Assessing the Potential of N-Butyl-\(L\)-deoxynojirimycin (\(L\)-NBDNJ) in Models of Cystic Fibrosis as a Promising Antibacterial Agent

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Cystic Fibrosis (CF; OMIM #219700) is a lethal and autosomal recessive disorder occurring in approximately 1 in 3,000–4,000 live births among Caucasian populations.\(^1\,2\) CF is caused by mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene that encodes a chloride and bicarbonate transporter, primarily located on the apical membrane of an epithelial cell.\(^3\) CFTR glycoprotein is the only known member of the large ATP-binding cassette (ABC) family that functions as an ion channel rather than as a pump and is responsible for the proper flow of chloride and other ions in and out of the cell membranes, in the lungs, and other organs. Even if more than 2100 CFTR mutations\(^4\) and polymorphisms have been detected to date, deletion of phenylalanine at position 508 (F508del), located in the first nucleotide-binding domain (NBD1) of the CFTR protein, is responsible for most CF cases and causes CFTR misfolding and retention in the endoplasmic reticulum quality control, rapid degradation by the ubiquitin-proteasome pathway, as well as defective channel function.\(^5\) CF is a multisystem disease involving many organs; however, the leading cause of morbidity and mortality in people with CF (pwCF) remains the respiratory disease.\(^6,7\) Subnormal CFTR activity mainly leads to dehydration of the airway surface liquid, accumulation of thick mucus, and decreasing of mucociliary clearance that promote the onset of a vicious cycle of airway inflammation and of recurrent and chronic bacterial infections.\(^8\) The CF airway inflammation is characterized by an excessive production of proinflammatory mediators, such as TNF-\(\alpha\), GM-CSF, G-CSF, IL-1\(\beta\), IL-6, and especially IL-8, mainly secreted by airway epithelial cells, and the presence of a massive number of neutrophils and macrophages. This dysregulated and excessive immune response in CF, together with the persistent bacterial infections, damages the lung tissues eventually leading to respiratory failure.\(^9,10\) Numerous attempts have been recently made to identify novel therapeutic agents, targets, and strategies to manage the exaggerated immune response and bacterial infections in pwCF without interfering with the natural host defenses.\(^11\,–\,13\)
decades of research, to date, high-dose ibuprofen remains the only anti-inflammatory protocol in CF, even if its use is limited by the onset of serious and numerous side-effects.\textsuperscript{14,15} On the other hand, although numerous anti-infective agents are nowadays available, the evolutionary pressure of bacteria to develop resistance hampers their effective use to treat and eradicate bacterial infection. A recent approach by using agents able to target and interfere with crucial pathogenicity factors or virulence-associated traits of the bacteria seems to offer a promising therapeutic alternative to conventional antibiotics.\textsuperscript{16} Accordingly, development of novel, safe, and effective anti-inflammatory and anti-infective agents remains an unmet need for ensuring therapeutic benefits to patients with more advanced stages of lung disease and in those for whom CFTR modulators are not appropriate or effective.

Iminosugars are sugar analogues in which an amino function replaces the endocyclic oxygen atom of carbohydrate skeleton.\textsuperscript{17} Due to their ability to interact with carbohydrate-processing enzymes, they have been identified as attractive therapeutic candidates for the management of several rare diseases, including CF.\textsuperscript{18,19} In this context, iminosugars have been investigated either as CFTR correctors or as agents able to control the onset of inflammatory events and bacterial infections characterizing CF lung disease. Particularly, the iminosugar drug D-NBDNJ (\(\text{N-butyl-D-deoxynojirimycin, Miglustat, 1}\), Figure 1), approved for the treatment of type I Gaucher and Niemann–Pick type C disease, has been identified as the first candidate able to restore CFTR functions \textit{in vitro} and \textit{in vivo}.\textsuperscript{20–22} Moreover, D-NBDNJ was able to significantly reduce the inflammatory response to \textit{Pseudomonas aeruginosa}, both in human bronchial epithelial cells \textit{in vitro} and in murine models of lung infection \textit{in vivo}, inhibiting the nonlysosomal \(\beta\)-glucosylceramidase (NLGase), an enzyme involved in the metabolism of sphingolipids (SLs).\textsuperscript{24,25}

Starting from the example of D-NBDNJ, other iminosugars have been evaluated for application in CF.\textsuperscript{18} In the frame of our studies aimed to explore the pharmacological properties of biomimetic compounds,\textsuperscript{26–32} we highlighted the role of sugar chirality\textsuperscript{3,34} on the anti-inflammatory and antibacterial properties of iminosugars. Particularly, our studies revealed the ability of unnatural \(\text{L-glucosyl-configured N-alkyl-deoxyminosugars to act as NLGase inhibitors, although less efficiently than their D-enantiomers and to strongly reduce the inflammatory response induced by \textit{P. aeruginosa in vitro}, either alone or in synergistic combination with their D-enantiomers.}\textsuperscript{35} This anti-inflammatory effect was confirmed in \textit{P. aeruginosa} acutely infected C57Bl/6NCr mice after treatment with L-NBDNJ (\textit{ent-1, Figure 1}), the enantiomer of D-NBDNJ (1).

Figure 1. D- and L-NBDNJ.

| Scheme 1. Synthesis of L-NBDNJ (\textit{ent-1}) by a Carbohydrate-Based Route |

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>1.</td>
<td>\text{AllOH, BF}_3\cdot\text{OEt}_2</td>
</tr>
<tr>
<td>2.</td>
<td>\text{BnBr, NaH}</td>
</tr>
<tr>
<td>3.</td>
<td>\text{PdCl}_2, 95%</td>
</tr>
<tr>
<td>4.</td>
<td>\text{BCl}_3 1M</td>
</tr>
<tr>
<td>5.</td>
<td>\text{LiAIH}_4</td>
</tr>
<tr>
<td>6.</td>
<td>\text{CH}_2\text{Cl}_2/\text{DMSO, TEA}</td>
</tr>
<tr>
<td>7.</td>
<td>\text{AcOH, NaBH}_3CN</td>
</tr>
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</table>

Particularly, oral treatment by gavage with \textit{ent-1} at a much lower dosage (40-fold) than that of its \(\text{D-counterpart reduced the recruitment of neutrophils in this murine model of pulmonary acute infection.}\textsuperscript{33} Inhibition of NLGase activity by \textit{ent-1} was also found \textit{in vivo}, confirming the participation of NLGase in the inflammatory response to \textit{P. aeruginosa} infection.\textsuperscript{34} This effect did not fully explain the observed anti-inflammatory activity, and the involvement of other targets could not be excluded. Eventually, while \textit{ent-1} did not work as the inhibitor of the most common glycosidases, thus being more selective compared to its \(\text{D-enantiomer, its action was efficacious toward enzymes involved in other rare diseases such as Pompe and Sanfilippo type B lysosomal diseases.}\textsuperscript{36,37}

Inspired by these findings, herein, we further investigated the anti-inflammatory properties of \(\text{L-NBDNJ}\) and its potential anti-infective activity in CF, using a murine model of \textit{P. aeruginosa} chronic infection that closely resembles the complexity of human CF lung disease.\textsuperscript{38,39} Particularly, recruitment of inflammatory cells, bacterial load, and cytokine/chemokine profiles have been explored. Further studies have been devoted to the analysis of the hydrolase activity and proteomic changes induced by \(\text{L-NBDNJ}\) in the tissues of \textit{P. aeruginosa}-infected mice. \textit{In vitro} anti-infective activity and the potential ability of \(\text{L-NBDNJ}\) to correct F508del-CFTR activity were also evaluated. Finally, the effect of \(\text{L-NBDNJ}\) on gene expression of the main virulence factors of the \textit{P. aeruginosa} strain was evaluated by RT-qPCR. All of these studies have contributed to suggest mechanistic insight about the observed antibacterial activity of \textit{ent-1}.

