Therapeutic Myeloperoxidase Inhibition Attenuates Neutrophil Activation, ANCA-Mediated Endothelial Damage, and Crescentic GN

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ABSTRACT

Background Myeloperoxidase released after neutrophil and monocyte activation can generate reactive oxygen species, leading to host tissue damage. Extracellular glomerular myeloperoxidase deposition, seen in ANCA-associated vasculitis, may enhance crescentic GN through antigen-specific T and B cell activation. Myeloperoxidase-deficient animals have attenuated GN early on, but augmented T cell responses. We investigated the effect of myeloperoxidase inhibition, using the myeloperoxidase inhibitor AZM198, to understand its potential role in treating crescentic GN.

Methods We evaluated renal biopsy samples from patients with various forms of crescentic GN for myeloperoxidase and neutrophils, measured serum myeloperoxidase concentration in patients with ANCA-associated vasculitis and controls, and assessed neutrophil extracellular trap formation, reactive oxygen species production, and neutrophil degranulation in ANCA-stimulated neutrophils in the absence and presence of AZM198. We also tested the effect of AZM198 on ANCA-stimulated neutrophil-mediated endothelial cell damage in vitro, as well as on crescentic GN severity and antigen-specific T cell reactivity in the murine model of nephrotoxic nephritis.

Results All biopsy specimens with crescentic GN had extracellular glomerular myeloperoxidase deposition that correlated significantly with eGFR and crescent formation. In vitro, AZM198 led to a significant reduction in neutrophil extracellular trap formation, reactive oxygen species production, and released human neutrophil peptide levels, and attenuated neutrophil-mediated endothelial cell damage. In vivo, delayed AZM198 treatment significantly reduced proteinuria, glomerular thrombosis, serum creatinine, and glomerular macrophage infiltration, without increasing adaptive T cell responses.

Conclusions Myeloperoxidase inhibition reduced neutrophil degranulation and neutrophil-mediated endothelial cell damage in patients with ANCA-associated vasculitis. In preclinical crescentic GN, delayed myeloperoxidase inhibition suppressed kidney damage without augmenting adaptive immune responses, suggesting it might offer a novel adjunctive therapeutic approach in crescentic GN.

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activated by hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and catalyzes the formation of reactive intermediates such as hypochlorous acid and other reactive oxygen species (ROS).\(^1\) Although MPO is a key component of innate immune defense,\(^2\) MPO deficiency does not appear to lead to a clinical phenotype.\(^3\) However, hypochlorous acid and other ROS can lead to host tissue damage by oxidizing/chlorinating a variety of targets, including proteins, lipids, and DNA.\(^4\)

Neutrophils are also known to release neutrophil extracellular traps (NETs), structures composed of decondensed chromatin decorated with granule-derived antimicrobial peptides and enzymes, such as neutrophil elastase (NE) and MPO.\(^5\) Recent data have shown that MPO is necessary for the formation of NETs.\(^6\) Neutrophils from donors who are completely deficient in MPO fail to form NETs in response to Candida infection,\(^7\) and the induction of NETs by TNF-\(\alpha\) in healthy individuals requires enzymatically active MPO.\(^8\)

NETosis has been linked to a wide array of autoimmune diseases (Systemic Lupus Erythematosus [SLE], Antineutrophil cytoplasmic antibody [ANCA]-associated vasculitis [AAV], rheumatoid arthritis, cystic fibrosis, and gout) and in recent years NETs have taken center stage in autoimmunity research as potential links in breaking immune tolerance to innate immune response molecules.\(^9\) NETs are intimately associated with CGN and have been found in the circulation of patients with AAV and SLE.\(^10\)–\(^12\) The finding that NETs are selectively loaded with antigens recognized by perinuclear ANCA,\(^13\) coupled with extracellular MPO deposition in inflamed glomeruli from patients with AAV,\(^14\) provides an explanation for how MPO present on NETs may act as an autoantigen to promote antigen-specific T and B cell reactivity, and glomerular targeting to enhance CGN. A causative role for MPO in mediating glomerular damage is also supported by murine models of CGN in which acute disease is attenuated in MPO-deficient animals, despite augmentation of adaptive (T cell) immunity.\(^15\)

Latterly, selective MPO inhibitors have been developed and in experimental models of heart failure,\(^16\) pulmonary hypertension,\(^17\) and vasculitis,\(^18\) they have been shown to ameliorate disease. In this study we have investigated the role of MPO in mediating glomerular damage and T cell activation using a novel pharmacologic MPO inhibitor, AZM198. AZM198 is a recently developed selective MPO inhibitor that effectively inhibits MPO bioactivity and NET formation by human neutrophils. It has been shown to block MPO activity within granules, with higher doses required for inhibition of intra-granular MPO when compared with doses needed to fully impede the activity of extracellular MPO.\(^6\)

METHODS

Patient Samples

Blood and tissue samples from patients presenting with AAV and other forms of GN, CKD disease controls, and healthy controls attending the Royal Free Hospital, London, UK were included in this study. All samples and biopsy specimens were obtained following informed consent (National Research Ethics Committee reference 05/Q0508/6). In all cases the diagnosis of AAV was confirmed by renal biopsy with a positive ANCA result. MPO activity during active AAV and at 2-month remission was measured in serum of proteinase 3 (PR3)-ANCA-positive patients enrolled in the Rituximab in ANCA-Associated Vasculitis (RAVE) trial.\(^19\) Eighteen renal biopsy specimens were obtained from patients with various forms of CGN (MPO-ANCA, \(n=5\); PR3-ANCA, \(n=3\); ANCA-negative pauci-immune GN, \(n=2\); IgA, \(n=4\); and SLE, \(n=4\)) and stained for CD15 (catalog no. 301902; BioLegend) and MPO (A0398; DAKO). Clinical and laboratory data were collected from hospital records and pathology archives.

