Neu-164 and Neu-107, two novel antioxidant and anti-myeloperoxidase compounds, inhibit acute cigarette smoke-induced lung inflammation

Thomas H. Thachter,1,2 Hsi-Min Hsiao,3 Elhanan Pinner,4 Moshe Laudon,4 Stephen J. Pollock,2,5 Patricia J. Sime,1,2,5 and Richard P. Phipps1,2,5

1Department of Medicine, University of Rochester, Rochester, New York; 2Lung Biology and Disease Program, University of Rochester, Rochester, New York, 3Department of Pathology, University of Rochester, Rochester, New York; 4Neurim Pharmaceuticals, Tel Aviv, Israel; and 5Department of Environmental Medicine, University of Rochester, Rochester, New York

Submitted 4 February 2013; accepted in final form 15 May 2013

Cigarette smoke (CS) is a strong inflammatory stimulus that induces production of proinflammatory cytokines such as IL-6, IL-8, and TNF-α and recruits macrophages and neutrophils to lung tissue (13, 37, 47). CS contains high levels of free radicals and other reactive oxygen species (ROS) that contribute to proinflammatory activation of lung cells via NF-κB and other redox-sensitive transcription factors (10, 35, 52, 53). Additional ROS, including superoxide, H2O2, and hypochlorous acid, are released by inflammatory macrophages and neutrophils via the action of enzymes, including xanthine/xanthine oxidase, myeloperoxidase (MPO), and NADPH oxidase (30, 69). Current smokers exhibit multiple indicators of chronic oxidative stress, including decreased levels of glutathione peroxidase in the saliva, higher levels of ROS in the blood, decreased circulating endothelial precursor cells, and higher levels of glutathione in epithelial lining fluid (24, 25, 50). Epidemiological studies have suggested a link between higher dietary antioxidant consumption and reduced severity of smoking-associated lung disease, and numerous antioxidants have been tested in animal models of cigarette smoking (12, 48). Significantly, 8-isoprostan, a marker of oxidative stress, was elevated in ex-smokers with COPD relative to both nonsmokers and “healthy” smokers (39), whereas an MPO inhibitor was recently shown to reduce airspace enlargement in mice chronically exposed to CS (14). Taken together, these results suggest that inflammatory cells in COPD contribute significantly to the overall burden of oxidative stress in smoking.

The role of leukotrienes in COPD has also been investigated. Cysteinyl leukotrienes are synthesized from arachidonic acid via the enzyme 5-lipoxygenase (5-LO) and are generally pro-inflammatory. Cysteinyl leukotrienes are potent bronchoconstrictors and also act to increase vascular permeability to cause edema and enhance mucus secretion (36). Leukotriene B4, a neutrophil chemoattractant and activator, is increased in exhaled breath condensate from COPD patients (21, 27). Inhibitors of the leukotriene pathway were found somewhat effective in several clinical trials in COPD (20, 54).

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD, chronic bronchitis, and emphysema) is the fourth leading cause of death worldwide and is projected to be the third by the year 2020 (44). In the United States alone, COPD affects 15 million people at an annual direct and indirect cost-of-illness of more than $66 billion (7). Cigarette smoking is the leading risk factor for developing COPD. Approximately 22% of American smokers. While significant gains have been made in reducing smoking rates among adults, teenage smoking is on the rise, and a disproportionate number of smokers are minorities (7, 44). COPD associated with cigarette smoking and burning of biomass fuels for heating and cooking is an increasing problem in less well-developed countries (63). There are no effective treatments to cure or reverse COPD; the disease can only be symptomatically managed. Thus, there is intense and ongoing interest in the development of novel therapeutic agents for smoking-related lung diseases.

Cigarette smoke (CS) is a strong inflammatory stimulus that induces production of proinflammatory cytokines such as IL-6, IL-8, and TNF-α and recruits macrophages and neutrophils to lung tissue (13, 37, 47). CS contains high levels of free radicals and other reactive oxygen species (ROS) that contribute to proinflammatory activation of lung cells via NF-κB and other redox-sensitive transcription factors (10, 35, 52, 53). Additional ROS, including superoxide, H2O2, and hypochlorous acid, are released by inflammatory macrophages and neutrophils via the action of enzymes, including xanthine/xanthine oxidase, myeloperoxidase (MPO), and NADPH oxidase (30, 69). Current smokers exhibit multiple indicators of chronic oxidative stress, including decreased levels of glutathione peroxidase in the saliva, higher levels of ROS in the blood, decreased circulating endothelial precursor cells, and higher levels of glutathione in epithelial lining fluid (24, 25, 50). Epidemiological studies have suggested a link between higher dietary antioxidant consumption and reduced severity of smoking-associated lung disease, and numerous antioxidants have been tested in animal models of cigarette smoking (12, 48). Significantly, 8-isoprostan, a marker of oxidative stress, was elevated in ex-smokers with COPD relative to both nonsmokers and “healthy” smokers (39), whereas an MPO inhibitor was recently shown to reduce airspace enlargement in mice chronically exposed to CS (14). Taken together, these results suggest that inflammatory cells in COPD contribute significantly to the overall burden of oxidative stress in smoking.

