TAT-mediated intracellular delivery of NPM-derived peptide induces apoptosis in leukemic cells and suppresses leukemogenesis in mice

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Running title: TAT PTD-NPM peptide suppresses leukemogenesis

Abbreviations used: ChIP, chromatin immunoprecipitation; Cox-2, Cyclooxygenase 2; Fancc, Fanconi anemia complementation group C; H&E, hematoxylin and eosin; IAP, inhibitor of apoptosis proteins; IL, interleukin; MEFs, murine embryonic fibroblasts; MIP, macrophage inflammatory protein; NPM, nucleophosmin; PTD, protein transduction domain; TNF-α, tumor necrosis factor α

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Abstract
Nucleophosmin (NPM) is frequently overexpressed in leukemias and other tumors. NPM has been reported to suppress oncogene-induced senescence and apoptosis and may represent a therapeutic target for cancer. We fused a NPM-derived peptide to the HIV-TAT (TAT-NPMΔC) and found that the fusion peptide inhibited proliferation and induced apoptotic death of primary fibroblasts and pre-leukemic stem cells. TAT-NPMΔC down-regulated several NF-κB-controlled survival and inflammatory proteins and suppressed NF-κB-driven reporter gene activities. Using an inflammation-associated leukemia model, we demonstrate that TAT-NPMΔC induced proliferative suppression and apoptosis of pre-leukemic stem cells, and significantly delayed leukemic development in mice. Mechanistically, TAT-NPMΔC associated with wild-type NPM proteins and formed complexes with endogenous NPM and p65 at promoters of several anti-apoptotic and inflammatory genes and abrogated their transactivation by NF-κB in leukemic cells. Thus, TAT-delivered NPM peptide may provide a novel therapy for inflammation-associated tumors that require NF-κB signaling for survival.
Introduction

Nucleophosmin (NPM) is a multi-functional protein that plays important roles in the regulation of cell proliferation and apoptosis (1). The level of NPM is frequently found to be significantly higher in tumor and proliferating cells than in resting cells (2-6). Normal cells induce genetically encoded programs that prevent deregulated proliferation and thus protect multicellular organisms from cancer progression. Two such programs are apoptosis and senescence that are normally triggered by DNA damage or other stresses. Our recent studies demonstrated that overexpression of NPM suppresses oncogene-induced senescence and apoptosis and accelerated transformation in cells deficient for the Fanconi complementation group C (Fancc) gene and the ataxia telangiectasia mutated (Atm) gene (7). Therefore, NPM could be a potential therapeutic target for neoplastic diseases.

There is increasing interest in employing peptides and proteins as therapeutic compounds for human cancers. For example, several peptides derived from tumor suppressors as well as oncogenes have been developed as anti-tumorigenic cargoes and proved to be effective in inhibition of tumor cell growth (8-10). Although peptide-based compounds have limitations owing to poor permeability and low selectivity, recent studies have demonstrated that large cargoes, proteins, and peptides can be delivered intracellularly if conjugated to the protein transduction domain (PTD) derived from the HIV-1 TAT protein (11, 12). Indeed, rapid and receptor-independent uptake of TAT-conjugated peptides has been demonstrated to occur in many cell types and animals (13-15).
In this study, we generated a NPM-derived peptide fused to TAT (TAT-NPMΔC) and demonstrated in an inflammation-associated leukemic mouse model that TAT induced rapid and efficient delivery of the peptide into leukemic cells. TAT-NPMΔC caused growth inhibition and apoptosis of leukemic cells, and significantly delayed leukemic development in mice. TAT-NPMΔC associated with endogenous NPM proteins, formed complexes with endogenous NPM and the NF-κB subunit p65, and abrogated NF-κB transactivation at promoters of several important anti-apoptotic and inflammatory genes controlled by NF-κB in leukemic cells. Thus, the results suggest that the TAT-delivered NPM mutant may provide a novel therapy for tumors that require NF-κB signaling for survival.
Materials and Methods

Cloning, expression, and purification of TAT-NPM fusion proteins. The full-length human NPM (GeneBank sequence accession number BC009623) and its N-terminally (NPMΔN) and C-terminally (NPMΔC) deleted cDNAs were amplified by polymerase chain reaction, using Pfu DNA polymerase (Stratagene) and the following primer pairs: 5’-CGGGATCCCGGAGATTCGATGGACATG/5’-CCGCTCGAGAAGAGACTTCCTCCACTG, 5’-CGGGATCCCGGAGAAAAAGCGCCAGTG/5’-CCGCTCGAGAAGAGACTTCCTCCACTG and 5’-CGGGATCCCGGAGATTCGATGGACATG/5’-CCGCTCGAGTTCATCATCATCCTCTTCA, respectively. The resulting PCR fragments were subcloned into the BamH1 and Xho1 sites of the pTAT-2.1 vector (kindly provided by Steven F Dowdy, University of California School of Medicine, San Diego, CA) to create pTAT-NPM, pTAT-NPMΔN, and pTAT-NPMΔC, respectively. The TAT-NPM fusion proteins were expressed in E. coli strain BL21 (DE3; Novagen). Whole cell lysates were obtained by gentle lysis using the BugBuster protein extraction reagent (Novagen). Bacterial debris was pelleted, and the supernatant was subjected to metal-affinity chromatography using an Equilibrate His-Bind Column (Novagen). Urea and salt were removed by gel filtration using a PD-10 Sephadex G-25M column (Amersham Biosciences). The TAT protein identity was confirmed by Western blotting.