Mice

C57BL/6 wild-type, DO11.10, and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were female, aged 10–12 weeks old, with an average weight of 25 g. All animals were bred at University College London (London, UK), housed in individually ventilated cages and fed a standard chow diet. Studies adhered to the Animals (Scientific Procedures) Act 1986 and University College London Animal Welfare and Ethical Review Body guidelines for animal experimentation.

Immunohistochemistry

Sections (2 \(\mu\)m) of formalin-fixed, paraffin-embedded tissue specimens were mounted on superfrost plus slides (Menzel), dewaxed, rehydrated, and pretreated with antigen retrieval solution Tris-EDTA (pH 9) in a pressure cooker for 20 minutes, blocked in 5% casein solution, and probed with antibodies against CD15 (catalog no. 301902; BioLegend) for 2 hours (1:50 dilution) and MPO (A0398; DAKO) overnight at 4°C. Sections were visualized with a VECTOR Red alkaline phosphatase substrate kit (SK-5100; Vector Laboratories) for CD15 and DAB substrate for MPO and counterstained with hematoxylin. MPO deposition was expressed as a percentage of total

Significance Statement

Myeloperoxidase released after neutrophil and monocyte activation can generate reactive oxygen species, leading to tissue damage. Free myeloperoxidase is deposited in glomeruli in various forms of crescentic GN and is elevated in ANCA-associated crescentic GN. The authors evaluated renal biopsy samples and studied the effect of a novel myeloperoxidase inhibitor, AZM198, in vivo and in vitro. They demonstrate that myeloperoxidase mediates neutrophil degranulation and neutrophil extracellular trap formation and contributes to ANCA-mediated endothelial damage. Their findings critically implicate myeloperoxidase in crescentic GN pathogenesis and show that use of AZM198 significantly attenuates these pathways and reduces disease severity in a preclinical crescentic GN model. These data suggest that clinical myeloperoxidase inhibition might represent a novel therapeutic strategy for diverse forms of crescentic GN.
captured area from ten random high power-fields using Color Deconvolution, FIJI software (National Institutes of Health, Bethesda, MD). The macro evaluated the area stained, correcting for background threshold and expressed results as area fraction percentage of assessed area. Intraleukocyte MPO was defined being associated with CD15 (CD15⁺MPO⁺ cells). Extracellular MPO was measured as MPO⁺CD15⁻ staining.

**Purification of Human Ig**

Human anti-PR3 IgG and healthy control IgG were purified using HiTrap Protein G column (GE Healthcare) chromatography. Each sample was diluted at a ratio of 1:1 in binding buffer (20 mM sodium phosphate, pH 7.0). Plasma from anti-PR3 and healthy control were processed on columns and IgG isolated from each sample, eluted by adding 5 ml of elution buffer (0.1 M glycine-HCl, pH 2.7), and neutralized with alkalizing agent (1 M Tris-HCl, pH 9.0). Subsequently, endotoxin was depleted (Detoxi-Gel Affinity Pak) and protein concentration was determined by spectrophotometry using a NanoDrop 8000 (Thermo Scientific) instrument. ANCA reactivity on isolated neutrophils was assessed using the dihydrorhodamine (DHR) assay described below (Supplemental Figure 1).

**Neutrophil Stimulation and Inhibition Experiments**

Peripheral blood anticoagulated with EDTA was mixed with 6% dextran and granulocytes isolated on a Percoll (Sigma) gradient. Human neutrophils were suspended in RPMI medium (2% AB serum [Sigma]), 500 U/ml penicillin-streptomycin [Gibco Life Technologies]). They were plated alone or stimulated with Phorbol 12-myristate 13-acetate (PMA) (20 μM) (Sigma) or TNF-α (2 ng/ml) (Bio-Rad Laboratories) and endotoxin-depleted ANCA or normal human IgG (both at 0.2 mg/ml) in the absence or presence of 10 μM AZM198, a concentration that has been reported to inhibit both intragranular and extracellular MPO activity, as well as NETosis. The supernatants were collected at 30 minutes and peroxidase activity measured as described below.

**ROS Production (DHR Assay)**

DHR 123 is a nonreduced, nonfluorescent molecule that, in the presence of H₂O₂, is converted to rhodamine 123, which fluoresces at a wavelength of approximately 534 nm and can therefore be detected in the FITC (FL-1) light channel on flow cytometry. Neutrophils (2.5×10⁶ cells/ml) were loaded with 17 μg/ml DHR123 (Calbiochem) together with 5 μg/ml cytochalasin B as well as 2 mM sodium azide and incubated in the dark for 10 minutes at 37°C. Cells were primed by incubation with 2 ng/ml TNF-α for 15 minutes, incubated with PR3-ANCA (200 μg/ml) for 45 minutes, and subsequently analyzed by flow cytometry.

**Circulating MPO and Human Neutrophil Peptide 1–3 Levels, MPO Enzymatic Activity, and ROS Production**

Circulating MPO was measured by ELISA (Abcam) in serum samples. In neutrophil supernatants, human neutrophil peptide (HNP) 1–3 levels were measured by ELISA (Hycult) and peroxidase activity was measured with 3,3′,5,5′-tetramethylbenzidine (Sigma), as described previously. A standard curve of MPO was made with human MPO (catalog no. M6908; Sigma).

