Rescue of functional delF508-CFTR channels in cystic fibrosis epithelial cells by the α-glucosidase inhibitor miglustat

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Abstract In the disease cystic fibrosis (CF), the most common mutation delF508 results in endoplasmic reticulum retention of misfolded CF gene proteins (CFTR). We show that the α-1,2-glucosidase inhibitor miglustat (*N*-butyldeoxynojirimycin, NB-DNJ) prevents delF508-CFTR/calnexin interaction and restores cAMP-activated chloride current in epithelial CF cells. Moreover, miglustat rescues a mature and functional delF508-CFTR in the intestinal crypts of ileal mucosa from delF508 mice. Since miglustat is an orally active orphan drug (Zavesca®) prescribed for the treatment of Gaucher disease, our findings provide the basis for future clinical evaluation of miglustat in CF patients.
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1. Introduction

The *CFTR* gene encodes the cystic fibrosis transmembrane conductance regulator (CFTR) that is mutated in the genetic disorder cystic fibrosis [1,2]. CFTR is an apical cAMP-activated Cl^- channel present in epithelial cells [3,4]. Most CF patients have the delF508 mutation leading to misfolding and retardation of the protein in the endoplasmic reticulum (ER) [4–6]. The ER quality control machinery monitors the folding and assembly of proteins, ensuring that only folded proteins proceed along the secretory pathway [7]. *N*-glycosylation allows the interaction of newly synthesized glycoproteins with the chaperone system in the ER [5,8,9]. The chaperone calnexin is a ER lectin-like protein binding monoglucosylated oligosaccharides, i.e., *N*-linked glycans of the form Glc-NAc2Man9Glc1 resulting from the removal of the two outer glucoses by glucosidases [8–10]. Recent evidences suggest that inhibitors of ER calcium pumps correct the delF508 trafficking defect through partial inhibition of delF508-CFTR/calnexin interaction [11,12]. Moreover, overexpression of calnexin leads to greater stability of delF508 in the ER [13] and a truncated form of calnexin partially reversed the misprocessing of functional delF508-CFTR [14].

In the present study, we hypothesized that by inhibiting the deglucosylation of delF508 protein in the ER, glucosidase inhibitors might prevent the interaction of delF508-CFTR with calnexin and hence its entry in the degradation pathway. To test this hypothesis, we used miglustat an *N*-alkylated imino sugar (*N*-butyldeoxynojirimycin, NB-DNJ) and castanospermine, inhibiting ER α-1,2-glucosidase, and one inactive imino-sugar analogue (*N*-butyldeoxygalactonojirimycin, NB-DGJ) [15,16].

2. Materials and methods

2.1. Cells

Human nasal epithelial JME/CF15 [17], tracheal gland serous CFKM4 [18] and pancreatic duct CFPAC-1 [19] cell lines were derived from delF508 homozygous patients. Non-transfected CHO-K1, stably transfected wt- and delF508-CHO cells and Cos-7 cells transiently transfected with GFP-delF508-CFTR cDNA or GFP alone (denoted Cos-7 mock) were used as described [20,21].

2.2. Immunoprecipitation and Western-blotting

CF15 cell lysates were incubated with monoclonal anti-human CFTR antibody (2 μg, IgG_{2a} clone 24-1, R&D Systems, USA) or polyclonal rabbit anti-calnexin antibody (2 μl/ml, SPA-860, Stressgen, USA). Immunoblots were probed with monoclonal mouse anti-CFTR antibody (10 μg/ml, IgG_1 M3A7, Chemicon, USA), polyclonal rabbit anti-calnexin antibody (2 μl/ml, SPA-860) or mouse anti-β tubulin antibody (1:200, Tebu-bio, USA). The protein levels were expressed as densitometry and percentage of controls. Other details [11].