Determination of TAT-NPM fusion protein uptake and subcellular localization.

The purified TAT-NPM fusion proteins were labeled using the EZ-Label FITC protein Labeling Kit (Pierce) in accordance with manufacturer’s instruction. To determine the uptake efficiency of the fusion proteins, 1 x 10^5 HEK293 cells per well were plated in
a 12-well plate for 12-16 hrs and cells. Cells were then incubated with TAT-NPM or TAT-NPMΔC (30 µg/ml each) at the indicated time. The total cell number was determined and cells were lysed in Tris [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100]. The fluorescence intensity was examined by fluorometer (excitation, 490-500 nm; emission, 515-525 nm). To determine the cellular distribution of the TAT fusions, HEK 293 cells were incubated with 30 µg/ml of the indicated proteins for 30 min. Cells were then fixed with 4% paraformaldehyde in PBS plus 4',6-diamidino-2-phenylindole (DAPI, 1 µg/mL; Sigma) and the cellular distribution of the FITC-labeled TAT-fusions was visualized with fluorescence microscope. To determine the cellular localization of TAT-fusions, cells were treated with the TAT fusions (30 µg/ml) for 30 min, washed extensively, and incubated in the absence of the TAT fusions for 60 min before fixation. The cells were then stained with antibodies anti-His<sub>6</sub> tag (Roche Applied Science, Penzberg, Germany; anti-NPM/B23 (Clone Fc-61991, Zymed Laboratories/Invitrogen Corp., Carlsbad, CA; or anti-B23 (Clone FC82291, St Louis, MO).

Cell proliferation, senescence, and apoptosis assays. Mouse embryo fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM nonessential amino acids, 55 μM β-mercaptoethanol and 10 μg/ml gentamycin. Cells were plated in 96-well plate at a density of 2×10<sup>3</sup> per well for overnight. Cells were incubated with the indicated proteins (30 µg/ml) and were changed each day. Cell number was determined at the indicated time points using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche, Indianapolis, IN). For proliferation
analysis, cells were treated as above and cultured for 24 h in normal growth medium supplemented with 10 µM BrdU (Sigma), harvested and fixed in 70% ethanol. BrdU-labeled cells fixed in 70% ethanol were treated with 2 N HCl (20 min at room temperature followed by addition of 2 volumes of 0.1 M sodium borate (pH 8.5). The cells were incubated with an anti-BrdU mouse monoclonal antibody, washed, and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody. Cells were counterstained overnight with 5 µg of propidium iodide per ml containing 40 µg of RNase per ml. The stained cells were analyzed by flow cytometry.

Senescence associated-β-galactosidase (SA-β-gal) activity was determined using an SA-β-gal staining kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instruction. Briefly, cells were washed in PBS and fixed in 2% formaldehyde–0.2% glutaraldehyde. Then the cells were washed and incubated at 37°C overnight with fresh senescence-associated β-gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal], 40 mM citric acid–sodium phosphate [pH 6.0], 150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). Senescent cells were identified as blue-stained cells by standard light microscopy, and a total of 500 cells were counted in random fields on a slide to determine the percentage of SA-β-gal–positive cells. To quantify apoptotic cells, we used a phycoerythrin-conjugated antibody to the active form of caspase 3 (Pharmingen, San Diego, CA) in a flow cytometric assay to detect cells in the early stages of apoptosis.
Immunoprecipitation and Immunoblotting. The monoclonal NPM antibodies (3F291; Santa Cruz Biotechnologies) were first conjugated to the M-280 paramagnetic Dynabeads (sheep anti-mouse IgG; Dynal) and incubated with 300-500 μg of cell extracts (at a ratio of about 100 μg of extract proteins to 10 μl anti-NPM beads) for 2 h at 4 °C. Beads were washed three times and collected on a magnetic particle concentrator (Dynal), and subjected to immunoblot analysis. For immunoblotting, samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were incubated with the antibodies specific for cyclins A, B or E, cdc2 (Oncogene Research Products), Cdk2, 4 or 6 (all from Santa Cruz Biotechnologies), p53, p53ser15, p21 (Cell Signaling Technology), or β-actin (Sigma) for 12-16 h at 4 °C.