**NET Visualization and Quantification**

After the neutrophil supernatants were collected, cells were stained with Sytox Green (0.5 μM) (catalog no. S7020; Invitrogen) or fixed and stained with MPO (DAKO) and elastase (M0752; DAKO) visualized by anti-rabbit FITC and anti-mouse Alexa Fluor 647 IgG respectively, and counterstained with DAPI (2 μg/ml). NETs were visualized under a fluorescence microscope.

Ten consecutive high-power fields at ×20 magnification were captured. For each image, DAPI and Sytox green (Alexa Fluor 488) were visualized. Acquired images were automatically analyzed by Fiji analysis software by determining the area of extracellular DNA, using a pixel threshold to exclude potential intracellular staining. Extracellular DNA of NETs was quantified as the cumulative area of positive Sytox Green. The ratio of Sytox Green stained area: number of nuclei was calculated, representing the NET area index corrected for the number of imaged neutrophils. A higher index ratio indicates a larger NET area present.

**Analysis of Endothelial Cell Damage during Endothelial Cell–Neutrophil Coculture**

Human umbilical vein endothelial cells (ECs), a gift from Dr Xu Shiwen, Centre for Rheumatology, University College London, were cultured on 0.1% gelatin-coated culture plates in basal EC medium containing low serum (2% FCS) and EC growth supplement (PromoCell). BrdU-labeled EC were seeded into 96-well microtiter plates at 10⁴ cells/well. Confluent EC were cultured for 18 hours in the presence of TNF-α (2 ng/well), then rinsed to remove all traces of TNF-α and cultured for an additional 18 hours under the following conditions: EC alone, EC plus unstimulated PMNs, EC plus activated PMN, EC plus activated PMN plus AZM198, and EC plus activated PMN plus DNase. The PMNs (2×10⁵ cells/well) were primed by TNF-α (2 ng/ml) and stimulated with PR3-ANCA (200 μg/ml). DNase (Sigma) was used at 500 U/ml (Sigma) and AZM198 at 10 μM. Supernatants were collected for analysis of BrdU-labeled DNA fragments using a cellular DNA fragmentation ELISA (Roche Diagnostics GmbH) and vWF release measured by ELISA (RayBiotech). Immunofluorescence staining of the EC monoculture and EC–PMN coculture was performed using vascular endothelial cadherin (MAB9381; R&D Systems) and MPO (DAKO) counterstained with DAPI.

**Nephrotic Nephritis**

Thirty two C57BL/6 mice were preimmunized subcutaneously with sheep IgG (0.2 mg) (catalog no. 15131; Sigma-Aldrich) in CFA (catalog no. F5881; Sigma-Aldrich), and 5 days later received intravenous sheep nephrotic serum (NTS; 200 μl)
mixed 1:1 with LPS (Escherichia coli R515; Hycult) diluted in 0.9% NaCl (5 μg/ml, final concentration). At 1 day post-immunization with NTS, 16 mice received AZM198 by gavage and the remaining animals were dosed with vehicle (0.5% hydroxypropyl methyl cellulose in water) every 12 hours for 7 days. Two different AZM198 dose regimens were used: 133 μmol/kg (n=8) or 400 μmol/kg (n=8). Both doses are predicted to inhibit at least 80% of the extracellular MPO activity over 12 hours, and the high dose is predicted to also inhibit intragranular activity more than 80% during 4 out of 12 hours.6 The allocation of animals to groups and the order of dosing were random. The animals were placed in metabolic cages on day 7 for urine collection and were euthanized on day 8. Proteinuria was quantified using the sulfosalicylic acid assay.21 Kidneys were collected in buffered formalin and subsequently stained for expression of CD4 (GK1.5), CD44 (10% FCS) with 10% R515; Hycult diluted 1:1 with LPS (Escherichia coli R515; Hycult) diluted in 0.9% NaCl (5 μg/ml, final concentration). At 1 day post-immunization with NTS, 16 mice received AZM198 by gavage and the remaining animals were dosed with vehicle (0.5% hydroxypropyl methyl cellulose in water) every 12 hours for 7 days. Two different AZM198 dose regimens were used: 133 μmol/kg (n=8) or 400 μmol/kg (n=8). Both doses are predicted to inhibit at least 80% of the extracellular MPO activity over 12 hours, and the high dose is predicted to also inhibit intragranular activity more than 80% during 4 out of 12 hours.6 The allocation of animals to groups and the order of dosing were random. The animals were placed in metabolic cages on day 7 for urine collection and were euthanized on day 8. Proteinuria was quantified using the sulfosalicylic acid assay.21 Kidneys were collected in buffered formalin and stained with periodic acid–Schiff and hematoxylin and eosin (Sigma) or paraformaldehyde-lysine-periodate for immunofluorescence, and stained for MPO (DAKO), macrophages (using anti-F4/80, catalog no. 14–4801–81; Invitrogen), CD4 cells (using rat anti-mouse CD4 clone GK1.5; BD Biosciences), and sheep IgG (Sigma). Spleens were removed and sieved, after which red cells were lysed for 5 minutes using RBC lysis buffer (catalog no. 420301) and single-cell suspensions were obtained. Splenocytes were cultured in DMEM (10% FCS) with 10 μg/ml sheep globulin for 72 hours, and subsequently stained for expression of CD4 (GK1.5), CD44 (1M7), and CD62L (MEL-14), and analyzed by flow cytometry (FlowJo software). Free-plasma AZM198 concentration was measured in all terminal samples.

