Plant-based oral delivery of β-glucocerebrosidase as an enzyme replacement therapy for Gaucher’s disease

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Summary

Gaucher’s disease (GD), a lysosomal storage disorder caused by mutations in the gene encoding glucocerebrosidase (GCD), is currently treated by enzyme replacement therapy (ERT) using recombinant GCD that is administered intravenously every 2 weeks. However, intravenous administration includes discomfort or pain and might cause local and systemic infections that may lead to low patient compliance. An orally administered drug has the potential to alleviate these problems. In this study, we describe the potential use of plant cells as a vehicle for the oral delivery of recombinant human GCD (prGCD) expressed in carrot cells. The in vitro results demonstrate that the plant cells protect the recombinant protein in the gastric fluids and may enable absorption into the blood. Feeding experiments, with rat and pig as model animals, using carrot cells containing prGCD, show that active recombinant prGCD was found in the digestive tract and blood system and reached both, liver and spleen, the target organs in GD. These results demonstrate that the oral administration of proteins encapsulated in plant cells is feasible. Specifically, carrot cells containing recombinant human prGCD can be used as an oral delivery system and are a feasible alternative to intravenous administration of ERT for GD.

Introduction

Oral administration of proteins is one of the challenges of the biotherapeutic industry, particularly due to early degradation of the proteins in the digestive tract. There have been many attempts to develop oral delivery methods for protein pharmaceuticals, most of them with limited success (Gupta et al., 2013; Park et al., 2011; Pinto Reis et al., 2013). Nevertheless, plant cell walls can provide natural protection for the cell content against the harsh conditions in the stomach. Several studies have demonstrated feasibility of utilizing plants as a vehicle to deliver recombinant pharmaceutical proteins through the digestive system (Choi et al., 2014; Daniell et al., 2009; Kwon et al., 2013b; Limaye et al., 2006; Sherman et al., 2014; Tremblay et al., 2010). Biopharmaceuticals expressed in plant cells are protected in the stomach and gut from acids and enzymes and may be absorbed into the circulatory system when the plant cell walls are digested in the gut (Kwon et al., 2013a). Freeze-dried plant cells containing pharmaceutical proteins for oral delivery have various advantages over fresh materials in regard to long-term storage, increase of therapeutic protein content, decreased risk of microbial contamination (Kwon et al., 2013b) and even development of oral immune tolerance (Chirino et al., 2004; Ma et al., 2004). This approach facilitates long-term storage at preferred conditions, in addition to eliminating the need of protein purification and sterile injections or infusions. Moreover, expression of therapeutic proteins within plant cells has the added advantages of biological containment and product uniformity (Shaaltiel et al., 2007).

Gaucher’s disease (GD) is the most prevalent lysosomal storage disorder, caused by the deficiency of the lysosomal enzyme glucocerebrosidase (GCD) (Bennett and Mohan, 2013; Grabowski et al., 2014; Rosenbloom and Weinreb, 2013). β-Glucocerebrosidase (GCD) is a lysosomal glycoside hydrolase enzyme that catalyses the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide. The accumulation of excessive glucocerebrosides in lysosomal compartments of macrophages causes hepatosplenomegaly, anaemia and thrombocytopenia as well as bone pain and fractures. Enzyme replacement therapy (ERT) with GCD has been successfully used for the clinical treatment of GD for two decades. All three currently approved ERT drugs are administered by intravenous infusion (Zimran, 2011). Although all enzymes are safe and efficient, the intravenous administration remains a limitation, which affects patients’ quality of life. ERT of the plant-derived β-glucocerebrosidase for GD is already a well-established therapy (Aviezer et al., 2009). Therefore, an oral alternative delivery method may have a definite advantage without the limitation of the intravenous administration, in addition to providing a continuous enzyme supply.