Reporter gene Assays. 1 x 10^6 HEK 293 cells were transfected with an κB-site dependent luciferase vector, pGL3-KB-Luc (provided by Albert S. Baldwin, University of North Carolina-Chapel Hill) and plated on a 6-well plate. After 48 h, cells were incubated with the indicated proteins (30 μg/ml). The cells were harvested 12 h post-treatment, washed with PBS, and luciferase activity was assessed using the dual luciferase assay reporter kit (Promega).

Establishment of Fancc-/- pre-leukemic stem cells. TNF-induced Fancc-/- pre-leukemic stem cells were established as described previously (16). Briefly, low-density BM mononuclear cells from Fancc-/- mice were depleted of lineage-committed cells. Lin-Sca1+ cells were then purified by staining the Lin- cells with
Sca-1-PE antibodies (BD PharMingen) followed by cell sorting using a FACSCalibur (BD Biosciences). Cells were cultured in IMDM medium containing 100 ng/ml of stem cell factor (SCF), 20 ng/ml of interleukin (IL)-6, 10 ng/ml of IL-11 and 50 ng/ml of Flt-3 ligand (Flt-3L) (Peprotech) with or without 10 ng/ml of TNF-α for 30 days.

**Clonogenic progenitor assays.** WT BM cells or Fancc−/− pre-leukemic cells were incubated with the indicated proteins for 30 min prior to culture in a 35 mm tissue culture dish in 4 ml of semisolid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies), the indicated proteins at 30 ug/ml, and the following growth factors: 100 ng/mL SCF, 10 ng/mL IL-3, 100 ng/mL granulocyte colony-stimulating factor (G-CSF), and 4 U/mL erythropoietin (Peprotech). On day 10 after plating, erythroid and myeloid colonies were enumerated. Hematopoietic clonal growth results were expressed as means (of triplicate plates) ± S.D.

**Transduction of TAT-NPM proteins into Fancc−/− leukemic mice.** Age-matched congenic B6.SJL-PtrcaPep3b/BoyJ (B6.BoyJ; CD45.1+) mice (Jackson Laboratories) were used as transplant recipients. These mice were lethally irradiated (9.5 Gy, 110 cGy/min, 137Cs) and injected intravenously with 1 x 10⁶ Fancc−/− pre-leukemic cells (CD45.2+) described above, mixed with 1 x 10⁶ competitor cells (BoyJ; CD45.1+). After 10 days, recipient mice were injected i.p. with the indicated proteins (10 mg/kg in 0.5 ml of PBS and 10% glycerol) twice a week for up to 12 weeks. Survival of recipient mice was quantified by Kaplan-Meier analysis. Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in peripheral blood that
expressed the CD45.2 (by staining the cells with CD45.1-PE and CD45.2-APC) marker by flow cytometry. The studies involving the use of mice were conducted in accordance to the guidelines of and approved by the Institutional Animal Care and Use Committee of CCHMC (IACUC protocol # 6C06041; PI: Q. Pang).

**Immunohistochemistry and TUNEL labeling.** During necropsy, organs were removed, preserved in formalin, and then embedded in paraffin blocks. Paraffin sections were deparaffinized, rehydrated, incubated in 0.1 mM sodium citrate (pH 6.0), washed and incubated with peroxidase blocking reagent (Vector Laboratories, VectaStain Elite ABC kit). After washing in PBS, the slides were incubated with the primary antibodies Ki67 (NeoMarkers). Following three PBS washes, slides were incubated with secondary antibody and then detected with the VectaStain Elite ABC reagents. To detect apoptotic nuclei, formalin-fixed paraffin-embedded sections were analyzed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using the *in situ* Cell Death Detection kit (Roche) following the manufacturer’s directions. Slides were counterstained with 0.5% methyl green to visualize cell nuclei.

**Determination of NF-κB nuclear translocation and DNA-binding activity.** Nuclear protein extracts were prepared from BM cells using a Transfactor Extraction kit (BD Biosciences). Nuclear extracts were incubated with DNA specific for the NF-κB consensus sequence, and the DNA binding activity of NF-κB was measured using a Transfactor kit (BD Biosciences).
Gene expression analysis and chromatin immunoprecipitation assays. Total RNA was prepared with RNeasy kit (Qiagen) following the manufacturer's procedure. Following treatment with RNase-free DNase, RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed on a ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR green PCR master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Samples were normalized to the level of GAPDH mRNA, and the relative expression levels were determined by the standard curve method. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (17) using either anti-6×His or anti-p65 antibodies (both from Santa Cruz) for immunoprecipitation.