2.3. Functional assay

Perforated whole-cell patch-clamp analysis was applied to CF15 cells. Patch electrodes (GC150-TF10, Harvard Apparatus, USA) filled with intracellular solution (resistances of 3–4 MΩ) were connected to the RK-400 amplifier (Biologic, France) through an Ag/AgCl pellet. External solution (mM): 145 NaCl, 4 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 D-glucose, 10 TES (pH 7.4, 315 mOsm). Intra-pipette solution (mM): 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 EGTA, 1 $MgCl_2$, 3 Mg-ATP (*ex-temporane*), 10 TES (pH 7.2 with CsOH, 285 mOsm) and amphotericin B (100 μg/ml) renewed every 2 h. Only cells with input resistance ⩽15 MΩ were analysed. The mean access resistance and whole cell capacitance were 12 ± 0.6 MΩ and 35 ± 4.3 pF ($n = 44$). Currents were obtained in response to voltage steps from −80 to +80 mV in 20 mV increment. Data were collected using pClamp 6.0.3 package software (Axon Instruments, USA).

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CFTR Cl⁻ channel activity was assayed on a cell population by the iodide (^{125}I) efflux technique as described [11]. All chemicals are from Sigma Chemicals (St. Louis, MO) exept NB-DNJ and NB-DGJ (Toronto Research Chemicals, Canada), forskolin and genistein (PKC Pharmaceuticals, USA). CFTR$_{inh}$–172 and TS-TM calix[4]arene were provided by Dr. Bridges, University of Pittsburgh. Results are expressed as mean ± S.E.M. of n observations. Sets of data were compared with Student's t test using GraphPad Prism 4.0 (GraphPad Software, USA). ns, non-significant difference; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

2.4. Ex vivo studies

Rotterdam delF508/delF508-CFTR mice (*Cftr$^{tm1\ Eur}$*), their littermate controls (FVB inbred, 14–17 weeks old, weight between 20 and 30 g, kept on solid food in a pathogen-free environment) and Cftr-KO mice (*Cftr$^{tm2\ Cam}$*) were used [22,23]. Muscle-stripped ileal mucosa was incubated in William's E-Glutamax medium supplemented with

insulin (10 μg/ml) and dexamethasone (20 μg/ml). At different time points, the compound was removed by repeated washings followed by short-circuit current (Isc) measurements in mini-Ussing chambers [24]. Western blotting was as described [25]. For immunohistochemistry, tissues were fixed in 4% (wt/vol) paraformaldehyde. Sections (5 μm) were stained with the antibody R3195 (1:500) as described [25].

