Exploring the Effect of Chirality on the Therapeutic Potential of *N*-Alkyl-Deoxyiminosugars: Anti-Inflammatory Response to *Pseudomonas aeruginosa* Infections for Application in CF Lung Disease

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ABSTRACT. In the frame of a research program aimed to explore the relationship between chirality of iminosugars and their therapeutic potential, herein we report the synthesis of *N*-akyl L-deoxyiminosugars and the evaluation of the anti-inflammatory properties of selected candidates for the treatment of *Pseudomonas aeruginosa* infections in Cystic Fibrosis (CF) lung disease. Target glycomimetics were prepared by the shortest and most convenient approach reported to date, relying on the use of the well-known PS-TPP/I_2 reagent system to prepare reactive alkoxyalkyl iodides, acting as key intermediates. Iminosugars *ent*-**1-3** demonstrated to efficiently reduce the inflammatory response induced by *P. aeruginosa* in CuFi cells, either alone or in synergistic combination with their D-enantiomers, by selectively inhibiting NLGase. Surprisingly, the evaluation in murine models of lung disease showed that the amount of *ent*-**1** required to reduce the recruitment of neutrophils was 40-fold lower than that of the corresponding D-enantiomer. The remarkably low dosage of the L-iminosugar, combined with its inability to act as inhibitor for most glycosidases, is expected to limit the onset of undesired effects, which are typically associated with the administration of its D-counterpart. Biological results herein obtained place *ent*-**1** and congeners among the earliest examples of L-iminosugars acting as anti-inflammatory agents for therapeutic applications in Cystic Fibrosis.

1. Introduction

Cystic Fibrosis (CF) is the most common life-threatening Mendelian disorder worldwide that affects respiratory, digestive, and reproductive systems. It is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, encoding for a cAMP-activated chloride channel, expressed at the apical membrane of most of the surface epithelial cells lining the airways, gastrointestinal tract, exocrine pancreas, airway submucosal and sweat glands [1,2]. CFTR protein plays a key role in hydrating airway secretions [3,4]. In the lung, the defective CFTR causes dehydration of the airway surface liquid and drives the cascade of pathological events characterized by progressive chronic infections and inflammation of the airways. The last ones then lead to irreversible lung damage and fibrosis, which represent the major cause of mortality in CF patients [3]. Accordingly, increasing attention is currently focusing on anti-inflammatory therapies, with the aim to ameliorate CF lung pathology [5]. High-dose ibuprofen is the only anti-inflammatory drug recommended to date for use in CF, however, it is not widely prescribed due to the onset of important side-effects as the gastrointestinal bleeding [6]. Hence, the development of novel and more potent anti-inflammatory drugs as well as candidate targets for precision therapies directed towards pathways regulating inflammatory response in CF airway disease remains a compelling need.

Sphingolipids (SLs) represent a large group of lipids with emerging importance for the pathophysiology of several human pathology including respiratory disorders [7-12]. They are one of the active constituents of the mucus secreted by alveolar epithelium, which protects the lung tissue from invading pathogens, thus representing effective novel specific drug targets for controlling pulmonary infections [13]. An increasing number of studies indicate that SLs play an important regulatory role in CF with respect to pulmonary infections and inflammation and support the use of modulators of SL metabolism as therapeutic agents for CF lung disease [11]. As a matter of fact, novel drugs are being developed to selectively target different enzymes involved in SL metabolism and, in this regard, iminosugars (sugar analogues having an amino group in place of the endocyclic oxygen) open up exciting new opportunities for therapeutic agent discovery, due to their good oral bioavailability and very specific immune modulatory and chaperoning activity [14].

As previously reported [15,16], the iminosugar *N*-butyl D-deoxynojirimycin (D-NBDNJ, **1**, Miglustat) exhibits anti-inflammatory effect *in vitro* and *in vivo* and reduces the *P. aeruginosa* induced immunoreactive ceramide expression by targeting non-lysosomal glucosidase 2 (GBA2-encoded NLGase) [17]. NLGase is an important player in the regulation of SL homeostasis at the plasma membrane level, due to its ability to modify the lipid organization at this site [11,18]. Therefore, by modulating plasma membrane ceramide levels, it is possible to determine the susceptibility to inflammatory stimuli. It must be noted that the pharmacological use of D-NBDNJ and its congeners has typically been associated to the onset of numerous (even severe) adverse effects [19], due to the side inhibition of a variety of carbohydrate-processing enzymes. In search for more selective NLGase inhibitors, a new generation of iminosugars, obtained either varying the *N*-alkyl chain length/structure [20] and/or changing the configuration of one or more stereogenic centres within the iminosugar core [21], is currently under development. On one hand, iminosugars bearing long alkyl chains including *N*-nonyl D-DNJ (D-NNDNJ, **2**), adamantanemethoxypentyl D-DNJ (D-AMP-DNM, **3**), *N*-hexyloxypentyl D-DNJ (D-HPDNJ, **4**) and *N*-nonyloxypentyl D-DNJ (D-NPDNJ, **5**) have demonstrated to be increasingly powerful NLGase inhibitors [21]. On the other hand, novel therapeutic opportunities are recently being offered by L-iminosugars and their *N*-alkylated derivatives [22,23]. While typically acting as weak [24] inhibitors of most glycosidases, in an increasing number of reports L-iminosugars have displayed more efficient pharmacological properties than their D-isomers toward specific enzymes, acting as either inhibitors [25] or activators [26-28]. As an example, *N*-alkyl iminosugars with L-*ido* [20,29] and L-*altro* [29] configuration have drawn attention because of their remarkably high selectivity and potency in the inhibition of various glycosidases and glycosyltransferases, including NLGase, compared to the activity of the corresponding D-*gluco* congeners. Inspired by these observations, we have conceived to widen the repertoire of therapeutic L-iminosugars, exploring the biological properties of *N*-alkyl iminosugars with L-*gluco* configuration *ent*-(**1-5**) (Figure 1). In preliminary studies, *ent*-**1** (i.e. L-NBDNJ) has already demonstrated to be able to increase the levels of exogenous GAA (acid α-glucosidase, Myozyme) in fibroblasts from patients with Pompe disease (PD), while not working as a glycosidase inhibitor [28]. Herein, the analysis of the pharmacological potential of *ent*-**1**, as well

as that of other *N*-alkyl congeners, has been extended to NLGase inhibition, with the aim to study the role of iminosugar chirality in the anti-inflammatory treatment of *P. aeruginosa* infections.