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Caffeic acid is a potent antioxidant and 5-LO inhibitor (26). Several indole-related compounds have been studied as reversible inhibitors of MPO activity (34). We hypothesized that combining these two functional groups in a single compound would have beneficial effects in smoking-related lung disease by attacking multiple pathological pathways, including oxidative stress, MPO, and 5-LO. Neu-164 and Neu-107 are novel small-molecule caffeic acid-indole conjugates with different bridges (Fig. 1, A and B). Here, we report that Neu-164 and Neu-107, delivered by inhalation, strongly inhibited acute CS-induced inflammation in a widely used preclinical mouse model.

MATERIALS AND METHODS

Ethics statement. All animal procedures were approved and supervised by the University of Rochester University Committee on Animal Resources (UCAR permit no. 2007-127). For euthanasia and tissue harvest, mice were anesthetized with an intraperitoneal injection of Avertin (2,2,2-tribromoethanol, 250 mg/kg), followed by exsanguination and tissue harvest once the animals reached a surgical plane of anesthesia. All efforts were made to minimize suffering.

Antioxidant assays. Inhibition of the oxidation of the fluorescent molecule scopoletin to a nonfluorescent product by horseradish peroxidase (HRP) was determined in the presence of 2 μM H2O2 and varying concentrations of Neu-164 (18) by Cerep (Paris, France). The reduction in fluorescence is reported as a percent of control.

Superoxide scavenging was measured using a commercial superoxide dismutase (SOD) assay kit (Cayman Chemical, Ann Arbor, MI). Superoxide (generated by xanthine oxidase and hypoxanthine) oxidizes a tetrazolium salt to a colored formazan dye. For this assay, the reactions were run without superoxide dismutase but with increasing concentrations of Neu-164, and the formation of the colored product was measured at 420 nm.

The MPO inhibition assay assessed the ability of the Neu compounds to inhibit the conversion of guaiacol to tetraguaiacol by purified human MPO (60). A large excess (44 μM) of H2O2 was included in the reaction to exclude H2O2 scavenging as the explanation for the activity decrease. The 5-LO inhibition assay measured the ability of the Neu compounds to inhibit the conversion of arachidonic acid to leukotriene B4 by 5-LO in human peripheral blood mononuclear leukocytes. The MPO and 5-LO assays were performed at MDS Pharma Services (Taiwan).

CS exposure. Adult female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Inhalation Core Facility at the University of Rochester. Mainstream CS exposures were as previously described (35, 61, 62). Mice were placed in individual compartments of a wire cage that was placed inside a closed plastic box connected to the smoke source. Research cigarettes (2R4F, University of Kentucky College of Agriculture Reference Cigarette Program) were smoked according to the FTC protocol (1 puff/min of 2-s duration and 35-ml volume) in a Jaeger-Baumgartner CSM2072i cigarette smoking machine (CH Technologies, Westwood, NJ). Mainstream CS was diluted with filtered air and directed into the exposure chamber. The smoke exposure (total particulate matter/cubic meter of air, TPM) was monitored by gravimetric sampling. The smoke concentration was set at a nominal value of 400 mg/m3 by adjusting the flow rate of the dilution air. The average actual exposure for these experiments was 418 ± 31 mg/m3. Mice received two 1-h exposures, 4 h apart, for three consecutive days and were harvested on the 4th day. Control mice were exposed to filtered air in an identical chamber according to the same schedule. All animal procedures were performed under the supervision of the University Committee on Animal Research.

