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Regulation of STAT3 and NF- κ B Activations by S-Nitrosylation in Multiple Myeloma

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Abstract

Numerous reports suggest that aberrant activations of STAT3 and NF- κ B promote survival and proliferation of multiple myeloma (MM) cells. In the present report, we demonstrate that a synthetic S-nitrosothiol compound, S-nitroso-N-acetylcysteine (SNAC), inhibits proliferation and survival of multiple MM cells via S-nitrosylation-dependent inhibition of STAT3 and NF- κ B. In human MM cells (e.g. U266, H929, and IM-9 cells), SNAC treatment increased S-nitrosylation of STAT3 and NF- κ B and inhibited their activities. Consequently, SNAC treatment resulted in MM cell cycle arrest at G1/S check point and inhibited their proliferation. SNAC also decreased the expression of cell survival factors and increased the activities of caspases, thus increased sensitivity of MM cells to melphalan, a chemotherapeutic agent for MM. In U266 xenografted mice, SNAC treatment decreased the activity of STAT3 and reduced the growth of human CD138 positive cells (U266 cells) in the bone marrow and also reduced their production of human IgE into the serum. Taken together, these data document the S-nitrosylation mediated inhibition of MM cell proliferation and cell survival via inhibition of STAT3 and NF- κ B pathways and its efficacy in animal model of MM.

Graphical abstract

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Keywords

Bcl-2; Cdk; Cyclin; multiple myeloma; NF- κ B; proliferation; S-nitroso-N-acetylcysteine (SNAC); S-nitrosylation; STAT3; U266; Xenograft; apoptosis

Introduction

Multiple myeloma (MM) is characterized by abnormal proliferation of malignant plasma B cells in the bone marrow. MM is diagnosed by the presences of paraprotein in the blood or urine, hypercalcaemia, renal insufficiency, anemia, and bone lesions. MM is treatable by chemotherapy (e.g. melphalan and bortezomib), steroid therapy (e.g. dexamethasone and prednisone), immunomodulatory therapy (e.g. thalidomide and lenalidomide), radiotherapy, and stem cell transplantation but rarely curable.

There is growing evidence that interleukin-6 (IL-6), produced in either an autocrine or paracrine manner, plays a critical role in growth and survival of MM cells and their malignant progression [1]. IL-6 binds its cognate receptor gp130 to trigger Janus kinase 2 (JAK2) activation. Subsequently, signal transducers and activators of transcription 3 (STAT3) is phosphorylated by JAK2, undergoes dimerization, and translocates to the nucleus [2]. STAT3 regulates target gene expression downstream to accelerate tumor cell proliferation, prevent apoptosis, and promote tumor angiogenesis [3]. MM cells also frequently exhibit a constitutive activation of nuclear factor- κ B (NF- κ B), a crucial transcription factor for cell cycle progression, inhibition of apoptosis and cell adhesion, thus promoting carcinogenesis and cancer progression [4]. NF- κ B was also reported to play a critical role in paracrine tumor necrosis factor α (TNF α)-induced IL-6 expression in MM cells [5], suggesting a cross-talk between NF- κ B and STAT3 pathways. In support, a significant number of primary and established MM cells expressed both constitutively activated NF- κ B and STAT3, and suppression of these transcription factors inhibited their survival [6]. Therefore, agents that suppress STAT3 and NF- κ B activities have potential for the treatment of MM.

In this study, we report that STAT3 and NF- κ B are post translationally modified by S-nitrosylation, a reversible and specific post-translational modification that regulate activities of a large number of target proteins [7]. The protein S-nitrosylation can be mediated through a direct reaction between NO and protein thiols in the presence of electron acceptors or by formation of transition metal adduct [8]. However, protein S-nitrosylation is also mediated via transnitrosylation through formation of intermediate low molecular mass S-nitroso compounds (RSNO), such as S-nitroso-L-cysteine (CysNO) and S-nitrosoglutathione

(GSNO) [9]. Recently, RSNOs have been demonstrated with potent anti-inflammatory, anti-oxidant, vasodilating, and antiplatelet properties [10–14]. We previously reported that anti-inflammatory activity of RSNO may be the result of S-nitrosylation-mediated inactivation of STAT3 and NF- κ B and thus inhibition of their downstream target genes [11, 12]. In addition, we also reported that GSNO treatment blocks NF- κ B and STAT3 pathways which are responsible for cell survival and proliferation of head and neck squamous cell carcinoma in *in vitro* cell culture models as well as *in vivo* mouse xenograft model [15].

