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Short Communication

Neoglycoconjugates Derived from Deoxynojirimycin as Possible Therapeutic Agents for Cystic Fibrosis Lung Disease, by Modulation of the Sphingolipid Metabolism

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Abstract

The identification and development of novel and more efficient anti-inflammatory drugs for management of Cystic Fibrosis (CF) airway disease remains a compelling need. Sphingolipids (SLs) play an important regulatory role in CF due to their function in pulmonary infections and inflammation. Given the emerging importance of SLs in much pathology, novel drugs are continuously developed to selectively target different enzymes involved in SL metabolism. Iminosugars disclose offer exciting and innovative opportunities for therapeutic agent discovery. Miglustat, the most popular iminosugar, has an anti-inflammatory effect in CF models through inhibiting non-lysosomal-β-glucosidase 2 (GBA2). A small library of neoglycoconjugates with an adamantane mojety (AMP-DNJ), characterized by differences in the length of the alkyl chain between the iminosugar and AMP, has been synthesized from the lead iminosugar deoxynojirimycin (DNJ) (Ardes-Guisot, 2011). This study was hence aimed to test the effect of these AMP-DNJ derivatives on the inflammatory response to *P. aeruginosa* in CF bronchial epithelial cells. Our original findings demonstrate that these AMP-DNJ derivatives reduce IL-8 mRNA expression in CF bronchial cells infected by *P. aeruginosa* at nanomolar concentrations. The selectivity towards β glucosidase seems to be modulated by variation of the length of the chain linking the iminosugar and AMP, which are key motifs for the therapeutic activity of these compounds. Our results further support the use of SL metabolism modulators for treating CF lung inflammation, thus providing useful hints on relevant targets and chemical structures that may be regarded as starting points for a drug discovery campaign.

ABBREVIATIONS

CF: Cystic Fibrosis; NSAIDs: Non-steroidal anti-inflammatory drugs; SLs: Sphingolipids; NB-DNJ: *N*-butyldeoxynojirimycin; GSL: Glycosphingolipid; GBA2: Non-lysosomal β-glucosidase 2; GlcCer: Glucosylceramide; AMP: Adamantane; CBE: Conduritol-B-epoxide

INTRODUCTION

Cystic Fibrosis (CF) lung disease is characterised by progressive chronic infection and inflammation of the airways, then leading to irreversible lung damage and fibrosis, which represent the major cause of mortality in patients with this condition [1]. Given the role of the inflammatory process as a driver of irreversible lung destruction, increasing attention is focused on anti-inflammatory therapies, with the aim to ameliorate CF lung pathology. High doses of non-steroidal anti-inflammatory drugs (NSAIDs), particularly ibuprofen, may efficiently counteract inflammation. However, the long-term effects due to prolonged use of high doses NSAIDs have yet to be determined [2]. Therefore, the identification and development of novel and more potent anti-inflammatory drugs for CF airway

disease remains a compelling need.

An increasing number of studies suggest that sphingolipids (SLs) play an important regulatory role in CF due to their favourable activity in pulmonary infections and inflammation, thus supporting the use of modulators of SL metabolism as therapeutic agents for CF lung disease [3-9]. Given the emerging importance of SLs in respiratory disorders, novel drugs are being developed to selectively target different enzymes involved in SL metabolism. Iminosugars (i.e., sugar mimics in which the endocyclic oxygen has been replaced by nitrogen) disclose exciting and innovative opportunities for therapeutic agent discovery, due to their favourable oral bioavailability and highly specific immune modulatory and chaperoning activity [10]. Among the most well known iminosugars, miglustat (*N*-butyldeoxynojirimycin, NB-DNJ) is a US Food and Drug Administration (FDA)-approved and European Medicines Agency (EMA)-designated orally bioavailable orphan drug, used in Europe and USA for the treatment of type I Gaucher's disease and other glycosphingolipid (GSL) storage diseases. We previously showed that miglustat has an anti-inflammatory effect *in vitro* and *in vivo*, and reduces the *P.aeruginosa*-induced immunoreactive ceramide expression [11,8] through inhibition of non-lysosomal-β-glucosidase 2 (GBA2) [12]. As a glucose- and short-chain ceramide-mimetic, miglustat inhibits a broad array of enzymes involved in carbohydrate, glycoprotein and glucosylceramide (GlcCer) metabolism. This observation may lead to putative off-target effects, thereby raising concerns about the efficacy of this treatment. Starting from the lead compound DNJ, a small library of adamantane neoglycoconjugates (AMP) has been prepared and mainly encompassing alkyl chains of various length [13]. The efficacy of these molecules has been already demonstrated for the modulation of the biological activity, especially regarding glycoenzyme inhibition profile and activity on the cellular enzymes involved in GlcCer metabolism [13]. Therefore, this study was planned to test the effect of this small library of AMP-DNJ derivatives on the inflammatory response to *P.aeruginosa* in CF bronchial epithelial cells.