Treatment. Neu-164 and Neu-107 were provided by Neurim Pharmaceuticals (Tel Aviv, Israel); dexamethasone was from Sigma (St. Louis, MO). The compounds were administered by oropharyngeal aspiration (29) under isoflurane anesthesia. The mice received Neu-164, Neu-107, or dexamethasone at 1 μg/day, or Neu-164 only at 5 μg/day in a 40-μl volume, corresponding to 40 or 200 μg/kg for a 25-g mouse. The mice were treated once per day, beginning 1 day before the first smoke exposure, and continuing daily 1 h before the first smoke exposure of each day, for the duration of the 3-day exposure protocol. The compounds were prepared as 10 mg/ml stock solutions in DMSO and were diluted on the day of use with sterile PBS; the final dilutions contained 2% DMSO, and 2% DMSO in PBS was used for vehicle controls.

Tissue harvest and analysis. Mice were anesthetized with 2,2,2-tribromoethanol (Avertin, 250 mg/kg ip) and killed by exsanguination. The heart and lungs were removed en bloc, and the lungs were lavaged two times with 0.5 ml PBS. The lavage fluid was centrifuged, and the cell-free supernatants were frozen for later analysis. The lungs were snap-frozen in liquid nitrogen for later analysis. The bronchoalveolar lavage (BAL) cell pellet was resuspended in PBS, and the total cell number was determined by counting on a hemacytometer. Dif-
ferential cell counts (minimum of 300 cells/slide) were performed on cytospin-prepared slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Behring, Newark, DE). Cytokines in BAL fluid were measured by cytokine multiplex analysis (Milliplex MAP; Millipore, Temecula, CA) and read on a Luminex 100 (Luminex, Austin, TX). Total protein in BAL fluid was measured by the bicinchoninic acid colorimetric assay (Thermo Scientific, Rockford, IL).

For histological sections, mouse lungs that had not been lavaged were fixed by inflation with 10% neutral buffered formalin at a pressure of 15 cm H2O. Tissues were embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin. To visualize neutrophils, sections were stained with a rat monoclonal anti-mouse neutrophil antibody (MCA771GA, 1:25 dilution; Serotec, Oxford, UK), developed with NovaRed (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. Oxidation of DNA was characterized by immunohistochemistry with a mouse monoclonal antibody against DNA (5-methyl-2'-deoxyuridine; MCA771GA, 1:25 dilution; Serotec, Oxford, UK), developed with NovaRed (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin.

Quantitative RT-PCR. Forty milligrams of lung were excised from total frozen lung, and RNA was extracted using a commercial kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). RNA (1 µg) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and the cDNA was subjected to quantitative real-time PCR using SYBR GreenER in a Bio-Rad iQ5 cycler. In brief, a 20-µl mixture was used containing 10 µl iQ SYBR Green Supermix, 0.5 µl forward and reverse primer, 7.5 µl sterile water, and 2 µl of the 1:5 diluted cDNA template. The real-time PCR was performed under the following conditions: 1 cycle at 95°C for 3 min, then 40 cycles at 95°C for 10 s, 55°C for 30 s, 95°C for 1 min followed by 55°C for 1 min. The intron-spanning primers were designed using sequence information from the NCBI database. The Ct values were normalized to the endogenous control (18S RNA). Primer sequences are listed as follows: m18s RNA, 5'-GCTGGCTGCTTCGCTTACCT-3'; m18s RNA, 5'-CTACTGTACCGGCCGTGCGTA-3'; mHo1 forward, 5'-AGGTACACATCCAAGCCGAGA-3'; mHo1 reverse, 5'-CATCCACACTCTCAAAGCAGGA-3'; mGlgc forward, 5'-CTCACCCAGCAGTCAAGGACC-3'; and mGlgc reverse, 5'-CCCTCATTCCAGTAAACCTGAGAC-3'.

Statistical analysis. All results are reported as means ± SE. Statistical significance was assessed by ANOVA using GraphPad Prism. A P value <0.05 was considered significant.

RESULTS

Neu-164 and Neu-107 have potent antioxidant and anti-MPO activity. Oxidative stress plays an important role in the pathology of CS-induced lung inflammation. The experimental anti-inflammatory compounds Neu-164 and Neu-107 were assayed for their antioxidant properties using antioxidant enzyme assays under standard conditions, with increasing amounts of the compounds. HRP catalyzes the conversion of scopoletin to a nonfluorescent product in the presence of H2O2. The H2O2 scavenging activity with micromolar affinity (Fig. 1C). Similarly, Neu-164 has potent scavenging activity against superoxide radical (generated by xanthine oxidase and hypoxanthine) and inhibited the superoxide-mediated oxidation of a tetrazolium indicator to its colored product (Fig. 1D). Large numbers of neutrophils are recruited to lung tissue following acute CS exposure, and it is believed that MPO released by neutrophils plays a causative role in lung tissue damage seen in emphysema (14, 34). Neu-164 and Neu-107 inhibit MPO activity with low-micromolar affinity (Table 1 and Fig. 1E). Finally, Neu-164 and Neu-107 were also effective 5-LO inhibitors with submicromolar affinity (Fig. 1F).