One of the approved ERTs, taliglucerase alfa, is expressed in carrot cells (Shaaltiel et al., 2007). This enzyme (prGCD; plant recombinant glucocerebrosidase) is expressed in the ProCellEx system, has the optimal glycosylation profile, with exposed mannose, needed for efficient uptake (Tekoah et al., 2013). Oral administration of carrot cells as a natural vehicle of human recombinant prGCD is particularly well suited as it uses edible carrot plant cells and provides ‘ready to use’ enzyme which requires no modifications to obtain optimal glycosylation suited for receptor-mediated uptake into target cells. As the treatment of Gaucher’s disease is based on intravenous bolus injection every 2 weeks, the level of glucosylceramide, the substrate of glucocerebrosidase, drops rapidly to the basal level, and then accumulates back to the original level until the next injection is administered (Aviezer et al., 2009). The development of oral...
administration of prGCD would optimally allow a daily treatment that would probably keep the substrate at the normal level and enable a more compliable procedure for patients. This study is the first step to evaluating the feasibility of an ERT treatment based on a plant-derived β-glucocerebrosidase, using carrot cells as a vehicle for oral administration.

**Results**

**Stability in gastric simulated fluid (GSF)**

To assess the plant cell ability to protect expressed proteins from the acidic environment of the stomach, reconstituted lyophilized plant-expressed human glucocerebrosidase (prGCD) (Shaaltiel et al., 2007) expressing carrot cells were subjected to incubation in a simulated gastric fluid at different pH values for various times, and GCD stability was determined by immunoblot analysis. The results (Figure 1a) show that prGCD encapsulated in the plant cells survived for as long as 30 min at pH as low as 3. Furthermore, prGCD was fully degraded only after 10 min in pH 2 and after 1 min at pH 1.6. The purified prGCD was totally degraded at the low pH levels of 1.6 and 2. At pH 3, the protein survived only up to 1 min. To further verify that this protection is not limited to carrot cells or to GCD, which is naturally located in the lysosome, and is stable at low pH, we have measured the stability of an additional 45-kDa plant recombinant protein with a pI of 5.37 expressed in tobacco BY2 cells. As can be seen in Figure 1b (top), the previously demonstrated effect is not plant specific, as tobacco cells succeeded to prevent protein degradation under simulated gastric conditions at all the pH values tested, down to pH 1.6, while the same 45-kDa purified protein was unstable at most pH values (Figure 1b, bottom).

The plant cell resistance to the harsh gastric conditions is depicted in Figure 1c. Staining with fluorescein diacetate (FDA), a cell-permeant esterase substrate that serves as a viability probe, demonstrates intact cells, while addition of calcofluor white indicates the existence of the cell wall. After 30-min incubation in simulated gastric fluid, at pH 4, no harmful effect on cell viability or cell wall was observed. However, further experiments with GSF at pH 1.2 showed that the cells do not survive and are not intact in these conditions (data not shown), similar to what has been shown in Figure 1a.

**Influence of simulated intestinal fluids (SIFs) on prGCD release from plant cells**

To assess the release of prGCD from the plant cells and its availability to reach the blood circulation, the cells were pre-incubated in simulated gastric fluids at pH 4 for 30 min (stomach conditions) and transferred to simulated intestinal fluids, mimicking either fasting or feeding conditions (Patel et al., 2006). Results show (Figure 2a,b) that prGCD is released from the cells and can retain its activity for up to 120 min when incubated in simulated intestinal fluid mimicking fast conditions. Results also show that prGCD content was reduced, both in the cells and in the media, while incubated in fed conditions. A similar pattern was seen while measuring activity of the enzyme, showing that fast conditions are optimal for prGCD release and survival in simulated intestinal fluid.