Pharmacokinetic Study for AZM198

Nine C57BL/6 mice received a single dose of 133 μmol/kg AZM198 via oral gavage. Free-plasma AZM198 was measured at 2, 12, and 19 hours postdose (n=3 at each time point) to define the maximal (Cmax) and trough (Cmin) levels, and to bridge the data to the terminal samples of the nephrotoxic nephritis (NTN) study.

Histologic Scoring

Murine kidney sections were scored for glomerular thrombosis by counting 25 consecutive glomeruli and scoring each glomerulus (number of glomerular quadrants with thrombosis, score 0–4). Glomerular MPO and F4/80 (both FITC) corrected total cell fluorescence (CTCF) and number of CD4-positive cells per glomerulus were counted using Fiji software on ten consecutive high-power fields of 25 consecutive glomeruli.

Glomerular Neutrophil Accumulation

Eight C57BL/6 mice were dosed with either vehicle (n=4) or AZM198 (n=4) twice a day at 133 μmol/kg for 48 hours before the injection of NTS as described above. Twelve hours after the last dosing and 2 hours after NTS injection, kidneys were collected and frozen sections were stained for Ly6g (ab25377), a murine neutrophil marker.

DO11.10 T Cell Adoptive Transfer Model

Lymphocytes were harvested from the lymph nodes of BALB/c DO11 mice, which are transgenic for an MHC class II–restricted T cell receptor (DO11.10) recognizing an ovalbumin peptide, and stained with KJ126, a mAb against the DO11.10 T cell receptor. KJCD4+ cells were subsequently injected intravenously into six BALB/c mice (1.2×106 cells/mouse). The next day, the mice were immunized subcutaneously with 50 μl ovalbumin peptide (2 mg/ml) mixed 1:1 in CFA on each side of the chest wall. Subsequently, the mice were dosed twice daily by oral gavage with vehicle or AZM198 (400 μmol/kg) for 7 days, after which they were euthanized, and their draining lymph nodes were harvested. Cells were stained for DO11.10, CD4, and CD44 and analyzed using flow cytometry (BD Biosciences) and FlowJo software.

Circulating Antibodies

Circulating serum levels of the mouse anti-sheep globulin IgG subclasses IgG1, IgG2b, and IgG3 were assessed by ELISA (Invitrogen) according to the manufacturer’s protocol.

Statistical Analyses

The results from animal and patient studies are expressed as medians (interquartile range). All statistics were performed using GraphPad prism 8.0 (GraphPad Software, San Diego, CA). Nonparametric and parametric tests of significance were applied. For comparing two groups, Mann–Whitney U test was used, and for groups of three or more, one-way ANOVA was used. Two-way ANOVA was used to analyze differences among groups from more than one experiment. Wilcoxon signed-rank test was used to compare nonparametric paired data. Correlations were assessed using the non-parametric Spearman rank correlation analysis. A significant value was defined P<0.05 with 95% confidence intervals.

RESULTS

Serum MPO Levels Are Elevated in Patients with Active ANCA-Associated Crescentic GN

Increased MPO levels were found in patients with active PR3–ANCA-associated crescentic GN (AAGN) at 482 (330.0–1397) ng/ml, compared with active MPO-AAGN at 204.3 (42.6–396.7) ng/ml (P=0.04) and healthy controls at 96.2 (53.5–176.3) ng/ml (P< 0.001) (Figure 1A). However, we found that MPO-ANCA–containing serum inhibited MPO detection in the immunoassay by up to 40% (Supplemental Figure 2), suggesting that the MPO concentration in MPO-AAGN samples is potentially underestimated. Therefore, we only included patients with PR3-AAGN when we analyzed differences between MPO levels in active AAGN and disease remission (Figure 1B).

We found that MPO levels in PR3-ANCA patients with active disease from the RAVE trial were significantly reduced when measured at 2 months after treatment (active AAGN,
Serum MPO levels are elevated in patients with active AAV and are reduced when disease is in remission. Median (interquartile range) plasma concentration of circulating MPO in (A) MPO (n=13) and PR3 (n=14) AAV ANCA subtypes, renovascular disease (RVD) controls (n=5), and healthy controls (HC; n=10); and (B) active PR3-ANCA AAV and PR3-ANCA AAV in remission (n=58). Nonparametric Kruskal–Wallis test and Dunn multiple comparison post-test, Wilcoxon test *P<0.05; **P<0.01; ***P<0.001.

Extracellular Glomerular MPO Deposition Is Seen in Various Forms of CGN

We stained for MPO and the neutrophil marker CD15 by immunohistochemistry using biopsies from patients with various forms of CGN due to MPO-ANCA (n=5), PR3-ANCA (n=3), ANCA-negative pauci-immune GN (n=2), crescentic IgA (n=4), and SLE (n=4) (Figure 2).

Total (leukocyte and nonleukocyte associated) whole-kidney MPO deposition significantly correlated with eGFR (r=-0.68; P<0.001; Figure 3A) and proteinuria (r=0.51; P=0.02; Supplemental Figure 3C), as well as percentage of glomeruli with active cellular crescents (r=0.58; P=0.01; Figure 3B) and interstitial fibrosis and tubular atrophy on the renal biopsy (r=0.66; P=0.001; Supplemental Figure 3A). Glomerular MPO deposition correlated with eGFR (r=-0.45; P=0.03; Figure 3C), percentage of active crescents (r=0.42; P=0.04; Figure 3D), and interstitial fibrosis and tubular atrophy (r=0.53; P=0.01; Supplemental Figure 3B). We detected the presence of extracellular glomerular MPO, defined as MPO staining (brown) that was not colocalized with CD15 (red). Extracellular MPO was found close to intraglomerular CD15+ cells, but was also deposited independently along the glomerular capillaries (Figure 2, A–D, yellow arrows) and on tubular epithelial cells. When we analyzed extracellular (nonleukocyte associated) glomerular MPO deposition, we found that it correlated inversely with eGFR (r=-0.58; P=0.03; Figure 3E) and positively with crescent formation (r=0.63; P=0.02; Figure 3F, Supplemental Table 1). Total tubulointerstitial MPO deposition correlated significantly with eGFR only (Supplemental Table 2).