Neu-164 and Neu-107 inhibit acute CS-induced lung inflammation. To investigate the anti-inflammatory effect of Neu-164 and Neu-107 in vivo, C57BL/6J mice were exposed to CS for 1 h, two times a day for 3 days. Neu-164 or Neu-107 was given by inhalation 1 h before the first smoke exposure on each day. Dexamethasone was included as a positive control for the inhibition of acute inflammation. The mice were killed on the 4th day, and the acute inflammatory response was determined.

As previously described with this model system, mice exposed to CS for 3 days develop acute lung inflammation, characterized by significant increases in the number of neutrophils recovered by BAL (23, 61). Neu-164 and Neu-107 each inhibited the CS-induced increase in total BAL cell numbers (Fig. 2A). Neu-164 and Neu-107 also significantly inhibited neutrophil accumulation. Dexamethasone had similar effects. There were no significant differences in the number of BAL lymphocytes or eosinophils between drug-treated and vehicle-treated mice (Fig. 2D and data not shown).

Neu-164 and Neu-107 reduce infiltration of lung tissue by neutrophils in CS-exposed mice. Lungs from mice exposed to CS or treated with Neu-164, Neu-107, or dexamethasone were fixed, and sections were stained with hematoxylin and eosin. Exposure to CS induced a significant perivascular inflammation compared with air exposure (Fig. 3, A and E). Neu-164 and Neu-107 at 40 µg/kg both reduced the number and size of these infiltrates (Fig. 3, F and G). Neu-164 at 200 µg/kg had a similar effect (data not shown). Neutrophils were also visualized by immunostaining with Vector Red. Neu-164 and Neu-107 (Fig. 3, N and O) also reduced the number of perivascular and tissue-infiltrating neutrophils in CS-exposed mice compared with CS-exposed vehicle-treated controls (Fig. 3M). The results are comparable to inhaled dexamethasone at the same dose.

Neu-164 and Neu-107 inhibit the production of inflammatory cytokines. We analyzed the levels in BAL fluid of several proinflammatory cytokines that are important to lung inflammation. IL-6, a key regulatory cytokine that is strongly upregulated by CS, was significantly inhibited by Neu-164 (Fig. 4A). Neu-107 reduced IL-6 levels by 33%. Although this reduction was not significant, neither was there significantly more IL-6 than with Neu-164. Interestingly, another key cytokine, TNF-α, which is also upregulated by CS exposure, was not affected by Neu-164 or Neu-107 (Fig. 4B). Macrophage inflammatory protein (MIP)-2 and keratinocyte-derived cytokine (KC) are the mouse homologs of IL-8 and recruit neutrophils by binding to CXCRII on the neutrophil surface (62, 71). Consistent with the reduction in neutrophil inflammation, both Neu-164 and Neu-107 inhibited production of KC, whereas Neu-164, but not Neu-107, inhibited MIP-2 (Fig. 4, C

Table 1. Antioxidant and MPO-inhibitory activities of Neu-164 and Neu-107

<table>
<thead>
<tr>
<th>Activity</th>
<th>Neu-164, µM</th>
<th>Neu-107, µM</th>
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<tr>
<td>MPO inhibition (IC50)</td>
<td>3.1</td>
<td>1.6</td>
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<tr>
<td>5-LO inhibition (IC50)</td>
<td>0.62</td>
<td>0.21</td>
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<tr>
<td>H2O2 scavenging (IC50)</td>
<td>0.83</td>
<td>ND</td>
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<tr>
<td>HRP/scopolin inhibition (IC50)</td>
<td>5</td>
<td>ND</td>
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MPO, myeloperoxidase; 5-LO, 5-lipoxygenase; HRP, horseradish peroxidase; IC50, half-maximal inhibitory concentration. *Assayed as described in MATERIALS AND METHODS. ND, not determined.
Fig. 2. Neu-164 and Neu-107 reduce accumulation of neutrophils in bronchoalveolar lavage (BAL). Mice were exposed to air or cigarette smoke (CS) and treated with Neu-164 (N.164), Neu-107 (N.107), dexamethasone (Dex), or vehicle (Veh) as described. The mice were killed, and the lungs were lavaged with PBS. Total and differential cell counts were performed. Neu-164 and Neu-107 block the CS-induced increase in total BAL cells (A) and neutrophils (C) but do not alter the accumulation of macrophages (B) or lymphocytes (D). Gray bars, air; black bars, CS. The data shown are means ± SE for n = 5 mice. *P < 0.05 and ***P < 0.001 compared with CS plus vehicle, 2-way ANOVA with Bonferroni posttest.