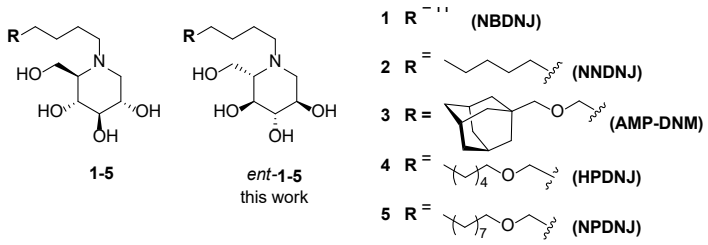


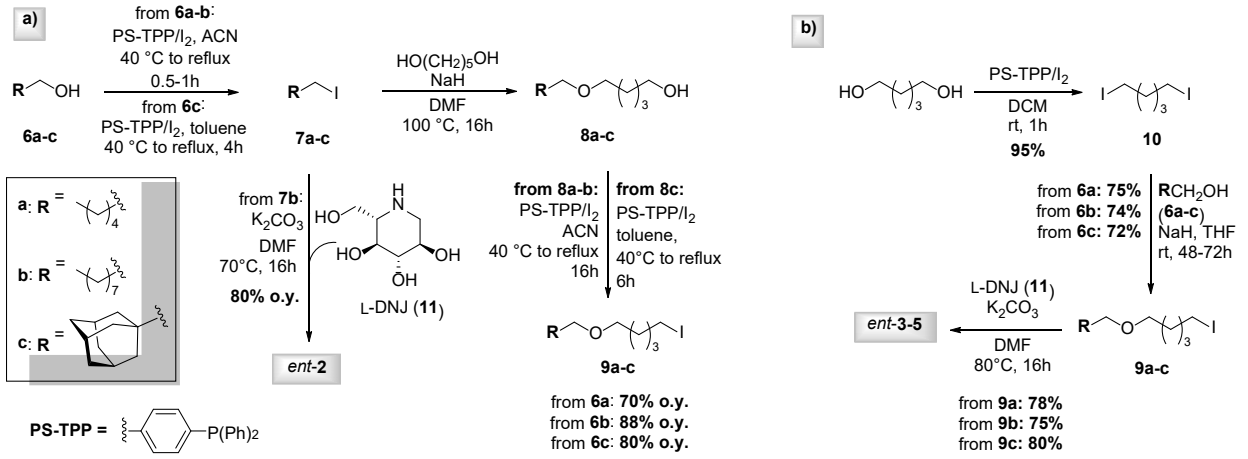
Figure 1. *N*-alkylated D- and L-DNJ derivatives.

2. Results and Discussion

2.1 Chemistry

The synthesis of *N*-alkyl L-deoxyiminosugars *ent*-(**2-5**) was devised as depicted in Scheme 1, whereas the preparation of *ent*-**1**, as well as the total synthesis of enantiomerically pure L-DNJ (**11**), was reported previously [28,30]. Particularly, herein our attention was focused on the preparation of alkyl iodides **7a-c** and on a straightforward route, leading to alkoxyalkyl iodides **9a-c**, to be used in the *N*-alkylation reaction of **11** by alternative procedures to the existing methods [20,21,29,31]. Our synthetic approaches are based on the use of the well-known polymer supported triphenylphosphine (PS-TPP) and iodine [32] as iodination system, which has been already used by us for other synthetic purposes [33]. The PS-TPP/I_2 reagent system, in this case, can be employed in the absence of the commonly used imidazole, thus enabling to devise a synthetic protocol with a sequence of steps not requiring extractive work-up and chromatographic purifications. Indeed, the only reaction by-product, i.e. the resin-bound phosphine oxide, can be easily filtered off and reduced to the original phosphine form by treatment with trichlorosilane [34]. In early studies, the procedure was based on two iodination reactions, the first one to convert alcohols **6a-c** into desired alkyl iodides **7a-c**, that were subsequently coupled with 1,5-pentandiol (NaH) to afford alcohols **8a-c**; the second one to obtain **9a-c** from **8a-c** (Scheme 1a). In order to find the best iodination conditions, different

solvents and temperatures were used: the use of refluxing ACN provided the best results for both **6a,b** and **8a,b**, whereas for the lower reactive adamantanemethanol (**6c**) and the corresponding derivative **8c**, refluxing toluene was the solvent of choice.



Scheme 1. Synthetic routes to *N*-alkylated L-DNJ derivatives *ent*-(**2-5**).

NMR analysis of the crude mixtures of **9a-c** demonstrated that high yield conversions (70-88% o.y.) could be obtained in all cases, with virtually no side products attributable to the limiting reagents. The strategy reported in Scheme 1a represents a faster and more convenient alternative to most procedures [20,21,29,31a-b] devised for the preparation of alkyl and alkoxypentyl chains to be used in the *N*-alkylation reaction of DNJ. In search for an even more expeditious protocol, in subsequent studies an alternative route was conceived, involving a PS-TTP mediated double iodination of 1,5-pentandiol, to provide 1,5-diiodopentane (**10**), followed by two sequential coupling reactions, with alcohols **6a-c** first and with L-DNJ (**11**) then (Scheme 1b). The efficiency of the double iodination reaction was markedly affected by the reaction conditions: over a variety of solvents tested (Table S1), the most convenient conversion (95%) was observed using DCM for 1h at rt. The subsequent coupling reaction of **10** with alcohols **6a-c** proceeded smoothly with hexanol (**6a**) and nonanol (**6b**), providing after 48h at rt the corresponding alkoxyalkyl iodides **9a,b** in 75 and 74% yields, respectively. On the other hand, the reaction with adamantanemethanol (**6c**) proceeded slower, and the corresponding iodide **9c** was obtained with

a 72% yield after 72h at rt (Scheme 1b) [35]. The eventual reaction of nonyl iodide **7b** and alkoxyalkyl iodides **9a-c** with L-DNJ (**11**) [28] was carried out under standard conditions (K_2CO_3), smoothly providing *ent*-**2** (80% o.y., Scheme 1a) and *ent*-(**3-5**) in satisfactory 75-80% yields (Scheme 1b), respectively.

2.2 Biochemistry

The effect of iminosugar chirality on NLGase inhibition and thereby on the *P. aeruginosa* triggered lung inflammation was then explored. In first studies, the inhibitory effect of the synthesized D- and L-iminosugars [**1-5**, *ent*-(**1-5**)] on NLGase activity was examined by *in vitro* assays in model SH-SY5Y cells, a human neuroblastoma cell line having high activity of both GCase (encoding the lysosomal enzyme glucocerebrosidase) and NLGase (encoding the non-lysosomal glucosylceramidase). As summarized in Table 1, L-iminosugars *ent*-(**1-5**) acted as NLGase inhibitors (entries 2, 5, 8, 11 and 14). Nevertheless, the inhibition potency was always lower (in the micromolar range) than those of D-iminosugars (IC_{50} down to 3 nM, entries 1, 4, 7, 10 and 13). *Ent*-**3** and *ent*-**5** displayed the highest inhibitory activity among L-iminosugars (IC_{50} 65 nM, entry 8; IC_{50} 137 nM, entry 14), in agreement with the role of the alkyl chain length regarding NLGase inhibition [20,21]. In several cases, L-iminosugars (entries 2, 5 and 12) when tested as GCase inhibitors, showed higher IC_{50} with respect to those observed against NLGase demonstrating, as expected, high enzymatic selectivity.