MATERIALS AND METHODS

Cells and bacteria

CuFi-1 cells [14] (a generous gift of A. Klingelhutz, P. Karp and J. Zabner, University of Iowa, Iowa City; USA) are human bronchial epithelial cells, grown as described elsewhere [11]. Reference *P. aeruginosa* strain, PAO1, kindly provided by A. Prince (Columbia University, New York; USA) was grown in trypticase soy broth (TSB) or agar (TSA) (Difco) as described elsewhere [11].

Inhibitors of SL metabolism

AMP-DNJ derivatives were synthesized as described elsewhere [13].

Inflammatory response *in vitro*

Cells were treated with different inhibitors or solvent alone, and then infected with PAO1 for 4 hours at 37^{o}C as described elsewhere [11]. The inflammatory response to PAO1 infection was studied at transcriptional level by measuring the expression of the chemokine IL-8, as previously specified [8].

Enzymatic activity

CuFi-1 cells seeded on T25 cm^2 flask were differently treated, scraped and pelleted. The cellular pellets were resuspended in water containing protease inhibitors and sonicated. An aliquot of cell lysate was used to detect the protein concentration by the DC Protein Assay (Biorad). The enzymatic activities associated with total cell lysates were determined using fluorigenic substrates as described elsewhere [15]. Aliquots of the cell homogenates were transferred to a 96-well microplate and enzymatic assays performed with three-fold replication. For GBA1 and GBA2 assay a pre-incubation in McIlvaine buffer (pH 6) containing 5nM of AMP-DNJ (which inhibits GBA2) and with 1mM of Conduritol-B-epoxide (CBE) (which inhibits GBA1) respectively, was performed for 30 minutes at room temperature.

Statistics

Results are expressed as mean ± standard error (SE). Comparisons between groups were performed using Student's t test. Statistical significance was set at $p<0.05$. In order to calculate IC50 values, experimental data were fitted by non linear regression, using software "R Core Team, 2013,"R: A language and environment for statistical Computing", R Foundation for Statistical Computing, Vienna, Austria.

RESULTS AND DISCUSSION

In this paper, we studied a small library of AMP derivatives (compounds #14-20) characterized by differences in the total length of the alkyl chain between the iminosugar and AMP. First, the effect of these compounds on expression of IL-8 induced by PAO1 infection in CuFi-1 cells was tested. Cells were treated with increasing doses (from 0.001 to 100 μ M) of AMP-DNJ conjugates or with solvent alone for 1 hour before infection. Doses higher than 5 μM of compound #20 were not tested, due to cellular toxicity. Cells were then infected with PAO1 for 4 h and the effect of treatment was tested by measuring the expression of IL-8 induced by PAO1. As shown in Figure (1), all the AMP-DNJ conjugates reduced PAO1-stimulated IL-8 mRNA expression in CuFi-1 cells. In order to calculate the IC50 values, experimental data reported in Figure (1) (panels A-G), were fitted by non-linear regression and summarized in Table (1). IC50 and maximal inhibition obtained with miglustat and Genz-529648 (*N*-(5-adamantane-1-yl-methoxy) pentyl)- deoxynojirimycin) [12] are also reported. All AMP-DNJ derivatives herein tested were more effective than miglustat, with IC50 values in the nanomolar range. As reported in Table (1), the IC50 values were found to be similar to that of Genz-529648, thus underpinning that the addition of AMP moiety to iminosugar increases the therapeutic potential in terms of anti-inflammatory effect in CF bronchial cells [12].