and D). Finally, we analyzed the production of macrophage chemotactic protein (MCP)-1 (CCL2), which is produced by fibroblasts and epithelial and endothelial cells and recruits monocytes and macrophages. Although Neu-164 and Neu-107 reduced levels of MCP-1 in BAL fluid to a lesser degree than that of KC, MIP-2, or IL-6, the reduction is statistically significant (Fig. 4E). The anti-inflammatory steroid dexamethasone has approximately the same effectiveness at Neu-164 at the same dose. Acute smoke exposure also increases total protein in BAL fluid, an indicator of edema (61). Here, acute CS exposure results in a significant upregulation of total BAL protein in vehicle-treated mice (Fig. 4F). Treatment with dexamethasone or the Neu compounds somewhat reduces total BAL protein, suggesting a partial protective effect against edema.

Activation of antioxidant genes is reduced in mice treated with Neu-164 and Neu-107. Because Neu-164 and Neu-107 are potent antioxidants, we examined whether drug treatment altered the expression of two key antioxidant genes known to be upregulated by acute CS exposure. Heme oxygenase-1 (HO-1) catalyzes the conversion of heme to biliverdin and carbon monoxide, both of which have free radical scavenging activity, and has been demonstrated to be critical for maintaining appropriate redox balance in CS exposure (8, 31). Glutamate-cysteine ligase (modifier subunit, GCLm) is the rate-limiting enzyme in the synthesis of glutathione, a key intracellular antioxidant (31). These oxidative stress response genes were shown to be strongly upregulated in mice exposed to CS (51). Here, RNA was extracted from lung tissue from mice exposed to air or CS treated with vehicle, Neu-164, or Neu-107. As shown in Fig. 5, GCLm and HO-1 were both significantly upregulated by CS. Neu-164 significantly inhibited upregulation of both HO-1 and GCLm. Neu-107 also prevented upregulation of both HO-1 and GCLm.

To further examine oxidative stress, we used immunohistochemistry to detect 8-oxoG, an important mutagenic DNA lesion generated under oxidative stress. Because the lungs are constantly exposed to oxygen, the airway epithelium of air-exposed mice exhibited a basal level of 8-oxoG staining (Fig. 5, C and D). Consistent with prior reports (68), acute CS exposure strongly induced 8-oxoG staining (Fig. 5E) in airway epithelial cells and also in parenchymal cells. Interestingly, 8-oxoG staining was reduced by treatment with Neu-164.

DISCUSSION

CS is a profound proinflammatory stimulus, causing inflammation within minutes of the first cigarette. CS contains numerous chemicals that activate proinflammatory pathways, including nicotine, acrolein, acetaldehyde, benzo[a]pyrene, and others (2, 38, 57, 58). CS also incites inflammation through oxidative stress. There are two principal sources of ROS in smoke. Tobacco smoke itself contains up to 1 × 10^{17} oxidants and free radicals per puff, and CS tar contains reactive aldehydes, quinones, and other organic molecules that can generate ROS in situ after deposition in the lungs (11, 42). A second significant source of ROS in cigarette smoking are inflammatory cells themselves, including monocytes/macrophages and neutrophils, that produce free oxygen and nitrogen radicals via the action of multiple enzymes, including NAPDH oxidase, xanthine/xanthine oxidase, and MPO (64, 69). ROS contribute to inflammation by activating multiple proinflammatory signaling pathways, including notably the activator protein-1 and NF-κB transcription factors (3, 10, 35, 40, 52, 67, 70). ROS...
also cause oxidation of proteins, lipids, and DNA, leading to cellular damage and death (via apoptosis or necrosis) and also contributing to inflammation and chronic tissue damage (1, 28, 53, 67, 70). Thus, there is great interest in the use of antioxidants to prevent and treat smoking-related lung disease. Thiol compounds such as N-acetylcysteine and carbocysteine have been extensively studied, as well as mimetics of superoxide dismutase and glutathione peroxidase, and inhibitors of nitric oxide synthase (4, 49).