Here we report a utility of synthetic low molecular mass RSNO, S-nitrosyl-N-acetylcysteine (SNAC) for inhibition of MM cell proliferation and survival. In cell culture model, treatment of MM cells with SNAC increased S-nitrosylation of STAT3 and NF- κ B (p65 and p50) and suppressed their constitutive activations. Consequently, SNAC inhibited MM cell proliferation by inducing cell cycle arrest pathways (i.e. Cyclins A/B1/E/D1, CDK1/2). SNAC in combination with melphalan, a type of chemotherapy for MM, also enhanced apoptotic MM cell death via inhibiting cell survival pathways (i.e. Mcl-1, cIAP2, and Bcl-xL) and/or by activation of pro-apoptotic cell death signal pathways (i.e. caspase-3/9 and p53). Overall, these data indicate that SNAC mediates inhibition of STAT3 and NF- κ B activities resulting in downregulation of STAT3 and NF- κ B downstream targets involved in cell proliferation and anti-apoptosis, thus inhibiting proliferation and induction of apoptosis of MM cells.

Materials and methods

Cell Culture

Human MM cell lines (U266, NCI-H929 [H929], and IM-9) were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), 100 U/ml penicillin and 100 μ g/mL streptomycin (Life Technologies) at 37°C under 5% CO₂/95% air.

SNAC preparation

SNAC was synthesized by mixing equimolar concentrations (200 mM) of N-acetylcysteine (Sigma-Aldrich, St. Louis, MO) and NaNO₂ (Sigma-Aldrich) in 0.5 N HCl for 1 hr at room temperature. The effective concentration of the SNAC was calculated from their optical absorbance at 338 nm and the reported molar extinction coefficients [16].

Assay of STAT3 and NF- κ B activation

The effect of SNAC on activity of STAT3 was analyzed by Western blot for phosphorylated (Tyr⁷⁰⁵) STAT3 (pSTAT3) and total STAT3 with specific antibodies (Cell Signaling Technologies, Danvers, MA). For nuclear localization assay of STAT3 and NF- κ B, total cell lysate or nuclear and cytoplasmic extracts from U266 cells were prepared using a previously published method [14, 17]. The total, cytoplasmic, and nuclear levels of STAT3 (or phospho-STAT3) and NF- κ B (p65 and p50) were analyzed by Western analysis using specific antibodies (Cell Signaling Technologies). H3 histone and β -actin were used for internal loading controls for nuclear and cytoplasmic proteins. The nuclear protein extracts were also

used for the gel-shift assay for detection of STAT3 or NF- κ B DNA binding activities as described previously [14, 17]. For STAT3 or NF- κ B reporter gene assay, U266 cells were transfected with STAT3 (or NF- κ B)-responsive luciferase construct (1.5 μ g/well; Panomics, Inc., Redwood City, CA), which encodes firefly luciferase reporter gene, and phRL-CMV (0.1 μ g/well; Promega, Madison, WI) construct, which encodes renilla luciferase under the control of a CMV promoter for an internal control for transfection efficiencies. Transfection was mediated by using lipofectamine-Plus (Invitrogen), according to the manufacturer's instructions. The activities of luciferases were assayed by using dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

Assay of S-nitrosylation of STAT3 and NF- κ B

Protein S-Nitrosylation was detected using the biotin-switch method as described in our previous reports [11, 14]. U266 cells were lysed in 250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethanesulfonylfluoride, 20 μ M methyl methanethiosulfonate (MMTS), 80 μ M carmustine, protease inhibitor mixture (Sigma-Aldrich), and mixed with an equal volume of 25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μ M neocuproine, 5% SDS, 20 μ M MMTS and incubated at 50°C for 20 min. After acetone precipitation, the precipitates were resuspended in 25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μ M neocuproine, 1% SDS and mixed with two volumes of 20 mM HEPES, pH 7.7, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100. The S-nitrosylated proteins were then modified with biotin in 25 mM HEPES, pH 7.7, 0.1 mM EDTA, 1% SDS, 10 μ M neocuproine, 10 mM ascorbate sodium salt, and 0.2 mM N-[6-(biotinamido)hexyl]-30-(20-pyridyldithio) propionamide (biotin-HPDP, Pierce). After acetone precipitation, biotinylated proteins were pull down with neutravidin-agarose and followed by Western blots for STAT3 and NF- κ B (p65 and p50).

Assay of cell proliferation, cell death, and cell cycle

For assay of cell proliferation and death, trypan blue staining and 5-bromo-2'-deoxyuridine (BrdU) DNA incorporation assay were performed. U266 cells cultured in 12-well plates (2×10^4) were incubated with SNAC and/or melphalan and the numbers of live cells (trypan blue negative cells) and dead cells (trypan blue positive cells) were counted in a counting chamber every 24hrs for 4 days. For BrdU DNA incorporation assay, U266, H929, and IM-9 cells were seeded on 96-well plates at a concentration of 1×10^4 cells/well and stabilized for 12 hours. Following the incubation for 2 hrs with SNAC treatment, the cells were treated with IL-6 or vehicle (culture media) for 4 hrs, and then treated with BrdU for 4 hours. Incorporated BrdU was detected with anti-BrdU monoclonal antibodies conjugated with horse-radish peroxidase. Sample absorbance was analyzed using ELISA reader at 370nm. Cell cycle analysis was performed by flow cytometry following the staining of cells with propidium iodide. Briefly, the ethanol fixed cells were treated with RNase solution and then stained with propidium iodide (50 μ g/ml). The samples were analyzed by FACSCalibur™ flow cytometer with Cell Quest™ software (BD Biosciences, San Jose, CA).

