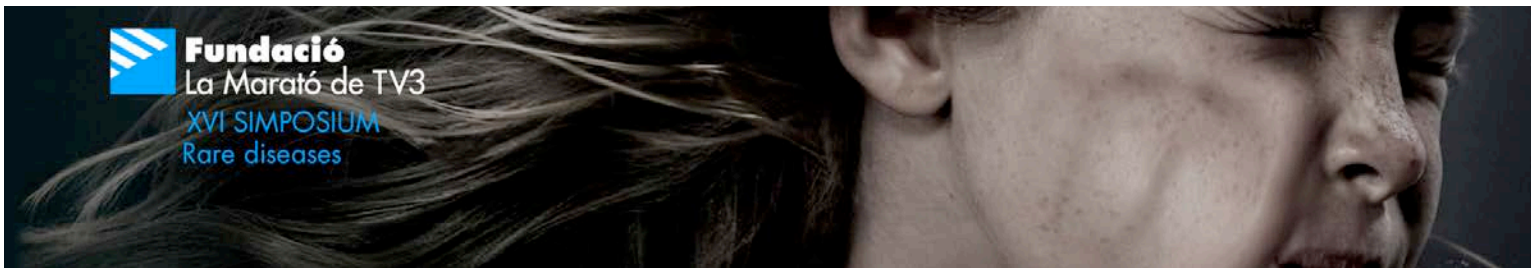

Development of nanomedicines for enzyme replacement therapy in Fabry disease

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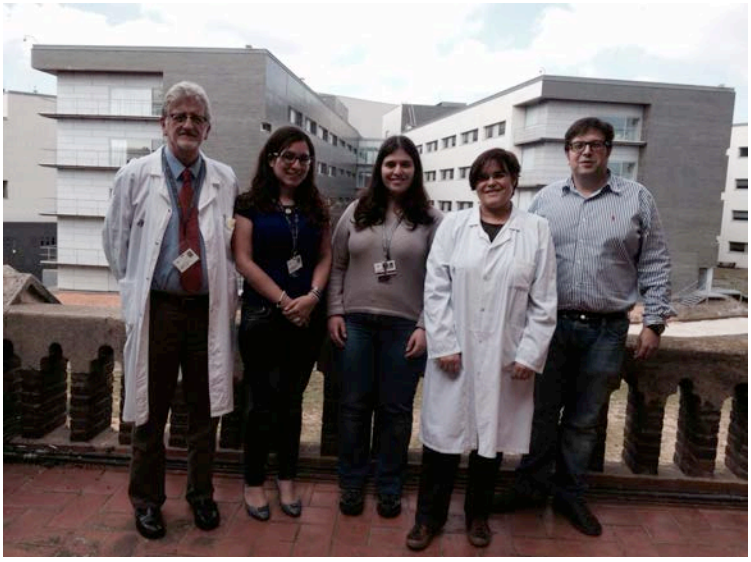

1. Summary of the Project

Fabry disease (FD) is an X-linked recessive disorder caused by a deficiency of lysosomal hydrolase α-galactosidase A (GLA). This enzymatic defect causes the progressive cellular accumulation of neutral glycosphingolipids, giving rise to a multisystemic clinical symptomatology. Current enzyme replacement therapy (ERT) has a limited treatment efficacy in patients with advanced stages of the disease. The objective of this project is to improve ERT by using new therapeutic nanoconjugates of GLA specifically targeted to the endothelial cells, one of the main cell types affected by GLA substrate accumulation.

The project proposes developing biodegradable GLA nanoconjugates that specifically release the defective enzyme into the endothelial cell lysosomes. The project contemplates obtaining and synthesizing recombinant GLA enzyme, incorporating the enzyme into three different nanocarriers (dendrimers, chitosan polyelectrolyte complexes and small unilamellar vesicles) and performing their alternative biofunctionalization against specific endothelial integrins. Obtained nanoconjugates will be validated using specific FD in vitro and in vivo models.

The most important and expected result of the project is for the GLA enzyme to be adequately protected from plasma proteases that will, in turn, increase its half-life in bloodstream. In addition, the use of an endothelial cell targeting moiety will significantly ameliorate tissue biodistribution and internalization of the recombinant enzyme. Overall, the use of nanotechnology based drug delivery system (DDS) with GLA is expected to significantly improve the therapeutic efficacy seen with the current ERT.
The general objective of the project is to improve the ERT of FD by using new therapeutic nanoconjugates of GLA specifically targeted to endothelial cells, one of the main cell types affected by Gb3 accumulation. The specific objectives to be developed during the project are:

1) Obtaining new drug delivery systems (DDS) loaded with GLA and targeting moieties against endothelial cells.