**Transcytosis of prGCD through intestinal epithelial barrier**

Purified prGCD was analysed in a permeability assay using a Caco-2 cell platform. The purified protein was applied at the apical chamber of the Caco-2 monolayer in intestinal simulation
fluid, representing the more extreme fed conditions. Active prGCD was measured in the basolateral chamber, up to 8 h. The rising activity at the basolateral side indicates that prGCD can cross the epithelial barrier with a Papp of $1.39 \times 10^{-7}$ cm/s. At the same time, the transepithelial electrical resistance (TEER) of the cell monolayer measured at the beginning and end of the incubation remained constant, which indicates that the integrity of the cell monolayer was preserved. Results clearly showed that prGCD can cross the epithelial barrier and is absorbed in a dose-responsive (Figure 3a) and time-dependent (Figure 3b) manner.

Biodistribution of prGCD after oral administration of plant cells in gastrointestinal tract (GIT) of rats

To evaluate the possibility of utilizing plant cells as a vehicle for oral administration of therapeutic proteins, rats were fed by gavage with carrot cells expressing prGCD. Three animals were sacrificed at each time point postfeeding, and plasma and the content of different sections of the GIT were collected. Total stomach content was measured. Results show that content decreases within the first 5 h after feeding (Figure 4a). In addition, active prGCD, normalized to content weight in stomach, intestine and colon, was evaluated as a function of time. Active prGCD in circulation was measured per ml of plasma (Figure 4b). Data demonstrate that rats fed with prGCD containing carrot cells show a decrease of stomach content and a concomitant decrease in concentration of active prGCD. Hence, the concentration of active prGCD decreases also within 5 h. After the 5-h time period, postfeeding, the concentration of active prGCD increases in the small intestine (maximum at 6 h) together with the increase found in plasma. Shortly after that, 6–12 h postfeeding, a decrease in activity is found both in the small intestine and in the plasma, while no increase in activity was found in the colon. Active prGCD in the plasma was still found after at least 24 h following the consumption of the cells.

Pharmacokinetics and biodistribution in target organs of rats and pigs

The pharmacokinetic (PK) profile of prGCD and organ biodistribution were investigated in rat and pig models. In a separate study, to assess the pharmacokinetics of orally administrated prGCD, rats and pigs were free fed, twice, with either prGCD-expressing carrot cells, or with nonexpressing cells. Plasma samples were collected at various time points, up to 2 h post-second feeding, and tested for concentration of active prGCD (Figure 5a,b). Results show an increase of active prGCD in plasma of rats with a peak at 6 h postfeeding and a peak between 6 and 8 h in plasma of pigs. In addition, IV administration of prGCD to 5 healthy pigs, used as a control, resulted in an average maximum
Values represent the mean ± SD.

Figure 4  prGCD uptake in gastrointestinal tract and plasma of rats. (a) Weight of stomach after oral administration of cells expressing prGCD, at various time points. Concentration of active prGCD measured at various time periods in (b) stomach, small intestine and colon and (c) in plasma. Values represent the mean ± SD.

concentration in plasma of 500 ng/mL after half an hour, which decreased to basal level within 1 h (data not shown). At the termination of the experiments, rats and pigs were sacrificed and their livers and spleens were extracted and analysed for concentration of active prGCD. The activity was compared with control groups, fed with carrot cells, not expressing prGCD. The results (Figure 5c,d) show a significant elevation in concentration of active prGCD in both organs of rat and a significant increase of active prGCD in the liver of pigs. Activity of prGCD in spleen of pigs was not found to be different.

In an additional study, we compared between orally administered and IV injected prGCD in rats. The relative amount of active prGCD that reached liver, out of the initial prGCD delivered, was measured after 2 h from feeding or 1 h after injection. Recovery of prGCD in liver of rats following IV administration was found to be 0.3%, compared to 0.03% in the feeding experiments, while the recovery in spleen was found to be 0.6% and 0.06% in IV injected and fed rats, respectively.