MPO Inhibition Inactivates Enzymatically Active MPO and Reduces Neutrophil Degranulation, ROS Production, and NET Formation in vitro

We measured the enzymatic activity of MPO, ROS production, HNP 1–3 levels, and visualized NET formation in neutrophils from patients with AAV and healthy controls in the absence and presence of AZM198. HNP 1–3, found in the azurophilic granules of human neutrophils, were chosen as a degranulation marker independent of MPO.

Neutrophils were stimulated with TNFα/PR3-ANCA or PMA, a synthetic activator of protein kinase C, in the presence or absence of AZM198. AZM198 at 2123 ± 643 AU reduced NETosis (Figure 4C) (NET area index PR3-ANCA 6.3 ± 3.3 AU versus unstimulated 2.7 ± 2.1 AU; P=0.004).

In TNFα-primed, PR3-ANCA–stimulated neutrophils, the addition of AZM198 led to a significant reduction in ROS production (Figure 4A) and HNP1–3 levels (Figure 4B) (MFI rhodamine 123: PR3-ANCA at 10,675 ± 4481 AU versus unstimulated at 1749 ± 3808 AU; P=0.004 and AZM198 at 2123 ± 643–6275 AU; P=0.04; HNP1–3 levels: PR3-ANCA at 135 ± 181 AU versus unstimulated at 0.003 ± 127 pg/ml; P=0.03).

TNFα/PR3-ANCA stimulation triggered NET formation in neutrophils from patients with active AAV (open squares) and healthy controls (open circles), whereas AZM198 reduced NETosis (Figure 4C) (NET area index PR3-ANCA 6.3 ± 3.3 AU versus unstimulated 2.7 [9.0–3.3] AU and AZM198 3.3 ± 1.9–4.2 AU; P=0.01). Representative images of NETosis induced by TNFα/PR3-ANCA and PMA stimulation in the presence and absence of AZM198 are shown in Figure 4, D–F and Supplemental Figure 4, B and C.
EC/PR3-ANCA–Stimulated Neutrophil Coculture Results in EC Damage that Is Reduced by MPO Inhibition

Recent reports have shown that activated EC can induce NET formation that can promote EC death.22–24

To quantify EC damage after coculture with healthy control neutrophils (n=5), we used TNFα-primed, BrdU-labeled EC and measured release of BrdU-labeled DNA fragments and vWF into the coculture supernatants. There was measurable EC death when cocultured with TNFα/PR3-ANCA–stimulated neutrophils (BRDU 0.44±0.04 AU), which was reduced with the inclusion of DNase (0.36±0.05; P=0.03), as well as AZM198 (0.34±0.04 AU; P=0.01; Figure 5A). However, we did not detect a significant reduction in supernatant vWF levels in the presence of AZM198 (PR3-ANCA 259±40 ng/ml versus AZM198 197±28 ng/ml; P=0.06; Figure 5B). EC morphology was visibly disrupted during the EC–PMN 18 hours coculture when PMN were stimulated, but this was attenuated in the presence of AZM198 (Figure 5, C–F).

MPO Inhibition Attenuates Glomerular Inflammation in the NTN Model

During the initial phase of NTN, injury is mediated by infiltrating neutrophils whose accumulation in glomeruli peaks...
2 hours after the administration of NTS. Subsequently, the disease develops over the next 7 days and is initiated by CD4 T cells against the immunizing antigen and macrophages. To confirm that MPO inhibition would also be protective in vivo, we assessed the effect of AZM198 in a 7-day and 2-hour mouse NTN model.

One day after NTS injection and after the time of early neutrophil entry into the kidney, mice received vehicle \((n=16)\) or AZM198, at 133 \(\mu\)mol/kg \((n=8)\) or 400 \(\mu\)mol/kg \((n=8)\) by gavage twice a day for 7 days. Two animals in the vehicle group (on day 6 and 7) and one in the 400 \(\mu\)mol/kg AZM198 group (day 2) died, and two vehicle animals were anuric. MPO inhibition using AZM198 at either dose led to a reduction in renal injury. In particular, median (interquartile range) glomerular thrombosis score in the 133 \(\mu\)mol/kg group was 0 (0–0.1) and in the 400 \(\mu\)mol/kg group 0.8 (0.5–1.3) and versus vehicle 2.6 (1.0–3.3) \((P<0.001 \text{ and } P=0.24\), respectively; Figure 6, C–E). There was also a reduction in proteinuria in both AZM198-treated groups compared with vehicle (133 \(\mu\)mol/kg, 1.3 [1.0–1.9] mg/24 h and 400 \(\mu\)mol/kg, 1.0 [0.1–1.2] mg/24 h versus vehicle, 3.5 [1.0–7.1] mg/24 h; \(P=0.22 \text{ and } P=0.02\), respectively; Figure 6A). Plasma creatinine was lower in both AZM198 groups compared with vehicle (plasma creatinine 133 \(\mu\)mol/kg, 12.5 [9.0–14.8] \(\mu\)mol/L and 400 \(\mu\)mol/kg, 6.0 [1.2–8.0] \(\mu\)mol/L and versus vehicle, 16.1 [8.4–52.8] \(\mu\)mol/L; \(P=0.26 \text{ and } P=0.04\), respectively; Figure 6B). Immunofluorescence staining showed a significant reduction in macrophage and CD4-positive T cell infiltration and glomerular MPO deposition in the kidneys of