### RESULTS

**Chemistry.** The synthesis of \(\text{L-NBDNJ}\), an unnatural enantiomer of \(\text{D-NBDNJ}\), has been performed through a carbohydrate-based route using \(\text{i-glucose (2)}\) as a chiral building block to obtain the iminosugar with the desired \(\text{l- and gluco-configuration (Scheme 1).}\)

As previously described by us,\textsuperscript{16} \(\text{2}\) was first converted into the corresponding allyl glucoside (\text{AllOH/BF}_3\cdot\text{OEt}_2), which was then reacted under standard conditions with \text{NaH/BrBr} to
mask the free hydroxyl groups with a benzyl ether function. The allyl group at the anomeric position was then removed (PdCl\textsubscript{2}) to afford the 2,3,4,6-tetra-O-benzyl-L-glucopyranose\textit{3} in 95% yield. With 3 in hand, LiAlH\textsubscript{4}-mediated reduction and subsequent Swern oxidation [(COCl)\textsubscript{2}/DMSO, then Et\textsubscript{3}N], followed by reductive amination [butylamine/AcOH/NaBH\textsubscript{3}CN], allowed us to obtain the protected N-buty-L-deoxynojirimycin\textit{4}. Notably, differently from our previous reports,\textit{36} here\in, the use of butylamine as a source of nitrogen for the reductive amination allows to introduce an already alkylated amino function in the ring. Eventually, treatment of 4 with 1 M BCl\textsubscript{3} in DCM at 0 °C gave access to the desired L-NBDNJ as a hydrochloride salt in 94% yield.

**Effect of L-NBDNJ in Murine Models of \textit{P. aeruginosa} Chronic Infection.** Our previous studies\textit{35} revealed the ability of L-NBDNJ to decrease the number of neutrophils recruited in the bronchoalveolar lavage fluid (BALF) of C57Bl/6NCr mice acutely infected by \textit{P. aeruginosa}. Importantly, the decrease in the recruitment of neutrophils did not increase the bacterial load in the airways; instead, it was accompanied by a dose-dependent decrease of bacteria within the lungs. To evaluate the potential anti-inflammatory efficacy of L-NBDNJ in CF, herein, it was tested in the mouse model of chronic lung infection that mimics the advanced stage of lung pathology in humans. The chronic infection in these models is typically established by including \textit{P. aeruginosa} CF-adapted variants in immobilizing agents, where they appear to grow in microcolonies under microaerobic/anaerobic conditions,\textit{40} similar to the mucus of pwCF. For the chronic infection, \textit{P. aeruginosa} MDR-RP73 clinical strain embedded in agar beads was prepared as described.\textit{39} 24 h before the infection, C57Bl/6NCr male mice were treated with 10 or 100 mg/kg L-NBDNJ (in water) by oral gavage. Mice were treated for 6 days and monitored daily for body weight.

![Figure 2](image-url) Effect of L-NBDNJ on the inflammatory response and bacterial burden in a murine model of \textit{P. aeruginosa} chronic infection. C57Bl/6NCr male mice were infected with \textit{P. aeruginosa} MDR-RP73 embedded in agar beads by intratracheal inoculation. Treatment with 10 and 100 mg/kg L-NBDNJ (in water) or vehicle (water) by oral gavage was started 24 h before and continued daily for 6 days after the infection. Total cells (a), number of neutrophils recruited in BALF (b), and total lung CFU 6 days after infection (c). Data are presented as mean ± SEM pooled from three independent experiments (\textit{n} = 22). One way ANOVA with Dunnett’s post test. *\textit{p} < 0.05; **\textit{p} < 0.01.

![Figure 3](image-url) Effect of L-NBDNJ on the hydrolase activity in the lung of a murine model of \textit{P. aeruginosa} acute infection. Activities of GCase (a), NLGase (b), β-galactosidase (c), and hexosaminidase (d) in the lung homogenate of C57Bl/6NCr mice infected with \textit{P. aeruginosa} PAO1 after oral treatment by gavage with L-NBDNJ (10 and 100 mg/kg). Data are presented as mean ± SD; for each condition, eight lungs in technical triplicate were analyzed. Student’s \textit{t}-test. *\textit{p} < 0.01; **\textit{p} < 0.001; ***\textit{p} < 0.0001.
Inflammatory cells and bacterial load (Figure 2) were estimated in the lung homogenate and in BALF 6 days post-infection. No significant differences in the recovery of body weight were found in mice treated with \( \text{L-NBDNJ} \) compared to mice treated with the vehicle (Figure S1). As shown in Figure 2, a trend toward the reduction of leukocyte recruitment in the BALF of mice treated with \( \text{L-NBDNJ} \) was observed. Although the total cells (Figure 2a) and neutrophils (Figure 2b) showed a dose-dependent decrease, this reduction did not reach statistical significance. These findings align with the results obtained in murine models of acute \( P. \text{aeruginosa} \) lung infection. To verify that the reduction of inflammatory cells in chronically infected mice does not impair the host defense or exacerbate the infection, bacterial load was evaluated in the

**Figure 4.** Effect of \( \text{L-NBDNJ} \) on the hydrolase activity in the lung of a murine model of \( P. \text{aeruginosa} \) chronic infection. Activities of GCase (a), NLGase (b), \( \beta \)-galactosidase (c), and hexosaminidase (d) in the lung homogenate of C57Bl/6NCr mice infected with \( P. \text{aeruginosa} \) MDR-RP73 after repeated oral treatment by gavage with \( \text{L-NBDNJ} \) (10 and 100 mg/kg). Data are presented as mean \( \pm \) SD; for each condition, eight lungs in technical triplicate were analyzed. Student’s t-test.

**Figure 5.** Filtering steps for the selection of a data set of significant proteins displaying a fold change >1.3 and <0.77 (V versus CTRL, 10 mg/kg \( \text{L-NBDNJ} \) versus V, and 100 mg/kg \( \text{L-NBDNJ} \) versus V) (a). Visualization of the number of up- or downregulated proteins (b). Heatmap hierarchical clustering of proteins detected along the biological replicates (each value is a result of two biological replicates and two technical replicates) (c).
A significant and substantial decrease in bacteria recovered from the airways of mice treated with both doses of L-NBDNJ was observed, thus indicating an ameliorating effect of ent-1 on infection (Figure 2c). This reduction correlated with an increase in bacterial clearance (Figure S2). To further assess the effect of L-NBDNJ on the host inflammatory response to *P. aeruginosa*, cytokine/chemokine/growth factor levels were measured in the supernatant of lung homogenates. Treatment of mice with 10 and 100 mg/kg L-NBDNJ showed a moderate reduction of some chemokines and cytokines activated by infection (Table S1).

