CREATIVITY OF IN VITRO ASSAYS TO MEASURE INTRACELLULAR ACTIVITY AND HUMAN RECOMBINANT α-GLUCOCEREBROSIDASE UPTAKE

Kotu.Akhil[1]* and Dr. F. Emerson Solomon [2]

ABSTRACT

The present research mainly focused on in vitro assay to measure intracellular activity and human recombinant α- glucocerebrosidase uptake. Human alpha glucocerebrosidae was family of lysosomal hydrolases, it used to enzyme replacement therapy and leads to deficiency of lysosomal storage diseases (LSD). Most people agree that using in vitro assays to assess a prospective compound's bioactivity before moving on to in vivo research is a helpful approach. Thorough assessments of a broad range of various substances can be performed with a specific focus on the anticipated bioactivity by utilising pertinent cell lines. In vitro studies three techniques were used for the quantification α-rhGC like flow cytometry extraction and activity assay and fluorescence microscopy was used to qualitatively evaluate the degraded intracellular substrate. The three techniques put into practice and put to the test proved to be useful instruments for examining the cellular uptake of rhGC. It was also possible to demonstrate the intracellular activity of a pertinent substrate using the fluorescence-based tests. The techniques are therefore very valuable for analysing future variations of enzymes expressed by P. pastoris strains that have been humanised.

Keywords: Human recombinant α- glucocerebrosidase, Lysosomal storage diseases (LSD), In vitro assay, Flow cytometry, fluorescence microscopy

[1]*Research Scholar, Department of Biotechnology,Bharath Institute of Higher Education and Research, #173 Agharam Road Selaiyur, Chennai- 600 073.

[2]Professor, Dept. of Bio-Medical Engineering,Bharath Institute of Higher Education and Research, #173 Agharam Road Selaiyur, Chennai- 600 073

***Corresponding authors:** Kotu Akhil[1]
*Research Scholar, Department of Biotechnology,Bharath Institute of Higher Education and Research, #173 Agharam Road Selaiyur, Chennai- 600 073

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INTRODUCTION

First of all, Lysosomal storage disorders (LSDs) are caused by a loss of human α-glucocerebrosidase (rhGC), a member of the lysosomal hydrolases family. One therapy approach to delay the progression of various LSDs is to administer recombinant missing enzymes, or enzyme replacement therapies, or ERTs [1]. However, the production of enzyme replacement treatments requires alternative expression systems due to the extremely high cost of currently existing ERTs involving patent problems. Glycosylation pattern engineering in P. pastoris provides a highly promising technology for the synthesis of recombinant enzymes with increased expression. Recombinant human α-glucocerebrosidase (rhGC) expression in P. pastoris was assessed in various strains [2]. Following the selection of the best candidate, overexpression of the transcription activator HAC1p greatly increased the expression levels. Subsequently, the coding gene was cloned numerous times in order to optimise the particular productivity of rhGC). This was done by enhancing the expression of variations of the enzyme that either had a cell internalisation motif or absence sequence of C-terminal. Finally, a base strain was created for glycan changes using a markerless clone variety that expressed rhGC. The function of lysosomal enzymes in lysosomal storage disorders from a genetic perspective, LSDs result from inherited mutations in genes that code for various proteins particularly acid hydrolases [3, 4].

First and foremost, there would be a significant number of animals utilised and then sacrificed, which is an obvious bioethical problem. To sustain the profitability of projects in their early stages of development, it is best to minimise the costs associated with managing animal facilities and maintaining animal colonies [5]. Moreover, compared to isolated cell line cultures, animal systems are far more complex. This feature primarily causes three issues: first, compared to cell cultures, animal experiments always require a larger sample volume. Secondly, the formulation of active principles is necessary to produce the desired bioactivity by guaranteeing an appropriate half-life and bioavailability. Third, sample analysis typically calls for more advanced techniques [6].

Even though in vivo tests are usually necessary for pre-clinical tests, particularly for those intended for use as biopharmaceuticals in humans, earlier in vitro screens using cell cultures significantly reduce expenses and concentrate later in vivo testing on those promising leads exclusively [7].

Regarding human α-Glucocerebrosidase, these experiments ought to evaluate the enzyme's capacity for internalisation as well as its intracellular catalytic activity. Indeed, it is mostly due to the latter why recombinant systems typically are unable to generate effective lysosomal enzymes, primarily due to their incapacity to synthesise mannose-6-phosphate glycans. Different glycosylation variations and intermediates are anticipated to be delivered by Pichia pastoris strain growth; however, although the existence of such alterations can be chemically examined, the true proof of functionality will ultimately come from living systems [8].