2) Evaluation of the internalization and in vitro activity of DDS in Fabry cell models.

3) In vivo toxicity and biodistribution studies of GLA-loaded DDS in Fabry mice models.

4) Efficacy studies of GLA-loaded DDS in Fabry mice. Reduction of Gb3 levels in vivo.

2. Results

The results achieved in each case are the following:

1) Production of active enzyme (α-GAL). The desired enzyme was provided when needed and requested by the consortium groups during the project. Production was by transient gene expression (TGE)-based protocol for the production of the human enzyme a-GAL in mammalian cells that was set up and optimized by the IBB-CIBER group. So, the routine production and purification of enough active α-GAL when needed was assured.

2) Obtaining new drug delivery systems (DDS) loaded with GLA targeting moieties against endothelial cells. Of the three different DDS that were proposed in the project, different stages of development were achieved. Specifically:

- Obtaining ***RGD targeted dendrimers*** loaded with GLA. Regarding the dendrimer-GLA-conjugates production, although synthesis with long oligo ethylene glycol (OEG) arms was achieved, the final product was impossible to detect by mass spectroscopy (MS) and because of that it was not possible to characterize the conjugate. It was decided to abandon this strategy. Regarding the second possible system, PEG-GLA-conjugates, the method of conjugating PEG units to GLA was achieved. The functionalization of these systems with three different targeting

peptides (RGD, P25 and P87) was also achieved. These compounds were conjugated to α-GAL and/or Replagal.

- Obtaining ***RGD targeted small unilamellar vesicles*** (SUVs) loaded with GLA. The Nanomol group was able to prepare SUVs composed of cholesterol, DPPC and cholesterol_RGD as a target moiety (6:10:1), with α-GLA incorporated or with commercial Replagal. It has been successfully demonstrated that the preparation of this SUV by the DELOS-SUSP method is optimal, obtaining from it batches of SUV-RGD-GLA and/or SUV-RGD-Replagal® nanoconjugates with homogenic particle size between 100-200 nm, with good stability in time and a high homogeneity and unilamellarity. The encapsulation efficiency of the GLA/Replagal within the SUVs was been determined via Western-Blot and was around 30 %. Finally, the formulations turned to be non-cytotoxic, non-haemolytic and sterile.
- Obtaining ***RGD targeted chitosan polyelectrolyte complexes*** loaded with GLA. This production of chitosan-based polyelectrolyte complexes (PECs) loaded with GLA was accomplished with a reproducible procedure and the suitability of the system to undergo liophilization processes has been demonstrated. An adequate freeze-drying protocol has been defined, thus ensuring the long stability of the PECs and allowing sample concentration, which is needed for the in vivo experiments.

3) Evaluation of the internalization and in vitro activity of the different DDS in Fabry cell models.

Internalization assays: In the case of the PEGylated systems (bound to a fluorochrome) it was demonstrated by flow cytometry and confocal microscopy techniques that the PEG-RGD and the PEG-P25 systems had a higher capacity to cross MAEC KO cell membranes than the other labelled PEG nanoconjugates. Moreover, both systems accumulated at lysosomes. In the case of the SUVs-RGD and SUVs-RGD- GLA nanoconjugates, the internalization of these systems in cells was demonstrated by laser scanning confocal microscopy, TIRF microscopy and flow cytometry. Finally, in the case of the PECs DDS the internalization in HMEC-1 cells was also demonstrated using flow cytometry techniques.

In vitro activity assay results: The efficacy of the different DDS systems, loaded with α-GAL or commercial enzyme (Replagal) was tested in primary cultures derived from the aorta of Fabry KO mice (MAEC cells). The activity of all three DDS systems was tested using the NBD-Gb3 assay. This assay relays in the ability of the α-GLA enzyme to reduce the cellular accumulation of a fluorescently labelled Gb3, namely NBD-Gb3 (N-dodecanoyl-NBD-ceramide trihexoside). This activity only occurs at low pH, within the acidic lysosomal compartment of the cell and therefore the NBD-Gb3 reduction reflects the internalization and final lysosomal localization of the GLA enzyme as well as its activity. So, it was demonstrated that in the case of the PEGylated systems loaded with GLA or Replagal, the activity of these systems was slightly worse than the GLA and/or Replagal free activity. However, it has been demonstrated that the addition of the targeting peptide REG to the PEGylated system can significantly improve its activity, by reverting the effect of the PEGylation. Therefore, these results also confirmed that the addition of a targeting moiety (specifically RGD) can significantly improve the internalization of the DDS (and therefore the final enzymatic activity). In the same way, it has been demonstrated that in the case of the PECs systems, the enzymatic activity of α–GLA is higher when delivered as a complex with the PECs than free, observing a higher reduction of the Gb3 levels in the cell incubating with PECs-GLA. Finally, in the case of the SUVs-RGD system loaded with GLA or Replagal it was demonstrated that the RGD-SUV-GLA system has a better performance regarding the enzymatic activity of the GLA than the free enzyme.