### Hydrolase Activity in the Lung of *P. aeruginosa*-Infected Mice after Treatment with L-NBDNJ

Several studies indicated that modulation of sphingolipid (SL) metabolism can be used to control the inflammatory response in CF, therefore highlighting attractive therapeutic applications of iminosugars in the CF lung disease. Hence, herein, we evaluated the inhibitory effect of L-NBDNJ on the activity of SL hydrolases in murine models of *P. aeruginosa* acute and chronic infection (Figures 3 and 4). C57Bl/6NCr mice were treated as previously described and as reported above, and the activity of NLGase and other enzymes involved in SL metabolism including β-glucocerebrosidase, β-galactosidase, and β-hexosaminidases was evaluated in the lung homogenate.

As shown in Figure 3, L-NBDNJ influenced the activity of all of the enzymes considered in the lung of acutely infected mice. Particularly, contrary to the results obtained by *in vitro* assays, the activity of GCase was significantly increased (Figure 3a), whereas those of NLGase, β-galactosidase, and hexosaminidase (Figure 3b–d) were inhibited. Different results were observed in the murine model of *P. aeruginosa* chronic infection (Figure 4) as no influence of L-NBDNJ on the activity of the selected enzymes was observed except for a slight inhibition of NLGase at a 10 mg/kg dose (Figure 4b). Overall, these data strengthen our hypothesis on the

**Table 1. String Analysis for a Data set of Samples Treated with 10 and 100 mg/kg L-NBDNJ Doses versus V by 3 k-Means Clustering**

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<thead>
<tr>
<th>treatment</th>
<th>node color</th>
<th>node number</th>
<th>edge number</th>
<th>expected edge number</th>
<th>PPI enrichment p-value</th>
<th>total protein number</th>
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<td>10 mg/kg</td>
<td>red</td>
<td>65</td>
<td>189</td>
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<td>&lt;1.0 × 10⁻¹⁶</td>
<td>146 (45%)</td>
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<td></td>
<td>green</td>
<td>36</td>
<td>43</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>blue</td>
<td>42</td>
<td>40</td>
<td>8</td>
<td>1.11 × 10⁻¹⁶</td>
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</tr>
<tr>
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<td>32</td>
<td>8</td>
<td>1.62 × 10⁻¹⁰</td>
<td>117 (30%)</td>
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<td>green</td>
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<td>84</td>
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<td>&lt;1.0 × 10⁻¹⁶</td>
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</tr>
<tr>
<td></td>
<td>blue</td>
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<td>25</td>
<td>6</td>
<td>2.17 × 10⁻⁸</td>
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<tr>
<td>10 mg/kg</td>
<td>red</td>
<td>58</td>
<td>89</td>
<td>23</td>
<td>&lt;1.0 × 10⁻¹⁶</td>
<td>177 (55%)</td>
</tr>
<tr>
<td></td>
<td>green</td>
<td>62</td>
<td>128</td>
<td>33</td>
<td>&lt;1.0 × 10⁻¹⁶</td>
<td></td>
</tr>
<tr>
<td></td>
<td>blue</td>
<td>54</td>
<td>88</td>
<td>27</td>
<td>&lt;1.0 × 10⁻¹⁶</td>
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<tr>
<td>100 mg/kg</td>
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<td>90</td>
<td>522</td>
<td>114</td>
<td>&lt;1.0 × 10⁻¹⁶</td>
<td>266 (70%)</td>
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<td></td>
<td>green</td>
<td>96</td>
<td>249</td>
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<td>&lt;1.0 × 10⁻¹⁶</td>
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<tr>
<td></td>
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<td>98</td>
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<td>&lt;1.0 × 10⁻¹⁶</td>
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involvement of other targets in addition to the modulation of SL metabolism underlying the observed anti-inflammatory activity of l-NBDNJ.

**Proteomic Studies.** A label-free quantification was performed for proteomic studies to observe changes in the abundance of significant proteins in the supernatant of lung homogenates of the non-infected (naïve) C57Bl/6NCr mice (control, CTRL) and by *P. aeruginosa* MDR-RP73-infected mice in the absence (vehicle, V) or presence of 10 and 100 mg/kg doses of l-NBDNJ. From 2912 proteins identified by MaxQuant software, a data set of 1888 proteins were figured out (Figure 5a), following the filtering process reported in the Experimental Section. Comparing samples from mice infected with *P. aeruginosa* MDR-RP73 (V) and noninfected C57Bl/6NCr mice (CTRL), 968 proteins (51%) displayed a significant *p*-value and fold changes higher than 1.3 or lower than 0.77 (V versus CTRL, Figure 5a). On the other hand, 322 (17%) and 383 (20%) proteins were significantly dysregulated in samples treated respectively with 10 and 100 mg/kg l-NBDNJ compared to the vehicle (10 mg/kg l-NBDNJ versus V and 100 mg/kg l-NBDNJ versus V, Figure 5a). The list of significant proteins is included in the Supporting Information for the first (V versus CTRL, Table S5), second (10 mg/kg l-NBDNJ versus V, Table S6), and third data set (100 mg/kg l-NBDNJ versus V, Table S7), including the *p*-values obtained by Student’s *t*-test performed by Perseus software. As shown in the histogram (Figure 5b), an upregulation of the most abundant proteins was mainly detected in infected mice compared to the naïve ones (V versus CTRL), while downregulation was mostly observed after treatment with l-NBDNJ (10 mg/kg l-NBDNJ versus V and 100 mg/kg l-NBDNJ versus V; Figure 5b). The hierarchical clustering heatmap performed by using Perseus highlighted a good sample correlation among all samples, suggesting that a higher dysregulation was observed after the infection with *P. aeruginosa* independently from the treatment with l-NBDNJ (Figure 5c). Moreover, proteins belonging to the three data sets were uploaded on the String software to pick up the most significant pathways where the upregulated or downregulated proteins were involved.

The data displaying significant changes after the treatment with 10 and 100 mg/kg l-NBDNJ doses versus V were summarized in Table 1, including the number of nodes and edges of 3 k-means clustering defined by each protein–protein interaction (PPI) enrichment *p*-value. The complete String analysis is reported in Table S8. As shown in Figure 6, pathways associated with the regulation of interleukin-l-mediated signaling, of type I interferon production, or of leukocyte-mediated immunity were significantly upregulated only in infected mice (vehicle) compared with the naïve ones (CTRL). Otherwise, no significant pathways were associated with chemokine production or immune system response in samples of mice treated with both doses of l-NBDNJ compared to the vehicle-treated mice, suggesting that iminosugar administration did not affect the dysregulation of such proteins as previously demonstrated (see above). As an example, 12 proteins were reported as a histogram to monitor the changes in abundance along all of the samples (Figure 6).

Interestingly, when the data set of samples from mice treated with l-NBDNJ (both 10 and 100 mg/kg doses) was compared to those from untreated infected mice (vehicle), other significant pathways resulted to be greatly affected. Indeed, among 43 proteins involved in cytoskeleton organization, 63%
of them were upregulated and 37% were downregulated following iminosugar treatments (Table S9). All of the structural proteins such as myosins, actins, troponins, laminins, and calponins (MYH6, MYH11, MYH9, MYH10, MYO1C, ACTS, ACTN4, ACTN1, TNNT3, LMNB1, and CNN2) were strongly downregulated following P. aeruginosa infection (V), whereas they were upregulated after treatment with L-NBDNJ, suggesting that the compound acted to counteract the damage caused by the infection, restoring the conditions prior to P. aeruginosa infection (CTRL) (Figure 7a). As an example, MYH11 and MYH9 expression levels were reported in Figure 8B, showing that the treatment with 10 mg/kg L-NBDNJ was greatly useful to restore MYH11 protein expression to a similar level to the control sample. On the other hand, an increase of MYH9 protein to levels higher than those in the control was highlighted in samples treated with 100 mg/kg L-NBDNJ dose (Figure 7b). Instead, for 16 proteins mostly involved in the regulation of cytoskeleton assembly, an upregulation was observed following P. aeruginosa infection, whereas a strong downregulation was observed as a consequence to both 10 and 100 mg/kg L-NBDNJ treatments (Table S9). In a total of 44 proteins, most of them displayed a dose-dependent dysregulation as a result of drug stimulation except for some, e.g., CISY for which the dose of 10 mg/kg resulted to be higher than of 100 mg/kg. This peculiar behavior could suggest a saturation mechanism triggered by L-NBDNJ so that the increase of the pharmacological dose did not induce further increment in abundance for that protein.