Discussion

Plant-based expression systems offer many advantages over mammalian expression systems, including low cost of manufacturing, easy scale up and minimal risk of contamination with human pathogens (Fischer et al., 2004). Significant advances to the oral delivery of plant cell-encapsulated proteins have been made in the last decade. Daniell and others showed that oral delivery of plants that produce recombinant vaccines can be successful (Alvarez et al., 2006; Arlen et al., 2008; Boyhan and Daniell, 2011; Daniell et al., 2005, 2009; Kapusta et al., 1999; Kwon et al., 2013a,b; Muller et al., 2003; Sherman et al., 2014; Verma et al., 2010) in addition to the delivery of small enzymes that do not require post-translational modifications (Arakawa et al., 1998; Boyhan and Daniell, 2011; Kwon et al., 2013a; Ruhlman et al., 2007; Xie et al., 2008). The current study, however, is the first successful attempt to orally deliver a plant cell-encapsulated glycosylated enzyme used for ERT.

Unlike intravenously injectable drugs that are delivered directly into the relatively stable environment of the circulatory system, the fluid contents of the GIT, the functioning environment (acid-induced hydrolysis in the stomach and enzymatic degradation in the intestine) and the transport across the intestinal epithelium make oral delivery extremely challenging (Choonara et al., 2014; Lee, 2002). Orally delivered drugs have to survive in these different, hostile conditions to be available for absorption in the gut.

In this study, we have shown that plant cells have proven to be suitable for the task, probably as a result of the presence of the cell wall, one of the most important features of plant cells. In addition, lyophilization of plant cells can increase their stability, as this brings to lowering of water activity and eliminates the development of unwanted micro-organisms. They can provide excellent protection from pH 2 and above (Figure 1a,b) in simulated gastric fluids. Feeding leads to the change in pH of the stomach from pH 1.8 to around pH 4 (Dressman et al., 1998), which leads to further protection by the cells due to the feeding conditions. At the same time, purified proteins seem to be degraded even at higher pH conditions. Experiments with prGCD expressed in carrot cells or an additional 45-kDa protein expressed in BY2 cells show the same quality of protection, regardless of cell type or protein expressed. The visualization of the protected cells in the simulated gastric fluid environment, at pH 4 (Figure 1c), showing cell viability and cell wall integrity, further supports these findings, while lower pH exposure of 1.2 resulted in cell damage (data not shown).

The therapeutic protein ordeal in the GIT does not end in the stomach. The small intestine is awash with proteinases waiting to degrade any proteins that have survived the stomach. The ability of prGCD to cross the epithelial barrier was tested in a cellular assay using Caco-2 cell platform. The Caco-2 monolayer is widely used as an in vitro model of the human small intestinal mucosa to predict the absorption of orally administered drugs. The correlation between the in vitro permeability across Caco-2 monolayers and the in vivo fraction absorbed is well established (Artursson and Karlsson, 1991). The type and amount of food entering from the stomach affect enzyme secretion from the pancreas, changing the conditions in the intestine (Brannon, 1990; Jahan-Mihan...
et al., 2011). Thus, it is interesting to note the opposite food effect on the successful survival of the recombinant proteins in our study. In the stomach, the pH is elevated in response to feeding, an elevation that reduces the degradation of the recombinant protein (Figure 1a,b). However, in the intestinal environment, pancreatic enzymes and bile salts are secreted in response to feeding. These enzymes and salts seem to help in releasing recombinant protein from the cells, as measured for their abundance by immunoassay and activity. But, at the same time, the GSF, after feeding, also enhances the protein degradation (Figure 2a,b). Therefore, it seems that fasting conditions would be more favourable. Considering the combined effects in both organs, stomach and intestine, we proposed that consumption of prGCD-expressing cells after fasting or, maybe after a light meal, can lead to the maximal amount of recombinant enzyme reaching the circulation. In addition, consumption of cells containing prGCD together with an appropriate formulation can further facilitate the protection of the cells in the stomach, before the release of the enzyme in the intestine.