**Figure 3.** Total (neutrophil-associated and extracellular) whole kidney and glomerular as well as extraleukocyte glomerular MPO deposition alone correlates with histological and clinical disease severity in patients with diverse forms of CGN (MPO-ANCA \(n=5\), crescentic IgA \(n=4\), SLE \(n=4\), PR3-ANCA \(n=3\), ANCA-negative \(n=2\)). Percentage of total whole kidney \((n=18)\) and glomerular intra- and extraleukocyte MPO deposition \((n=11)\) (percentage of MPO-stained area per whole kidney section or glomerulus) with eGFR (ml/min per 1.73 m²) (A, C, and E, respectively) and percentage of active cellular crescents (B, D, and F, respectively). Nonparametric Spearman rank correlation analysis, \(*P<0.05; \**P<0.01; \***P<0.001.**
the animals that were treated with AZM198 133 μmol/kg compared with vehicle controls: F4/80 CTCF in AZM198, 41.1 (24.0–42.3) AU versus vehicle, 68.2 (58.3–75.6) AU (P=0.01); number of T cells per glomerulus AZM198, 0 (0–0.4) versus vehicle, 1 (0–2.1); and MPO CTCF in AZM198, 45.9 (40.6–51.6) AU versus vehicle, 67.7 (61.0–102.0) AU (P=0.01) (Figure 7, A–G, Supplemental Figure 5, C and D).

To assess the effect of AZM198 on glomerular neutrophil accumulation, we dosed mice with either vehicle (n=4) or AZM198 133 μmol/kg (n=4) for 48 hours. On day 3, NTS was administered and kidneys were collected 2 hours later, for assessment of glomerular neutrophil infiltration (Figure 7H, Supplemental Figure 5, A and B). We found a non-significant reduction in glomerular neutrophil infiltration in those treated with AZM198 (Ly6g CTRF AZM198, 26.5 [23.0–70.7] AU versus vehicle, 71.0 [45.3–152.8] AU; P=0.11).

Effect of MPO Inhibition on Adaptive Immunity

Previous reports have suggested that in murine models of CGN, acute glomerular disease is attenuated in MPO-deficient animals, but there is augmentation of adaptive T cell immunity. To investigate if this also occurs after pharmacologic inhibition of MPO, we determined the effect of AZM198 on T and B cell responses in context of two different models. We investigated the effect on humoral and cellular immunity in the Th1- and Th17-mediated and immune complex–driven NTN model described above, and the effect on antigen-specific T cell responses in an adoptive transfer model of T cell receptor transgenic T cells recognizing an ovalbumin peptide, i.e., the DO11.10 mice.

There was no significant increase in the concentration of IgG1, IgG2b, and IgG3 subclasses in the AZM198-treated animals compared with vehicle controls: IgG1, 354.5 (33.6–465.2) μg/ml versus vehicle, 347.3 (212.5–994.5) μg/ml (P=0.38); IgG2b, 191.8 (73.9–305.3) μg/ml versus vehicle, 163.0 (30.9–297.7) μg/ml (P=0.51); and IgG3, 8.4 (5.1–50.6) μg/ml versus vehicle, 16.0 (3.4–46.6) μg/ml (P>0.99) (Figure 8, B–D).

CD4+ splenocytes from the NTN mice that received vehicle or AZM198 133 μmol/kg were isolated and restimulated with
sheep globulin in vitro for 72 hours, after which expression of CD44 and CD62L were assessed by flow cytometry (Figure 8A). There was no difference in the median frequency of CD44^high CD62L^low T cells among the vehicle and treatment group, suggesting that AZM198 attenuated glomerular inflammation without an increase in adaptive T cell responses (median CD44^high CD62L^low % of CD4^+ cells: AZM198, 49.6% (42.9%–55.9%) versus vehicle, 47.6% (38.6%–58.2%) (P=0.78).

**Figure 5.** PR3-ANCA stimulated PMNs induce EC damage that is reduced by pharmacological MPO inhibition with AZM198. (A) Median (interquartile range) BrdU release and (B) vWF release in the supernatants of EC/PMN coculture. BrdU-labeled ECs were primed with TNFα for 18 hours. After rinsing to remove all traces of TNFα, ECs were cultured without or with inclusion of PMN in the absence or presence of TNFα/PR3-ANCA, and with AZM198 or DNAse for a further 18 hours, after which vWF release and BrdU-labeled DNA fragments from ECs was analyzed; Friedman test **P<0.01; ***P<0.001. Merged microscopic images of TNFα-primed ECs in monoculture (C) or coculture (D–F) with unstimulated PMN (D), TNFα/PR3-ANCA–stimulated PMNs in the absence (E) or presence (F) of AZM198 (10 μM). ECs are stained with vascular endothelial (VE) cadherin (red), PMNs are stained with MPO (green), and nuclei are stained with DAPI (blue).
Finally, we tested the effect of AZM198 on antigen-specific T cell responses, using adoptive transfer of DO11.10 lymphocytes into OVA-immunized mice (Figure 8, E and F). The frequency of activated CD4 cells and activated OVA-specific T cells in draining lymph nodes were determined by staining for CD4, CD44, and the DO11.10 T cell receptor. Treatment with AZM198 did not result in higher frequency of activated total (CD4, CD44) or OVA-specific (CD44, KJ) T cells (CD44 high% on CD4+ cells: AZM198, 12.6% (11.8%–13.8%) versus vehicle, 19.35% (16.5%–19.9%) (P=0.10); CD44 high KJ% on CD4+ cells (AZM198, 1.9% [1.64%–3.14%] versus vehicle, 4.1% [3.9%–5.0%]; P=0.05). These results suggest that pharmacologic MPO inhibition, unlike genetic MPO deficiency, does not augment antigen-specific T cell responses in the draining lymph nodes.