Expression of cell cycle and cell survival regulators

Effect of SNAC treatment on nuclear expression of cell cycle regulators in U266 cells was performed by Western analysis using antibody specific to Rb (Cell Signaling Technology),

phospho-(Ser⁷⁸⁰)-Rb (p-Rb; (Cell Signaling Technology), Cdk1 (Cell Signaling Technology), Cdk2 (Cell Signaling Technology), Cdk5 (Cell Signaling Technology), Cyclin A1 (Abcam, Beverly, MA), Cyclin B1 (Cell Signaling Technology), Cyclin D1 (Cell Signaling Technology), or Cyclin E1 (Cell Signaling Technology). Antibody specific to histone H3 was used for assay of internal loading control. The expression and subcellular localization of p-Rb and Cyclin D1 were also analyzed by immunofluorescent staining of U266 cells. For this, U266 cells cultured on chamber slides (LabTek, Nunc, Inc., Naperville, IL) were fixed in cold methanol and incubated with primary antibodies against p-Rb or Cyclin D1 and then secondary antibody (goat polyclonal Secondary antibody to rabbit IgG conjugated with Alexa Fluor 647) (Abcam). 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for nuclei counter-stain. For analysis of cellular expression of anti-/pro-apoptotic factors, Western analysis using antibody specific to Bax, Bid, Bad, phospho-(Ser¹¹²)-Bad, Bcl-2, Bcl-xL, c-IAP2, cleaved caspase 3 and 9, p53, phospho-(Ser¹⁵)-p53, or phospho-(Ser³⁷)-p53 (all antibodies were purchased from Cell Signaling Technology) was performed.

Generation of xenograft mouse model for MM using U266 cells

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care & Use Committee (IACUC) of the Medical University of South Carolina. The MM xenograft mouse model was generated as reported previously [18]. Briefly, NOD-*scid* IL2R γ (NOD.Cg-*Prkdc*^{*scid*} *IL2rgtm1*^{*Wjl*}/SzJ; The Jackson Laboratory, Sacramento, CA), also known as NSG mice, were irradiated with 2.4 Gy using an Clinac 21EX Linear Accelerator (Varian Medical Systems, Atlanta, GA). The mice were then randomly divided to control, engrafted, and SNAC treated engrafted groups (n=5). Shortly after irradiation, 2×10^6 U266 cells were intravenously inoculated to engrafted groups. Following the stabilization period for 2 weeks, the engrafted mouse groups received daily phosphate buffered saline (PBS) or SNAC (20mg/kg/i.p/in 0.1ml phosphate buffered saline) for 4 weeks and then sacrificed by euthanasia injection (pentobarbital). For analysis of STAT3 phosphorylation, bone marrow cells were isolated from femur. For analysis of serum levels of human IgE secreted from U266 cells, bloods were collected by cardiac puncture and analyzed for human IgE using ELISA kit (Abcam). For analysis of bone marrow U266 cell growth, the isolated femur were fixed in neutral buffered formalin and decalcified. Following the paraffin embedding and sectioning, morphology of bone marrow cells was assessed by hematoxylin and eosin (H&E) staining and the expressions of human CD138 in U266 cells were analyzed by immunohistochemistry.

Statistical analysis

Statistical analysis was performed with Graphpad Prism5. Values are expressed as mean $\pm$ SD. Comparisons among means of groups were made with a two-tailed Student's t-test for unpaired variables. Multiple comparisons were performed using one-way ANOVA followed by Bonferroni test.

Results

SNAC inhibits STAT3 activation and proliferation of MM cells

Initially, we examined the inhibitory activity of SNAC on basal and IL-6 -induced STAT3 activity and cell proliferation. Fig. 1 shows that treatment of human MM cells (U266, H929, and IM-9 cells) with SNAC (200 μ M) at 2 hrs before IL-6 treatment (20 ng/ml/0.5hr) significantly decreased the IL-6 induced STAT3 phosphorylation at Tyr⁷⁰⁵ residue without altering total STAT3 levels. In addition, SNAC treatment also decreased IL-6 induced proliferation of these cell lines in a dose dependent manner as shown by BrdU incorporation assay (Figs. 1A-ii, B-ii, and C-ii). Autocrine or paracrine effect of IL-6 in proliferation of MM cells (e.g. U266, H929, and IM-9) was well established previously [19, 20]. Since SNAC treatment also reduced STAT3 phosphorylation and MM cell proliferation in the absence of IL-6 stimulation, these data suggest an inhibitory role of SNAC in autocrine/paracrine IL-6 loop mediated MM cell proliferation. Overall, these data indicate that SNAC is a potential inhibitor of IL-6/STAT3 pathway and an anti-proliferative agent in those MM cells.