GI epithelial cell membranes strictly limit the penetration of peptides and proteins (Morishita and Peppas, 2006) as the transport of drugs across the intestinal epithelial cell barrier is a major determinant of drug bioavailability. The possible mechanisms by which proteins and peptides are absorbed from the GIT include passive transport, active transport and endocytosis (Choonara et al., 2014). Several approaches have been attempted to facilitate crossing the intestinal epithelial barrier (Carino and Mathiowitz, 1999; Devriendt et al., 2012; Müller, 2010; Ziv et al., 1987), and the most commonly used approach uses encapsulation of the protein drug in liposomes, microparticles, or nanoparticles. In this study, the plant cell proved to be an appropriate vehicle to deliver prGCD to the intestine (Figure 2a,b). The protection supplied by the plant cell to the recombinant prGCD from the acidic environment of the stomach (Figure 1) and the subsequent prGCD release from the cells in the intestine (Figure 2) enable absorption into the circulation and uptake to the relevant organs. As can be seen both in in vitro (Figure 3) and in vivo experiments (Figure 4), once reaching theintestinal barrier, prGCD can cross this barrier (Figure 3) and can be found in the circulatory system. The properties of prGCD, being a relatively lipophilic, lysosomal protein with mannose-terminated glycans, may facilitate the protection in stomach. These characteristics may even facilitate the protein crossing of the intestinal epithelial layer. But, an overall study dedicated to finding the mechanism of uptake is needed to fully understand this. Furthermore, the addition of microbial enzymes to the intestinal simulated fluid may further facilitate the release and uptake of prGCD from the plant cells in the GI tract.

Once in the blood system, the efficient uptake of the prGCD into the target organs is similar to that of the intravenously administered prGCD, taliglucerase alfa. The uptake is mediated

Figure 5  Biodistribution of orally administered prGCD in blood and organs of rats and pigs. Relative concentration of active prGCD measured in plasma of (a) rats and (c) pigs. Activity of prGCD in (b) rat liver and spleen, and (d) pig liver. Organ prGCD values represent the mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
by the mannosine receptors that bind the exposed mannosine residues on prGCD glycans (Tekoah et al., 2013). In the case of prGCD, the plant cells offer a unique advantage over other production platforms, in that they enable the production of recombinant proteins with mannosine-terminated glycans, providing efficient uptake into target organs without further manipulation (Shaaltiel et al., 2007). Although intravenously administered prGCD in pigs shows a prGCD peak in plasma after half an hour and human experiments report a mean half-life (t 1/2) of approximately 15 min (range: 8–32 min) (Aviezer et al., 2009), oral administration using carrot cells leads to an extended release of the drug, as was measured in the plasma (Figures 4 and 5). This extended release can reach between 6 and 8 h. In this study, the amount of prGCD in plasma and organs was found to be low, compared to the initial available dose. In addition, uptake to liver and spleen in orally administered prGCD in rats was found to be 10-fold lower than the parallel IV administered rats. Although optimization is still needed, it is obvious that the change in pharmacokinetics together with the option of daily delivery of prGCD may drastically change patient compliance to treatment and set a slow release of prGCD to circulation.

Furthermore, studies using rats and pigs fed with carrot cells expressing prGCD showed the biodistribution of prGCD in the digestive tract, leading to elevated levels of active prGCD in both plasma (Figure 5a,b) and target organs (liver and spleen) (Figure 5c,d). Although a high increase of prGCD was measured in rat organs (Figure 5b), the increase of prGCD in pig liver was less dramatic (Figure 5d). We propose that the limited increase in active prGCD found in pig organs may be due to lower initial doses of prGCD and/or to high levels of endogenic prGCD found in pigs, leading to high background levels. In recovery experiments, prGCD was found to be 10-fold higher after IV compared to oral administration. Such a difference can be expected due to the challenge of oral administration.

A preliminary study of 6 months using minipigs did not show any major adverse symptoms up to date, indicating that prGCD can be safe and well tolerated by the animals and caused no major adverse symptoms.