The effect of the combined incubation of D- and L-iminosugars [26], leading to *rac*-(**1-5**), was also considered. While no significant synergistic effect was found for *rac*-**1** and *rac*-**2**, conversely *rac*-**3-5** displayed a similar activity to that exerted by **3-5** when taken alone (entries 9, 12 and 15) with a total inhibition of the NLGase activity.

Table 1. Effect of iminosugar chirality on glycohydrolase activity.

entry	Compound	GCase		NLGase	
		IC_{50} (μM)	M.I. (%)	IC_{50} (μM)	M.I. (%)
1	**1**	118	57	0.014	100
2	*ent*-**1**	62	24	7.7	100
3	*rac*-**1**	N.D.	N.D.	0.260	100
4	**2**	5.6	100	0.003	100
5	*ent*-**2**	136	51	4.6	100
6	*rac*-**2**	N.D.	N.D.	0.035	100
7	**3**	N.D.	N.D.	0.003	100
8	*ent*-**3**	N.D.	N.D.	0.065	10
9	*rac*-**3**	N.D.	N.D.	0.004	100
10	**4**	5.4	100	0.0004	100
11	*ent*-**4**	603	65	36.7	100
12	*rac*-**4**	N.D.	N.D.	0.001	100
13	**5**	N.D.	N.D.	0.034	100
14	*ent*-**5**	N.D.	N.D.	0.137	10
15	*rac*-**5**	N.D.	N.D.	0.044	100

The assays were performed on lysates of SH-SY5Y cells, using different concentrations of each compound (from 1nM to 1mM) in the presence of 5nM AMP-DNM or 750μM CBE. IC_{50}: inhibitor concentration that produces 50% inhibition. M.I.: maximal inhibition. N.D.: Not Determined.

2.3 Biological evaluation

2.3.1 In vitro studies

A selection of *N*-alkylated iminosugar enantiomers were tested for their effect on *P. aeruginosa* stimulated IL-8 mRNA expression in CF bronchial epithelial cells. CuFi-1 cells were treated with either **1**, **2**, **3**, *ent*-**1**, *ent*-**2**, *ent*-**3**, or the corresponding racemic mixtures (*rac*-**1**, *rac*-**2**, *rac*-**3**) 1 h before infection with *P. aeruginosa* and for the following 4 h. We found that both *ent*-**1** and *ent*-**3** (0.1 μM) reduced the IL-8 mRNA expression (up to a 20% and 29% reduction, respectively), with an effect comparable to that of the corresponding enantiomers. Conversely, *ent*-**2** exhibited a smaller effect (16% reduction) under the same conditions, although it was greater than that of **2** (8% reduction). Interestingly, the combination of iminosugar enantiomers (0.05 + 0.05 μM) produced an even more pronounced effect. As an example, the reduction of IL-8 mRNA expression by *rac*-**1** and *rac*-**3** was down to 30% and 42%, respectively. Analogously, *rac*-**2** inhibited the expression of IL-8 down to 22% (Figure 2). These results suggest that the combination of D and L enantiomers could reduce the concentration of D enantiomers, thus minimizing the undesired side effects.

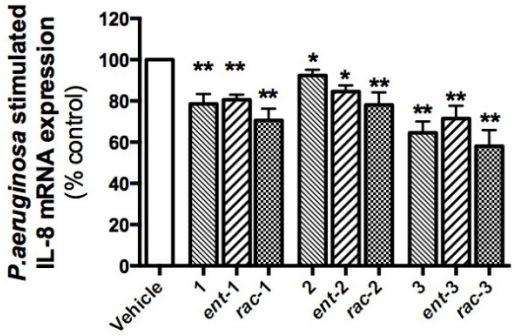


Figure 2. Effect of sugar chirality of NBDNJ (**1**, *ent*-**1**), NNDNJ (**2**, *ent*-**2**) and AMPDNM (**3**, *ent*-**3**) on the inflammatory response to *P. aeruginosa* in CF bronchial epithelial cells. CuFi-1 cells were treated with the compounds (0.1 μM) for 1 h before *P. aeruginosa* infection. The inflammatory response was evaluated by studying the expression of IL-8 mRNA, measured by Real-time qPCR and obtained by comparing the ratio IL-8 and the housekeeping gene β-actin between not infected and infected cells. The results, expressed as percentage of untreated cells, are mean ± standard error of the mean of four independent experiments in duplicate. Comparisons between groups were made by using Anova.

Subsequently, dose-response experiments were performed. IC_{50} values related to the inhibition of IL-8 mRNA expression were calculated by adding increasing doses of *rac*-**1**, *rac*-**2** and *rac*-**3** (from 1 nM to 1 mM) to CF bronchial cells 1 h before infection (Table 2). We found that *rac*-**1** inhibited at very low concentration (IC_{50} 0.95 nM; CI 0.54-1.36), with a maximal inhibition of 36% (CI 29-38). The IC_{50} value of *rac*-**2** was even lower (IC_{50} 0.6 nM; CI 0-2), although maximal inhibition was only 12% (CI 7-15). Similarly, the IC_{50} value of *rac*-**3** was 1.84 nM (CI 0.3-2.1), while maximal inhibition was 29% (CI 27-33). In the same cell model, **1** alone displayed an IC_{50} value of about 2 μM and a maximal inhibition of 50% [17]; conversely, **3** alone displayed an IC_{50} value very similar to that obtained using the racemic mixture.

The effect of racemic mixtures reported in Figure 2 was also tested in CF bronchial primary cells infected by *P. aeruginosa* (Figure 3). Primary airway epithelial cells, i.e., mainstem human bronchi, derived from CF individuals [36], were treated with *rac*-**1**, *rac*-**2** or *rac*-**3** (10 nM) before the infection with *P. aeruginosa* strain PAO1. As shown in Figure 3, *rac*-**1** and *rac*-**3** significantly reduced the inflammatory response to *P. aeruginosa* in CF primary cells.

Whether as single enantiomer or as racemic mixtures, *N*-alkylated iminosugars did not induce significant apoptosis in CF bronchial cells when compared with vehicle after 48h treatment (Figure S1). In addition, all tested iminosugars did not significantly decrease cell viability after 48h incubation in the same experimental model (Figure S2).