**In Vitro Antibacterial and Antibiofilm Activity of L-NBDNJ against P. aeruginosa.** Based on the results about the ability of L-NBDNJ to reduce the bacterial load and to increase the bacterial clearance in mice infected by P. aeruginosa, the antibacterial properties of L-NBDNJ were investigated evaluating the effect on bacterial growth of planktonic and sessile P. aeruginosa cells. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against P. aeruginosa MDR-RP73 and five clinical isolates from pwCF (PA01018, PA66148, PA66148, PA90553, PA90224) were determined by broth microdilution assay.

Data revealed that L-NBDNJ did not exert any appreciable inhibition on the planktonic growth for all of the strains considered up to the concentration of 1 mg/mL (see the Supporting Information for details). In subsequent experiments, the combined effect of L-NBDNJ with conventional antibiotics was also investigated to determine any synergistic, additive, or antagonistic interactions. Antibacterial activity was tested in combination with tobramycin, ciprofloxacin, and amikacin against P. aeruginosa MDR-RP73 or PA90224 isolates using broth microdilution checkerboard assay. No interactions between L-NBDNJ and antibiotics were observed, suggesting that it does not influence their effectiveness against P. aeruginosa infections (Table S2). The effect of L-NBDNJ on the biofilm formation of P. aeruginosa MDR-RP73 and clinical isolate PA90224 was eventually evaluated. As shown in Figure S3, subinhibitory concentrations of L-NBDNJ were not able to affect biofilm formation of both the considered strains compared to untreated control.

**Antivirulence Activity of L-NBDNJ.** As L-NBDNJ was not able to alter the in vitro viability of P. aeruginosa, but its antibacterial activity was clearly detected in vivo, we wanted to verify whether this activity could be explained by a different mechanism of action. Therefore, the effect of L-NBDNJ on gene expression was evaluated by RT-qPCR analysis of the MDR-RP73 strain. The gene expression of virulence factors, encoding extracellular enzymes, toxins, and quorum-sensing signals of the bacteria, in a planktonic state in the presence or absence of 1 mg/mL L-NBDNJ, were compared. The results are summarized in Figure 8 and Tables S3 and S4. Only genes showing an absolute fold change in transcript equal to or greater than 2 and a p-value less than 0.05 were considered as having a significant change in the transcript level in response to L-NBDNJ. Most interestingly, the obtained results showed that the genes encoding for main exotoxins were downregulated, with fold changes of −2.414, −5.011, and −2.031 observed for exoT, exoS, and exoY, respectively. The expression of lasI was downregulated with a fold change of −2.525. Another interesting regulation change was the decreased expression of genes encoding for the main pigments of P. aeruginosa, as pydA and phzA that had fold changes of −2.173 and −2.461, respectively. A downregulation of alkaline protease (aprA) and hydrogen cyanide synthase (hcnA) genes was noted, with fold changes of −2.456 and −5.135, respectively.

**Cell Adherence of P. aeruginosa MDR-RP73 after Treatment with L-NBDNJ.** Eventually, the effect of L-NBDNJ on the adhesion of the pathogenic strain to the host cells was evaluated. Therefore, human cell adhesion/invasion assays were performed using human type 2 pneumocyte A549 cells infected by pretreated (L-NBDNJ) or untreated P. aeruginosa MDR-RP73. After removal of nonadherent bacteria, A549 cells...
were lysed and analyzed by colony counts to determine the number of viable bacterial cells (both adherent and internalized). Our results showed that the adhesion efficiency of *P. aeruginosa* MDR-RP73 was significantly affected by treatment with L-NBDNJ (Figure 9) being reduced by roughly 5-fold compared to that of untreated bacteria.

**Figure 9.** Effect of L-NBDNJ (1 mg/mL) on the adherence of *P. aeruginosa* MDR-RP73 to A549 human alveolar epithelial cells. Experiments were carried out in triplicate and repeated 3 times. The mean value is shown with standard deviation. ***p < 0.001.

**Evaluation of L-NBDNJ as a CFTR Corrector.** Over the last years, iminosugars have also been evaluated in CF as pharmacological correctors of mutant CFTR. With the iminosugar drug D-NBDNJ as the first candidate showing the ability to rescue F508del-CFTR function *in vitro* and *in vivo* by inhibition of α-glucosidase I and II of the endoplasmic reticulum (ER), even though this activity was then not confirmed in clinical trials. Based on these data, we wondered whether L-NBDNJ could have the ability to correct F508del-CFTR activity. Tests were performed on CF bronchial epithelial cells CFBE41o- expressing F508del-CFTR and using the halide-sensitive yellow fluorescent protein assay (HS-YFP). Before the assay, cells were treated for 24 h with vehicle (DMSO) or VX-809 corrector (1 μM), taken as negative and positive controls, respectively, and with L-NBDNJ (5, 20, 50, 100 μM), with and without VX-809 (Figure 10).

As shown in Figure 10, when L-NBDNJ was considered alone, no significant correction activity was observed compared to that of the vehicle-treated cells. A modest improvement in the CFTR activity was observed only when L-NBDNJ was used in combination with the VX-809 corrector, with a significant increase, compared to VX-809 alone, by using 20 and 100 μM L-NBDNJ.

**Figure 10.** Evaluation of L-NBDNJ as a CFTR corrector. F508del-CFTR activity was determined with the HS-YFP assay in CFBE41o-cells treated for 24 h with vehicle alone (DMSO), VX-809 (1 μM), or L-NBDNJ (5, 20, 50, 100 μM) plus/minus VX-809. Bars report the average and SD of the quenching rate (QR) from multiple experiments. \(*\ast p < 0.01; \ast\ast p < 0.001 vs\) VX-809 alone (Kruskal-Wallis and Dunn’s tests).