**DISCUSSION**

We have shown that MPO can be detected intra- and extracellularly in inflamed glomeruli of biopsy specimens from patients with the most common form of CGN in adults, AAV, as well as in ANCA-negative disease, crescentic IgA nephropathy, and lupus nephritis. Total, neutrophil-associated, and extracellular MPO deposition in the whole kidney, and total glomerular MPO deposition, correlated with clinical and histologic disease severity in the initial renal biopsy specimen. Extracellular glomerular MPO deposition alone correlated with eGFR and crescent formation, suggesting that extracellular MPO could be an important driver mediating renal inflammation. Our data on the distribution of extracellular and intracellular MPO within kidneys of patients with AAV confirm and extend previous observations.10,14,27,28 Additionally, we measured circulating MPO levels and showed increased MPO levels in patients with AAV with active disease compared with disease in remission, confirming it as a modifiable marker of disease activity.

We also showed that MPO inhibition inactivated enzymatically active MPO and reduced neutrophil degranulation, ROS production, and NET formation in cytokine- and...
Figure 7. MPO inhibition with AZM198 reduces glomerular MPO deposition as well as macrophage and T cell infiltration in nephrotic nephritis with no significant effect on neutrophil recruitment. Median (interquartile range) glomerular CTCF and immunofluorescence staining for (A, C, and D) F4/80-expressing macrophages, (B, E, and F) MPO, and (G) glomerular CD4-positive cells in the glomeruli of mice with NTN treated with (C and E) vehicle or (D and F) MPOi at 133 μmol/kg. Mann–Whitney test, *P<0.05; **P<0.01. (H) Effect of MPOi on glomerular neutrophil accumulation: mice were dosed with either vehicle (n=4) or MPOi (n=4) twice a day at 133 μmol/kg for 48 hours after the injection of NTS (12 hours after the last dosing). There is no significant effect on neutrophil accumulation at 2 hours after NTS injection; yellow lines outline glomeruli.
ANCA-stimulated neutrophils from patients and healthy controls.

Recent studies support the central role for NETs in the pathophysiology of different forms of CGN. Impaired NET degradation in patients with SLE has been associated with higher double-strand DNA titers and frequency of flares.29 In MPO-ANCA CGN a reduced degradation of NETs has been observed, implicating NETs as a means of breaking tolerance to ANCA autoantigens.30 The latter was corroborated in animal studies where presentation of extracellular DNA derived from NETotic neutrophils to myeloid dendritic cells led to MPO-ANCA and PR3-ANCA production, with subsequent vasculitis-like renal lesions in C57BL/6 mice.31 In addition, augmented NET formation after infection in dectin−/− mice leads to a vasculitic phenotype, which can be attenuated by inhibiting NET generation.32

Although there have been recent insights into the processes regulating neutrophil degranulation and NETosis, the exact

Figure 8. MPO inhibition with AZM198 did not increase cellular and humoral responses in nephrotoxic nephritis (A–D): (A) median (interquartile range) frequency of CD44^{high} CD62L^{low} CD4^{+} splenocytes from mice with NTN (n=8 per group). (B–D) Effect of MPO inhibition on serum humoral immune responses to sheep globulin: median (IQR) IgG subclass concentrations IgG1, IgG2b, and IgG3, respectively (n=8 per group). (E and F) Antigen-specific T cell responses using adoptive transfer of DO11.10 lymphocytes into OVA-immunized mice: median (interquartile range) frequency of CD44^{high}CD4^{+} cells (E) and CD44^{high} KJ^{+} CD4^{+} cells (F). Mann–Whitney test, P=NS.
molecular mechanisms remain to be elucidated. Upon neutrophil stimulation, the oxidative burst generates H$_2$O$_2$ that triggers the activation of MPO, which subsequently catalyzes the formation of ROS. ROS stimulate MPO to trigger the activation and translocation of NE from azurophilic granules to the nucleus, where NE proteolytically processes histones to disrupt chromatin packaging. Subsequently, MPO binds chromatin and synergizes with NE in chromatin decondensation and NET formation.

One of the hallmarks of CGN is endothelial injury and rupture of the glomerular capillary loops due to aberrant leukocyte activation. Cytokine-activated EC produce IL-8, which has a significant role in promoting neutrophil recruitment and migration. Recent reports have shown that activated EC not only interact with neutrophils during transmigration, but may also induce NETosis, leading to EC death. Both ROS and serine proteases released during neutrophil degranulation and presented on NETs have been previously proposed as initiators of EC damage in vasculitic lesions in vivo. We found that TNFα/PR3-ANCA–stimulated neutrophils led to EC injury that was attenuated by enzymatic NET degradation using DNase as well as pharmacologic inhibition of MPO.

We used the accelerated NTN model of CGN, which is dependent on adaptive and innate immunity and representative clinically of a rapidly progressive GN due to immune-complex disease. Despite the initiation of treatment 1 day after the glomerular neutrophil influx, AZM198 at two different doses attenuated glomerular inflammation clinically and histologically. When we assessed the effect of AZM198 on neutrophil influx, we found no significant difference in neutrophil influx at an early time point. Acknowledging the small sample size of animals tested in this experiment (n=4 in each group), we may have missed a biologic effect on neutrophil recruitment, which has been shown in previous reports. Alternatively, our results may be explained by an effect of the delayed MPO inhibition on neutrophil activation/degranulation and inhibition of the biologic effects of enzymatically active MPO, in addition to any effect on neutrophil recruitment.