3. Results

3.1. Miglustat prevents delF508-CFTR/calnexin interaction in CF15 cells

Analysis of CFTR immunoprecipitation on calnexin Western blot showed that miglustat and castanospermine prevent, by ≈75% and ≈50%, respectively, the delF508-CFTR/calnexin interaction as compared to cells at 37 °C, treated by NB-DGJ
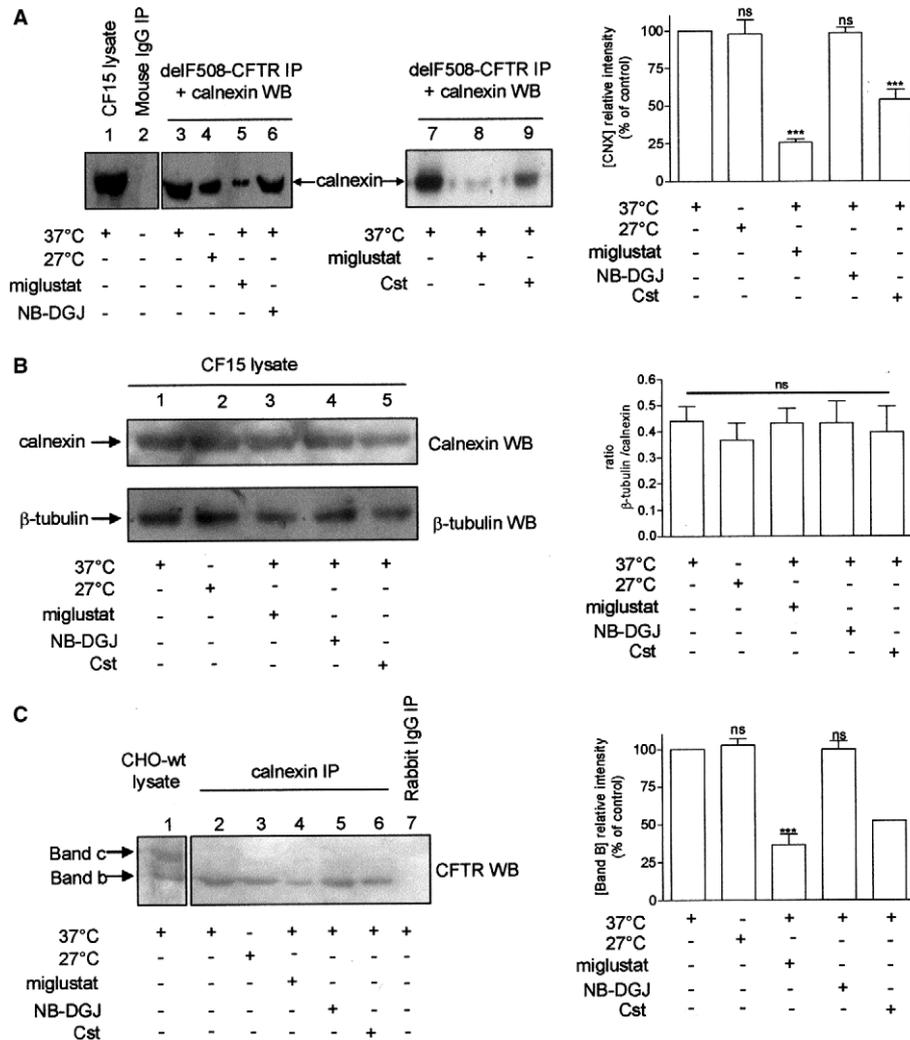

Fig. 1. Miglustat prevents delF508-CFTR/calnexin interaction. (A) CFTR immunoprecipitation (IP) on calnexin Western-blot (WB) in treated CF15 cells or not. Calnexin WB (lane 1) and non-immune mouse IgG (lane 2) were used as positive and negative controls. Two separate experiments are presented. From lanes 3 to 6 we compared IP CFTR/WB calnexin from cells incubated 2 h at 37 °C with miglustat (lane 5), with NB-DGJ (lane 6) or incubated at 27 °C (lane 4) to the control 37 °C (lane 3). In the second experiment, lanes 7–9, we compared miglustat (lane 8) and castanospermine denoted Cst (lane 9) to the control 37 °C (lane 7). The calnexin intensity band for each experimental condition was expressed as function of its own IP control (i.e., 37 °C: lane 3 for the first gel and lane 7 for the second gel). Then the histograms report the mean ± S.E.M. of these