Search of the clinical trials database revealed that only two trials were conducted on orally delivered therapeutic proteins: prGCD that is described here (ClinicalTrials.gov Identifier: NCT01973920), but no data are yet available to evaluate these experiments. This demonstrates that the transfer of these encapsulation methods into clinical development is a major hurdle.

The majority of currently diagnosed patients with Gaucher’s disease are treated with bi-weekly intravenous infusions of enzyme replacement therapy. Having a safe and effective orally available enzyme agent would dramatically improve patients’ quality of life without compromising on the benefits of enzyme therapy. Current clinical studies will further help in evaluating this potential.

**Experimental procedures**

**Materials**

Talgulcerase alfa (prGCD) produced in carrot cells and an additional 45-kDa protein produced in BY2 cells were obtained from in-house production, from Protalix Biotherapeutic (Carmiel, Israel). All other reagents were purchased from Sigma-Aldrich, unless otherwise indicated.

**Concentration of active enzyme after feeding**

Concentration of active prGCD was measured by the hydrolysis of 4MU-G (4-methylumbelliferyl β-D-glucopyranoside), following the method by Urban and colleagues (Urban et al., 2008). Briefly, the reaction was initiated by the addition of 10 μL of purified enzyme, cell extract or medium, containing prGCD to 50 μL activity buffer (50 mM citrate, 176 mM potassium phosphate, 10 mM taurocholic acid, 0.01% Tween-20, pH 5.5) (Urban et al., 2008). The reactions were incubated for 45 min at 37 °C. After incubation, 10 μL aliquots from the reaction mixture were added to 90 μL of stop solution (1 mM NaOH, 1 mM glycine, pH 10) and the fluorescence of the product methylumbelliferone was measured at excitation/emission of 370/440 nm (TECAN Infinite M200, Austria). Standard curve with known concentrations of standard prGCD (50-3.125 ng/mL) was used to translate the obtained absorbance data to active enzyme concentrations.

**Western blot analysis**

Preparation of cell lysates and protein blotting were carried out as described previously (Shaaltiel et al., 2007). prGCD and 45-kDa protein were detected by specific antibodies produced at Gene-Script (Piscataway, NJ) against these proteins.

**Stability assessment of prGCD bio-encapsulated in carrot cells in simulated gastric and intestinal fluids**

Carrot cells expressing prGCD were lyophilized by freezing to −40 °C and gradually heating until 20 °C. Cells and prGCD content were stable up to 6 months at room temperature. For the analysis of the protective effect of cell wall on prGCD, lyophilized cells were incubated in simulated gastric fluid (SGF) (70 mM sodium chloride, 50 mM potassium chloride, 2.2 mM D-glucose, 0.14 mM pepsin, 1.1 mM lactic acid, 1.5 mM sodium thiocyanate and 0.14 mM catechin) with an extensive shaking in pH ranging from 1.6 to 6 for 1, 10 and 30 min at 37 °C. At each time point, reaction was stopped by adding Na2CO3. Results were compared to purified prGCD which was exposed to the same conditions.

In addition, cells incubated in pH 4 after 30 min in SGF were separated from the media by vacuum and the pellet was resuspended in either fasted or fed simulated intestinal fluid (SIF). Fasted SIF: 49 mM monobasic potassium phosphate, 15.4 mM sodium hydroxide, 0.2 mM KCI, 3 mM sodium taurocholate, 1.5 mM lecithin, 70 mg pancreatin, at pH 6.5; fed SIF: 49 mM monobasic potassium phosphate, 15.4 mM sodium hydroxide, 0.2 mM KCl, 15 mM sodium taurocholate, 1.5 mM lecithin, 1 g pancreatin, at pH 6 (Patel et al., 2006). Samples were incubated at either condition at predetermined time points (30 min, 60 min and 120 min) at 37 °C. Following the second incubation, all samples were centrifuged. For the measurement of content and enzymatic activity in cells, pellet was resuspended in extraction buffer (20 mM phosphate buffer pH 7.2, 20 mM EDTA, 20 mM l-ascorbic acid, 1% Triton X-100). Cell lysate was analysed for protein content by Western blot and enzymatic activity with 4MU-G. Total prGCD content and activity in media were also measured.