AZM198 is a membrane-permeable compound that acts as a suicide substrate for MPO and has been shown to inhibit enzymatically active extracellular MPO, whereas at higher concentrations it also inhibits intragranular MPO, together with NET formation. The rationale for the dose setting in the current experiments was to yield exposures that would mainly inhibit extracellular MPO (133 μmol/kg) or extracellular as well as intragranular MPO (400 μmol/kg). A satellite group of mice was dosed with 135 μmol/kg AZM198 and sampled at 2, 12, and 19 hours to define the maximal and trough levels, and to bridge the data with the terminal samples of the efficacy study. The observed pharmacokinetic responses are shown in Supplemental Figure 6, which also shows the concentration of AZM198 predicted to inhibit 80% of extracellular MPO and 80% peroxidase activity in human whole blood (the latter corresponding to the intragranular MPO potency). The reported IC$_{50}$ of AZM198 is 0.015 μM.

Therapeutic AZM198 levels were achieved throughout the study. Our PK data suggest that the peak plasma concentrations our MPOi achieved in the current experiments were in the range found to inhibit intragranular MPO, even using the lower dose of AZM198. However, the limited benefit of 400 μmol/kg over 133 μmol/kg may argue that some beneficial effect of AZM198 on glomerular inflammation in this model might also be due to the inhibition of extracellular MPO activity.

The NTN model dissociates the effect of the AZM198 from the induction and perpetuation of autoreactivity. As there is no autoimmunity in this model and the treatment is delayed, we can reasonably assume that this is testing MPO inhibition largely for its effects in the kidney and not on the induction or maintenance of immunity to sheep Ig. Our data confirm and extend previous observations made by Zheng et al., which showed that prophylactic MPO inhibition attenuated disease in a mild, noncrescentic murine anti-GBM model and a model of lung vasculitis.

Importantly, our methodology differs in that we used an accelerated severe NTN model with features of glomerular thrombosis and administered delayed MPO inhibition, reflecting clinical presentations to a greater extent. Moreover, we demonstrate for the first time that there is no effect on adaptive T cell responses, a critical requirement for translation of such therapy to the clinic in the setting of autoimmune CGN.

As the 1-year survival of patients with AAV has improved from 20% to over 80% over the past 5 decades, current clinical needs have focused on the long-term outcome. There is still significant morbidity in vasculitis patients associated with current treatments, and there are also forms of CGN such as IgA nephropathy, for which we have limited effective treatments. Glucocorticoid therapy is associated with significant long-term morbidity and recent trials in CGN have been designed to address the need for steroid minimization (PEXIVAS, CLEAR, ADVOCATE, and our recent steroid-free maintenance AAV cohort study).

In conclusion, we have demonstrated that therapeutic MPO inhibition reduced degranulation and NET formation in neutrophils from patients with AAV, and attenuated kidney damage in preclinical models of CGN without augmenting adaptive immune responses, suggesting that MPO inhibition may be an effective adjunctive therapy in various forms of CGN.

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Prof. Salama, Dr. Antonelou, and Prof. Walker designed the study. Dr. Antonelou, Dr. Wang, and Dr. Evans carried out experiments. The RAVE ITN research group provided patient samples. Dr. Antonelou, Dr. Michaèlsson, and Dr. Henderson analyzed the data. Dr. Antonelou and Dr. Evans made the figures. Dr. Antonelou, Dr. Michaèlsson, Prof. Salama, and Prof. Unwin drafted and revised the paper. All authors approved the final version of the manuscript.
DISCLOSURES

Dr. Michaelsson has a patent 1-[2-(AMINOMETHYL) BENZYL]-2-THIOXO-1, 2, 3, 5-TETRAHYDRO-4H-PYRROLO [3, 2-d] PYRIMIDIN-4-ONES AS INHIBITORS OF MYELOPEROXIDASE issued. Dr. Unwin reports employment with AstraZeneca Pharmaceutical R&D (declared in the paper), which provided the MPO inhibitor and was involved collaboratively in the work.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2019060618/-/DSSupplemental.

Table 1. Clinical and histological features and percentage area of MPO deposition in the renal biopsy specimens of patients with various forms of CGN.

Table 2. Correlation of intrarenal MPO with clinical and histological parameters.

Figure 1. Purified human Ig-induced neutrophil degranulation.

Figure 2. Inhibition studies of MPO by MPO-ANCA-containing serum.

Figure 3. Correlation of intrarenal MPO deposition with interstitial fibrosis and tubular atrophy and urinary protein-to-creatinine ratio.

Figure 4. Effect of AZM198 on PMA-induced NET formation.

Figure 5. Immunofluorescence staining for CD4-positive cells and neutrophils in the NTN model.

Supplemental Figure 1. Purification of neutrophils.

Supplemental Figure 2. Inhibition studies of MPO by MPO-ANCA-containing serum.

Supplemental Figure 3. Correlation of intrarenal MPO deposition with interstitial fibrosis and tubular atrophy and urinary protein-to-creatinine ratio.

Supplemental Figure 4. Effect of AZM198 on PMA-induced NET formation.

Supplemental Figure 5. Immunofluorescence staining for CD4-positive cells and neutrophils in the NTN model.

Supplemental Figure 6. Pharmacokinetic studies of AZM198.

REFERENCES


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