data; $n = 5$ for each treatment except for castanospermine $n = 2$. (B) Calnexin and β-tubulin WB from untreated (lane 1) and treated cell (lanes 2–5) lysates (50 μg of total protein). Histograms show the β-tubulin/calnexin ratio density band ($n = 3$). (C) Calnexin IP on CFTR WB in cells treated (lanes 3–6) or not (lane 2). CFTR WB in CHO-wt lysates (lane 1) and non-immune rabbit IgG IP (lane 7) were used as positive and negative controls. Bar graph reports the CFTR band B intensity expressed in % of control ($1 < n < 2$). Treatments are indicated below each lane or bar with a positive sign. Treatment: 37 °C (control), low-temperature (24 h, 27 °C), miglustat (2 h, 100 μM, 37 °C), castanospermine denoted Cst (2 h, 100 μg/ml, 37 °C) or NB-DGJ (2 h, 100 μM, 37 °C).

or maintained at 27 °C, a procedure known to rescue delF508-CFTR to the cell surface [26] (see Fig. 1A). Glucosidase inhibitors have no direct effect on the production of calnexin itself because the β-tubulin/calnexin ratio remains unchanged (see Fig. 1B). Finally, the reverse co-immunoprecipitation (see Fig. 1C) confirms that delF508-CFTR and calnexin form a complex in the ER prevented by miglustat and to a lesser extent by castanospermine.