**DISCUSSION**

Over the past years, the therapeutic potential of iminosugars in CF has been investigated either for their use as correctors of defective CFTR mutants or as anti-inflammatory agents for the symptomatic treatment of CF lung disease with the drug D-NBDNJ as the first candidate to be evaluated. In this context, we recently highlighted the anti-inflammatory properties of an unnatural mirror image of D-NBDNJ, i.e., L-NBDNJ and its congeners revealing their ability *in vitro* to reduce the inflammatory response to *P. aeruginosa*. This effect was validated *in vivo* for L-NBDNJ in a murine model of *P. aeruginosa* acute infection pointing out its ability to significantly reduce the recruitment of neutrophils in BALF of infected mice without increasing the bacterial load at a lower dosage than in its enantiomer D-NBDNJ. Moreover, an interesting antibacterial activity was also observed for some derivatives of this class of glycomimetics. On this basis, in this work, we further evaluated the therapeutic potential of L-NBDNJ as an anti-inflammatory/antibacterial agent for application in CF lung disease treatment. Herein, the effect of L-NBDNJ was evaluated in a murine model of *P. aeruginosa* chronic infection that mimics the advanced stage of CF lung disease, revealing a dose-dependent tendency to reduce the neutrophils recruited in the airways of mice. This action was accompanied by an unexpected significant decrease in the level of bacteria recovered in the lung along with an increase in the bacterial clearance. These findings suggested a potential anti-inflammatory effect of L-NBDNJ associated with a modulation of pulmonary inflammation. The action of L-NBDNJ on the production of inflammatory mediators and hydrolase activity in the lung of infected mice was also studied, showing no significant changes in cytokine/chemokine levels, as determined by both immunoassays and proteomic studies. Regarding hydrolase activity, our previous *in vitro* data on the cell lysate revealed that L-NBDNJ exhibited a selective inhibitory activity toward NLGase, an important player in the regulation of SL homeostasis. From *in vivo* studies carried out herein, two different outcomes were highlighted. The activity of the major glycohydrolases involved in sphingolipid metabolism was significantly reduced, even if to a slight extent, in the acute phase of infection by treatment with L-NBDNJ, whereas the level of GCase activity was increased. These effects were lost when lung samples from chronically infected mice were analyzed. Different results obtained from *in vitro* and *in vivo* studies are not surprising, since the metabolic fate and compartmentalization of the molecule administered to living tissues are unknown. Future analyses will therefore be aimed at studying the role of L-NBDNJ in the control of the inflammatory response in CF through modulation of the hydrolases involved in sphingolipid (SL) metabolism.

The observed *in vivo* antibacterial activity prompted us to carry out further experiments that could be useful to elucidate the mechanism of action with which this molecule acts. Indeed, there are only few examples of L-iminosugars endowed of antimicrobial properties, even for the most well-known D-iminosugars, for which there are no clear indications about the molecular targets involved.

In this context, an untargeted proteomic approach has been chosen as this type of analysis gave a broader view of the changes, induced by the drug treatment, in the relative...
abundances of the totality of proteins in the sample. Data obtained from proteomic analysis of mouse lung homogenate following the infection with *P. aeruginosa* revealed a marked dysregulation of many metabolic pathways compared to those following the infection with *P. aeruginosa* obtained from proteomic analysis of mouse lung homogenate. Data were strongly downregulated in the vehicle, whereas their abundances of the totality of proteins in the sample. Furthermore, we also performed in vitro studies to assess the effect of L-NBDNJ on *P. aeruginosa* MDR-RP73 and other clinical isolates. As highlighted by MIC values, L-NBDNJ was not able to inhibit the growth of *P. aeruginosa*, thus excluding any bacteriostatic/bactericidal activity. Similarly, no inhibition of biofilm formation was observed. On these bases, to explain the observed in vivo anti-inflammatory activity, we reasoned whether an antivirulence activity of L-NBDNJ could be invoked thanks to which only virulence-associated traits are targeted without interfering with bacterial survival or fitness. RT-qPCR analysis revealed that L-NBDNJ was able to downmodulate *P. aeruginosa* virulence factors involved in the host response, suggesting it could facilitate the key host defense mechanism playing a protective role against bacterial attack. Indeed, after treatment with L-NBDNJ, we observed a significant reduction of gene expression of *apmA*, encoding for alkaline protease, a zinc-dependent metalloprotease, that inactivates C1q, C2, and C3 complement system components and cytokines as interferon-γ, IL-6, and TNF-α, useful to bacteria to escape from phagocytes.52,53 The secretion of alkaline protease also reduced airway surface liquid volume and mucociliary clearance of pulmonary bacteria.54 Furthermore, in pwCF, the decrease in sputum alkaline protease titer has been correlated with an improvement in clinical prognosis. Another relevant virulence factor in pwCF correlated with a worsening prognosis is the cyanide production, a potent poison that can inhibit the respiratory electron transport chain and several metalloenzymes (e.g., catalase, peroxidase, superoxide dismutase) of eukaryotic cells.55 We have found that MDR-RP73 cells treated with L-NBDNJ expressed 5-fold lower levels of the *hcnA* gene, encoding hydrogen cyanide synthase, compared to nontreated cells. Therefore, the reduction of cyanide production could improve lung function by limiting tissue destruction. Moreover, *P. aeruginosa* uses a large repertoire of exoenzymes to cause the disease.56 Indeed, high concentrations of the greenish-blue pigment pyocyanin, encoded by the *phzA* gene and involved in neutrophil apoptosis,57 assist *P. aeruginosa* to evade the immune response, causing long periods of pulmonary exacerbation in pwCF.58 After treatment with L-NBDNJ, the expression of this gene decreased 2.5-fold. Also, L-NBDNJ treatment resulted in the downregulation of pyoverdine gene (*pvdA*), the major fluorescent yellow-green siderophore produced and secreted by *P. aeruginosa*, that provides the bacterium with iron during infection.59 The RT-qPCR results also revealed that three toxic effectors injected via T3SS genes were significantly downregulated upon L-NBDNJ exposure, namely, *exoS*, *exoT*, and *exoY*. These cytotoxins can cause cell death and also affect host defense by blocking phagocytosis and bacterial clearance.60 *ExoS* is the main effector protein involved in the escape of *P. aeruginosa* from the phagosome to the cytoplasm and is required for macrophage lysis.61 Both ExoS and ExoT also promote bacterial survival in neutrophils favoring *P. aeruginosa* persistence in host tissues.62 *ExoY* inhibits the expression of proinflammatory genes including cytokines, chemokines, and other inflammatory mediators during *P. aeruginosa* infection.63 In addition, these exoenzymes play a key role in the pathogenesis of *P. aeruginosa* infection by interfering with cytoskeletal regulation. Particularly, they are known to target cytoskeleton components of host cells causing morphological changes and leading to loss of barrier integrity.64,65 The downregulation of *exoS*, *exoT*, and *exoY* genes achieved after L-NBDNJ treatment could therefore reduce bacterial-induced tissue damage. These results are in agreement with those obtained by proteomic analysis, showing a beneficial effect by L-NBDNJ on the regulation of metabolic paths involved in cytoskeleton assembly and organization. In addition, a decreased adherence efficiency of *P. aeruginosa* to host cells was also induced by L-NBDNJ that, together with the antivirulence effect, could contribute to explain the antiinfective activity observed in vivo in the absence of a bacteriostatic/bactericidal effect in vitro. As many glycometabolisms have shown to be able to reduce pathogen adhesion to the host cell,54,66 acting directly against the pathogen itself or the host cell, even for L-NBDNJ a possible role as antiadhesive agent could be recognized as responsible of the observed reduction in virulence factors. Further adherence experiments could be helpful to clarify the exact mechanism of action.

Eventually, based on previous reports on D-NBDNJ, we also considered the possibility that L-NBDNJ is endowed with a CFTR corrector activity, i.e., the ability to promote F508del-CFTR escape from degradation and to promote its trafficking to the plasma membrane. We found no effect with L-NBDNJ alone. A modest effect on CFTR rescue was only observed when L-NBDNJ was tested in combination with VX-809, a known F508del-CFTR corrector.