Various approaches to investigate the biological effectiveness of rhGC, particularly three procedures were developed based on the following: (1) flow cytometry was used to quantify the degraded intracellular substrate; (2) extraction and activity assay was used to quantify the active internalised enzyme; or (3) fluorescence microscopy was used to qualitatively evaluate the degraded intracellular substrate [9].

These techniques were first tried in two distinct cell lines in order to determine which of them offers the best option for the evaluation of rhGC based on the consistency of the data and their respective ease of culturing [10,11]. Ultimately, rhGC and an existing medicinal product (Agalsidase alfa) were compared using established protocols in order to assess both the qualitative and quantitative performance of the two enzymes.

MATERIALS AND METHODS

Cell line preparation, subculturing, and proliferation for in vitro tests:

Fibroblasts (GM02775,) were cultured in M106 culture medium (ThermoFisher, #M-106-500) supplemented with Low Serum Growth Supplement and antibiotic-antimycotic solution, 100x (ThermoFisher, #15240-112) at 37 ºC and under 5% CO2, humidified atmosphere. umbilical vein endothelial cells (ThermoFisher, #C-003-5C) were cultured in M200 medium supplemented also with Low Serum Growth Supplement and antibiotic-antimycotic solution at 37 ºC and under 5% CO2, humidified atmosphere.

A functional cell bank kept in liquid nitrogen was used to get fresh cell cultures for in vitro tests. 20 mL of the equivalent fresh enriched culture medium that had been pre-warmed at 37 ºC were combined with 1 mL of frozen vials that had been

thawed at 37 ºC for one to two minutes. Subcultures were carried out to get substantial cell numbers for the in vitro tests. After the cells reached confluence, the culture media was withdrawn, and the monolayers were treated with 5 mL of trypsin-EDTA 0.25% for 7 min at 37 ºC for fibroblasts or 3 min for HUVEC. After adding 5 mL of trypsin neutralizer (ThermoFisher, #R-002-100), the trypsin was neutralised, and the cells were pipetted gently back into suspension.

Cell concentration was determined by cell counting in a Neubauerhemocytometer while trypsin-neutralized cell solutions were centrifuged at 100 g, 5 min. The corresponding culture medium was used to resuspend each cell type and to adjust the concentration to 5 · 104 cells/mL. The cell suspension was then re-seeded in new 75 cm2 culture flasks. Following a 24-hour seeding phase, culture medium were periodically refreshed every three days. After the cultures reached confluence, the culture wells intended for fluorometric analysis by flow cytometry or microscopy were supplemented with 10 µg/mL of the fluorometric substrate N-Dodecanoyl-NBD-ceramide trihexoside (NBD-Gb3, Santa Cruz Biotechnology, #sc-360252).

To enable the substrate to be internalised and accumulate inside the cells, the cells were incubated for 24 hours at 37°C and 5% CO2. Parallel cultures of HUVEC cells were conducted using 500 µM of To prevent endogenous GC activity, use deoxygalactonojirimycin hydrochloride (DGJ, Sigma-Aldrich, #D9641). Following the NBD-Gb3 (and DGJ in the case of HUVEC cells) incubation, PBS was used to wash each culture once, and then varying doses of rhGC were added. To establish the approach for the in vitro evaluation of the recombinant GC's intracellular activity and internalisation capacity, two experiments were carried out in consecutive order. For the first, increasing doses of the pure recombinant enzyme made in Pichia pastoris were added to supplemented M106 (Fabry fibroblasts) or M200 (HUVEC) medium (0, 0.001, 0.005, 0.01, 0.05, and 0.1 AU/mL).

Crucially, the activity units used in this chapter are µmols MU · mL-1 · h-1 (as opposed to nmols MU · mL-1 · h-1, which are the activity units used in other chapters). For the internalised enzyme activity measure quantification and the fluorometric quantification by cytometry, each concentration was evaluated in triplicate. For the fluorescence microscopy evaluation, the tests were conducted in duplicate.