Although these AMP-DNJ conjugates have been already evaluated in terms of glycoenzyme inhibition profiling, including glucosidases, galactosidases and mannosidases [13], there is no information concerning their effect in CF bronchial cells, in particular on GBA2 activity. Therefore compounds #14, 16, 17, and 18 were tested on total β-glucosidase, GBA1 and GBA2 activities in cell lysates obtained from CuFi-1 cells, at two different concentrations: [0.5 μM] (Figure 2, panels A-C)

and [5 μM] (Figure 2, panels D-F). The shorter compound #14 reduced relevantly only GBA2 (Figure 2, panels C and F), whereas derivatives #16, #17 and #18 impacted total β–glucosidase other than GBA2 (Figure 2, panels A and D) and GBA1 activity (Figure 2, panels B and E). These findings were more evident when CuFi-1 cells were treated with the AMP-DNJ conjugates at the highest concentration (Figure 2, panels D and E), indicating that only the shorter analogue #14 was selective for GBA2, as it did not affect total β–glucosidase and GBA1 activities even when was used at [5μM]. The other compounds # 16, #17 and #18 completely inhibited GBA2 activity, even at the lower concentration, although they were either effective on GBA1.

Our findings demonstrate that AMP-DNJ derivatives herein tested exhibit interesting anti-inflammatory activity in CF bronchial cells infected by *P. aeruginosa* (Figure 1 and Table I). The selectivity towards β–glucosidase seems to be modulated by variation of the length of the chain linking the iminosugar and AMP (Figure 2). Compound #14, displaying the shortest spacer in this library, seems to be selective for GBA2 (Figure 2, panels C and F) and, even more interestingly, it does not inhibit ER α-glucosidases I and II [13], whereas longer derivatives, which were poor selective inhibitors of GBA2 at least in our experimental conditions, were found to be potent α-glucosidase inhibitors [13]. In addition #14 has been found to restore CFTR