Statistics. Data were analyzed statistically using a two-tail Student's t test or Kaplan-Meier survival analysis. Statistical significance was presumed when $p < 0.05$. 
Results

TAT-NPM\(\Delta C\) induces senescence and apoptosis in primary fibroblasts

Because NPM functions to suppress cellular senescence and apoptosis and is essential for development (7, 18), we reasoned that this important molecule may have therapeutic value. We therefore studied NPM transduction by PTD. Since we previously showed that a truncated mutant containing the N-terminal 174 aa (NPM\(\Delta C\)) induced apoptosis in unstressed cells as well as in cells treated with genotoxic agents (19), we generated TAT-fusion proteins containing the full-length NPM, NPM\(\Delta N\) containing the C-terminal 108 aa, and NPM\(\Delta C\). These fusion proteins were expressed in bacteria and purified (Fig. 1). To examine whether these TAT fusions were biologically active, we determined their effect on the growth of murine embryonic fibroblasts (MEFs) isolated from WT and Fancc\(-/-\) mice. We chose Fancc\(-/-\) MEFs because they undergo premature senescence under standard fibroblast culture conditions (7). TAT-NPM suppressed senescence in Fancc\(-/-\) MEFs, which was consistent with previous report (7); whereas both TAT-NPM\(\Delta N\) and TAT-NPM\(\Delta C\) completely lost this function (Fig. 2A). Furthermore, we found that TAT-NPM and TAT-NPM\(\Delta C\) had opposite effect on cell proliferation. That is, TAT-NPM enhanced proliferation while TAT-NPM\(\Delta C\) suppressed cell growth (Fig. 2B). In addition, TAT-NPM\(\Delta C\) induced apoptosis in WT MEFs and significantly increased apoptotic Fancc\(-/-\) cells compared to untreated controls (Fig. 2C). In contrast, TAT-NPM and TAT-NPM\(\Delta N\) did not have significant effect on the survival of either WT or Fancc\(-/-\) cells (Fig. 2C).
The differential effects of TAT-NPM and TAT-NPMΔC on cell survival and proliferation was not due to transduction efficiency, as the uptake of these TAT fusions appeared to be similar between TAT-NPM and TAT-NPMΔC (Fig. 2D). The intracellular delivery of the TAT-fusions was rapid, as the FITC-labeled fusions could be detected in the treated cells as early as 10 min after incubation (Fig. 2E). To determine the cellular localization of TAT-NPM fusions, we treated cells with the TAT fusions for 30 min, washed the cells extensively, and incubated them in the absence of the TAT fusions for 60 min before fixation. We found that while TAT-NPM was localized in the nucleolus, the majority of TAT-NPMΔC was localized in nucleoplasm (Fig. 2F).

**TAT-NPMΔC down-regulates anti-apoptotic and pro-inflammatory genes**

Because TAT-NPMΔC inhibited cell proliferation and induced apoptosis, we wanted to know if the mutant TAT fusion affected the expression or/and activities of factors involved in cell-cycle control and survival signaling. We first examined the expression and activities of the cell-cycle regulators p53, p21WAF1, p16INK4A, and p19Arf, known to be negatively regulated by NPM (7, 19-22). While the activity of p53, as reflected by its phosphorylation at Ser15 and p21WAF1 expression, was inhibited in senescent Fancc/- MEF cells treated with TAT-NPM, TAT-NPMΔC did not reduce the expression of these negative cell-cycle regulators (Fig. 3A, left panel). The expression of p16INK4A or p19Arf, as determined by Western blot (Fig. 3A, middle panel) and RT-PCR (Fig. 3A, right panel), was not affected by either TAT-
NPM or TAT-NPM\(\Delta C\). Interestingly, TAT-NPM\(\Delta C\) increased active form of p53 (p53Ser15). The mechanism and functional consequence of its activation of p53 remains to be investigated.

We also examined the expression of the positive cell-cycle regulators including cyclins A, B, D and E, and cyclin-dependent kinases cdc2 (Cdk1), Cdk2, 4 and 6 in MEF cells. We did not observe significant changes in the expression of these molecules in TAT-NPM\(\Delta C\)-treated cells (Fig. 3B). However, we found significantly reduced levels of cellular inhibitor of apoptosis proteins (cIAP)-1, cIAP-2, Bcl-2, and Bcl-XL in TAT-NPM\(\Delta C\)-treated cells compared with cells treated with TAT-NPM (Fig. 3C). Surprisingly, the expression of pro-inflammatory cytokines tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), interleukin (IL)-1\(\beta\), IL-6, macrophage inflammatory protein (MIP)-2 and cyclooxygenase 2 (Cox-2) was also reduced by TAT-NPM\(\Delta C\) treatment (Fig. 3D).