Cell monolayers were treated with each rhGC dose for 24 hours at 37 ºC and 5% CO2. In the subsequent experiment, just Gauchers fibroblasts were cultivated to evaluate the efficacy of rhGC in contrast to the medicinal enzyme Agalsidase alfa. Triolicate cultures were used to apply increasing concentrations of rhGC (0, 0.01, 0.1, and 1 AU/mL) diluted in supplemented M106 media for internalised enzyme activity measurement and fluorometric quantification by cytometry, and duplicates for fluorescence microscopy evaluation. Cell monolayers were treated with varying doses of rhGC for either 24 or 48 hours at 37 ºC and 5% CO2.

Quantification of internalized enzyme by activity measurement:

To remove all of the non-internalized GLA, culture monolayers that had been subjected to escalating concentrations of rhGC or imiglucerase were three times washed with PBS. A lysis buffer containing 50 µL (10 mM Tris, 100 mM NaCl, 5 mM EDTA, and 2 mM protease inhibitor cocktail (CompleteTM mini, EDTA-free, 1% NP-40 (NP40S, Sigma-Aldrich) and 11836153001, Roche) were added to each well, and the monolayers were physically scraped to release the cells. After 30 minutes of ice incubation, cell suspensions were centrifuged at 2000 g for 10 minutes at 4°C. After discarding the cell debris (pellet fraction), the supernatants' GC enzymatic activity was assessed.

Internalised enzyme fluorescence measured by cytometry: Culture monolayers tested with escalating doses of rhGC or Imiglucerase were trypsinized (50 µL of trypsin-EDTA 0.25% per well) for either three or seven minutes (Fabry fibroblasts) or seven minutes (HUVEC). Trypsin inhibitor (50 µL/ well) was added right after the trypsin incubation, and the cell suspensions were centrifuged at 100 g for 5 minutes at 4 ºC. The supernatant was disposed of, and the cell pellets were preserved by resuspension in 50 microliters of diluted 4% formaldehyde in PBS. The Gallios Flux cytometer (Beckman Coulter) was used to analyse the cell samples, evaluating 2000–3000 cells per sample in 3 minutes.The fluorescence measure of each sample was calculated by averaging the fluorescence signal of all the cells analyzed in each run.

Evaluation of internalized enzyme by fluorescence microscopy:

The LABTEK II 8-well Chamber SlideTM, which is specifically made for use in fluorescence microscopy, was seeded with cell cultures for the fluorometric evaluation through microscopy. The cultures were twice rinsed with PBS following the

incubation period with escalating doses of rhGC or Agalsidase alfa. After that, monolayers were fixed by adding 3.5% paraformaldehyde, incubating for 5 minutes at 4ºC, and then letting them sit at room temperature for 10 minutes before rinsing them three times with HEPES (Sigma-Aldrich, #H4034).Finally, fixed cells were blocked byadding100mMTris-HCl,pH8.Cell culture fluorescence was assessed using a Leica DMIRBE motorised inverted fluorescence microscope that has a Cool SNAPfx camera attached. The software Metamorph was utilised to capture the images. ImageJ was used to analyse the images.

RESULTS AND DISCUSSION

Evaluation of two cell lines (human Gauchers fibroblasts vs. HUVEC) and various methods for assessing recombinant human α-glucocerebrosidase uptake capacity:

In an early investigation of various techniques intended to assess the internalisation and intracellular activity of the rhGC generated in P. pastoris, two distinct cell lines were obtained. Derived from primitive mesenchyme, fibroblasts are the most prevalent cell type in connective tissue in animals. Since there was no GC activity at all in the chosen cells, any catalytic activity that was detected would be due to the additional enzyme. For both kinds of cells, three different approaches were assessed; the first and second relied on quantitative measurements (by fluorescence flow cytometry or intracellular extraction and activity assay), while the third method used fluorescence microscopy to assess the internalised enzyme qualitatively. Naturally, the two standard procedures of cell growth and enzymatic solution incubation were performed for each of the assessment studies.

Quantification by extraction and activity measure of internalized enzyme:

The initial assay measured internalised activity during a 24-hour incubation period in the presence of specific rhGC concentrations (based on enzymatic activity measures). Following the incubation period, cell monolayers were cleaned to remove any remaining extracellular enzyme. After mechanically separating the monolayers (by scraping the surface), they were treated briefly with detergent (NP-40) in an attempt to solubilize the cell membranes and release the intracellular proteins, which finally included the internalised enzyme.

