SUPPLEMENTAL MATERIALS

SUPPLEMENTARY METHODS

TABLE 1.

SUPPLEMENTARY METHODS SECTION

Clinical Study Design

36 patients with stable disease were enrolled in double-blind placebo-controlled treatment trial with N-acetylcysteine (NAC; FDA approval, IND No: 101,320; clinicaltrials.gov identifier: NCT00775476). The mean (± SE) age of patients was 44.6 (± 1.8) years, ranging between 25-64 years (Table 1). 34 of the patients were females including 30 Caucasians, two African-Americans, and two Hispanic. 2 patients were Caucasian males. 42 healthy subjects were individually matched for each patient blood donation for age within ten years, gender, and ethnic background and freshly isolated cells were studied in parallel as controls for immunological studies. The mean (± SE) age of controls was 44.4 (± 1.7) years, ranging between 22-63 years. 39 of the controls were females including 36 Caucasians, two African-Americans, and one Hispanic. 3 controls were Caucasian males.

We randomized 36 SLE patients equally to receive either placebo or NAC in one of three treatment arms of increasing doses: 600 mg, 1,200 mg, or 2,400 mg twice daily for three months. We enrolled 12 patients per dosing group, 9 were randomized to receive NAC while 3 were randomized to receive placebo in each group. We expected a 15% dropout, resulting in 8 active and 2 placebo patients per group. We employed the following dose-progression rule: 6 of 8 active patients needed to tolerate each dose or show no worsening of SLE as defined in the Data Safety and Monitoring Plan (DSMP) to proceed to the next higher dosing group.

Inclusion criteria: age > 18 yr, male or female, SLE with $\geq$ 4 of eleven diagnostic criteria approved by the American College of Rheumatology [1], clinically stable disease on prednisone ($\leq$ 10 mg/day), anti-malarials, azathioprine or mycophenylate mofetil as allowable immunosuppressant medications.

Exclusion criteria: Patients who were pregnant or lactating, had moderately serious or serious co-morbidities (e.g., diabetes mellitus, congestive heart failure, chronic obstructive pulmonary disease, chronic renal insufficiency), history of chronic infections (e.g., HIV, hepatitis B virus, hepatitis C virus, mycobacteria, bronchiectasis), infections in the past month, history of severe or recurrent infections, and smokers were excluded. Patients taking over-the-counter antioxidants that can enhance the effect of NAC were excluded. Alternatively, patients taking acetaminophen (Tylenol) which is metabolized by hepatic cytochrome P450 enzymes, primarily CYP2E1, to a toxic intermediate compound (N-acetyl-para benzoquine imide), requiring detoxification by hepatic GSH [2], were also excluded. One daily dose of multivitamin, containing $\leq$ 500 mg of vitamin C and $\leq$ 30 IU of vitamin E was allowed for each patient. Patients with acute flare of SLE threatening vital organs and requiring intravenous cyclophosphamide treatment, were excluded. Patients receiving biologicals (rituximab, abatacept) and enrolled in other clinical trials were also be excluded.

Study materials: Identical appearing capsules containing NAC or placebo (dextrose) were manufactured by the compounding pharmacy within the Department of Pharmacy at SUNY Upstate Medical Center. Both NAC and dextrose were obtained from Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ). Each capsule contained 600 mg NAC or placebo. All capsules were rolled in NAC to equalize smell. Each bottle contained capsules needed for 32 days. The pills were counted when the bottles were returned to ascertain

compliance. Our study biostatistician worked closely with the Department of Pharmacy so that PI and research staff remained blinded to participants' randomized condition.

Patient visit schedules
Screening visit: patients were evaluated for inclusion and exclusion criteria.
Visit No. 1: Baseline assessment of clinical disease activity, blood draw for routine laboratory parameters and immunological parameters and GSH were performed before the first NAC dose. Additional blood samples were drawn 3 h and 6 h after the initial NAC dose for measurement of GSH by HPLC. First monthly supply of NAC or placebo was provided.
Visit No. 2: One-month visit: clinical assessment was performed and blood was drawn for routine laboratory tests and measurement of immunological parameters and GSH levels before morning NAC dose. Second monthly supply of NAC or placebo was provided.
Visit No. 3: Two-month visit: clinical assessment was performed and blood was drawn for routine laboratory tests and measurement of immunological parameters and GSH levels before morning NAC dose. Third monthly supply of NAC or placebo was provided
Visit No. 4: Three-month visit: clinical assessment was performed and blood was drawn for routine laboratory tests and measurement of immunological parameters and GSH levels before morning NAC dose.
Visit No. 5: Four-month visit (end of 1 month washout): clinical assessment was performed and blood was drawn for routine laboratory tests and measurement of immunological parameters and GSH levels.