**CONCLUSIONS**

Our previous investigations highlighted the potential anti-inflammatory properties of L-NBDNJ in murine models of *P. aeruginosa* acute infection; instead, following the current study, the same iminosugar exhibited a promising antibacterial effect in models of chronic infection. Particularly, the synthesis of L-NBDNJ has been herein realized by a carbohydrate-based route, and in vitro and in vivo assays have been carried out to assess the anti-inflammatory/antibacterial activity and to provide mechanistic insights into the observed activity. In our opinion, as bacterial growth or survival was not affected in vitro by treatment with L-NBDNJ, a bacteriostatic or bactericidal action could be excluded. On the other hand, L-NBDNJ was able to interfere with the metabolic paths involved in cytoskeleton assembly and organization of the host cell, downregulate the main virulence factors of *P. aeruginosa* involved in the host response, and affect pathogen adhesion to human cells, thus outlining a possible role of L-NBDNJ as a novel antivirulence agent. Further studies will be carried out by RT-qPCR and proteomic experiments to deeply evaluate the gene and protein expression changes induced by L-NBDNJ in vivo models of *P. aeruginosa* infection. These data and those obtained so far could concur to validate a possible employment of L-NBDNJ as an alternative candidate to treat drug-resistant bacteria and infection states for which no therapeutic solutions are still available.
**EXPERIMENTAL SECTION**

**Chemistry. General Information.** All commercially available reagents and solvents were purchased at the highest degree of purity from commercial sources and used without purification. Reactions were monitored by thin-layer chromatography analysis with F254 Merck silica gel plates, followed by exposure to ultraviolet radiation, iodine vapor, and spraying with ethanolic p-anisaldehyde solution and ninhydrin. Compounds were purified by column chromatography with silica gel (70–230 mesh, Merck Kieselgel 60) and characterized by NMR analysis (Varian Inova 500 MHz and Bruker AVANCE 400 MHz). The purity of L-NBDNJ was assessed by absolute quantitative nuclear magnetic resonance (qNMR) experiments following the “general guidelines for quantitative 1D 1H NMR (qHNM R) experiments,” provided by the Journal of Medicinal Chemistry and was ≥95% (see the Supporting Information for details). 2,3,4,6-Tetra-O-benzyl-L-glucopyranose (3) has been synthesized as previously reported. 36

2,3,4,6-Tetra-O-benzyl-N-butyI-L-deoxynojirimycin (4). Step i: To a solution of 2,3,4,6-tetra-O-benzyl-L-deoxynojirimycin (3; 8.0 g, 14.8 mmol) 36 in anhydrous THF (84 mL), LiAlH₄ (1.1 g, 29.6 mmol) was slowly added at 0 °C and under argon atmosphere. The reaction mixture was stirred at rt, and after the complete disappearance of 2 (TLC monitoring), H₂O was slowly added until achieving a neutral pH. The suspension was extracted with EtOAc and washed with brine. The organic layers were dried (Na₂SO₄) and concentrated under reduced pressure to give the corresponding glucitol (8.0 g, 14.8 mmol), which was used in the next oxidation step without further purification. Step ii: a solution of DMSO (5.2 mL, 74.0 mmol) in DCM (44.0 mL) was slowly added to a solution of (COCl)₂ (1.1 g, 29.6 mmol) in DCM (4.8 mL, 56.2 mmol) at 0 °C. After the complete disappearance of the starting glucitol (8.0 g, 14.8 mmol) in DCM (40 mL) at −78 °C under an argon atmosphere, the resulting solution was stirred at the same temperature for 30 min, and then a solution of the crude glucitol (8.0 g, 14.7 mmol) in DCM (40 mL) was slowly added dropwise. The resulting solution was stirred for 2 h at −78 °C. Then, triethylamine (27.0 mL, 195 mmol) was added, and the mixture was stirred for further 3 h at the same temperature. Eventually, the solution was warmed to rt, dissolved in MeOH (0.24 L), and butyamine (1.46 mL, 14.8 mmol), AcOH (0.97 mL, 16.2 mmol), and NaBH₄CN (1.37 g, 22.0 mmol) were added at 0 °C. The suspension was stirred for 16 h at rt and then the volatiles were removed under reduced pressure. The organic layers were dried (Na₂SO₄), concentrated under reduced pressure, and chromatographed over silica gel (hexane/EtOAc = 8:2) to give pure 2,3,4,6-tetra-O-benzyl-N-butyI-L-DNJ (4; 5.7 g, 67% overall yield). 1H and 13C NMR spectra were fully in agreement with those reported for its d-enantiomer. 58

N-Benzyl-L-DNJ HCl (ent-1). BCl₃ (1 M solution in DCM, 38 mL, 38.2 mmol) was added to a stirred solution of 4 (5.7 g, 9.8 mmol) in DCM (0.29 L) at 0 °C. The mixture was stirred for 12 h at the same temperature, quenched with MeOH (0.23 L) at 0 °C, and concentrated under reduced pressure. The crude residue was purified by chromatography over silica gel (DCM/MeOH = 85:15) and then converted into the corresponding hydrochloride salt by the addition of 1 M HCl (1.0 equiv). Evaporation of volatiles under reduced pressure afforded pure L-NBDNJ-HCl (ent-1) (2.4 g, 94% yield). NMR data were consistent with those reported by us elsewhere. 36 1H NMR (400 MHz, CD₃OD) δ: 4.12 (d, J = 12.4 Hz, 1H), 3.90 (d, J = 12.4 Hz, 1H), 3.73–3.64 (m, 1H), 3.60 (t, J = 9.7 Hz, 1H), 3.44 (dd, J = 4.8, 12.0 Hz, 1H), 3.41–3.34 (m, 2H), 3.19 (td, J = 5.4, 12.4 Hz, 1H), 3.05 (td, J = 9.7 Hz, 1H), 2.99 (t, J = 12.0 Hz, 1H), 1.83–1.64 (m, 2H), 1.50–1.38 (m, 2H), 1.02 (t, J = 7.3 Hz, 3H). 13C NMR (100 MHz, D₂O) δ: 76.5, 67.9, 66.8, 65.2, 54.7, 53.5, 52.5, 24.6, 19.5, 12.9. (α)D = +5.6 (c = 0.41, H₂O). Purity was ≥95% by qNMR.

**Bacterial Strains and Growth Conditions.** For in vitro assays, P. aeruginosa PAO1 strain, multidrug-resistant (MDR)-RP73 strain, and five clinical isolates of P. aeruginosa, obtained from the respiratory tract of pwCF and belonging to a collection previously established at the Department of Molecular Medicine and Medical Biotechnology (University of Naples Federico II), were used. Epidemiological features of strains were in accordance with previous publications 95-70 and no ethical approval was required for the study because there was no access to patient data. All isolates were identified by the BD Phoenix (BD Diagnostics) and confirmed by MALDI-TOF. All strains were grown in trypticase soy broth (TSB).