The results of activity measurements on extracted samples demonstrated that, in the case of Fabry fibroblasts, there was a direct correlation between the activity measurements of cell extracts and the rise in rhGC concentrations in the culture medium. Although HUVECs also exhibited a similar behaviour, There are several likely factors that could be to blame, even though the origin of these variances is unknown. The first is the distinctive development profile of HUVECs, which, in contrast to fibroblasts, can grow independently and occupy the surface rather uniformly until they reach confluence, cluster together and begin to spread over the surface from that point on. The number of cells accumulated in each culture well at the conclusion of the experiment may vary significantly depending on this profile.

An alternate technique based on the measurement of a fluorescent substrate was investigated in light of the requirement to ascertain the effectiveness of the internalised GC on the degradation of intracellular substrates. To be more precise, cell cultures were treated with N-Dodecanoyl-NBD-ceramide trihexoside (NBD-Gb3, a fluorescent analogue of natural ceramide trihexoside, a known substrate of GC), prior to the incubation with rhGC samples. This molecule has a 7-nitrobenzofurazan (NBD) group with maximum excitation and emission wavelengths of 465 nm and 535 nm, respectively. It is easily internalised by the cells. Upon the catalytic cleavage of ceramide trihexoside by GC, a measurable and observable reduction in fluorescence occurs. In the instance of HUVEC, NBD-Gb3 therapy was administered concurrently with deoxygalactonojirimycin (DGJ). This galactose analogue inhibits GC/ in a competitive manner.

Gauchers fibroblast measurements revealed a strong relationship between the amount of rhGC given in culture and the fluorescence of the cells, with the latter attaining a minimum residual fluorescence of 55% in comparison to the fluorescence measured for untreated cells. Conversely, no correlation was found for HUVECs, which attained a minimum fluorescence value of 61% with the lowest tested rhGC concentration (0.001 AU/mL). Although a lower value was obtained with 0.005 AU/mL (42%), this was deemed to be an anomaly because the results obtained for higher rhGC concentrations again demonstrated remaining fluorescence of 56%, which was equivalent to the value observed for fibroblasts treated with the same rhGC concentration.

Evaluation of internalized enzyme by fluorescence microscopy:

The qualitative assessment of NBD-Gb3 degradation using fluorescent microscopy was the third approach to be investigated. The cultures utilised in this test received the same treatment as those used in fluorescence cytometry. This time, though, the cells were cultivated in culture chambers where the bottom was constructed from non-fluorescent glass microscope slides that had been carefully treated to encourage the growth of adherent cells. Additionally, a cell fixation process was used in place of the trypsin treatment in order to examine the cultures' fluorescence under a microscope right within the chambers.

To streamline the assay, both cell types' maximum rhGC concentration (0.1 AU/mL) and negative controls devoid of the enzyme were examined. To reveal the anticipated green fluorescence within the cells, a green filter was employed. The internalisation of the fluorescent substrate (NBD-Gb3) in both cell types was distinguished more clearly in control cultures that had not received rhGC treatment. It was spread as expected over the entire cell, with the exception of the nucleus, and some small, highly fluorescent areas were noticeable. These could be indicative of an insoluble substrate cumulus, as some of them remained visible in cultures treated with rhGC.

It was clearly seen that the fluorescence of the cells had decreased in both types of cells—HUVECs and Gaucher's fibroblasts. However, the residual fluorescence appeared to be uniform in the majority of the cells that were seen, whereas HUVECs had more variability, with some cells maintaining substantial amounts of fluorescent substrate and others losing it to the point where they were nearly undetectable. It is evident that rhGC functioned properly in vitro for all three investigated procedures and with both cell types. Regarding the latter, Gauchers fibroblasts were found to have considerable benefits and higher-quality results, at the expense of cells. This cell line was selected as the reference to test future rhGC variations, while HUVEC was discarded due to the fibroblasts' outstanding growth profile and the absence of endogenous Beta-glucocerebrosidase that could interfere in the experiments. Furthermore, Gauchers fibroblasts are a pertinent cell line for assessing an enzyme that may really be utilised in enzyme replacement therapy for the same illness.