Table 2. IC_{50} and Maximal Inhibition of racemic mixtures on IL-8 mRNA expression

Compound	IC_{50} (nM)	CI (nM)	M.I. (%)	CI (%)
rac-**1**	0.95	0.54-1.36	36	29-38
rac-**2**	0.6	0-2	12	7-15
rac-**3**	1.84	0.3-2.1	29	27-33

CuFi-1 cells were treated as indicated in Figure 2. Data obtained in three independent experiments in duplicate were used to calculate IC_{50} values by "Graph Pad Prism" 6.0.

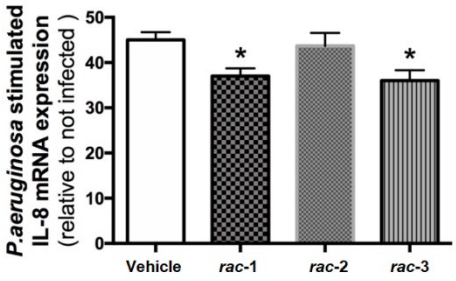


Figure 3. Effect of racemic mixtures on the inflammatory response to *P. aeruginosa* in CF primary cells. CF bronchial primary cells were treated with the racemic mixtures (0.01 μM) for 1 h before *P. aeruginosa* infection. The inflammatory response was evaluated as indicated in Figure 2. The results, expressed as relative to not infected cells, are mean ± standard error of the mean of three independent experiments in duplicate. Comparisons between groups were made by using Anova.

2.3.2 In vivo studies

The combined effect of iminosugar enantiomers was eventually studied in murine models of lung inflammation. Since we were interested to evaluate the role of chirality on anti-inflammatory activity of such compounds, we needed of a complete overview of the *in vitro* and *in vivo* behavior, as well as of pharmacokinetics and safety profile data of a reference iminosugar. Among all the synthesized compounds, *N*-butyl iminosugar derivative *rac*-**1** was chosen for preliminary studies since **1** was already widely studied [15,37-40]. Treatment with 100 mg/kg of **1** for 3 days reduced the recruitment of neutrophils into the bronchoalveolar space after intranasal instillation of LPS [15]. Moreover, an oral dose of 400 mg/kg of **1** given to mice before the intra-tracheal inoculum with *P. aeruginosa* was effective in reducing strongly the inflammatory response associated with acute pneumonia in terms of leukocyte recruitment and myeloperoxidase activity in the airways [16]. Therefore, we evaluated at first the effect of *rac*-**1** on acute infection and inflammation in C57Bl/6NCr male mice. Considering the IC_{50} values obtained *in vitro* with *rac*-**1** (Table 2), lower doses than those previously described [15] were used in these studies. Mice were treated by gavage 24 and 1 h before the infection with *rac*-**1** at 10 mg/kg or 100 mg/kg (Figure 4). After infection (6h), mice were killed with an overdose of carbon dioxide, and murine lungs tested for lung infection, while BAL was processed for inflammation as previously described [41]. We found that treatment of mice with *rac*-**1** at 100 mg/kg provided a slight reduction of the amount of neutrophils, although this decrease was not

statistically significant (Figure 4B). Interestingly, we also found a marked increase of alveolar macrophages in mice treated with the lower dose of *rac*-**1** (Figure 4C). In line with this last result, treatment with *rac*-**1** at 10 mg/kg was accompanied by a reduction of CFU both in BAL and in lungs (Figure S3).

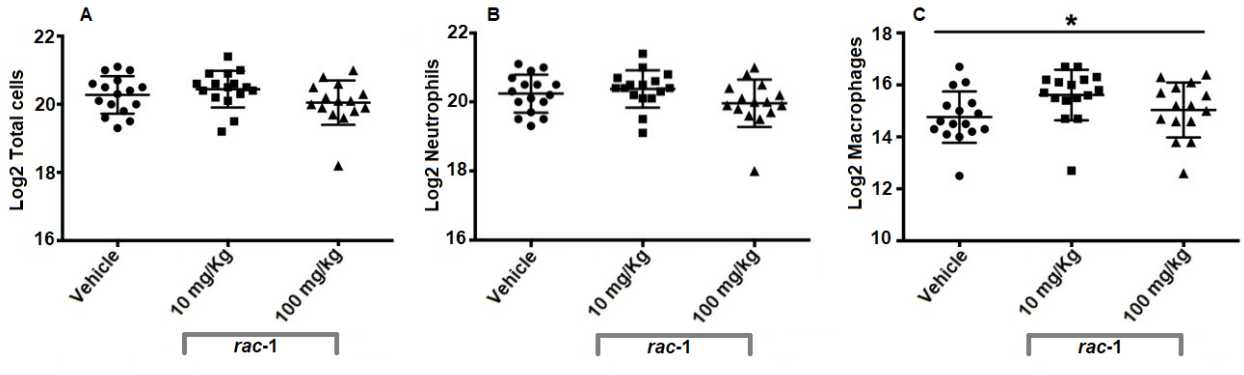


Figure 4. Effect of *rac*-**1** on cells recruited in BAL of C57Bl/6NCr mice infected by *P. aeruginosa.* A. Total cells; B. Neutrophils; C. Alveolar macrophages.

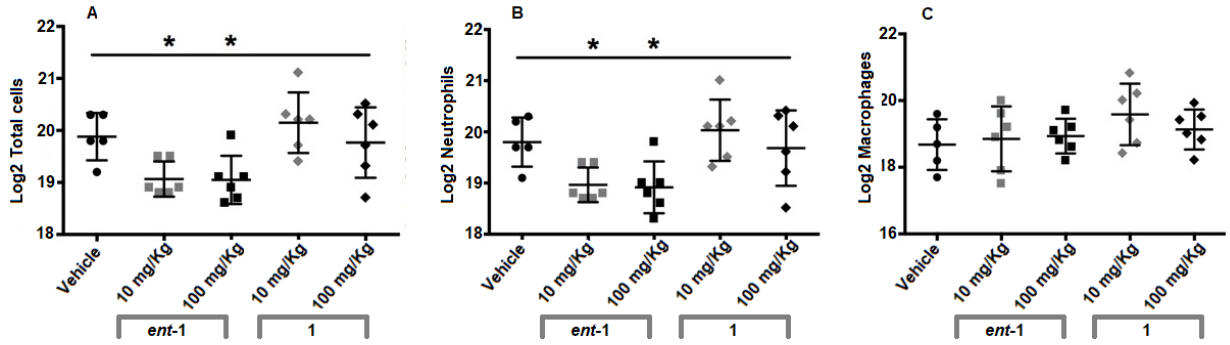


Figure 5. Effect of **1** and *ent*-**1** on cells recruited in BAL of C57Bl/6NCr mice infected by *P. aeruginosa.* A. Total cells; B. Neutrophils; C. Alveolar macrophages.