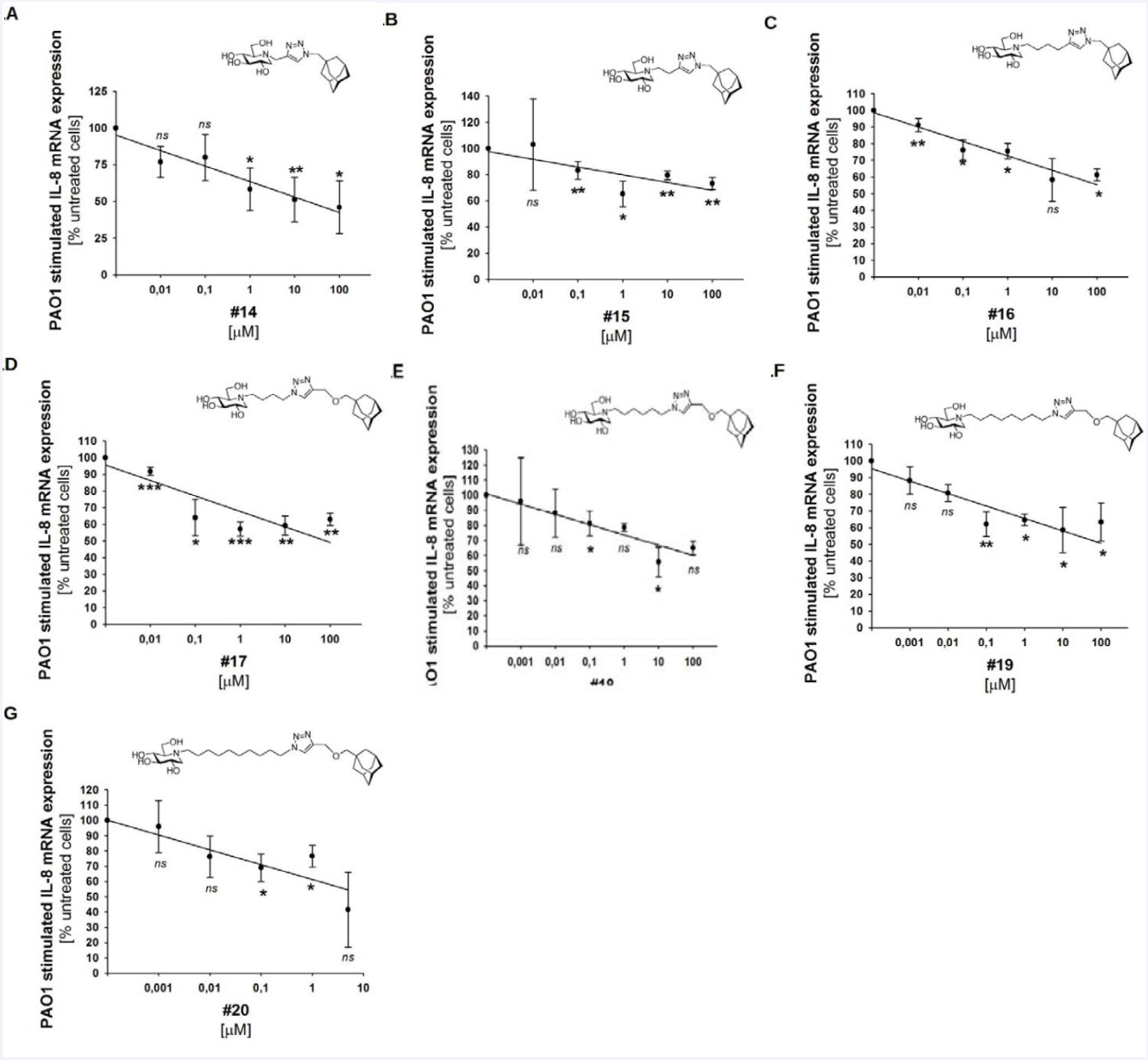

Figure 1 Effect of AMP-DNJ derivatives on IL-8 mRNA expression in CF bronchial cells infected by PAO1. CuFi-1 cells were treated with increasing doses (from 0.001 to 100μM) of compounds #14 (A), #15 (B), #16 (C), #17 (D), #18 (E), #19 (F), (0.001-5μM) #20 (G) or solvent alone for 1 hour and then infected with PAO1 (50 CFU/cell) for 4 hours at 37°C. The inflammatory response was evaluated by as IL-8 mRNA expression, measured by Real-time qPCR and normalized on housekeeping gene β-actin. Results, expressed as % of untreated cells are mean ± standard error of 3 independent experiments in duplicate. Comparisons between groups were performed using Student's t test; $p<0.05$ (*); $p<0.01$ (**); $p<0.001$(***).

dependent chloride transport [13]. Although Genz-529648 seems to be more effective than #14 in reducing IL-8 production, at least in our experimental model, the selectivity for GBA2 and its effect as CFTR corrector makes #14 a compound to be further explored as an anti-inflammatory drug for CF lung inflammation, acting through the modulation of SL metabolism.

CONCLUSION

Our previous findings [12] support the contention that GBA2 is involved in the inflammatory response to *P. aeruginosa* (Figure 3). The results herein presented further propose the use of inhibitors of GBA2 activity to reduce the inflammatory response to *P. aeruginosa* in CF bronchial cells. This study provides a clear support for the development of therapeutic options for CF lung inflammation using iminosugars, which can be effective at even low doses, thus limiting potential adverse and/or toxic effects. In this respect, all the alkylated iminosugars herein tested reduced IL-8 mRNA expression in CF bronchial cells infected by *P. aeruginosa* and inhibited GBA2 activity. Notably, they were even more effective than miglustat, wherein needing lower

Table 1: Inhibition of P.aeruginosa stimulated IL-8 mRNA expression in CuFi-1 cells.