SNAC inhibits activation of STAT3 and NF- κ B in U266 cells

U266 is an immortal human B lymphocyte cell line that expresses high levels of the anti-apoptotic proteins through autocrine IL-6 induced STAT3 activation [21] and constitutive NF- κ B activation [22]. Studies from our laboratory previously reported that STAT3 and NF- κ B activities in microglia and endothelial cells are regulated by GSNO, an endogenous low molecular mass RSNO, via S-nitrosylation dependent mechanisms [11, 12, 14, 17]. Based on these studies, we assessed the therapeutic potential of S-nitrosylation-mediated targeting of STAT3 and NF- κ B activities in U266 MM cells using SNAC, a synthetic low molecular mass RSNO.

Fig. 2A shows that untreated U266 cells expressed high levels of phosphorylated STAT3 (Tyr⁷⁰⁵) and nuclear NF- κ B (p50 and p65). Treatment of U266 cells with SNAC for 2hrs decreased the protein levels of phosphorylated STAT3 both in nucleus and cytoplasm as well as total protein levels of NF- κ B (p50 and p65) in nucleus in a dose dependent manner (Fig. 2A). Accordingly, SNAC treatment also decreased the STAT3 and NF- κ B DNA binding activities in U266 cells as shown by gel shift assay (Fig. 2B-i). To further establish the role of SNAC in the regulation of STAT3 or NF- κ B trans-activity, U266 cells transfected with STAT3 (or NF- κ B) responsive luciferase reporter construct were treated with SNAC. As shown in Fig. 2B-ii, SNAC treatment also decreased STAT3 and NF- κ B-mediated transcription activity in U266 cells. As we reported previously with GSNO [11, 14], SNAC treatment increased cellular levels of S-nitrosylated proteins (see supplementary data Fig. S3) as well as levels of S-nitrosylated STAT3, p50, and p65 in U266 cells in a dose dependent manner (Fig. 2C). As SNAC treatment inhibited STAT3 phosphorylation in U266 cells (Fig. 2A), treatment of U266 cells with other S-nitrosylating agents (e.g. GSNO) also decreased the STAT3 Tyr⁷⁰⁵ phosphorylation, whereas the related compounds lacking protein S-trans-nitrosylation activity, such as NAC, GSH, NaNO₂, and pre-decomposed SNAC under UV light (UV-SNAC) had no effect on STAT3 phosphorylation (Fig. 2D). These data indicate that SNAC regulates STAT3 and NF- κ B activation in U266 MM cells

via S-nitrosylation mediated mechanisms as we reported previously for other cell types with GSNO [11, 12, 14, 17]. However, the observed increase in total S-nitrosylated proteins by SNAC treatment (Fig. S3) also suggests a possible involvement of S-nitrosylation mechanisms in other cell signaling pathways regulating U266 cell proliferation.

SNAC inhibits proliferation of U266 cells by inducing G1-S cell cycle arrest

Since STAT3 and NF- κ B have been implicated in control of gene expressions for cell proliferation and survival [23–26], we next investigated the effect of SNAC treatment on proliferation and death of U266 cells. Fig. 3A showing trypan blue exclusion test for counting live cells indicates that daily treatment of U266 cells with SNAC decreased the number of live cells in a dose dependent manner. At concentration of 200 μ M, SNAC completely inhibited the time-dependent increase in viable cell number (Fig. 3A). At 500 μ M SNAC, the number of viable cells was dropped below the initial plating density at 72 and 96 hrs of treatment (Fig. 3A) and accordingly the number of dead cells (trypan blue positive cells) increased at those time points (Fig. 3A). However, lower concentrations of SNAC (~200 μ M) had no obvious effect on U266 cell death (Fig. 3A). These data indicate the efficacy of SNAC in inhibiting proliferation and survival of U266 MM cells.

We next investigated mechanism of SNAC mediated cell cycle arrest. Fig. 3B showed that treatment of U266 cells with SNAC (200 μ M/12hrs) increased the number of cells in G0/G1 phase and decreased the numbers of cells in S phases, indicating that SNAC treatment inhibits U266 cell proliferation by inducing cell cycle arrest at G1-S checkpoint located between the end of the cell cycle's G1 phase and just before entry into S phase. Rb is a key player in the G1-S checkpoint system. At the G1-S checkpoint, phosphorylation of the Rb protein releases Rb from the transcriptional regulator E2F and activates E2F function for gene expression required for initiation of S phase (see review [27, 28]). In U266 cells, SNAC treatment decreased the levels of phospho-Rb in nucleus as shown by Western blot (Fig. 3C-i) as well as fluorescent staining of the cells for phospho-Rb (see supplementary data Fig S5A), indicating the participation of Rb in SNAC-induced cell cycle arrest at G1-S checkpoint. In G1 phase, phosphorylation of Rb is initiated by activated Cdk4 and Cdk6 complexes with D-type cyclins (Cyclin D) and then Rb phosphorylation is completed by Cdk2/cyclin E complexes in late G1 phase [27, 28]. Fig. 3C-ii shows that the nuclear expressions of Cdk2 and Cdk4 and the expressions of their binding partners, such as cyclin E and Cyclin D (Fig. 3C-iii and supplementary data Fig. S5B) were decreased by SNAC treatment. In addition, nuclear expression of Cdk1, which regulates G2 and M cell cycle phases [27, 28], was also reduced by SNAC treatment. However, the expressions of cyclin A and cyclin B which regulates S, G2, and M phases were not affected by SNAC treatment (Fig. 3C-iii). These data indicate that SNAC induces cell cycle arrest at G1-S checkpoint by inhibiting CDK4/Cyclin D and Cdk2/Cyclin E complexes, thus inhibiting Rb phosphorylation.