For each patient visit, we obtained blood from healthy donors matched for age (within one decade), gender, and ethnicity, to be used as control for flow cytometry measurement of mitochondrial function, T-cell activation and death pathway selection, Ca^{2+} flux, production of nitric oxide (NO) and reactive oxygen intermediates (ROI), activation of mTOR and expression of Foxp3 in subsets of T cells and B cells. GSH was measured in whole blood and isolated peripheral blood lymphocytes (PBL) by HPLC. Each patient provided seven blood samples (visit 1/0h, visit 1/3 h, visit 1/6 h, visit 2 in 1 month, visit 3 in 2 months, visit 4 in 3 months, visit 5 in 4 months (after one month washout). 42 healthy controls have also donated blood to use as control for HPLC analysis of GSH, flow cytometry of live cells as well as for the gene expression and signaling studies. We have recorded ~384 flow cytometry data points for each of the five patient visits, both for the patients and the matching controls. DNA, RNA, and protein lysates have been saved and catalogued for each visit. Individual controls gave blood on multiple occasions.

Clinical outcomes and assessments
1. Tolerance: common side effects (nausea, bloating, bad taste) seen in prior trials were specifically asked for at each visit and reviewed by our Data Safety and Monitoring Board (DSMB) bi-annually. Tolerance and safety were primary clinical outcomes.
2. Blinding: smell or taste was specifically asked for.
3. Clinical assessments: a complete physical examination of the cardiovascular, respiratory, gastrointestinal, musculoskeletal, neurological systems, skin, head, neck, sinuses, nasal and oral cavities were performed at each visit. SLE disease activity was assessed by using the British Isles Lupus Assessment Group (BILAG) [3] and SLE Disease Activity Index (SLEDAI) [1]. The

concurrent use and dosage of prednisone and other medications were documented.
4. Fatigue was assessed by using a validated Fatigue Assessment Scale (FAS), a self-questionnaire that provides a subjective measurement of fatigue severity and has shown to have a high degree of internal consistency, validity, and sensitivity to changes in clinical condition [4].
5. Routine blood tests included complete blood count, liver and kidney function test, urinalysis and lupus-relevant laboratory tests, such as anti-double-stranded DNA, C3, and C4.
6. Compliance: compliance of patients was assessed based on self-reporting and pill counts. Pill counts in returned study drug vials indicated a compliance rate of 98.4 ± 1.0%. The PI did not participate in scoring of patients during enrollment or follow-up visits to avoid bias stemming from potential knowledge of GSH levels due to oversight of immuno-biological and HPLC studies. Upon review of source documents, the PI discovered that two patients received prednisone in excess of 10 mg/day during the study: patient AIM1-005 in the placebo group (for attacks of asthma): 15 mg (visit 2), 20 mg (visit 5); patient AIM1-007 in the 1st NAC dosing group of 1.2 g/day: 15 mg (visit 1), 13 mg (visit 2), 13 mg (visit 3), 11 mg (visit 4), 11 mg (visit 5). We also analyzed the data leaving out these two patients which did not affect the significant improvement in SLEDAI, BILAG, and FAS. However, as tolerance and disease activity, but not prednisone dosage, were clinical outcomes, these two patients, both of whom well tolerated the study drug, were retained for the intent-to-treat analysis.

Immuno-biological outcomes and assessments

The primary immunobiological outcome was a measurable increase or normalization of GSH previously found to be diminished in PBL by HPLC [5]. The secondary immunobiological outcomes were the modulation of $\Delta\psi_m$, ROI production (oxidative stress), activation-induced apoptosis [5], activation of mTOR[6] and expression of FoxP3 [7].