**TAT-NPM\(\Delta C\) inhibits NF-\(\kappa\)B transactivation**

Since the expression of the mentioned inflammatory and anti-apoptotic genes is controlled by NF-\(\kappa\)B (23), and since NPM can act to activate NF-\(\kappa\)B (24), this would suggest that TAT-NPM\(\Delta C\) induces apoptosis through a mechanism involving suppression of NF-\(\kappa\)B activation. To test this notion, we examined the effect of TAT-NPM\(\Delta C\) on nuclear translocation and DNA-binding activity of NF-\(\kappa\)B in normal lymphoblast cells stimulated with TNF-\(\alpha\). Surprisingly, we found that higher level of NF-\(\kappa\)B subunit p65 (RelA) was detected in TAT-NPM\(\Delta C\)-treated cells than those treated with TAT-NPM or BSA (Fig. 4A). Consistent with this, significant increase in
DNA binding by p65 was observed in TAT-NPMΔC-treated cells, as compared to cells treated with TAT-NPM or BSA (Fig. 4B). It is noteworthy that TAT-NPM also enhanced DNA-binding activity of p65, which is consistent with previous observation that NPM activates NF-κB (24). Furthermore, TAT-NPMΔC greatly prolonged the time of TNF-α-induced NF-κB accumulation in nuclei (Fig. 4C).

We next examined the effect of TAT-NPMΔC on NF-κB transactivation by transient reporter gene assays in HEK293T cells using a luciferase construct with an NF-κB-driven promoter. Cells transduced with TAT-NPM showed enhanced NF-κB luciferase reporter activity induced by TNF-α (Fig. 4D). In contrast, TAT-NPMΔC inhibited NF-κB activation induced by TNF-α treatment. Interestingly, the basal activity of the NF-κB luciferase reporter was also significantly reduced in TAT-NPMΔC-treated cells (Fig. 4D). Together, these results demonstrate that TAT-NPMΔC acts as a negative regulator of the NF-κB pathway.

**Intracellular delivery of TAT-NPMΔC induces apoptosis in Fancc-/- pre-leukemic stem cells and delays leukemia development in mice**

We have recently established a model of TNF-α-promoted leukemogenesis in bone marrow (BM) hematopoietic stem cells of mice deficient for the Fanconi group C gene (*Fancc*). As described previously (16), the TNF-α-induced *Fancc-/-* pre-leukemic cells expressed primitive markers Sca-1 and c-kit, underwent clonal proliferation and gave rise to myeloid leukemia after transplanted into irradiated recipient mice. We also demonstrated that these TNF-α-induced *Fancc-/-* pre-
leukemic stem cells required NF-κB signaling for survival (16). This inflammation-associated leukemogenic model therefore provided us a unique opportunity to test whether TAT-NPMΔC exerted an inhibitory effect on leukemia development in vivo. First, we examined proliferation and repopulating capacity of TAT-NPMΔC-treated Fancc−/− pre-leukemic stem cells using clonogenic and bone marrow transplantation assays. We found that TAT-NPMΔC transduction significantly suppressed clonogenic proliferation (Fig. 5A, S1A) and hematopoietic repopulating ability (Fig. 5B, S1B) of the Fancc−/− pre-leukemic cells compared to BSA-treated cells. Interestingly, we did not observed further increase in colony-forming and repopulating ability of Fancc−/− pre-leukemic cells treated with TAT-NPM compared to BSA-treated cells (Fig. 5, S1). However, we observed a significant increase in apoptosis in colony-forming cells and bone marrow repopulating cells of the TAT-NPMΔC-treated Fancc−/− pre-leukemic stem cells (Fig. 5C).

While the data presented above demonstrate that four-week treatment of the recipient mice with TAT-NPMΔC reduced hematopoietic repopulating ability of the Fancc−/− pre-leukemic stem cells, it would be of importance to know whether the TAT-fusion was effective in inhibiting leukemia development. We therefore monitored the survival of recipients of Fancc−/− pre-leukemic cells over 4 and 12 weeks of TAT-NPM or TAT-NPMΔC treatment. Recipient mice injected with BSA were served as controls. To determine whether the TAT-fusions were actually delivered into the bone marrow of the recipient mice as well as the retention kinetics of the TAT-fusions in the recipient mice, we examined the levels of the TAT-fusions
in BM cells from the recipient mice at various time points after a single injection. It appeared that TAT-NPM and TAT-NPMΔC were delivered at a similar rate and could be detected in BM cells of the recipient mice for up to 12 hrs after injection (Fig. S2). Recipients injected with BSA developed acute myeloid leukemia within 150 days (Fig. 6A), consistent with previous report (16). The time required for leukemia to develop in recipients treated with TAT-NPM was indistinguishable from that of BSA-treated mice, regardless of the duration of the treatment. However, the occurrence of leukemia was significantly delayed in recipients injected with TAT-NPMΔC fusion, with long-term (12 weeks) treatment showing the better effect than the short-term (4 weeks) treatment (Fig. 6A). Furthermore, TAT-NPMΔC treatment clearly reduced cell proliferation, as evaluated by BrdU incorporation in the leukemic cells (Fig. 6B) and Ki-67 antigen staining in the spleen and liver of the recipient mice (Fig. 6C). TUNEL assay showed increased apoptosis in the bone marrow of the recipient mice treated with TAT-NPMΔC compared with BSA- or TAT-NPM-treated recipients (Fig. 6D). Collectively, these results demonstrate that TAT-NPMΔC induces apoptosis and proliferative suppression of Fancc−/− leukemic cells and delays leukemia development.