SNAC enhances melphalan-induced U266 cell death signaling via inhibition of STAT3 and NF- κ B pathways

Melphalan is one of widely used chemotherapy drugs for MM but it has dose-limiting toxicity and drug resistance, thus limiting its application in many clinical settings [29]. In

cell culture study, treatment of U266 cells with melphalan inhibited cell proliferation at low concentrations (5 μ M) and induced cell death at higher concentrations (10 μ M) (Fig. 4A-i). Interestingly, the treatment of U266 cells with subtoxic concentration of SNAC (100 μ M) and subtoxic concentration of melphalan (5 μ M) increased cell death (Fig. 4A-ii), indicating that SNAC increases the sensitivity of MM cells to melphalan.

Constitutive activations of IL-6/STAT3 [21, 30] and NF- κ B [31, 32] are known to confer resistance to melphalan in MM therapy. We observed that melphalan itself slightly increased both levels of constitutive active STAT3 (nuclear levels of phospho- Tyr⁷⁰⁵-STAT3 in Fig. 4B-i) and NF- κ B (nuclear p50 and p65 levels in Fig. 4B-i and NF- κ B DNA binding activity in Fig. 4B-ii). SNAC treatment decreased both STAT3 and NF- κ B activations either alone as well as in combination with melphalan (Fig. 4B), indicating that SNAC increases MM cell sensitivity to melphalan by inhibitions of STAT3 and NF- κ B. Consistent with the inhibitions of STAT3 and NF- κ B activities, SNAC treatment alone or in combination with melphalan decreased the phosphorylation of Bad at Ser¹¹² residue and thus its activity (Fig. 4C-i). Pim-2 is known to phosphorylate Bad on Ser¹¹² residue [33] and its expression is upregulated by increased activities of STAT3 and NF- κ B in MM cells [34]. SNAC treatment decreased the Pim-2 expression in U266 cells (Fig. 4C-i). Therefore, these data indicate the role of STAT3 and NF- κ B inhibition by SNAC in reductions in Pim-2 expression and Bad phosphorylation (Ser¹¹²) and thus induction of proapoptotic Bad activity. However, neither melphalan nor SNAC had effect on the expression of Bid and Bax, known pro-apoptotic members of the Bcl-2 proteins family (Fig. 4C-i).

SNAC treatment also decreased expression of anti-apoptotic members of the Bcl-2 family proteins, such as Bcl-2, Bcl-xL, and Mcl-1, and cIAP2 (Fig. 4C-ii) which are also known to be regulated by STAT3 and NF- κ B [35–44]. Melphalan by itself decreased the expressions of Bcl-xL and Mcl-2, and its combination with SNAC further decreased the expression of these proteins. Both pro-apoptotic and anti-apoptotic Bcl-2 family members act as checkpoint upstream to caspases and mitochondrial dysfunction [45]. Fig. 4C-iii shows that treatment of U266 cells with SNAC alone did not induce any obvious activations of caspase 3 and caspase 9 as observed by Western analysis for full length (proenzyme) and cleaved (activated) caspases while melphalan treatment slightly increased the levels of activated (cleaved) caspase 3 and 9. Interestingly, SNAC in combination with melphalan greatly enhanced the activations of caspase 3 and 9, thus documenting the role of SNAC activity in increasing MM cell sensitivity to melphalan-induced cell death. Since expression of p53, a critical cell cycle and apoptosis regulator [46], is regulated by STAT3 and NF- κ B [40, 47], we also investigated the expression and activity of p53 in SNAC/melphalan treated U266 cells. Fig. 4C-iv shows that treatment of U266 cells with either SNAC or melphalan increased the expression of p53 and its activity as phosphorylation on Ser¹⁵ and Ser³⁷ residues. The phosphorylation of p53 on Ser¹⁵ and Ser³⁷ residues are known to induce p53 activity under DNA damaged conditions [48, 49]. Moreover, combination of SNAC and melphalan further enhanced the expression and activity of p53 (phosphorylation of p53 on Ser¹⁵ and Ser³⁷), suggesting the role of SNAC in enhancement of p53 activation for cell cycle arrest and cell death under normal and melphalan treated conditions.