**Mouse Models of P. aeruginosa Chronic Infection.** Immunocompetent C57BL/6NcrlBr male mice (8 and 10 weeks; Charles River Laboratories, Calco, Italy) were used. The mice were housed under specific pathogen-free conditions in sterile cages placed in a ventilated isolator. The light cycle was set at 12 h on and 12 h off, and the ambient temperature (23 ± 1 °C) and relative humidity (40–60%) were controlled. Mice were fed with standard rodent autoclaved chow and autoclaved tap water. For the infection procedure, P. aeruginosa MDR-RP73 strain isolated from a pwCF, was embedded in agar beads as previously described. 36 Mice were anesthetized by an intraperitoneal injection of a solution of Avertin (2,2,2-tribromothanol, 97%) in 0.9% NaCl at a volume of 0.015 mL/g body weight. The mice were placed in a supine position, and the trachea was directly visualized by ventral midline exposure and intubated with a sterile flexible 22 g cannula attached to a 1 mL syringe. An inoculum of 50–70 μL of a suspension of agar beads, embedding 4–5 × 10⁶ colony-forming units (CFUs) of the MDR-RP73 strain, was implanted into the lung via cannula. After inoculation, the incisions were sutured. Mice were treated with 10 and 100 mg/kg L-NBDNJ or vehicle by gavage using flexible plastic tubes to reduce trauma (Instech Laboratories) starting 24 h before infection. The mice were weighted and treated daily for 6 days. CFUs and cell counts in the bronchoalveolar lavage fluid (BALF) were analyzed as previously described 95,71,72 at 6 days after infection (see the Supporting Information for details). Cytokine/chemokine levels were measured in the supernatant of lung homogenates. After quantification of total protein content with Bradford’s assay (Bio-RAD) to ensure the same amount of protein in all samples (700 μg/mL), cytokine/chemokine levels in supernatants of lung homogenates were analyzed using the Bio-Plex Protein Array System (Bioplex Pro Mouse Cytokine 23-Plex Immunoassay, Bio-Rad).

**Ethics Statement.** Animal studies strictly adhered to the Italian Ministry of Health guidelines for the use and care of experimental animals (protocol #733). The research involving the P. aeruginosa multidrug-resistant (MDR)-RP73 isolate from a CF individual and storage of biological materials was approved by the Ethics Commission of Hannover Medical School, Germany.

**Hydrolase Activity in Lungs.** Enzyme activities of glycohydrolases were determined in lung homogenates obtained by mechanical trituration and sonication with the...
addition of an equal volume of McIlvaine Buffer 2X (0.2 M citric acid, 0.4 M NaHPO₄, pH 6) containing a cocktail of protease inhibitors. For the evaluation of β-glucocerebrosidase (GCase) and nonlysosomal β-glucosylceramidase (NLGase) activities, aliquots of the lysates corresponding to 150 μg of proteins were preincubated for 30 min at rt in a 96-well plate with a reaction mixture composed of 25 μL of McIlvaine buffer 4X (0.4 M citric acid, 0.8 M NaHPO₄, pH 5.2) and 5 nM AMP-DNM (N-[5-(adamantan-1-yl-methoxy)-pentyl]-1-deoxy-xynojirimycin) to assay GCase or the same buffer at pH 6 containing 0.5 mM conduritol B epoxide to assay NLGase. In each case, water was added to reach a final volume of 75 μL. The enzymatic reaction was then started adding 25 μL of the specific fluorogenic substrate 4-methylumbelliferyl-β-d-glucopyranoside (MUB-β-Glc, Glycosynth) at a final concentration of 6 mM. For the activities of the β-galactosidase and β-hexosaminidase, aliquots of the lysates corresponding to 35 μg of proteins were incubated with a reaction mixture composed of 25 μL of McIlvaine buffer 4X, pH 5.2, 50 μL of the specific fluorogenic substrate 4-methylumbelliferyl-β-d-galactopyranoside (MUB-β-Gal) for β-galactosidase and 4-methylumbelliferyl-β-d-glucosaminide (MUG) for β-hexosaminidase, and H₂O to a final volume of 100 μL. Each sample has been assayed in triplicate, and as background for each enzymatic assay, wells have been set up with the same experimental conditions without the tissue lysate. Each reaction mixture has been put at 37 °C with gentle stirring for 1 h, and then 10 μL of the reaction mixture has been transferred to a black 96-well plate (Black, 96-well, OptiPlate 96 F, PerkinElmer) and added with 190 μL of 0.25 M glycine pH 10.7. Plates were analyzed through a microplate reader (Victor, PerkinElmer). Picomoles of converted substrate were calculated through interpolation on a calibration curve of the MUB standard at a known concentration. Specific activity of the enzymes was expressed as picomoles of converted substrate/h/mg of proteins.

**Proteomic Analysis. In-Solution Digestion.** Lung homogenates from seven individual subjects were pooled for each condition to obtain two biological replicates of noninfected (naïve) C57BL/6NCr mice (control, CTRL) and of mice infected by *P. aeruginosa* MDR-RP73 in the absence (vehicle, V) or presence of 10 and 100 mg/kg doses of 1-NBDNJ were subjected to an in-solution digestion protocol after the protein dosage by Bradford assay. Proteins of each sample (200 μg) were precipitated with four volumes of cold acetone, each centrifuged (10 min at 10.000 rpm), and the pellet washed twice with cold acetone. Each protein pellet was resuspended in 50 μL of lysis buffer (5% SDS, 50 mM AMBIC) and the reaction of cysteine reduction was performed by using 20 mM dithiothreitol. The reaction was carried out at 95 °C in a thermostatic bath for 10 min. After the samples were cooled, the alkylation reaction was performed by using 40 mM iodoacacetamide. The samples were incubated in the dark for 30 min at room temperature. Then, 1% phosphoric acid was added to the reaction mixture to stop the alkylation process. After centrifuging at 12.000 rpm for 5 min, the mixture was diluted with 350 μL of S-trap buffer (100 mM AMBIC in 90% MeOH and 10% H₂O) and loaded on S-trap columns. The mixture was centrifuged at 5000 rpm for 3 min and washed 3 times with 250 μL of S-trap buffer, centrifuging at 5000 rpm for 3 min after each addition. For enzymatic digestion, an AMBIC solution of trypsin (0.12 μg/μL) was added to each sample, and the mixture quickly centrifuged to allow trypsin to penetrate the S-trap membrane and to perform the protein digestion. The gathering tubes were replaced, and the reaction was conducted for 2 h at 47 °C in a thermostatic bath. After tryptic digestion, the peptides were recovered and collected by sequential washes by using different solutions: 10 mM AMBIC, 0.2% HCOOH, 50% aqACN, and 80% aqACN, both acidified with 0.5% HCOOH. The eluate was dried using a speed-vac concentrator for further mass spectrometry analysis. For each condition, two technical replicates were obtained and injected into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) apparatus.

**LC-MS/MS Analysis. Orbitrap.** The mixture was analyzed by LC-MS/MS on an LTQ-Orbitrap XL system (Thermo Fisher) equipped with a nano-LC Proxeon nanoEasy-II system. Peptides were fractionated onto a C18 reverse-phase capillary column (5 μm biosphere, 75 μm ID, 200 mm length) at a flow rate of 250 nL/min. A linear gradient from 10 to 60% of eluent B (0.2% HCOOH, 95% ACN LC-MS grade) was used over 120 min. Mass spectrometer analyses were carried out in Data Dependent Acquisition mode (DDA): from each MS scan, spanning from 300 to 1800 m/z, the five most abundant ions were selected and fragmented in collision-induced dissociation (CID) modality.