Administration of *rac*-**1** proved to be safe as demonstrated by the weight of mice after treatment. Furthermore, while infection typically induces a weight loss in untreated mice, this effect was

not observed in mice treated with *rac*-**1** at the higher dose (Figure S4). In order to investigate whether this effect was due to the combined treatment of NBDNJ enantiomers or to singularly administered iminosugars, in subsequent studies we separately treated mice with **1** or *ent*-**1** before infection. Surprisingly, we found that *ent*-**1** decreased the amount of neutrophils recruited in BAL at a concentration as low as 10 mg/kg, i.e. 40-fold lower than that of **1** (Figure 5B). Very importantly, the decreased recruitment of neutrophils did not increase the amount of bacteria recovered in the airways (Figure S5). It was also noteworthy that in mice treated with *ent*-**1** a dose-dependent decrease of bacteria was detected in lungs, although it was not statistically significant (Figure S5). Treatment with both **1** and *ent*-**1** was safe as shown by the weights of mice monitored during the experiment (Figure S6). Lastly, the NLGase activity in the lungs of mice infected by *P. aeruginosa* in presence of **1**, *ent*-**1** and *rac*-**1** was evaluated (Figure 6). If *rac*-**1** does not influence the enzymatic activity, *ent*-**1** deeply inhibits NLGase. These results, differently from the *in vitro* assays (Table 1) suggested, as previously observed for **1** [17], the involvement of NLGase inhibition in the inflammatory response to *P. aeruginosa* infection.

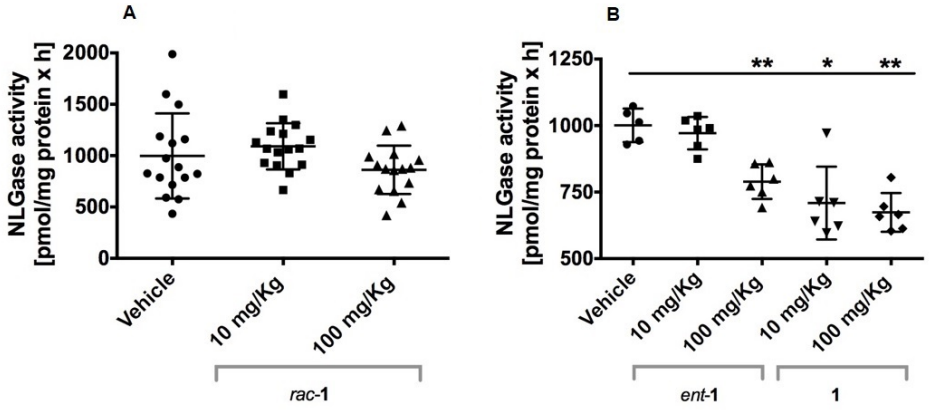


Figure 6. NLGase activity in the lungs of mice infected by *P. aeruginosa.*

Mice were treated as indicated in Figure S3 (A) or S5 (B). Lung lysates were diluted in Mc Ilvaine buffer (pH 6.0) and treated for 30 min at rt with 1mM CBE to inhibit GCase. Then lysates were incubated with 6mM methylumbelliferyl-β-D-glucopyranoside (MUB-Glc) at 37 °C under gently shaking. After 1h incubation, the developed fluorescence was detected diluting an aliquot of the assay mixture with 20 vol of 0.25 M glycine, pH 10.7. As background, we used boiled aliquots of each lung lysate. Data were expressed as pmoles×mg tissue proteins^{-1}× h^{-1}. Data reported are Mean ± Standard Error Comparisons between groups were made by Anova.

3. Conclusion

The role of iminosugar chirality in the anti-inflammatory treatment of CF lung disease has been herein explored through the analysis of the therapeutic potential of novel *N*-alkyl L-DNJ derivatives – from the workbench to *in vivo* studies. From the synthetic standpoint, the shortest approach reported to date has been tuned, leading to the insertion of the bioactive alkoxypentyl chains onto the iminosugar core through the efficient preparation of alkyl iodides **7a-c**, **10** and alkoxyalkyl iodides **9a-c** by the PS-DPP/I_2 reagent system. L-Iminosugars *ent*-(**1-3**) have demonstrated to act as selective NLGase inhibitors and they are able to reduce the inflammatory response to *P. aeruginosa* in CF bronchial cells at very low concentration. In addition, a synergistic effect deriving from the combined use of enantiomeric iminosugars has been suggested both in NLGase inhibition and in the reduction of the IL-8 mRNA expression in CF bronchial cells CuFi-1 cells. Importantly, the use of *ent*-**1** in C57Bl/6NCr mice (which was totally safe under our experimental conditions) has led to a decrease in the amount of neutrophils recruited in BAL at a concentration 40-fold lower than **1**, without increase of the amount of bacteria recovered in the airways.

Taken together, these findings provide for the first time interesting indications on the therapeutic potential of L-iminosugars as anti-inflammatory agents in CF lung disease. Their activity (especially that of *ent*-**1**, which has been evaluated in greater detail) appears even preferable to that of the corresponding D-iminosugars. In particular, on the basis of these data, strong indications that *ent*-**1** could be a promising molecule to reduce the inflammatory response in CF patients with no side inhibition of glycosidases and without impairing host defences have been given. Further studies aimed to more extensively define scope and limitation of the anti-inflammatory properties of *ent*-**1** and its congeners are currently ongoing and will be published in due course.

4. Experimental Section

4.1 Chemical Synthesis

General information. All chemicals and solvents were purchased with the highest degree of purity (Sigma-Aldrich, Alfa Aesar, VWR) and used without further purification. All moisture-

sensitive reactions were performed under nitrogen atmosphere using oven-dried glassware. The reactions were monitored by TLC (precoated silica gel plate F254, Merck) and the products were detected by exposure to ultraviolet radiation, iodine vapor, chromic mixture and ninhydrin. Column chromatography: Merck Kieselgel 60 (70-230 mesh); flash chromatography: Merck Kieselgel 60 (230-400 mesh). The purity of the synthetic intermediates and the final compounds was determined by CHNS analysis and was ≥ 95% in all cases. NMR spectra were recorded on NMR spectrometers operating at 400 MHz (Bruker DRX, Bruker AVANCE) or 500 MHz (Varian Inova), using $CDCl_3$ solutions unless otherwise specified. Combustion analyses were performed using a CHNS analyzer.