SNAC inhibits U266 cell growth in mouse xenograft model of MM

To assess the *in vivo* efficacy of SNAC on inhibition of MM cell growth in bone marrow, we used U266-transplanted NSG mice based on previous study [18]. Following 2 weeks of cell transplantation, the mice received daily SNAC (20mg/kg/i.p/in 0.1ml phosphate buffered saline) or phosphate buffered saline for 4 weeks, and then serum levels of human IgE, which is produced by transplanted U266 cells, was analyzed by ELISA. Fig. 5A shows that the increased serum human IgE levels in U266 transplanted mice were significantly reduced by daily SNAC treatment. In addition, SNAC treatment also attenuated phospho-(Tyr⁷⁰⁵)-STAT3 levels in the bone marrows (Fig. 5B) and decreased human CD138 positive cells (U266 cells) in femoral bone marrow (Fig. 5C). Taken together, these *in vivo* mouse studies and the data from *in vitro* cell culture studies document that the inhibition of STAT3 and NF- κ B activities by SNAC-mediated S-nitrosylation mechanisms induces cell cycle arrest and apoptotic death of U266 cells thus attenuates the development of MM-like pathology in mouse xenograft model.

Discussion

Protein S-nitrosylation, a post-translational modification of cysteine thiols into S-nitrosothiols, is now regarded as a principle mechanism of NO-based cellular signaling [7]. Studies from our laboratory and others have documented that cellular activities of STAT3 and NF- κ B are tightly regulated by NO-based cellular signaling mechanisms [11, 12, 14, 50]. In microglia, we reported that endogenous NO produced by inducible NOS (iNOS) or exogenous GSNO treatment inhibited STAT3 activation (phosphorylation of Tyr⁷⁰⁵) via S-nitrosylation of STAT3 at Cys²⁵⁹ residue [14]. We and others also reported that endogenous NO or exogenous GSNO also inhibited NF- κ B activation induced by proinflammatory cytokines via direct S-nitrosylation of NF- κ B components (Cys³⁸ of p65 and Cys⁶² of p50) [11, 51, 52] or S-nitrosylation of upstream kinase IKK β (Cys¹⁷⁹) [50]. STAT3 plays key roles in regulation of gene expressions for cell survival (i.e. Bcl-xL, cIAP, survivin, and Mcl-1), cell cycle (i.e. c-myc, Cdk2, Cyclin-E, Cdk1, and Cyclin-B), and tumor angiogenesis (i.e. vascular endothelial growth factor; VEGF) [35–40, 53]. Similarly, NF- κ B also participates in regulation of gene expressions for cell survival (i.e. cIAP, Bcl-2, Bcl-xL, and XIAP), cell cycle (i.e. c-Myc and Cyclin D), and multidrug resistance (e.g. MDR1) [41–43, 54]. Due to these key regulatory roles, the regulatory inhibition of STAT3 and NF- κ B is considered as a promising strategy for therapy against various cancer treatment including MM.

Recently our laboratory and others reported that GSNO inhibited growth of head and neck squamous carcinoma and ovarian cancer via inhibition of STAT3 and NF- κ B activities [15, 55]. GSNO is the most abundant endogenous small molecular mass S-nitrosothiol in mammalian cells and widely used as an S-nitrosothiol donor in many experimental setups. Studies have documented that amino acid transport system (L-AT) is a major mechanism of S-nitrosothiol delivery into cells [56, 57]. L-AT transports single un-substituted amino acid based S-nitrosothiols (e.g. CysNO and S-nitrosohomocysteine) across cell membrane [56, 57]. GSNO itself is not a ligand of L-AT and thus its cellular effect requires additional mechanisms [56]. For this reason, the cell permeable CysNO has been widely used to study

NO biology and signaling. However, CysNO has a significantly shorter half-life than other S-nitrosothiols, such as GSNO and SNAC [58–60], and thus limits its extensive use. Therefore, we tested *in vitro* and *in vivo* efficacy of SNAC in inhibition of MM cell proliferation and survival. Similar to GSNO, SNAC is not a natural ligand for L-AT [56, 57]. However, SNAC has a better cellular effect than GSNO [61] and much longer half-life than CysNO [59, 60].

In this study, SNAC efficiently inhibited STAT3 and NF- κ B activation in MM cells and thus inhibits U266 MM cell proliferation and survival in *in vitro* cell culture studies as well as *in vivo* animal studies. This suggest the therapeutic potential of SNAC for MM therapy. Previous studies reported that S-nitrosylating agents, such as GSNO and ($\pm$)-S-Nitroso-N-acetylpenicillamine (SNAP), had no obvious cytotoxicity to human peripheral blood mononuclear cells (hPBMCs) over 250 μ M while they inhibited mitogen activated hPBMC proliferation [62]. Similarly, we also observed that SNAC treatment of hPBMCs did not induce any obvious cell death up to 200 μ M while it inhibited proliferation of hPBMCs in a dose dependent manner (Fig. S7A). However, high concentration of SNAC (500 μ M) caused slight cell death (Fig. S7A) as seen in U266 cells (Fig. 3A). Therefore, the effect of SNAC on hPBMC and U266 is similar. In addition, combination of subtoxic concentrations of melphalan (5 μ M) and SNAC (100 μ M) cooperatively inhibited hPBMC proliferation (Fig. S7B). However, melphalan and SNAC combination did not induce any obvious cell death (Fig. S7B) contrary to U266 cells (Fig. 4A-ii), indicating a tumor cell specific cytotoxic activity of SNAC and melphalan combination.