3.2. Rescue of functional delF508-CFTR by miglustat

Perforated patch-clamp technique was applied to 37 °C (see Fig. 2A), low-temperature (see Fig. 2B), miglustat (see Fig. 2C) or NB-DGJ-treated (see Fig. 2D) CF15 cells. Cl^- currents were first recorded in resting cells (see Fig. 2A–D, top traces) and after stimulation by forskolin/genistein [11] (see Fig. 2A–

D, middle traces). A linear non-voltage-dependent Cl^- current was recorded only for cells cultured at 27 °C or incubated with miglustat (see Fig. 2B and C, middle traces). This current was fully inhibited by glibenclamide (see Fig. 2B and C, bottom traces). Untreated or NB-DGJ treated cells failed to respond to forskolin/genistein (see Fig. 2A and D, middle traces). The current densities measured at +40 mV with cells stimulated were 0.9 ± 0.19 pA/pF (see Fig. 2A), 28.75 ± 3.2 pA/pF (see Fig. 2B), 14.3 ± 1.05 pA/pF (see Fig. 2C) and 1.06 ± 0.03 pA/pF (see Fig. 2D).

Iodide efflux experiments performed in untreated or NB-DGJ treated CF15 cells confirm absence of response after forskolin/genistein stimulation (see Fig. 2E, top panel). On the contrary, the bottom panel demonstrates stimulation of iodide efflux after low-temperature, miglustat or castanospermine
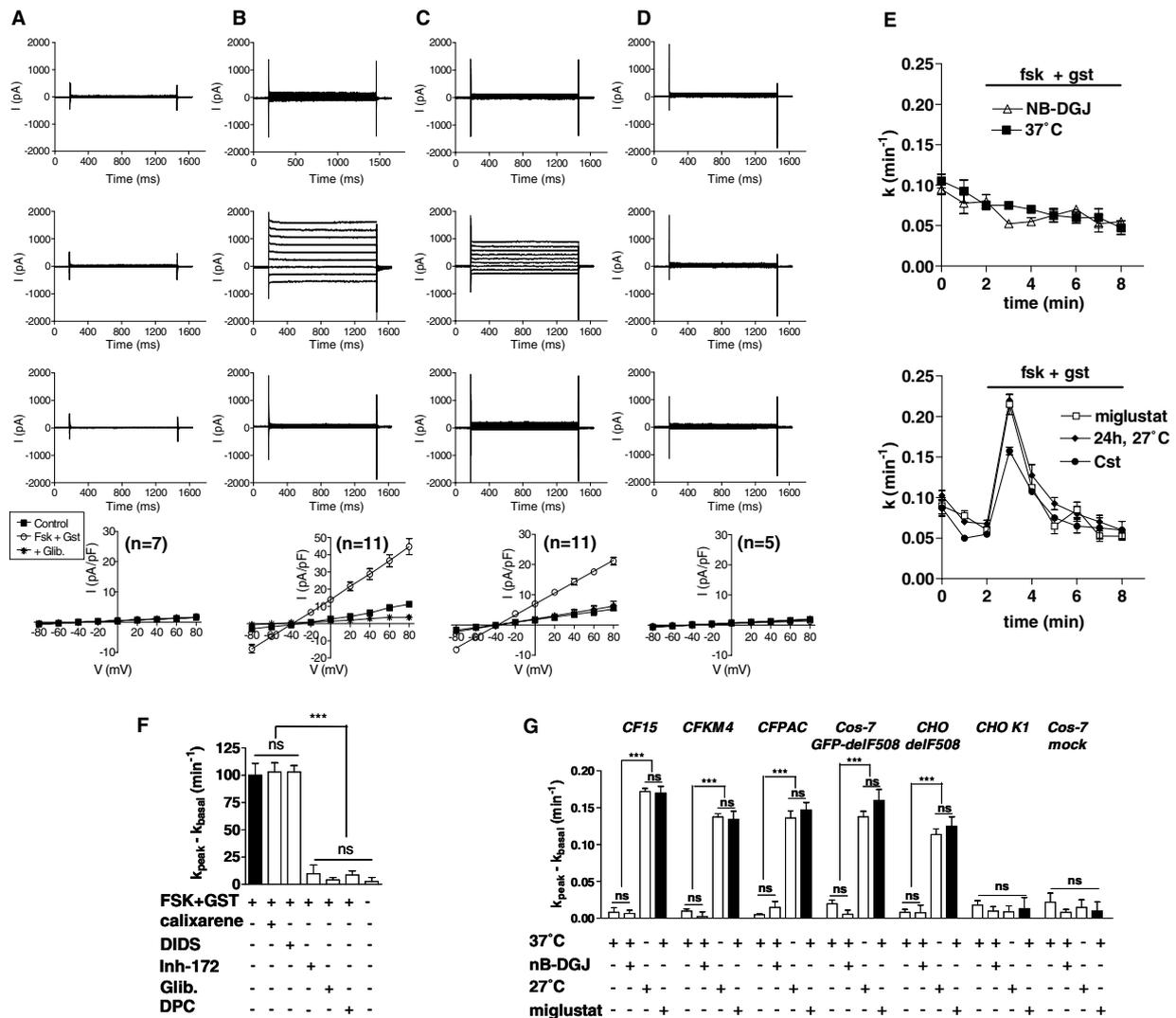