*General procedure for **9a-c**: PS-TPP/I_2 as the iodination reagent. Step 1*: formation of iodides **7a-c.** Alcohol **6** (1.0 mmol) was dissolved in the appropriate anhyd solvent (**6a** and **6b**: ACN; **6c**: toluene), then polymer supported triphenylphosphine (PS-TPP; 100-200 mesh, extent of labeling: ~3 mmol/g triphenylphosphine loading) (2.0 equiv) was added. The mixture was warmed to 40 °C and I_2 (2.0 equiv) was added. After warming the reaction to reflux temperature and leaving the suspension for the appropriate time (**6a**: 0.5h; **6b**: 1h; **6c**: 4h), the mixture was cooled at rt, filtered and the solvent removed under reduced pressure at rt, affording iodide **7**. **1-Iodohexane (7a)** and **1-iodononane (7b):** ^{1}H and ^{13}C NMR spectra were fully in agreement with those reported in the literature [42]. **Adamantanemethyl iodide (7c)**: ^{1}H NMR (500 MHz): δ 1.51 (*d*, J = 2.5 Hz, 6H), 1.64-1.67 (*m*, 3H), 1.72-1.75 (*m*, 3H), 1.99 (*s*, 3H), 3.20 (*s*, 2H). ^{13}C NMR (125 MHz): δ 27.1, 28.8, 36.7, 42.2. Anal. calcd for $C_{11}H_{17}I$: C, 47.84; H, 6.20. Found: C, 47.77; H, 6.22.

Step 2: formation of alkoxyalcohols **8a-c**. An appropriate amount of 1,5-pentandiol (**7a** and **7b**: 1.0 equiv; **7c**: 5.0 equiv) was dissolved in dry DMF (**7a** and **7b**: 1.0 mL; **7c**: 5.0 mL) and NaH (60% dispersion in mineral oil; **7a** and **7b**: 1.2 equiv; **7c**: 5.0 equiv) was added. The mixture was stirred for 30 min at room temperature. Then a solution of the crude iodide **7** in dry DMF (0.5 mL) was added dropwise. After 16h at 100 °C, the mixture was concentrated under reduced pressure to furnish **8**. For **8c**, the crude was washed repetitively with hexane in order to remove the excess of 1,5-pentandiol. NMR analysis of a small aliquot of the crude reaction mixture confirmed the structure of desired alkoxyalcohols **8**. **5-Hexyloxyl-1-pentanol (8a)**: ^{1}H NMR

(400 MHz): δ 0.88 (*t*, J = 6.4 Hz, 3H), 1.25-1.35 (*m*, 6H), 1.41-1.47 (*m*, 2H), 1.52-1.64 (*m*, 6H), 3.40 (*q*, J = 6.7 Hz, 4H), 3.65 (*t*, J = 6.5 Hz, 2H). ^{13}C NMR (100 MHz): δ 14.2, 22.7, 22.8, 26.0, 29.6, 29.9, 31.9, 32.7, 63.0, 70.9, 71.2. Anal. calcd for $C_{11}H_{24}O_2$: C, 70.16; H, 12.85; O, 16.99. Found: C, 70.06; H, 12.89. **5-Nonyloxyl-1-pentanol (8b)**: ^{1}H NMR (400 MHz): δ 0.87 (*t*, J = 6.6, 3H), 1.21–1.36 (*m*, 12H), 1.38–1.46 (*m*, 2H), 1.51–1.65 (*m*, 6H), 3.39 (*q*, J = 6.6, 4H), 3.65 (*t*, J = 6.5 Hz, 2H). ^{13}C NMR (125 MHz): δ 14.2, 22.5, 22.7, 26.3, 29.3, 29.5, 29.6, 29.6, 29.8, 32.0, 32.6, 63.0, 70.8, 71.1. Anal. calcd for $C_{14}H_{30}O_2$: C, 72.99; H, 13.12; O, 13.89. Found: C, 73.10; H, 13.06. **5-Adamantanemethoxypentanol (8c)**: ^{1}H NMR (500 MHz): δ 1.39-1.45 (*m*, 2H), 1.52-1.53 (*m*, 6H), 1.56-1.61 (*m*, 4H), 1.63-1.72 (*m*, 6H), 1.95 (*bs*, 3H), 2.96 (*s*, 2H), 3.39 (*t*, J = 6.5 Hz, 2H), 3.66 (*t*, J = 6.6 Hz, 2H). ^{13}C NMR (125 MHz): δ 22.6, 28.5, 29.4, 32.7, 34.3, 37.4, 39.9, 63.1, 71.7, 82.1. Anal. calcd for $C_{16}H_{28}O_2$: C, 76.14; H, 11.18; O, 12.68. Found: C, 76.05; H, 11.22.

Step 3: formation of iodides **9.** Alcohol **8** was dissolved in the appropriate anhyd solvent (**8a** and **8b**: ACN, 2.5 mL; **8c**: toluene, 5 mL), then PS-TPP (2.0 equiv) was added. The mixture was warmed to 40 °C and I_2 was added (2.0 equiv). After warming the reaction to reflux temp and leaving the suspension under stirring at the same temp for the appropriate time (**8a** and **8b**: 16h; **8c**: 6h), the mixture was filtered and the filtrate evaporated under reduced pressure. Column chromatography of the crude residue (hexane/EtOAc) gave the pure alkoxyalkyl iodide **9** (**9a**: 70 % o.y.; **9b**: 88% o.y.; **9c**: 80% o.y.). **1-Iodo-5-hexyloxyl-pentane (9a)**: ^{1}H NMR (400 MHz): δ 0.89 (*t*, J = 6.7 Hz, 3H), 1.25-1.37 (*m*, 5H), 1.42-1.50 (*m*, 2H), 1.52-1.63 (*m*, 5H), 1.82-1.89 (*m*, 2H), 3.19 (*t,* J = 7.0 Hz, 2H), 3.39 (*t*, J = 5.0 Hz, 2H), 3.41 (*t*, J = 4.4 Hz, 2H). ^{13}C NMR (100 MHz): δ 7.1, 14.2, 22.7, 26.0, 27.4, 28.8, 29.9, 31.9, 33.5, 70.6, 71.2. Anal. calcd for $C_{11}H_{23}IO$: C, 44.30; H, 7.77; I, 42.56; O, 5.37. Found: C, 44.22; H, 7.44. **1-Iodo-5-nonyloxyl-pentane (9b)**: ^{1}H NMR (500 MHz): δ 0.88 (*t*, J = 6.8 Hz, 3H),1.25-1.34 (*m*, 12H), 1.43-1.49 (*m*, 2H), 1.53-.63 (*m*, 4H),1.82-1.88 (*m*, J = 7.1 Hz, 2H), 3.19 (*t*, J = 7.0 Hz, 2H), 3.4 (*q*, J = 6.4 Hz, 4H). ^{13}C NMR (125 MHz): δ 7.3, 14.5, 23.1, 26.6, 27.7, 29.1, 29.7, 29.9, 30.0, 30.2, 32.3, 33.8, 70.9, 71.5. Anal. calcd for $C_{14}H_{29}IO$: C, 49.41; H, 8.59; I, 37.29; O, 4.70. Found: C, 49.50; H, 8.56. **1-Iodo-5-adamantanemethoxyl-pentane (9c)**: ^{1}H NMR (500 MHz): δ 1.41-1.50 (*m*, 2H), 1.50-1.54 (*m*, 6H), 1.54-1.60 (*m*, 2H), 1.60-1.74 (*m*, 6H), 1.80-1.89 (*m*, 2H), 1.92-1.99 (*m*, 3H), 2.94