Mechanistically, SNAC inhibited U266 cell proliferation by inducing cell cycle arrest at the G1-S checkpoint. The detailed mechanisms for SNAC-induced cell cycle arrest is not well understood at present, but the observed SNAC-induced decreased expressions of cell cycle regulators for G1-S checkpoint (e.g. Cdk2, Cdk4, Cyclin D, Cyclin E) and phosphorylation of Rb (Fig. 3) suggest that SNAC induces cell cycle arrest at the G1-S checkpoint by inhibiting Cdk4/Cyclin D and Cdk2/Cyclin E mediated Rb phosphorylation [27, 28]. It is of interest to note that NF- κ B and STAT3 play critical roles in regulation of these cyclins and cdks [63], thus supporting the role of SNAC-mediated inhibition of NF- κ B and STAT3 in cell cycle arrest of MM cells.

Melphalan is the most commonly used chemotherapeutic agent against multiple myeloma. However, due to its dose-limiting toxicity and development of drug resistance [29], successful melphalan treatment for MM requires additional mechanisms for increasing sensitivity to the drug and/or decreasing the drug resistance of MM cells to melphalan. Constitutive activations of IL-6/STAT3 [21, 30] and NF- κ B [31, 32] have been documented to be major signals conferring resistance to melphalan-induced apoptosis in MM cells. Therefore, inhibition of STAT3 and NF- κ B mediated cell survival pathways are critical for increasing the drug sensitivity and/or for decreasing the drug resistance of MM cells to melphalan. In this study, we observed that SNAC decreased the expression of cell survival factors, such as Pim-2, Bcl-2, Bcl-xL, and Mcl-1, and cIAP2, but increased the expressions and activities of proapoptotic factors (e.g. p53 and caspase3/9) and these changes were well correlated with the increased sensitivity of U266 cells to melphalan induced cytotoxicity

(Fig. 4). These data, therefore, indicate the potential of SNAC-mediated mechanisms in complement the melphalan for MM therapy.

Presently, a number of pre-clinical animal models of MM have been developed to assess the efficacy of therapeutic agents for the treatment of MM. Among these the immune-suppressed NOD-*scid* IL2R γ strain of mice (NSG) has been used successfully in human xenograft models of MM for bone and bone marrow disease [18]. In this study, we assessed the potential therapeutic efficacy of SNAC for MM therapy using U266 xenografted NSG mouse model. In agreement with *in vitro* cell culture study, SNAC treatment inhibited STAT3 activity and decreased the growth of U266 in bone marrow of U266 xenografted mice (Fig. 5). Accordingly, SNAC treatment also decreased serum levels of human IgE produced by U266 cells, suggesting the potential efficacy of SNAC in MM therapy either alone or in combination with present day therapies for MM.

In summary, this study reports that SNAC efficiently modulates STAT3 and NF- κ B activities in MM cells in *in vitro* cell culture model. The SNAC induced inhibition of STAT3 and NF- κ B activities resulted in inhibition of cell cycle progression and cell survival, thus inhibiting MM cell growth in animal model. However, SNAC also S-nitrosylated other cellular proteins and thus contribution of other mechanisms in SNAC-mediated regulation of MM cell proliferation cannot be excluded. This study also reports that SNAC increases sensitivity of MM cells to melphalan induced cell death, thus indicating that SNAC-mediated mechanisms complement with melphalan therapy for MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CysNAC	S-nitroso-L-cysteine
MM	multiple myeloma
SNAC	S-nitroso-N-acetylcysteine

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Highlights

- S-nitroso-N-acetylcysteine (SNAC) is a cell permeable S-nitrosothiol compound.
- SNAC S-nitrosylates and inhibits STAT3 and NF κ B in multiple myeloma (MM) cells.
- SNAC inhibits STAT3 and NF κ B-mediated cell cycle and thus MM cell proliferation.
- SNAC inhibits STAT3 and NF κ B-mediated anti-apoptotic pathways in MM cells.
- SNAC treatment inhibits MM cell growth in mouse xenograft model of MM.