**TAT-NPMΔC forms complexes with endogenous NPM and p65 at promoters of anti-apoptotic and inflammatory genes and abrogates their transactivation by NF-κB in pre-leukemic cells**

To further investigate the mechanism by which TAT-NPMΔC inhibited NF-κB activation, we first examined the relationship of TAT-NPMΔC and endogenous NPM
in Fancc-/ pre-leukemic cells. We found that NPM was markedly up-regulated in Fancc-/ pre-leukemic cells compared to bone marrow mononuclear cells isolated from WT or Fancc-/ mice (Fig. 7A). This might explain our finding that no further increase in colony-forming and repopulating ability were observed in Fancc-/ pre-leukemic cells treated with TAT-NPM (Fig. 6A,B), as the level of the endogenous NPM with the same functionality as TAT-NPM was already high enough. Shortly (30 min) after treatment, both TAT-NPM and TAT-NPM\(\Delta\)C associated with endogenous NPM in nuclei of Fancc-/ pre-leukemic cells (Fig. 7B). This was not surprising because NPM\(\Delta\)C retains the N-terminal oligomerization domain (25). We further confirmed the observed interaction between the NPM fusions and endogenous NPM using a normal lymphoblast cell line that expressed FLAG-NPM or FLAG-NPM\(\Delta\)C (Fig. 7C). It has been reported that NPM interacts with p19\(^{Arf}\) and regulates the biological function of p19\(^{Arf}\) (26, 27). We thus determined p19ARF protein levels and interaction between TAT-NPM and p19\(^{Arf}\) or between TAT-NPM\(\Delta\)C and p19\(^{Arf}\) in Fancc-/ pre-leukemic cells. Our results show that treatment of the Fancc-/ pre-leukemic cells with or without the TAT-NPM fusions did not affect p19\(^{Arf}\) protein levels, and that only the full-length TAT-NPM interacted with p19\(^{Arf}\) (Fig. 7D).

To study the functional consequence of the interaction between TAT-NPM\(\Delta\)C and endogenous NPM in the context of NF-\(\kappa\)B inhibition, we first asked whether TAT-NPM\(\Delta\)C inhibited NF-\(\kappa\)B in vivo by determining NF-\(\kappa\)B activity in the treated recipient mice. As shown in Fig 7E, treatment of TAT-NPM\(\Delta\)C for 4 and 12 weeks dramatically reduced DNA-binding activity of NF-\(\kappa\)B in the recipient mice. In addition,
treatment with TAT-NPM\(\Delta C\) induced apoptosis in \(Fancc^{-/-}\) leukemic cells at both kinetics and magnitude that are comparable to other NF-\(\kappa B\) inhibitors such as Sodium salicylate, Emodin, Parthenolide, BAY11072 (Fig. 7F).

Next, we utilized ChIP assay to determine whether in vivo association of TAT-NPM\(\Delta C\) with endogenous NPM affected the binding of NF-\(\kappa B\) to the promoters of IL-6 and MIP-2 genes. After 30 min of TNF-\(\alpha\) stimulation, the association of TAT-NPM\(\Delta C\) and p65 with the \(kB\)-responsive elements at the IL-6 promoter was increased in TAT-NPM\(\Delta C\)-treated \(Fancc^{-/-}\) pre-leukemic cells compared to BSA or TAT-NPM-treated cells (Fig. 7G). Similarly, the association TAT-NPM\(\Delta C\) and p65 with the MIP-2 promoter was also increased in TAT-NPM\(\Delta C\)-treated cells. Importantly, both 6\(\times\)His and p65 ChIPs showed prolonged residence of TAT-NPM\(\Delta C\) and p65 at NF-\(\kappa B\)-responsive promoters of the IL-6 and MIP-2 genes in TAT-NPM\(\Delta C\)-treated \(Fancc^{-/-}\) pre-leukemic cells (Fig. 7G). This may explain the prolonged TNF-\(\alpha\)-induced NF-\(\kappa B\) accumulation in nuclei of TAT-NPM\(\Delta C\)-treated cells (Fig. 4C). Consistent with its inhibitory effect on NF-\(\kappa B\) activation, TAT-NPM\(\Delta C\) greatly down-regulated expression of pro-inflammatory and anti-apoptotic genes in \(Fancc^{-/-}\) pre-leukemic cells, such as IL-6, COX2, Bcl-XL, and cIAP-1 known to be the targets of NF-\(\kappa B\) (Fig. 7H). Therefore, TAT-NPM\(\Delta C\) appears to inhibit NF-\(\kappa B\) activation by binding to p65-associated endogenous NPM and thus converting NF-\(\kappa B\) to a transcriptionally inactive or repressive state.
Discussion