**Cell viability staining with Fluorescein diacetate**

Cell suspension (0.5 mL) was stained with 10 μL of 0.5% fluorescein diacetate (FDA) dissolved in acetone for 10 min. The cells were rinsed three times with 1 mL isotonic medium to remove excess of the staining solution. Viability was assessed immediately using an Olympus BX50 fluorescence microscope equipped with a
suitable barrier filter (excitation 460–490 nm and emission 510 nm). FDA staining of viable cells results in bright green fluorescence, while nonviable cells remain colourless.

**Cell wall staining by calcofluor white**

Cell suspension (0.5 mL) was stained for 5 min with 0.5 mL staining solution containing 0.1% calcofluor white dissolved in 0.4% m mannitol. The cells were rinsed three times with 1 mL medium to remove excess of the staining solution. The blue staining of the cell walls was observed using an Olympus BX50 fluorescence microscope equipped with a UV filter with excitation at 350 nm and emission at 420 nm.

**Cell culture and transcytosis**

Human intestinal Caco-2 cells purchased from the American Type Culture Collection were grown as described (Fossati et al., 2008). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Life technologies, Grand Island, NY) supplemented with 1% nonessential amino acids (Biological Industries, Beit Haemek, Israel) and 5% foetal bovine serum (Biological Industries, Kibbutz Lahav, Israel), were handled according to the US NIH guidelines.

Male Wistar rats 6–8 weeks old (Pharmaseed Laboratories, Revohov, Israel) and female Lancer White Swine (Sus scrofa domestica), about 1 month old (Lahav Research Institute (LRI), Kibbutz Lahav, Israel), were handled according to the US NIH (National Institutes of Health) and the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines.

**Timeline of carrot cells passing through the GI tract**

Rats (n = 3 per group, seven time points) were gavage fed with carrot cells expressing prGCD (3 mg active prGCD/kg). Each group was sacrificed at predetermined time points (1, 2, 4, 6, 8, 12 and 24 h). Plasma samples were collected and analysed for enzymatic activity, by the 4MU-G assay. The contents of stomach, small intestine and colon were collected and weighed. prGCD from the gastrointestinal contents was extracted and analysed for enzymatic activity, as described above.

**Biodistribution in Rats and Pigs**

Rats (n = 21) or pigs (n = 3) were allowed a free access to lyophilized carrot cells containing prGCD for a duration of 1 h (two exposures with a 6-h interval for rats and one exposure for pigs). Rats were orally administered with a total dose of 12 mg of prGCD/kg, while pigs were orally administered at a dose of 5 mg prGCD/Kg. Single infusion of purified prGCD was administered intravenously to pigs by a single bolus injections of 2.6 mg/kg. This dose is comparable to the currently used intravenous doses in humans. Whole blood was taken before feeding and at various time points as indicated up to 12 or 9 h post-first feeding of rats and pigs, respectively. Plasma samples were evaluated for concentration of active prGCD using 4MU-G.

In the organ biodistribution study, rats were administered at a dose of 16 mg/kg, and pigs were administered at a dose of 2 mg/kg. Rat organs (n = 12, 6 for feeding and 6 control) were processed partially by the Office of the Chief Scientist of the Ministry of Industry, Trade and Labor, Israel. This study was supported by Protalix Biotherapeutics and funded partially by the Office of the Chief Scientist of the Ministry of Industry, Trade and Labor, Israel.

**Conflict of interest**

All of the authors were employees of Protalix Biotherapeutics Inc., the sponsor of this study and as such have vested commercial interests.

**References**


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