HPLC assay of NAC and GSH. Reduced glutathione (GSH) was measured by reverse phase ion-exchange HPLC using UV detection at 365 nm, as earlier described [5, 8]. We measured GSH in whole blood and PBL before (visit 1; 0 h) and after the first NAC/placebo dose (visit 1; 3 h and 6 h) and upon each monthly follow-up visit (visits 2-4: between 9-11 am after having taken the last NAC/placebo capsule 8 pm the night before), and after 1 month wash-out (visit 5). HPLC analysis required ~ 0.25 ml of whole blood and 5 x 10^6 PBL which were obtained from a total of 10 ml of blood collected at each time point.

Assessment of viability, mitochondrial transmembrane potential ($\Delta\psi_m$), mitochondrial mass, Ca^{2+} levels, NO and ROI production, mTOR activity, and Foxp3 expression in resting and activated T cell subsets and B cells by flow cytometry. Cell viability was monitored with annexin V-FITC, annexin V-PE, or annexin V-Cy5 matched with emission spectra of propidium iodide (PI) to detect Annexin V+/PI- apoptotic cells, mitochondrial potentiometric ($DiOC_6$, 40 nM, excitation: 488 nm, emission: 525 nm recorded in FL-1; TMRM, 100 nM, excitation: 543 nm, emission: 567 nm recorded in FL-2), potential-insensitive mitochondrial dyes MitoTracker Green-FM (MTG, 100 nM; excitation: 490 nm, emission: 516 nm recorded in FL-1) or nonyl acridine orange (NAO, 50 nM; excitation: 490 nm, emission: 540 nm recorded in FL-1), superoxide sensing hydroethidine (HE, 1 μM) and H_2O_2-sensing dichlorofluorescein diacetate (DCF-DA, 1 μM), nitric oxide sensor 4-amino-5-methylamino-2',7'-difluoroflourescein diacetate (DAF-FM, 1 μM, excitation: 495, emission: 515 nm recorded in FL-1), or cytosolic (Fluo-3, 1 μM, excitation: 506 nm, emission: 526 nm recorded in FL-1) and mitochondrial Ca^{2+}-sensitive

fluorescent probes (Rhod-2, 1 μM), respectively. All metabolic and mitochondrial sensor dyes were obtained from Invitrogen (Carlsbad, CA) and used as earlier described [6, 9-12].We examined unstimulated cells and cells stimulated with CD3/CD28 for 16 h and measured cell death pathway selection in T cells by concurrent staining with annexin V-Alexa 647 and PI as well as cell type-specific antigens. T-cell subsets were analyzed by staining with antibodies to CD4, CD8, and CD25. B cells were identified by CD19 staining. mTOR activity was assessed by phosphorylation of its downstream substrate S6 ribosomal protein (pS6-RP) using a monoclonal antibody to pS6-RP (Cell Signaling; Beverly, MA; Cat. No. 4851) in cells permeabilized with Cytofix/CytopermPlus (BD Biosciences). Foxp3 expression was measured in permeabilized cells using Alexa-647-conjugated antibody from BioLegend (San Diego, CA; cat No 320014). We recorded up to 11 parameters simultaneously using a Becton Dickinson LSRII flow cytometer equipped with 20 mW solid-state Nd-YAG (emission at 355 nm), 20 mW argon (emission at 488 nm), 10 mW diode pumped solid state yellow-green (emission 561 nm) and 16 mW helium-neon lasers (emission at 634 nm). Each patient's cells were processed and analyzed in parallel with a matched control.

Statistics

Power/Sample Size. Sample size requirements for this study were based on a type I error rate of 0.05, two-tailed testing, and a minimal power level of .80, using Sample Power v2 software (SPSS Chicago, Ill). Estimates of effect size were based on our preliminary data [5] and the relevant literature to assess/compare mean values of GSH across treatment groups (placebo, lowest NAC, medium NAC, highest NAC dose). Our analysis suggested that administration of NAC to a minimum of 8 patients per treatment arm should have 83.7%power to detect a 42% elevation of intracellular GSH in SLE patients (3.60 ± 0.30 ng/μg protein) to reach those in normal donors (5.11 ± 0.50 ng/μg protein [5]. This study compared the longitudinal effects of three different doses of NAC and a placebo control condition, before (visit 1), during (visit 2, after 1 month; visit 3, after 2 months; visit 4, after 3 months) and following a 3-month intervention (visit 5, after 1 month washout). Thus, we were employing a double-blinded longitudinal trial design comparing 4 groups on observations collected at intervals pre, during and post intervention.