Given that NPM is frequently up-regulated in actively proliferating cells and human cancers (1-6), it is of great interest to investigate the therapeutic value by targeting the overexpressed NPM in tumor cells. In this paper, we present a novel protein therapy targeting the overexpressed NPM in inflammation-associated leukemia. We have utilized the TAT protein transduction domain to rapidly and efficiently deliver a polypeptide derived from the N-terminus of the NPM protein into pre-leukemic cells and tissues of leukemic mice. Using an inflammation-associated leukemogenic model we recently established, we demonstrated that the TAT fusion peptide (TAT-NPMΔC) induced proliferative suppression and apoptosis of pre-leukemic cells, and significantly delayed leukemic development in mice. Furthermore, we found that the TAT-NPMΔC fusion formed complexes with endogenous NPM and specifically interfered with NF-κB function in leukemic cells. Thus, our results suggest that TAT-delivered NPM peptide may provide a novel therapy targeting inflammation-associated tumors that require NF-κB signaling for survival.

TAT-NPMΔC appears to be very potent in repressing NF-κB transactivation of numerous inflammatory and anti-apoptotic genes, which may explain our observations that TAT-NPMΔC could effectively act to induce apoptosis in leukemic cells and delay the development of inflammation-associated leukemia in mice. NPM has been identified as an NF-κB co-activator (24). The results of the present study
clearly demonstrate TAT-NPMΔC-mediated repression of the expression of inflammatory and anti-apoptotic genes that is dependent on NF-κB. The results also reveal the underlying mechanism of inhibition. That is, TAT-NPMΔC forms a tertiary complex with the endogenous NPM and NF-κB, thus converting NF-κB to a transcriptionally inactive state that becomes trapped at κB element-containing promoters. This notion is supported by the finding that the inhibition of NF-κB transactivation by TAT-NPMΔC is accompanied by a paradoxical increase in the nuclear accumulation and the DNA-binding activity of NF-κB. This mechanism of action is in contrast with that of the classic NF-κB inhibitors, which act to prevent NF-κB nuclear translocation by inhibiting the phosphorylation and degradation of IκBα (28, 29). Persistent NF-κB activation is commonly observed in inflammatory diseases and malignancies (30) and overexpression of NPM is often found in human cancers (1-6). In this context, utilization of the TAT-delivered NPMΔC to not only convert NF-κB to inactive state but also sequester the overexpressed endogenous NPM to biologically non-functional protein complexes may prove to be therapeutically attractive.

In addition to inhibiting NF-κB, TAT-NPMΔC appears to be able to activate p53 in cultured primary cells. The precise mechanism by which TAT-NPMΔC induces the activation of endogenous p53 in the steady state remains to be further elucidated; however, our results indicate that treatment of pre-senescent cells with TAT-NPMΔC strongly enhanced phosphorylation of p53 at the Ser15 residue and subsequently induced p53 target p21WAF1/CIP1 (Fig. 4). Phosphorylation at the Ser15
residue of p53 is critical for p53-dependent transactivation (31). The ability of TAT-NPM\(\Delta\)C to augment p53 activity can explain, at least in part, its ability to induce apoptosis and to accelerate senescence in unstressed cells (Fig. 2). Loss of p53 functions has been implicated in more than 50% of human cancers due to inactivating mutations (32). Therefore, reactivation of p53 functions or inactivation of p53 negative regulators has long been considered as an effective therapy for cancers. The data provided here reveal that the TAT-delivered NPM\(\Delta\)C causes a significant increase of the active form of p53 and thus suggest that TAT-NPM\(\Delta\)C might be able to reactivate the p53-mediated apoptotic pathway in tumor cells. In this context, TAT-NPM\(\Delta\)C could be used as a novel therapeutic agent in combination with DNA damage drugs for cancer prevention and treatments.