Fig. 1. SNAC inhibits STAT3 activation and proliferation of MM cells

The effect of SNAC (200 μM/2 hr pretreatment) on basal or IL-6 (20 ng/ml/0.5 hr treatment)-induced phosphorylation of STAT3 (Tyr⁷⁰⁵) and expression of STAT3 in U266 (A-i), H929 (B-i), and IM-9 (C-i) MM cells were analyzed by Western blot (see supplementary data Fig. S1 for quantitative densitometry). BrdU incorporation assay was performed for analysis of cell proliferation (A-ii, B-ii, and C-ii). For this, the cells were pretreated with SNAC (0, 100, 200, and 400 μM) for 2 hrs, treated with 20 ng/ml IL-6 (or vehicle) for 4 hrs, and then treated with BrdU for 4 hrs. Each column (or dot) and vertical line represent the mean ± SEM (n = 3).

Fig. 2. SNAC inhibits activation of STAT3 and NF-κB in U266 MM cells
(A) The effect of SNAC (50~500 μM/2hr) on phosphorylation of STAT3 (Tyr⁷⁰⁵) and expression of STAT3 and NF-κB (p65 and p50) and their subcellular localization (cytoplasmic vs. nucleus) were analyzed by Western blots (see supplementary data Fig. S2A for quantitative densitometry). The levels of β-actin or histone H3 were used for loading control for total/cytoplasmic or nuclear proteins. **(B)** The effect of SNAC (200μM) on STAT3 and NF-κB DNA binding activities **(i)** and their trans-activities **(ii)** were analyzed by gel-shift assay and luciferase assay (N.S.: nonspecific band). Each column and vertical line represent the mean ± SEM (n = 3). **(C)** SNAC-induced S-nitrosylation of STAT3 and NF-κB was analyzed by biotin-switch assay and Western blot as described under experimental procedures (see supplementary data Fig. S2B for quantitative densitometry). **(D)** The effects of N-acetylcysteine (NAC), SNAC, glutathione (GSH), S-nitrosoglutathione (GSNO), NaNO₂, and pre-decomposed SNAC under UV light (UV-SNAC) on phosphorylation of STAT3 were analyzed by Western blot.

Fig. 3. SNAC inhibits U266 cell proliferation by inducing G1-S cell cycle arrest
 (A) Effect of various concentrations of SNAC (50~500 μM) on numbers of live cells (trypan blue negative cells) or dead cells (trypan blue positive cells) was analyzed by trypan blue exclusion test of U266 cells. (B) Assay for cell cycle status was performed in untreated control U266 cells or in SNAC (200 μM) treated U266 cells using fluorescent cell flow cytometry analysis and represented by bar graph. (C) Effects of SNAC on nuclear expression and phosphorylation (Ser⁷⁸⁰) of Rb (C-i), nuclear expression of Cdks (Cdk1, Cdk2, and Cdk4) (C-ii) and cyclins (Cyclin A1, Cyclin B1, Cyclin D1, and Cyclin E) (C-iii) were analyzed by Western blot (see supplementary data Figs. S4A, B, and C for quantitative densitometry). Each dot and vertical line represent the mean ± SEM (n = 3). The level of histone H3 was used for internal loading control.

Fig. 4. SNAC enhances melphalan-induced U266 cell death by inhibiting STAT3 and NF- κ B pathways

(A) Effects of various concentrations of melphalan (2–20 μM) (i) and SNAC (100 μM) combination with melphalan (5 μM) (ii) on cell proliferation and death were analyzed by trypan blue exclusion test. Each dot and vertical line represent the mean $\pm$ SEM ($n = 3$). (B) Effects of SNAC (100 μM) combination with melphalan (5 μM) on subcellular localization (cytoplasm vs. nucleus) of phospho-STAT3 (pY⁷⁰⁵), STAT3, and NF- κ B (p50 and p65) (B-i) and NF- κ B DNA binding activity (B-ii) were analyzed by Western blot and gel-shift assay. See supplementary data Fig. S6A for quantitative densitometry of Western blots. (C) Effects of SNAC and melphalan combination (24hrs) on the expression of proapoptotic factors (e.g. Bax, Bid, Bad, and phospho-Ser¹¹²-Bad) and Pim-2 (i), anti-apoptotic factors (e.g. Bcl-2, Bcl-xL, Mcl-1, and cIAP2) (ii), and activated (cleaved) caspases (caspase 3 and 9) (iii), and p53/phospho-p53 (Ser¹⁵ and Ser³⁷) (iv) were analyzed by Western blot. See supplementary data Figs. S6B and C for quantitative densitometry of Western blots.

Fig. 5. SNAC inhibits U266 cell growth in mouse xenograft model of MM

U266 inoculated NOD-*scid*IL2R γ (NSG) mice were treated with daily SNAC or vehicle (phosphate buffered saline), then the effect of SNAC treatment on serum levels of human IgE (A), protein levels of phospho-STAT3 in the bone marrow (B), bone marrow expression of human CD138 (C) were analyzed by ELISA (A), Western blot (B), immunohistochemistry (C). Each column and vertical line represent the mean $\pm$ SEM (n 5). * $P < 0.05$ compared to the vehicle treated control groups. + $P < 0.05$; compared to the vehicle treated U266 inoculated NSG mice.