Caffeic acid derivatives are potent antioxidants and have shown some protective effects in CS-exposed rats and rabbits (19, 46, 55). Caffeic acid is also a potent inhibitor of 5-LO (26), which is upregulated in COPD patients (21, 27). Indole compounds such as N-acetylcysteine and carbocysteine have been extensively studied, as well as mimetics of superoxide dismutase and glutathione peroxidase, and inhibitors of nitric oxide synthase (4, 49).

Caffeic acid derivatives are potent antioxidants and have shown some protective effects in CS-exposed rats and rabbits (19, 46, 55). Caffeic acid is also a potent inhibitor of 5-LO (26), which is upregulated in COPD patients (21, 27). Indole compounds also have antioxidant properties and have been developed as MPO inhibitors (34). Neu-164 and Neu-107 were developed as caffeic acid-indole conjugates and evaluated for their antioxidant and enzyme inhibitory activities. Neu-164 and Neu-107 are potent scavengers of superoxide and inhibit MPO and 5-LO at micromolar or submicromolar concentrations (Fig. 1 and Table 1). We did not specifically study the mechanism of action, but we note that indoles are reversible inhibitors of MPO that act as poor peroxidase substrates and inhibitors of the chlorination reaction (reviewed in Ref. 34). This inhibition can be partially lifted by superoxide, which reduces the heme group in the enzyme back to its native state. In this context, the superoxide scavenging function provided by the caffeic acid functional group (22) may potentiate the efficacy of the Neu compounds by limiting their reversibility by in situ superoxide. We also note that, while the scopoletin assay is generally described as an assay for H2O2 scavenging by antioxidants (6), we cannot determine whether inhibition of the assay by Neu-164 (Fig. 1C) was due to H2O2 scavenging or inhibition of the HRP used in the assay. However, Neu-164 and Neu-107 clearly are potent superoxide scavengers and inhibitors of MPO and 5-LO.

Next, we investigated the effects of Neu-164 and Neu-107 in an acute model of CS exposure. Both Neu-164 and Neu-107 inhibited acute neutrophilia in mice exposed to CS for 3 days (Figs. 2 and 3). Neu-107, which was more effective at inhibiting MPO and 5-LO enzyme activity, was also more effective at reducing cellular inflammation. The compounds also inhibited upregulation of proinflammatory cytokines, including IL-6, MIP-2, KC, and MCP-1, and total BAL protein (Fig. 4). The efficacy of Neu-164 and Neu-107 at 40 μg/kg was similar.
to dexamethasone. Neu-164 did not show an increased dose-response when tested at 200 μg/kg, suggesting that its maximum effect was achieved with the lower dose. Interestingly, Neu-164 and Neu-107 did not affect TNF-α levels upregulated by CS exposure, and only weakly inhibited MCP-1. This may indicate that IL-6 is more closely regulated by oxidative stress while upregulation of TNF-α is less dependent on oxidative stress and more dependent on other proinflammatory components of CS and suggests that, for maximum effect, it might be desirable to combine these compounds with other drugs that target other proinflammatory pathways.

It should be noted that, while dexamethasone (included as a positive control for the inhibition of inflammation) was effective at inhibiting acute CS-induced inflammation, corticosteroids alone are not effective in long-term management of COPD (65). Additionally, COPD patients and animals exposed to chronic CS develop steroid resistance due to epigenetic changes to histone acetylation (4, 9). This is one of the factors that has prompted the search for novel anti-inflammatory compounds in the treatment of CS-induced lung disease.

In rodent models of CS exposure, neutrophilic inflammation predominates the acute phase, whereas both macrophages and neutrophils are important in the chronic phase (15, 17). Macrophages and neutrophils are also both important in human COPD. COPD patients have chronic neutrophilia, and it is believed that neutrophil elastase is one of the key proteases responsible for lung tissue destruction in emphysema (16, 56). Indeed, direct instillation of elastase into the lungs of rodents is a long-recognized experimental model of emphysema. However, recent evidence suggests that MPO, produced by both neutrophils and macrophages, contributes significantly to the pathology of inflammatory diseases and emphysema (34). For