Inhibitor	IC50	CI	Maximal Inhibition	CI
	(μM)	*(μM)*	(%)	(%)
#14	0.012	0.002-0.06	43	29-57
#15	0.005	0.0001-0.23	23	18-28
#16	0.045	0.008-0.24	36	29-43
#17	0.035	0.018-0.066	40	36-44
#18	0.62	0.19-0.27	36	24-48
#19	0.007	0.003-0.016	36	32-40
#20	0.002	0.0001-0.02	28	17-39
Miglustat*	1.9	1.4-2.7	51.5	51-57
Genz-529648*	0.002	0.002-0.003	46.0	38-53

IC50 values (*i.e.* inhibitor concentration resulting in 50% inhibition) were calculated by fitting experimental data with a non-linear regression, according to the formula:
$-\log(I) = pKi + \log(V-v)/v$
I= inhibitor concentration; v= % inhibition; *pKi*= IC50; V= maximal inhibition; CI=confidence interval 95%
*(Loberto, 2014)

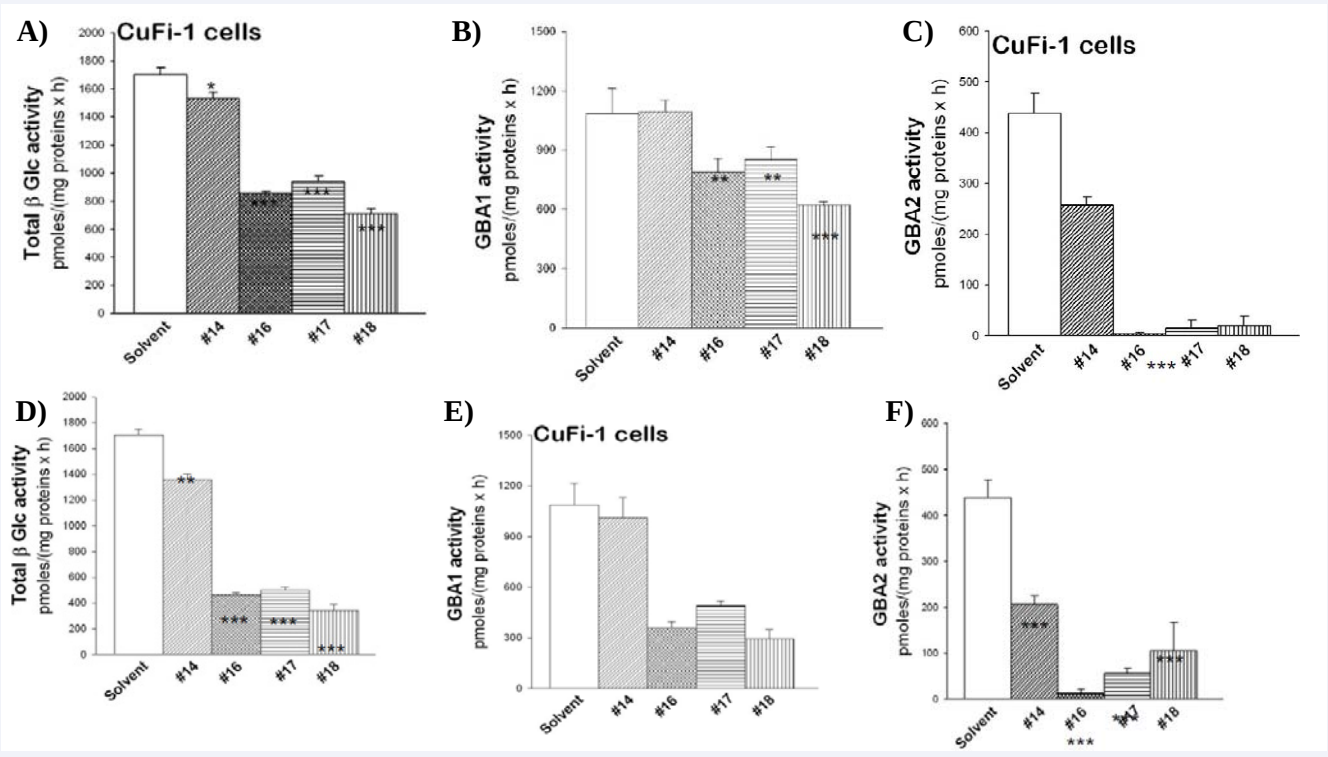

Figure 2 Effect of AMP-DNJ derivatives on β-glucosidase activity in CF bronchial cells. CuFi-1 cells were treated with #14, #16, #17 and #18 [0.5μM] (A-C) or [5μM] (D-F) for 1 hour and β-glucosidase activity measured as described elsewhere (12). Results reported in the Figure are mean ± standard error of 3 independent experiments. Comparisons between groups were performed using Student's t test; $p<0.05$ (*); $p<0.01$ (**); $p<0.001$(***).