(*s*, 2H), 3.19 (*t*, J = 7.1 Hz, 2H), 3.37 (*t*, J = 6.3 Hz, 2H). ^{13}C NMR (125 MHz): δ 7.2, 27.4, 28.4, 28.5, 28.6, 28.7, 29.8, 31.4, 32.2, 33.5, 34.2, 37.4, 37.5, 39.9, 71.4, 82.1. Anal. calcd for $C_{16}H_{27}IO$: C, 53.04; H, 7.51; I, 35.03; O, 4.42. Found: C, 53.16; H, 7.54

General synthetic procedure for ***9a-c*** *via bis-iodination*. *Step 1*: **1,5-diiodopentane (10)**. To a solution of PS-TPP (100-200 mesh, extent of labeling: ~3 mmol/g triphenylphosphine loading) (4.0 equiv) and iodine (4.0 equiv) in anhyd DCM, 1,5-pentandiol (1.0 equiv) was added dropwise. The reaction was stirred at room temperature for 1 h, then filtered, to remove triphenylphosphine oxide, and washed with saturated aq $Na_2S_2O_3$, brine and extracted with DCM. Organic layers were dried (Na_2SO_4) and evaporated under diminished pressure at room temperature to give the pure 1,5-diiodopentane (**10**) as a pale yellow oil (95% yield). ^{1}H NMR (500 MHz): δ 1.49-1.55 (*m*, 2H), 1.82-1.88 (*m*, 4H), 3.19 (*t*, J = 7.0 Hz, 4H). ^{13}C NMR (100 MHz), δ: 6.3(2C), 31.1, 32.5(2C). Anal. calcd for $C_5H_{10}I_2$: C, 18.54; H, 3.11; I, 78.35. Found: C, 18.62; H, 3.12. *Step 2*: NaH (60% dispersion in mineral oil; 1.0 equiv) was added to a magnetically stirring solution of alcohol **6** (1.5 equiv) in dry THF (1.5 mL) at 0 °C. The reaction mixture was stirred at the same temperature for 2 h. *Bis*-iodide **10** (0.70 mmol) was then added and the mixture warmed to rt. After the appropriate time (**6a** and **6b**: 48h; **6c**: 72h) the mixture was diluted with DCM and washed with aq NH_4Cl and brine. The organic layer was dried with Na_2SO_4 and the solvent evaporated under reduced pressure. Chromatography of the crude residue over silica gel provided the pure iodide **9** (**9a**: 75% yield; **9b**: 74% yield; **9c**: 72% yield).

*General synthetic procedure for ent-***2-5**: iodide **7b, 9a-c** (1.2 equiv) was dissolved in anhyd DMF (3.5 mL) and added dropwise to a solution of L-DNJ (1.0 equiv) and K_2CO_3 (3.0 equiv) in DMF (3.5 mL). The reaction was warmed to the appropriate temperature (**7b**: 70 °C; **9a-c**: 80 °C) and stirred for 16 h. Afterwards, the solvent was removed under diminished pressure. Chromatography of the crude residue over silica gel (*ent*-**2-4**: acetone/MeOH = 8:2; *ent*-**5**: acetone/MeOH = 9:1) afforded the DNJ derivative *ent*-**2-5** as an oil. The compound, dissolved in water, was further purified with Dowex® 1X8, 50-100 mesh, ion-exchange resin.

L-*N*-nonyl DNJ (*ent*-2): 80% yield; ^{1}H NMR (400 MHz, CD_3OD): δ 0.90 (*t*, J = 7.0 Hz, 3H), 1.22-1.38 (*m*, 12H), 1.45-1.57 (*m*, 2H), 2.18-2.20 (*m*, 1H), 2.24 (*t*, J = 10.9 Hz, 1H), 2.60-2.66 (*m*, 1H), 2.81-2.87 (*m*, 1H), 3.02 (*dd*, J = 4.8, 11.3 Hz, 1H), 3.15 (*t*, J = 9.1 Hz, 1H), 3.37 (*t*, J =

9.3 Hz, 1H), 3.49 (*dt*, J = 4.9, 5.3 Hz, 1H), 3.86 (*bs*, 2H). ^{13}C NMR (100 MHz, CD_3OD), δ: 14.4, 23.7, 24.5, 27.9, 30.3(2C), 30.6, 33.0, 54.1, 55.5, 56.4, 67.4, 68.6, 69.7, 78.7. Anal. calcd for $C_{15}H_{31}NO_4$: C, 62.25; H, 10.80; N, 4.84; O, 22.11. Found: C, 62.40; H, 10.76; N, 4.81. LC-TOF MS: *m/z* $[M + H]^+$, calcd: 290.23; found: 290.23.

L-*N*-Adamantanemethoxypentyl-DNJ (L-AMP-DNM) (*ent*-3): 80% yield; ^{1}H NMR (500 MHz, CD_3OD): δ 1.31-1.40 (*m*, 2H), 1.50-1.64 (*m*, 10H), 1.69 (*bd*, J = 11.9 Hz, 3H), 1.76 (*bd*, J = 12.0 Hz, 3H), 1.95 (*bs*, 3H), 2.09-2.15 (*m*, 1H), 2.16-2.22 (*m*, 1H), 2.55-2.63 (*m*, 1H), 2.77-2.83 (*m,* 1H), 2.97 (*s*, 2H), 2.99 (*dd*, J = 4.8, 11.3 Hz, 1H), 3.13 (*t*, J = 9.1 Hz, 1H), 3.35 (*t*, J = 8.9 Hz, 1H), 3.39 (*t*, J = 6.2 Hz, 2H), 3.45-3.50 (*m*, 1H), 3.84 (*dd*, J = 1.7, 12.0 Hz, 1H), 3.87 (*dd*, J = 2.3, 12.2 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD), δ: 24.2, 24.7, 29.7, 30.2, 33.4, 38.3, 40.8, 55.1, 55.7, 67.4, 68.1, 69.2, 72.2, 72.6, 78.4, 83.1. Anal. calcd for $C_{22}H_{39}NO_5$: C, 66.47; H, 9.89; N, 3.52; O, 20.12. Found: C, 66.37; H, 9.92; N, 3.51. LC-TOF MS: *m/z* $[M + H]^+$, calcd: 398.29; found: 398.29.