Fig. 2. Rescue of functional delF508-CFTR in miglustat-treated cells. (A)–(D) Perforated whole-cell patch-clamp recordings with CF15 cells treated by low-temperature (B), miglustat (C), NB-DGJ (D) or untreated (A). Top traces: cells are unstimulated. Middle traces: stimulation by forskolin/genistein. Bottom traces: stimulation by forskolin/genistein + glibenclamide. The corresponding current density/voltage relationships are given below each series of experiments. Numbers of analyzed cells are indicated. The bath contained calixarene (200 nM) and DIDS (200 μM). (E)–(F) Iodide efflux curves in 37 °C, low-temperature-, miglustat-, castanospermine- or NB-DGJ-treated CF15 cells. Iodide effluxes are stimulated by forskolin/genistein as indicated by the horizontal bar above the traces. Stimulation of miglustat-treated CF15 cells in presence of 10 μM CFTR$_{inh}$-172, 100 μM glibenclamide, 500 μM DPC, 500 μM DIDS or 100 nM calixarene (F). $n = 4$ for each condition. (G) Iodide efflux experiments with CF15, CFKM4, CFPAC-1, Cos7 GFP-delF508, Cos 7 mock, CHO-K1 and CHO delF508 cells treated as indicated below each bar with a positive sign ($n = 4$). Functional experiments with 37 °C-, low-temperature- (24 h, 27 °C), miglustat- (2 h, 100 μM, 37 °C), castanospermine denoted Cst (2 h, 100 μg/ml, 37 °C) or NB-DGJ- (2 h, 100 μM, 37 °C) treated CF cells. Stimulation of CFTR: forskolin (10 μM), genistein (30 μM).
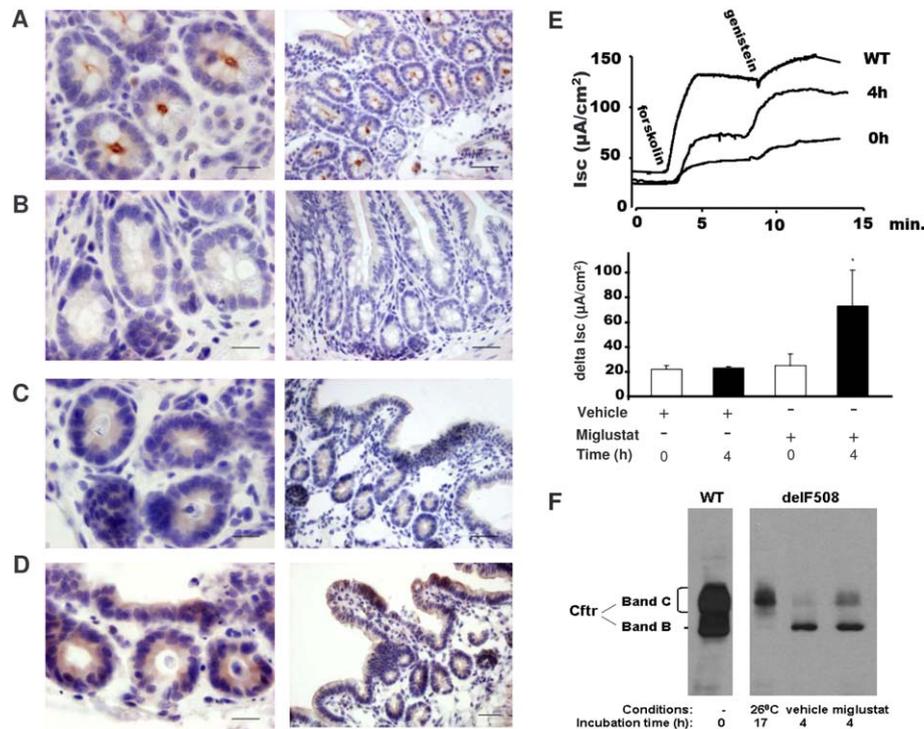
Fig. 3. Cellular localization, maturation and functionality of delF508-CFTR protein in ileal mucosa from delF508 mice. (A)–(D) Ex vivo exposure of ileal mucosa from delF508 mice to miglustat results in the appearance of CFTR protein (brown immunostaining) in the intestinal crypts. (A) WT mice, untreated; (B) Cftr$^{-/-}$ mice, untreated; (C) Cftr$^{delF508/delF508}$ mice, PBS treated (4 h, 37 °C); (D) Cftr$^{delF508/delF508}$ mice, miglustat treated (100 μM, 4 h, 37 °C). Scale bars: 12 μm (left), 30 μm (right). (E) Rescue of forskolin/genistein-activated Cl^{-} current response (Isc) in ileal mucosa from delF508-CFTR mice by miglustat (4 h, 100 μM). (top) Isc tracings showing the effect of miglustat pretreatment on the response to forskolin (10 μM) and genistein (100 μM) of delF508-CFTR vs. WT mice. (bottom) summary data from duplicate measurements on 8 delF508-CFTR mice; delta Isc represents the combined response to forskolin + genistein; *$P < 0.05$, Student t-test. (F) Detection of core-glycosylated (band B) and mature (band C) CFTR by western blotting in wt- and low-temperature- or miglustat-treated delF508 CFTR ileum.