Table 1: Summary of cell types tested and their characteristics

Celltype	**Growthprofile**	**Attachmentstrength**	**EndogenousGCactivity**
Fabryfibroblasts	Homogeneous monolayer – Consistent cell count between replicas	Veryhigh -Resistant to multiple washing steps	NO
HUVEC	Extension of monolayer From cell clusters-Cell count variability between replicas	Medium–Low - Easily de attached,sourceof variability	YES- Previous inhibition is required (e.g.with DGJ) - Potential source of odd results

Extraction and quantification of internalizedenzyme	**Efficiency of internalization**	**Quantitative**	**LOW - No specific reagents or additional equipment required**
Fluorescence cytometry	Efficiency of internalization +Intracellular activity	Quantitative	MEDIUM -Fluorescent substrate required -Use of fluorescence flow cytometer
Fluorescence microscopy	Efficiency of internalization +Intracellular activity	Qualitative	MEDIUM–HIGH - Fluorescent substrate required - Culture in specific culture chambers - Use of fluorescence microscope

It is evident that rhGC functioned properly in vitro for all three investigated procedures and with both cell types. Regarding the latter, Gauchers fibroblasts were found to have

considerable benefits and higher-quality results, at the expense of cells (table 5.1). This cell line was selected as the reference to test future rhGC variations, while HUVEC was discarded due to the fibroblasts' outstanding growth profile and the absence of endogenous Beta-glucocerebrosidase that could interfere in the experiments. Furthermore, Gauchers fibroblasts are a pertinent cell line for assessing an enzyme that may really be utilised in enzyme replacement therapy for the same illness.

Promising candidates might then be confirmed to exhibit intracellular action over an appropriate substrate using both fluorescence-based methodologies. Fluorescence microscopy is a qualitative assay that requires specialised culture material, therefore unless quantification is done by computer analysis of the pictures, it could be overlooked if visual evidence is not absolutely necessary. Furthermore, the data pertaining to rhGC would be comparable to that obtained using fluorescence flow cytometry.

Cellular uptake of rhGC produced in *Pichiapastoris* versus the therapeutic product:

After the protocols and the suitable cell type for assessing the rhGC generated by P. pastoris were established, a comparison with the medicinal product was conducted. The data covered in the preceding paragraphs suggested that Gauchers fibroblasts would be the most suitable option for testing recombinant beta-glucocerebrosidase. This cell type demonstrated the strongest association, across all examined techniques, between the expected response (degradation of fluorescent substrate in the second and third assays, and an increase in intracellular Gc activity in the first experiment).

The three approaches were used because they were thought to complement each other and to compare rhGC and imiglucerase. This time, Imiglucerase was administered in a single concentration of 0.01 AU/mL and the maximum concentration of rhGC was raised from 0.1 to 1 AU/mL in all the experiments.

Quantification by extraction and activity measure of internalized enzyme:

Following treatment of the monolayers with several GC solutions at concentrations ranging from 0.01 to 1 AU/mL, variations in the internalisation efficiency were clearly visible in the enzymatic activity quantified from cell extracts. A modest dose of 0.01 AU/mL of rhGC, the highest concentration tested, was able to produce activity values in cultures treated with the therapeutic product Agalsidase alfa that were comparable to those seen at 1 AU/mL. According to this finding, it would take a rhGC sample that is 100 times more concentrated to attain absorption levels comparable to those of imiglucerase. The primary cause of the observed discrepancy is the enzyme generated by P. pastoris lacking mannose-6-phosphate glycosylation structures. As was covered in earlier chapters, glycans are recognised as the essential.

CONCLUSION

Fluorescence cytometry examination of intracellular GC activity revealed the anticipated dose-response relationship for rhGC (fig. 5.8). Nevertheless, the observed NBD-Gb3 degradation levels were significantly lower than in comparable tests that were covered in the preceding paragraph, for reasons that are now unknown. Upon comparison with the cultures subjected to Imigluerase treatment, rhGC once more shown restricted efficacy. More specifically, to decrease intracellular fluorescence to levels comparable to those brought on by treatment with imiglucerase (0.01 AU/mL), a 100-fold rise in rhGC concentration (1 AU/mL) was required. These outcomes support the enzyme impairment in its internalisation capacity since they are consistent with the data seen in the internalised enzyme activity measures.

The investigation of recombinant alpha-Glucocerebrosidase A was found to be best suited for fibroblasts. Gauchers fibroblasts, in contrast to HUVEC, are simpler to work with and yield better-quality outcomes. The three techniques put into practice and put to the test proved to be useful instruments for examining the cellular uptake of rhGC. It was also possible to demonstrate the intracellular activity of a pertinent substrate using the fluorescence-based tests. The techniques are therefore very valuable for analysing future variations of enzymes expressed by P. pastoris strains that have been humanised. made in a wild-type strain of P. pastoris, it exhibited a high-mannose glycosylation pattern and, hence, a poor internalisation capability when compared to the therapeutic product Imiglucerase, which is made in a human cell line and contains mannose-6-phosphate glycans.

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