L-*N*-[5-(Hexoxy)pentyl]-DNJ (*ent*-4): 78% yield; ^{1}H NMR 500 MHz, CD_3OD): δ 0.93 *(t*, J = 6.7 Hz, 3H), 1.31-1.39 (*m*, 8H), 1.52-1.65 (*m*, 6H), 2.15-2.24 (*m*, 2H), 2.59-2.65 (*m*, 1H), 2.81-2.87 (*m*, 1H), 3.02 (*dd*, J = 4.9, 11.2 Hz, 1H), 3.15 *(t*, J = 9.1 Hz, 1H), 3.37 (*dd*, J = 9.4, 12.0 Hz, 1H), 3.45 (*q*, J = 6.2 Hz, 4H), 3.49 (*dt*, J = 4.9, 10.3 Hz, 1H), 3.86 (*dd*, J = 2.7, 12.0 Hz, 1H), 3.89 (*dd*, J =2.6, 12.0 Hz, 1H). ^{13}C NMR (500 MHz, CD_3OD): δ 14.4, 23.7, 23.9, 24.5, 26.9, 30.1, 30.7, 32.8, 54.3, 54.9, 54.9, 67.4, 67.8, 68.8, 71.4, 72.1, 78.1. Anal. calcd for $C_{17}H_{35}NO_5$: C, 61.23; H, 10.58; N, 4.20; O, 23.99. Found: C, 61.13; H, 10.62; N, 4,21. LC-TOF MS: *m/z* $[M + H]^+$, calcd: 334.26; found: 334.25.

L-*N*-[5-(Nonyloxy)pentyl]DNJ (*ent*-5): 75% yield;. ^{1}H NMR (500 MHz, CD_3OD): δ: 0.92 (*t*, J = 6.6 Hz, 3H), 1.29-1.41 (*m*, 14H), 1.51-1.64 (*m*, 6H), 2.13 (*bd*, J = 9.5 Hz, 1H), 2.19 (*dd*, J = 6.7, 10.8 Hz, 1H), 2.57-2.63 (*m*, 1H), 2.79-2.85 (*m*, 1H), 3.01 (*dd*, J = 4.9, 11.2 Hz, 1H), 3.14 (*t*, J = 9.1 Hz, 1H), 3.37 (*t*, J = 9.0 Hz, 1H), 3.44 (*q*, J = 6.3 Hz, 4H), 3.49 (*dt*, J = 4.8, Hz, 1H), 3.85 (*dd*, J = 2.2, 11.9 Hz, 1H), 3.89 (*dd*, J = 1.9, 11.9 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD), δ: 14.4. 23.7, 24.0, 24.5, 27.3, 30.2, 30.4, 30.6, 30.7, 30.8, 33.0, 54.3, 54.8, 54.9, 67.4, 67.8, 68.8, 71.4, 72.1, 78.2. Anal. calcd for $C_{20}H_{41}NO_5$: C, 63.96; H, 11.00; N, 3.73; O, 21.30. Found: C, 64.07; H, 10.96; N, 3,72. LC-TOF MS: *m/z* $[M + H]^+$, calcd: 376.31; found: 376.30.

4.2 Biochemical Studies.

General. SH-SY5Y cell line was cultured in DMEM High Glucose containing 10% inactivated FBS (fetal bovine serum), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. At 80% of confluence, cells were scraped and pelleted and lysed in water containing protease inhibitors. An aliquot of the cell homogenate was used to evaluate the protein concentration by DC Protein Assay (Biorad). The enzymatic activity of GCase and NLGase associated with total cell lysate were determined by an *in vitro* assay based on the use of the florigenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (MUB-Gluc) as previously described with some modifications [18,43,44]. Aliquots corresponding to the same amount of cell lysate were transferred in a 96-well plate and incubated or not (control), for 30 minutes at rt, in McIlvaine buffer pH 5.8 containing the compounds at the concentrations of 1nM, 10nM, 5µM, 100µM, and 1mM respectively. For the detection of NLGase, the reaction mixtures were then incubated with 5nM AMP-DNM; whereas, for GCase were incubated with 750µM conduritol B epoxide, in both cases for 30 minutes at the rt. The assay was started by adding MUB-Glc at the final concentration of 6mM. After 2 h at 37°C of incubation, 10 µl of the reaction mixtures were transferred to other 96-well plates and 190 µl of 0.25 M glycine at pH 10.7 was added to each reaction. The plates were fluorometrically analyzed using a microplate reader (Victor, Perkin Elmer). By the use of a free MUB standard the pmoles of the formed product were calculated and the specific enzymatic activity was expressed as pmoles of product formed/mg proteins/hour. The percentages of inhibition were calculated as ratio between the enzymatic activity measured in presence of the different compounds with respect to that measured in their absence. IC_{50} and maximal inhibition were determined by interpolating the data, expressed as % of inhibition with respect to the inhibitor concentration, by one phase non-linear exponential regression using the software Graphpad Prism 5.0.

In vitro and in vivo assays. *Cells*- CF bronchial epithelial CuFi-1 cells were grown as previously described [17]. Primary airway epithelial cells, i.e., mainstem human bronchi, derived from CF individuals, obtained from “Servizio Colture Primarie” of the Italian Cystic Fibrosis Research Foundation were cultured as previously described [17].

Inflammatory response in vitro- Cells were infected with *P. aeruginosa* laboratory strain PAO1 as detailed [16]. As readout of the inflammatory response, IL-8 mRNA expression was measured by Real-time qPCR, as described [17].

Toxicity and apoptosis- Annexin V and Dead Cell assays were performed with the Muse cell analyzer (Millipore, Billerica, MA, USA) method, as described [15]. Data were acquired and recorded utilizing the Annexin V and Dead Cell Software Module (Millipore, Billerica, MA, USA). Cell counting and viability assays were carried out with the automated Muse cell analyzer method. Data from samples were acquired and recorded utilizing the Count & Viability Software Module.

Inflammatory response in vivo- C57Bl/6NCr male mice were treated by gavage 24 and 1 hour before infection with 10 mg/kg or 100mg/kg **1** or *ent*-**1** or *rac*-**1**. 6 hours after infection, mice were killed with an overdose of carbon dioxide, and murine lungs tested for lung infection, and BAL processed for inflammation as described [41].

Statistics- Statistical analysis was carried out withone-way ANOVA, using Prism 6.0 Software (GraphPad Software, San Diego, CA). *P<0.05, **P <0.01, ***P <0.001and ****P <0.0001).

Conflicts of interest

The authors have no competing interests to declare and have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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Graphical abstract