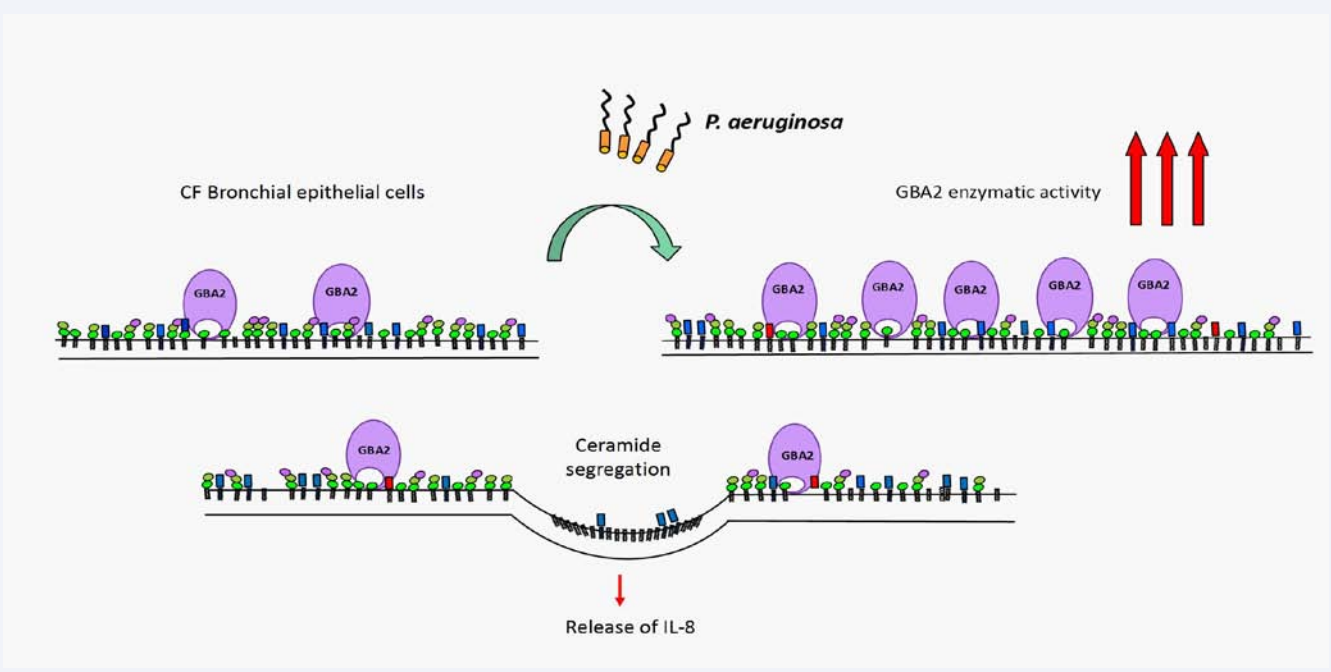

Figure 3 Schematization of GBA2 involvement in the inflammatory response due to P. aeruginosa infection in CF bronchial cells. In CF bronchial epithelial cells, P. aeruginosa infection increases GBA2 enzymatic activity. This augments ceramide at the plasma membrane thus leading to an increased release of the inflammatory chemokine IL-8.

concentrations. The AMP moiety and DNJ are key motifs for the therapeutic potential of these iminosugars. The novelty of our findings is that these classes of molecules which are inhibitors of GBA2 activity are all effective in reducing the expression of IL-8 in CF bronchial epithelial cells. Our results further support the use of modulators of SL metabolism for CF lung inflammation and provide useful hints on relevant targets and chemical structures that may be used as foundation for a drug discovery campaign. Efforts to develop therapeutic strategies for CF should encompass immune cells as well as other cell types. Because SLs are known to be important modulators of immune cell function [16], our results suggest that iminosugars, and the signalling pathways they modulate, merit further exploration as possible targets for therapeutic interventions on innate immune function aimed to ameliorate CF lung inflammation.

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