Overall clinical effectiveness of NAC relative to placebo was analyzed with multilevel modeling as implemented in the STATA routine XTMIXED (from StataCorp, College Station, TX), with the three nested levels being drug group, subject within drug group and study visit within subject. All models included fixed effects for drug group, study visit and the drug group by study visit interaction along with random intercepts at each design level. Our test for efficacy was the fixed effect for the drug group by study visit interaction which, if significant indicated that the change in outcome scores over time was significantly different about drug groups. The reduction in lupus disease activity SLEDAI scores was greater for the NAC than placebo group, as indicated by a significant visit by drug interaction ($z = -2.14$, $p = 0.033$). The reduction in BILAG scores was also greater for NAC than placebo group. The overall effect was statistically significant as indicated by a significant visit by drug interaction ($z = -2.62$, $p = 0.009$). Two-tailed paired t-test was used to assess the effects of placebo and of each and all NAC doses on clinical indices and biomarkers recorded on visits 2-5 relative to visit 1; $p < 0.05$ was considered

significant. Patients and controls were compared with two-tailed unpaired t-test.

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Table 1. Demographic data of SLE patients enrolled into placebo and three NAC treatment arms:
Dose 1, 0.6 g twice daily; Dose 2, 1.2 g twice daily; Dose 3, 2.4 g twice daily

Placebo	Dose	Duration (days)	Side effect	Gender	Age	Ethnicity	Age of onset	Disease duration
AIM1-003	0	90	0	Female	48	White	41	8
AIM1-005	0	90	0	Female	35	White	25	10
AIM1-009	0	90	0	Female	31	White	23	9
AIM1-015	0	90	0	Female	61	White	52	9
AIM1-017	0	<30	0	Female	26	White	21	5
AIM1-021	0	90	0	Female	53	White	37	17
AIM1-027	0	90	0	Female	54	White	48	6
AIM1-029	0	90	0	Male	51	White	49	3
AIM1-033	0	90	0	Female	38	White	32	11
Mean					44.11		36.44444444	8.666666667
NAC	Dose							
AIM1-001	1	90	0	Female	49	White	43	6
AIM1-002	1	90	0	Female	33	White	21	12
AIM1-004	1	90	0	Female	56	White	53	3
AIM1-006	1	90	0	Female	42	AA	33	9
AIM1-007	1	90	0	Female	33	White	22	11
AIM1-008	1	90	0	Female	40	White	35	5
AIM1-010	1	90	0	Female	54	White	46	8
AIM1-011	1	90	0	Female	59	White	56	2
AIM1-012	1	90	0	Female	44	White	39	6
AIM1-013	2	90	0	Female	25	White	24	2
AIM1-014	2	90	0	Female	41	White-Hispanic	27	14
AIM1-016	2	60	0	Female	50	White	48	2
AIM1-018	2	90	0	Female	46	AA-Hispanic	30	5
AIM1-019	2	90	0	Female	64	White	60	3
AIM1-020	2	90	0	Female	37	White	32	4
AIM1-022	2	90	0	Female	52	White	48	4
AIM1-023	2	90	0	Female	48	AA	43	5
AIM1-024	2	90	0	Female	31	White	30	1
AIM1-025	3	90	0	Female	33	White	31	1
AIM1-026	3	19	Heartburn	Female	49	White	29	19
AIM1-028	3	60	Nausea	Male	25	White	23	2
AIM1-030	3	90	0	Female	43	White	24	14
AIM1-031	3	90	0	Female	50	White	38	12
AIM1-032	3	42	Nausea	Female	56	White	54	3
AIM1-034	3	90	0	Female	60	White	56	2
AIM1-035	3	90	0	Female	44	White	40	3
AIM1-036	3	90	0	Female	45	White	41	5
Mean					44.78		38	6.037037037

DOB, date of birth

Age, age of onset, and disease duration are expressed in years

AA, African-American