treatments. Note the lower efficacy of castanospermine vs. miglustat. Stimulation of miglustat-treated cells was inhibited by DPC, glibenclamide and CFTR$_{inh}$-172 [27] but neither by calixarene nor DIDS (Fig. 2F) as expected for CFTR [11]. The response to forskolin/genistein after treatment with miglustat or NB-DGJ was analyzed in different delF508-CFTR expressing cell types. In all these cell types, a delF508-CFTR dependent iodide efflux was rescued by miglustat to the same level as cells cultured at 27 °C (see Fig. 2G). Fig. 2G also shows that miglustat has no effect in CFTR-minus cells (e.g., parental CHO-K1 and mock Cos-7).

3.3. Correction of abnormal processing and function of delF508-CFTR in Cftr$^{delF508/delF508}$ mice

We studied the effect of miglustat in ileal tissues from Cftr$^{+/+}$, Cftr$^{-/-}$ and Cftr$^{delF508/delF508}$ mice. Immunostaining study localized CFTR in apical membrane of intestinal crypt cells of Cftr$^{+/+}$ mice (see Fig. 3A). CFTR is absent in preparations from Cftr$^{-/-}$ mice (see Fig. 3B). On contrary of ileal tissues from Cftr$^{delF508/delF508}$ mice maintained in PBS (see Fig. 3C), exposure to miglustat shows that delF508 proteins have clearly moved to the apical membrane (see Fig. 3D). However, the immunolocalization signal in intestinal crypt cells differs from Cftr$^{+/+}$; the rescued protein is also localized throughout the cell interior (see Fig. 3D). Ex vivo exposure of intestinal mucosa from Cftr$^{delF508/delF508}$ mice to miglustat resulted in a threefold increase in forskolin/genistein-stimulated current (see Fig. 3E), indicating restoration

of transepithelial chloride secretion up to ≈55% of the secretory response in Cftr$^{+/+}$-mice (140 ± 26 μA/cm^2, n = 28). This gain in CFTR function is in good agreement with the delF508-CFTR localization and was accompanied by an increase (∼1.8-fold) in the band C/band B ratio as detected by western blotting corresponding to ≈12% mature WT cftr (Fig. 3F). Functional rescue of delF508 CFTR up to WT levels by low-temperature incubation [28] is accompanied by a gain in mature band C CFTR protein expression from ∼4% to ∼25% of WT.

4. Discussion

Our data show a relatively fast rescue of functional delF508-CFTR by miglustat in human and mice epithelial cells. Miglustat prevents the delF508-CFTR/calnexin interaction in the ER suggesting that inhibition of deglucosylation of nascent proteins may be the molecular mechanism of the miglustat effect. Importantly, inhibition of N-linked oligosaccharide trimming by the glucosidase inhibitors 1-deoxynojirimycin and castanospermine did not interfere with surface expression of the vesicular stomatitis virus G [29] or influenza virus hemagglutinin [30] proteins, suggesting that these molecules, like delF508-CFTR are transported to the cell surface despite the presence of glucosidase inhibitors.

Loss of function and/or accumulation of mutant proteins in the ER leads to the development of numerous protein-misfolding diseases [31]. Because the molecular mechanism of

retention of the mutant proteins associated with these diseases may have (at least in part) in common the recognition of mutant proteins by calnexin and retention in the ER, glucosidase inhibitors might be valuable candidates for pharmacological prevention of protein-misfolding diseases [31–34].

Miglustat is an orally bioavailable orphan drug approved in Europe and USA for use in patients with type I Gaucher disease (Zavesca®) [15]. Human studies show that miglustat is well tolerated at 100 or 300 mg once or three times daily [15]. Imino sugar therapy has also been proposed for other diseases [16], like Fabry disease [35].

Partial correction of CFTR channel activity may have significant clinical impact. For example, with the G480C trafficking mutant ≈8% of mature CFTR protein is associated to ≈40% residual cAMP-stimulated chloride secretion in mice intestine [25]. We found that treatment with miglustat restored in CF mice ≈12% mature CFTR and ≈55% of WT chloride secretion. This is in reasonable good agreement with the estimation that 100% chloride secretion corresponds with 20% mature CFTR [28].

In conclusion, we found that miglustat rescues partially the abnormal processing of functional delF508-CFTR in human epithelial airway, tracheal gland serous and pancreatic duct cells as well as in intestinal cells of delF508 mice. The mechanism of action involves, at least in part, the prevention of delF508/calnexin interaction in the ER. Because miglustat is a medicament prescribed in another orphan disease, it holds a great promise not only for CF therapy but also for an increasing number of protein-misfolding diseases.

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