. During the investigation of the phenomenon of hyperchromicity we found that the ionic strength and the pH are the two parameters of the formulation which have crucial impact. We concluded from the results that hyperchromicity can be identified as the measure of the degree of association of the components; if pDNA and PEIm form more bonds, hyperchromicity is higher. Our hypothesis was confirmed by AFM images, gel retardation- and nuclease-resistance assays. Our results show that for potent biological activity the nanoparticle must be stabile enough to survive the endosome after cellular uptake but then in the proximity of the nucleus it has to release the pDNA in time so that it can enter the nucleus to express the encoded antigens. This delicate balance needs the optimal degree of association – the optimal hyperchromicity which is fulfilled in case of the new formulation. As the DermaVir nanoparticle is formed by the electrostatic interaction of the two components, the ionic character of their solution is crucial in determining the degree of association and also in determining the number of unprotonated nitrogens in PEIm backbone, which will buffer the low pH of the endosome. In the present clinical formulation the majority of the phosphate groups of the pDNA are in sodium-salt form, and even the highly cationic PEIm cannot form compact nanoparticles as seen in AFM images. In addition to this, the highly cationic PEIm cannot buffer the low pH of the endosome as it has very few unprotonated nitrogens. In contrast, the new formulation with low ionic strength pDNA contains much less sodium thus more phosphate group can interact with PEIm, and the polymer with optimized cationic degree has enough unprotonated amine groups to survive the endosome this way showing higher biological activity. We also observed similar but inverse correlation in case of the pH; at low pH the dissociation of the weak acid pDNA is suppressed and although the protonation of PEIm is advanced, low number of bonds can be formed between them because of the pDNA, and the number of unprotonated nitrogens is low. At high pH, pDNA dissociation is advanced, and even though PEIm protonation is suppressed, more bonds will be formed as PEIm is in four time molar excess. Besides, as PEIm has more unprotonated nitrogens, it can efficiently buffer the endosome. The most convincing evidence for this is that when the new formulation is buffered to pH ~3, like the present clinical formulation, its potency decreases to the level of the clinical formulation, independently from the ionic character of the components.

During the development of the stabile nanomedicine formulation we have optimized the components, the formulation solvent, set up and verified the intracellular mechanism of action and the structure-activity relationship of the nanomedicine. We implemented a new, simple quality control method that is in connection with the biological activity and can detect the differences between nanoparticle fine structures and compactness. We expanded the specification of the components and the nanomedicine with the newly found critical parameters, thus DermaVir will start the phase III clinical trials with the new, optimized formulation. Developments and results related to the thesis are important milestones in both chemical and clinical development of DermaVir nanomedicine which is confirmed by scientific publications and a submitted patent.

PUBLICATIONS RELATED TO THE THESIS

- Tőke, E.R., Lőrincz, O., Somogyi, E., Lisziewicz, J. Rational development of a stable liquid formulation for nanomedicine products, *Int. J. Pharm*. 2010, 392, 261-267.

- Lőrincz, O., Tőke, E.R., Somogyi, E., Horkay, F., Chandran, P.L., Douglas, J.F., Szebeni, J., Lisziewicz, J. Structure and biological activity of pathogen-like synthetic nanomedicines. *Nanomedicine: NBM*. 2012, 8, 497–506.

- Lőrincz, O., Lisziewicz, J. Transdermal immunotherapy with synthetic pathogen-like nanomedicines and its clinical application towards the cure of HIV. *Handbook of Clinical Nanomedicine: From Bench to Bedside*. Pan Stanford Publishing, Editors: Raj Bawa, Gerald F. Audette, ISBN 9789814316170. With editor.

- Lisziewicz, J., Lőrincz, O. HIV-specific immunotherapy with DermaVir, the first pDNA/PEIm pathogen-like nanomedicine. *Eur. J. Nanomed*. 2012, 4, 81–87.

- Edina Garaczi, E., Szabó, K., Francziszti, L., Csiszovszki, Zs., Lőrincz, O., Tőke, E.R., Molnár, L., Bitai, T., Jánossy, T., Bata-Csörgő, Zs., Kemény, L., Lisziewicz, J. DermAll nanomedicine for allergen-specific immunotherapy. *Nanomedicine: NBM*. 2013. DOI: 10.1016/j.nano.2013.05.011

INTELLECTUAL PROPERTY

Immunogenic nanomedicine composition.
Inventors: Tőke R. Enikő, Lőrincz Orsolya, Somogyi Eszter, Pandur József, Lisziewicz Julianna. Registry number: 0910727. Submitted: 2009.05.14.

PUBLICATIONS NOT RELATED TO THE THESIS

Jakab, I.N., Lőrincz, O., Jancsó, A., Gajda, T., Gyurcsik, B. Approaching the minimal metal ion binding peptide for structural and functional metalloenzyme mimicking. *Dalton Transactions*, 2008, 6987–6995.

CONFERENCE PARTICIPATIONS

Presentation:

Lőrincz, O., Tőke, E.R., Somogyi, E., Lisziewicz, J. Relationship between biophysical and biological properties of NanoComp. 3rd annual meeting of Vaccine Therapy Cluster. International Conference, 2008, Mátraháza.

Posters:

- Tőke, E.R., Lőrincz, O., Somogyi, E., Lisziewicz, J. Liquid nanomedicine formulation for plasmid DNA-based vaccine products. Rational design of HIV vaccines and microbicides, Network Annual Conference, 2009, Budapest.
- Lőrincz, O., Tőke, E.R., Somogyi, E., Lisziewicz, J. Structure and biological activity of pDNA-based nanomedicine vaccines. Rational design of HIV vaccines and microbicides, Network Annual Conference, 2009, Budapest.
- Tőke, E.R., Lőrincz, O., Somogyi, E., Lisziewicz, J. Nanotechnology to improve the biological activity of DNA vaccines. XVIIIth International Aids Conference, 2010, Vienna, Austria.
- Lőrincz, O., Tőke, E.R., Somogyi, E., Lisziewicz, J. Formulation development and structure-activity relationship of DermaVir nanomedicine. 9th International Symposium on Polymer Therapeutics, 2012, Valencia, Spain.
- Lisziewicz, J., Csiszovszki, Zs., Lőrincz, O., Lajos Kemény, L., Garaczi, E. Dermall Nanomedicine for Allergen-Specific Immunotherapy (ASIT). *J. Allergy Clin Immunol*, vol. 131, Nr. 2. American Academy of Allergy, Asthma & Immunology Annual Meeting 2013, San Antonio, Texas, USA.

- Garaczi, E., Szabó, K., Csiszovszki, Zs., Lőrincz, O., Kemény, L., Lisziewicz, J. DermAll ASIT: a new nanomedicine-based allergen-specific immunotherapy. European Academy of Allergy and Clinical Immunology & World Allergy Organization, World Allergy and Asthma Congress, 2013, Milan, Italy (Poster Discussion).