Fig. 4. Neu-164 and Neu-107 reduce expression of proinflammatory cytokines in BAL fluid. Mice were exposed to air or CS and treated with Neu-164, Neu-107, dexamethasone, or vehicle as described. The mice were killed, and the lungs were lavaged with PBS. Cytokines were determined in BAL fluid by multiplex assay. A: IL-6; B: TNF-α; C: macrophage inflammatory protein (MIP)-2; D: keratinocyte-derived cytokine (KC); E: macrophage chemotactic protein (MCP)-1; F: total BAL protein. Gray bars, air; black bars, CS. The data shown are means ± SE for n = 5. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with CS plus vehicle; ††P < 0.01 between treatment groups, 2-way ANOVA with Bonferroni posttest. NS, not significant.
example, ex-smokers with COPD exhibit levels of oxidative stress in their lungs twofold higher than healthy current smokers (39), and MPO in human COPD sputum is correlated with poor clinical outcome (45), suggesting that inflammatory cells are a significant contributor to oxidative stress in situ. Recently, an MPO inhibitor successfully prevented emphysema in a guinea pig model of chronic CS exposure (14).

We hypothesize that Neu-164 and Neu-107 inhibit CS-induced acute inflammation by at least two mechanisms (Fig. 6). First, by acting as antioxidants, they scavenge ROS present in smoke. Second, by blocking MPO and scavenging superoxide produced by inflammatory cells, they interrupt the positive feedback loop.

Fig. 6. Hypothesis of the role of Neu-164 and Neu-107 in inhibiting CS-induced inflammation. Reactive oxygen species (ROS) in CS leads to inflammation by upregulating ROS-sensitive transcription factors, and also by recruiting inflammatory macrophages and neutrophils. These macrophages and neutrophils produce additional ROS through the actions of enzymes, including xanthine oxidase, NADPH oxidase, and MPO, which contribute to further oxidative stress and further recruitment of inflammatory cells. Neu-164 and Neu-107 potentially inhibit inflammation by two pathways: by scavenging the ROS found in CS and reducing the initial oxidative stress response, and by blocking MPO and scavenging superoxide produced by inflammatory cells, interrupting the positive feedback loop. PMN, polymorphonuclear neutrophils.

Fig. 5. Neu-164 and Neu-107 inhibit the upregulation of antioxidant genes and reduce oxidative stress. A and B: mice were exposed to air or CS and treated with Neu-164 and Neu-107 (40 μg/kg) as described. RNA was purified from whole lung and mRNA for heme oxygenase-1 (HO-1), and glutathione-cysteine ligase modifier subunit (GCLm) was amplified by quantitative RT-PCR and normalized to 18S rRNA. The data shown are means ± SE for n = 3 mice (in the air + vehicle and all CS groups) or n = 5 mice (in the air + Neu-164 and air + Neu-107 groups). *P < 0.05 and ***P < 0.001 compared with air + vehicle; †P < 0.05, ††P < 0.01, and †††P < 0.001 compared with CS + vehicle by 2-way ANOVA with Bonferroni posttest. NS, not significantly increased compared with air + vehicle and not significantly decreased compared with CS + vehicle. C–F: lung sections were stained with an antibody that detects 8-oxoguanine. C: air + vehicle; D: air + Neu-164 (40 μg/kg); E: CS + vehicle; F: CS + 40 μg/kg Neu-164 as described. a, Airways; triangles, positively stained parenchymal cells. No staining was detected in the negative control (primary antibody omitted, data not shown).
and smoke tars, which reduces proinflammatory signaling and recruitment of inflammatory cells, including neutrophils. Second, by inhibiting MPO and scavenging superoxide produced by neutrophils and macrophages, Neu-164 and Neu-107 reduce the burden of oxidative stress produced by inflammatory cells, resulting in reduced tissue damage and dampening the positive feedback loop between ROS, ROS-sensitive proinflammatory transcription factors, and inflammatory cell recruitment. In support of this hypothesis, we demonstrated that Neu-164 reduced the level of DNA oxidation in CS-exposed lung tissue (Fig. 5). We also investigated the upregulation of endogenous antioxidant enzymes HO-1 and GCLm. Acute CS exposure upregulated both HO-1 and GCLm. Treatment with Neu-164 significantly inhibited the upregulation of both antioxidant enzymes. Neu-107 inhibited upregulation of GCLm and partially inhibited HO-1 (Fig. 5). HO-1 and GCLm are regulated by the transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) (33). Nrf2 is regulated by its binding partner Kelch-like-ECH-associated protein 1 (Keap1), which acts as a sensor for oxidative stress and retains Nrf2 in the cytoplasm until it becomes oxidized, at which point Keap1 dissociates from Nrf2 and is degraded, allowing Nrf2 to translocate to the nucleus (41). However, Keap1 and Nrf2 recycle so quickly (43) that they would not be informative in these experiments in which the mice were harvested 24 h after the last exposure, hence the need to investigate their downstream targets. Overall, these results suggest that Neu-164 and Neu-107 did indeed reduce the oxidative stress burden associated with acute CS exposure, resulting in reduced Nrf2 activity and reduced up-regulation of antioxidant response genes.