**Proteomic Data Analysis.** Raw data files were processed by using MaxQuant software (1.6.8.0 version). The following parameters were used for raw data processing: trypsin enzyme specificity, three missed tryptic cleavages, oxidation of methionine, formation of pyroGlu from N-terminal glutamine (Q) or glutamic acid (E), as variable modifications, and carbamidomethylation of cysteine (C) as a fixed modification. Identification parameters included a minimum peptide length of six amino acids and a minimum of one peptide (both razor and unique peptide). Peptide tolerance was of 10 ppm, and fragment mass tolerance was of ±0.2 Da. All proteins were filtered according to a false discovery rate (FDR) of 0.01% applied at both peptide and protein levels and a maximum peptide posterior error probability (PEP) of 1. The derived peak list generated by Quant.exe (the first part of MaxQuant) was searched using the Andromeda search engine integrated into the MaxQuant against the reviewed *Mus musculus* fasta file obtained from the UNIPROT Web site.

**Data Visualization.** MaxQuant output files were subsequently processed using Perseus (version 1.6.8.0) software platforms. An experimental design template was used to get merged replicate experiments (each data set contained two technical replicates) into a single column containing all of the proteins into every sample. Contaminants, reverse, and only identified by site hits were filtered out. Expression values of LFQ intensity were log2 transformed, and only the protein rows containing a minimum of 3 valid value were maintained within the Perseus matrix.

**String Pathways Software.** The STRING software was used to integrate all known and predicted associations between upregulated proteins, including both physical interactions as well as functional associations. A full STRING network (the edges indicate both functional and physical protein associations), a medium confidence of 0.4, and k-means clustering (the network is clustered to a specified number of clusters) were set.

**RNA Purification and Real-Time RT-PCR.** The extraction of total RNA was performed as previously described with slight modifications. Total RNA was isolated from three independent cultures by using a TRIzol kit according to the manufacturer’s instructions. The RNA concentration was
determined spectrophotometrically by using a Nanodrop instrument (Nanodrop Technologies). First-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer’s protocol. Transcript-level changes were evaluated using quantitative real-time PCR, performed on an ABI7500 FAST instrument (Applied Biosystems) and using a SYBR Green master mix (Applied Biosystems). The genes and primer sequences used for the real-time PCR analysis are listed in Table S3. The housekeeping gene, RNA polymerase sigma factor rpoD (PA0576), was used as an endogenous control. Changes in the gene expression in each sample relative to the control were analyzed by using the threshold cycle method \(2^{-\Delta\Delta C_T}\). All statistical analyses were carried out using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA). All experiments were performed at least 3 times and the results are shown as means ± SD. Differences between mean values were tested for significance by performing two-tailed Student’s \(t\)-tests. A \(p\)-value < 0.01 was considered to be statistically significant.

**Cell Adherence Assays.** Adhesion assays were carried out as previously described \(^{77}\) with the following modifications. *P. aeruginosa* MDR-RP73 from overnight cultures in TSB medium, grown in the absence of \(\nu\)-NBDNJ, were further subcultured up to 0.5 \(\text{OD}_{600}\). Subsequently, cells were treated with or without 1 mg/mL of \(\nu\)-NBDNJ for 1 h, washed twice, and resuspended in DMEM (without FBS and 1% penicillin/streptomycin) to a density of \(\sim1 \times 10^7\) CFU/mL (defined as the original bacterial CFU). AS49 human lung epithelial cells (ATCC CCL 185) were seeded into 12-well plates and grown to 90% confluence at 37°C and 5% \(\text{CO}_2\). Following infection at a multiplicity of infection (MOI) of 10:1 (2 \(\times\) 10\(^6\) bacteria: 2 \(\times\) 10\(^5\) cells) for 1 h, AS49 cells were then washed 3 times with PBS and lysed by the addition of distilled water (1 mL). To calculate the total number of cell-associated bacteria, corresponding to adherent and intracellular bacteria, serial dilutions of cell lysates were plated on LB agar and incubated at 37°C overnight. Controls were wells pretreated with medium alone (without \(\nu\)-NBDNJ) and considered having a 100% adhesion. Adhesion was then normalized against controls according to the following equations:

\[
\text{relative adherence} = \frac{[\text{adhered} \& \text{internalized} \text{ bacteria CFU of sample/original CFU of sample}] - ([\text{adhered}\&\text{internalized} \text{ bacteria CFU of control/original CFU of control}])}{[\text{adhered}\&\text{internalized} \text{ bacteria CFU of control/original CFU of control}])}
\]

Each experiment was performed in triplicate and at least 3 times. The relative adhesion values were statistically analyzed by two-tailed Student’s \(t\)-tests, using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). \(p < 0.001\) was considered statistically significant.

**CFTR Functional Assay.** CFTR-dependent anion transport was determined as previously described. \(^{77}\) Briefly, CFB841o-cells coexpressing F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP) were plated in 96-well microplates (35,000 cells/well). After 24 h, cells were treated with vehicle (DMSO), VX-809 (1 \(\mu\)M), or \(\nu\)-NBDNJ at multiple concentrations, plus/minus VX-809. After further 24 h, the culture medium with vehicle or test compounds was removed and the cells were washed 3 times with 200 \(\mu\)L of PBS. After the washes, each well received 60 \(\mu\)L of PBS containing an activating cocktail (20 \(\mu\)M forskolin, 50 \(\mu\)M genistein) to maximally stimulate CFTR activity. After 30 min, the microplate was transferred to a FLUOstar Omega microplate reader (BMG LABTECH) equipped with excitation/emission optical filters optimized for Enhanced Yellow Fluorescent Protein EYFP (Chroma Technology Corporation). The assay was done in “well mode”, by continuously reading the fluorescence in each well for 14 s. At 2 s from the start, a syringe pump injected 165 \(\mu\)L of a modified PBS in which NaCl was replaced with NaI. The final I\(^-\) concentration in the well was 100 mM. The fluorescence recording from each well was background subtracted and then normalized for the initial value. The fluorescence decay resulting from I\(^-\) influx and HS-YFP quenching, reflecting the CFTR activity in the plasma membrane, was fitted with an exponential function to derive the maximum quenching rate (QR). Data analysis was done with the Igor Pro software (WaveMetrics).

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**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.4c00044.

1\(^H\) and 13\(^C\) NMR spectra of \(\nu\)-NBDNJ and details for qNMR; additional figures and information concerning *in vitro* and *in vivo* studies; molecular formula strings of compounds (PDF)

Details of proteomic analysis (XLSX)

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■ ABBREVIATIONS
ACT, actin; AlloH, allyl alcohol; BALF, bronchoalveolar lavage fluid; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; CF, Cystic Fibrosis; CFU, colony-forming unit; CTRL, control; DNJ, deoxynojirimycin; ER, endoplasmic reticulum; GM-CSF, granulocyte-macrophage colony-stimulating factor; GCase, β-glucocerebrosidase; HS-YFP, halide-sensitive yellow fluorescent protein; IL, interleukin; LMNB1, lamin B1; MDR, multidrug-resistant; MUB-β-Glc, 4-methylumbelliferyl-β-D-glucopyranoside; MUG, 4-methylumbelliferyl-β-D-glucosaminide; MYH, myosin; NBNDJ, N-butyloxynojirimycin; NLGase, nonsylosomal β-glucosylceramidase; PPI, protein–protein interaction; P. aeruginosa, Pseudomonas aeruginosa; PS-TPP, polymer-supported triphenylphosphine; pwCF, patients with CF; RT-qPCR, real-time quantitative polymerase chain reaction; SL, sphingolipids; TNNT3, troponin T3; TNF, tumor necrosis factor; V, vehicle

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