4) In vivo toxicity and biodistribution studies of GLA-loaded DDS in Fabry mice models.

In vivo toxicity. The purpose of this objective was to demonstrate the adequate toxicity profile of the different DDS. Cytotoxicity and haemocompatibility of naked enzyme, PECs (targeted with and without RGD) and SUVs (targeted or non-targeted with RGD) were assayed. The results showed that no signs of toxicity were observed after 72 hours of incubation of HeLa and HMEC-1 cells. with PECs-RGD or

PECs alone, SUVs or SUVs-RGD. Cell viability was maintained above 80%. Moreover, no significant haemolysis was observed.

Biodistribution assays: a PK/PD assay of RGD-SUVs has been performed, along with that of free GLA enzyme and free Replagal. The reason of selecting RGD-SUVs for the assay (and not the other DDS) was that the RGD-SUVs was the more advanced system from a chemical point of view (characterization, patent production, purification process etc.), and with the best results in vitro (by the NBD-Gb3 assay). The results, although preliminary, clearly indicate that the half-life of the enzyme (in this case, the commercial one, Replagal) is significantly increased when it is administered conjugated to SUVs-RGD. These results would further suggest that the in vivo activity of RGD-SUV-Replagal could be significantly better than the activity of naked enzyme.

5) Efficacy studies of GLA-loaded DDS in Fabry mice. Reduction of Gb3 levels in vivo.

The goal of this activity is to provide a good proof of concept that the DDS are able to deliver active GLA into the lysosomal compartment of endothelial cells and restore GLA activity in KO Fabry mice model. To that end, several tasks were performed throughout the project, specifically, the development of a quantitative method for detection of Gb3 levels in plasma and tissue samples (kidney, spleen, and liver). At the start of the project the expectation was to set up a method for quantification of LysoGb3 but this was discarded because the detection limit of the HPLC detector used was not reached. Therefore, it was decided to determine extracted Gb3 levels from WT and KO Fabry mice by an HPTLC method. During this last period different tissues and plasma from WT and KO Fabry mice were studied in order to set the analytical conditions and start the methodology. This made it possible to confirm that Gb3 levels in tissues from KO mice are higher than those from WT mice. Although the method is almost validated, more experiments have to be performed to confirm the findings.

3. Relevance and possible clinical implications of the final results

From the scientific point of view, the potential use of nanocarriers to effectively deliver an active enzyme at the endothelial lysosomes represents an important challenge. On the one hand, it requires the cooperation of several groups and experts with different scientific backgrounds such as chemical engineering, molecular biology, biochemistry, biotechnology and pharmacology, as well as good biomedical and clinical knowledge. Networking is therefore necessary. Such is the case of this proposal. On the other hand, there is still much information missing about the procedures that have to be implemented concerning the chemical synthesis of the drug delivery systems (DDS) planned in the project and also on their in vitro and in vivo validation. In this context, our project has certainly helped to improve knowledge of the groups involved in DDS chemical-related methodology. Furthermore it has provided new experimental data on cellular and animal models for DDS preclinical validation. All these aspects are crucial for future translation to the clinics and industrial transfer of intellectual property developed in this project. It is worth mentioning that during the course of this project, two patents were granted, and one of them has already been transferred to industry. In addition, the continuation of this project is ensured, since the coordinating group (VHIR) and partners Nanomol-CSIC and IBB-UAB are continuing the work developed in this project within two additional projects, namely TERARMET (ref: RTC-2014-2207-1; from 01/11/2014 to 31/12/2017, financed by the Spanish "Ministerio de Economía y Competitividad" under the programme "Retos-Colaboración del Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad" and LIPOCELL (cofunded by CIBER-BBN and Praxis Biopharmaceuticals). In detail, the new nanomedicine candidate for FD, will be tested at preclinical regulatory level under the LIPOCELL technology transfer project and the scale-up for the production of this new nanodrug under Good Manufacturing Practices (GMP) will be done under the TERARMET project.