Neu-164 and Neu-107 also have potent inhibitory activity against 5-LO. Neutrophils express 5-LO, which participates in the synthesis of leukotrienes that cause bronchoconstriction, and have been found to be upregulated in COPD patients (32, 36). Therefore, it is likely that 5-LO inhibition would contribute to the beneficial effects of these compounds in patients. Unfortunately, leukotrienes were not upregulated in our mouse model of acute CS exposure (data not shown), so our study cannot address the possible benefits of 5-LO inhibition by Neu-164 and Neu-107. Studies of other leukotriene pathway inhibitors have shown beneficial effects (20).

The potential therapeutic role of antioxidants in smoking-related lung disease is complex. There are numerous studies suggesting antioxidants such as N-acetylcysteine have some benefit in COPD (reviewed in Ref. 4). However, there is also some evidence that dietary antioxidants may promote cancer (5). In an ex-smoker with COPD, the predominant source of ROS in the lungs is ongoing chronic inflammation (macrophages and neutrophils) that does not resolve for several years after smoking cessation (59, 66). We suggest that treatment with compounds such as Neu-164, which have antioxidant and MPO-inhibitory effect, may act to reduce the overall burden of ROS on the lungs, interrupting the positive feedback loop between ROS and inflammatory cell recruitment. As such, the treatment might be able to be discontinued after downregulating the inflammation, thus mitigating possible procarcinogenic effects of antioxidants.

Taken together, our results demonstrate that Neu-164 and Neu-107, novel therapeutic candidates with antioxidant and anti-MPO activity, have potent anti-inflammatory effects in vivo in an acute cigarette smoking model. This is an important first proof-of-principle study on the efficacy of dual MPO inhibitor/antioxidant compounds in smoking-related inflammation. While we believe these compounds could be useful prophylactically (such as in acute smoke inhalation), or in other non-smoking-related lung inflammatory conditions such as asthma, the main goal would be to treat the effects of cigarette smoking that persist after smoking cessation. As such, further experiments in chronic smoke exposure models are needed. We also note that, although the Neu compounds did not appear to have short-term side effects (such as nonspecific inflammation in mice exposed to air and treated with the compounds), long-term toxicological data are also needed. Given the recent report that an orally delivered MPO inhibitor prevents emphysema in a guinea pig model of chronic CS exposure (14), we expect that Neu-164, with strong anti-inflammatory activity and multiple disease-relevant functions (anti-MPO, anti-5-LO, and antioxidant), will have similar results in chronic CS exposures. Importantly, these effects were seen when the compounds were delivered by inhalation, which may result in increased availability at the target organ and decreased systemic side effects. This demonstrates the utility of small molecule inhibitors with multiple targets in treating disease and suggests the potential of these compounds in long-term studies of chronic CS exposure.

ACKNOWLEDGMENTS

We thank Kathryn Sewernyiak for expert technical assistance and Tirumalai Rangasamy for advice and comment.

GRANTS

This work was supported in part by National Institutes of Health Grants 8UL1-TR-000042, R01-HL-088325, and P30-ES-001247. These funders had no role in study design, data collection; and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

This study was funded in part by Neurim Pharmaceuticals. E.P. and M.L. were employed by Neurim Pharmaceuticals and performed the experiments shown in Fig. 1 and Table 1. M.L. is an inventor on patents covering Neu-164 and Neu-107 that are assigned to Neurim Pharmaceuticals. E.P. consulted on the design of the other experiments. While these authors concur in the overall submission of the manuscript, neither these authors nor Neurim Pharmaceuticals had veto power over the manuscript or presentation of the results. R.P.P. has served as a consultant to Neurim. T.H.T., H.H., S.J.P., and P.J.S. have no conflicts to declare.

AUTHOR CONTRIBUTIONS


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