It is worth mentioning that because rare diseases have a low incidence in the overall population (defined as lower than 1:10,000 people) the development of specific treatments raises questions related to the potential operating profits for the pharma market. However, in this specific project we have succeeded in transferring results to industry (patent transferred to Praxis Biopharmaceutical) and we even have the possibility of partnering Shire (producer of one of the two currently available treatments for FD patients) as they are interested in participating in our LIPOCELL project.

4. Generated Literature

I Cabrera, E. Elizondo, O. Esteban, J.L. Corchero, M. Malgarejo, D. Pulido, A. córdoba, E. Moreno, U. Unzueta, E. Vázquez, I. Abásolo, S. Schwartz Jr., A. Villaverde, F. Albericio, M. Royo, M.F. García-Parajo, N. Ventosa, J. Veciana. "Multifunctional nanovesicle-bioactive conjugates prepared by a one-step scalable method using compressed fluids". Nano Letters., 13(8), 3766-3774 (2013).

I Cabrera, I. Abásolo, J.L. Corchero, E. Elizondo, P. Rivera, s. Sala, M. Rivas, M. Malgarejo, D. Pulido, M. Royo, F. Albericio, A. Villaverde, M.F. García-Parajo, S. Schwartz Jr, N. Ventosa, J. Veciana. "α-Galactosidase A loaded nanoliposomes with enhanced enzymatic activity for Fabry's disease (under review at Nano Letters).

JL. Corchero, E. Vázquez, E. Garcia Fruitos, N. Ferrer-Miralles, A. Villaverde. "Recombinant protein materials for bioengineering and nanomedicine". Nanomedicine 9(18): 2817-2828, (2014).

E. Rodríguez-Carmona, R. Mendoza, E. Ruiz-Cánovasc, N. Ferrer-Miralles, I. Abásolo, S. Schwartz, A. Villaverde, JL. Corchero. "A novel bio-functional material based on mammalian cell aggresomes". Under Revision on Acta Biomaterialia (2015).

L. Ferrer-Tasies, E. Moreno-Calvo, M. Cano-Sarabia, M. Aguilera-Arzo, A. Angelova, S. Lesieur, S. Ricart, J. Faraudo, N. Ventosa, J. Veciana. “Quatsomes: vesicles formed by self-assembly of sterols and quaternary ammonium surfactants”. Langmuir, 29 (22), 6519-6528. (2013).

JL. Corchero, R. Mendoza, J. Lorenzo, V. Rodríguez-Sureda, C. Dominguez, E. Vázquez, N. Ferrer-Miralles, A. Villaverde. “Integrated approach to produce a recombinant, His-tagged human α-galactosidase A in mammalian cells”. Biotechnol. Prog. 2011. 27(5):1206-17.

N. Ventosa. “Materiales moleculares nanoestructurados en el desarrollo de nuevos fármacos”. SEBBM. 2011. 168.

F. Temelli, A. Córdoba, E. Elizondo, M. Cano-Sarabia, J. Veciana, N. Ventosa. “Phase behavior of phytosterols and cholesterol in carbon dioxide-expanded ethanol”. J. of Supercritical Fluids. 2012. 63, 59.

E. Elizondo, J. Larsen, N. S. Hatzakis, I. Cabrera, T. Bjornholm, J. Veciana, D. Stamou, N. Ventosa. “Influence of the preparation route on the supramolecular organization of lipids in vesicular system”. J. Am. Chem. Soc., 2012, 134, 1918.

M. I. Gianotti, O. Esteban, M. Oliva, M.F. García-Parajo, F. Sanz. “pH-responsive polysaccharide-based polyelectrolyte complexes as nanocarriers for lysosomal delivery of therapeutic proteins”. Biomacromolecules 12, 2524-2533. (2011).

JL. Corchero, B. Gasser, D. Resina, W. Smith, E. Parelli, F. E. Vázquez, I. Abásolo, M. Giuliani, J. Jäntti, P. Ferrer, M. Saloheimo, D. Mattanovich, S. Schwartz Jr., L Tutino, A. Villaverde. “Unconventional microbial systems for the cost-efficient production of high-quality protein therapeutics”. Biotechnol. Adv. 2013. 31(2):140-53.