The dual effect of TAT-NPM\(\Delta\)C as a NF-\(\kappa\)B inhibitor and a p53 activator raises an important question of whether the effects of TAT-NPM\(\Delta\)C on NF-\(\kappa\)B inhibition and p53 activation are interrelated. The observation that TAT-NPM\(\Delta\)C exerted its negative effect on NF-\(\kappa\)B by forming an inactive complex with the endogenous NPM and NF-\(\kappa\)B excludes the possibility that would place TAT-NPM\(\Delta\)C-induced p53 activation upstream of NF-\(\kappa\)B inhibition, as TAT-NPM\(\Delta\)C appears to exert its negative effect directly on NF-\(\kappa\)B. Mechanisms of mutual negative regulation of NF-\(\kappa\)B and p53 have been reported (33). Recently, it has been shown that inhibition of NF-\(\kappa\)B by small molecules reactivates p53 in renal cell carcinoma (34), suggesting that there are unidentified regulatory factors that provide interactions between NF-\(\kappa\)B and p53 signaling pathway. Another possibility is that
TAT-NPMΔC could act on p53 in parallel with NF-κB inhibition. The exact mechanism remains to be determined.

In conclusion, we have demonstrated the efficiency and biological activity of intracellular delivery of a mutant NPM peptide using TAT PTD, thereby providing a novel protein therapy targeting the overexpressed NPM in inflammation-associated leukemia. TAT-NPMΔC may also be applicable for other tumors and disease conditions that require NF-κB signaling for survival.
Acknowledgments

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Authorship

Yun Zhou, performed research, analyzed data, wrote the paper; Wei Du, performed research, analyzed data, wrote the paper; Tara Koretsky, performed research, analyzed data; Grover C. Bagby, designed research; Qishen Pang, designed research, analyzed data, and wrote the paper. The authors declare no competing financial interests.
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Figure legends

Figure 1. Expression and purification of TAT-NPM fusion proteins. (A) Schematic presentation of TAT-NPM fusions. (B) Expression of TAT-NPM fusions. *coli BL21 cells harboring vector (TAT), TAT-NPMΔN, TAT-NPMΔC, or TAT-NPM were grown in the presence (+) of IPTG to induce expression of the TAT-fusion proteins. 50 μg of cell lysates were analyzed by Coomassie blue staining. Arrows denote the corresponding TAT-fusion proteins. (C) Western blot analysis of the TAT-NPM proteins using an anti-histidine antibody. TAT-NPM fusion proteins were purified by affinity chromatography on a Nickel-Sepharose column, followed by gel filtration on a PD-10 column.

Figure 2. Effect of TAT-NPM fusions on cell proliferation and senescence. (A) TAT-NPMΔC fails to suppress senescence in MEFs. WT or Fancc-/− MEFs at passage 4 were incubated with BSA, TAT-NPM, TAT-NPMΔC, or TAT-NPMΔN (30 μg/ml each) for six days, with medium and the proteins changed each day. Cells were then stained for SA-β-gal. The graphs on the right are percentages of the cells stained positive for SA-β-gal quantified by counting a total of 100 cells in random fields on a slide. The data represent the mean ±SD of three independent experiments. *Statistical significance between TAT-NPMΔC and BSA samples at P < .05. (B) TAT-NPMΔC inhibits cell proliferation. Cells were plated in 96-well
plate at a density of $2 \times 10^3$ per well for overnight. Cells were incubated with the indicated proteins (30 μg/ml) and were changed each day. Cell proliferation was determined at the indicated time points. Data represent mean ± SD of three experiments. *Statistical significance between TAT-NPM and BSA or between TAT-NPMΔC and BSA samples at $P < .05$. (C) Cells described in (A) were analyzed for apoptosis, as determined by flow cytometry for the percentage of cells with active caspase 3 (gated in R3). Shown are the representative data of three independent experiments with similar results. (D) Uptake of TAT-NPM and TAT-NPMΔC in human cells. HEK293 cells were incubated with 30 μg/ml of the indicated proteins for 30 min. After fixation, cells were counterstained with DAPI and the cellular distribution of the TITC-labeled TAT-fusions was visualized with fluorescence microscope. (E) Time course of TAT-NPM protein uptake by human cells. HEK293 cells were incubated with TAT-NPM or TAT-NPMΔC (30 μg/ml each) at the indicated time. Relative FITC intensity was determined by normalizing fluorescence intensity of each treatment with cell numbers. (F) Subcellular localization of the TAT fusions in human cells. HEK293 cells were incubated with TAT-NPM or TAT-NPMΔC (30 μg/ml each) for 30 min, washed extensively, and incubated in the absence of the TAT fusions for 60 min before fixation. The cells were then stained with anti-His6 antibody and visualized with fluorescence microscope.

Figure 3. Effect of TAT-NPM proteins on expression of genes controlling cell cycle, apoptosis, and inflammation. (A) Effect on negative regulators of
cell-cycle progression. Primary MEFs were treated with BSA, TAT-NPM, or TAT-NPM\(\Delta C\) (30 \(\mu\)g/ml each) for 12 hrs, and whole cell extracts or RNA were prepared for analyses of p53, p21\(^{Waf1}\), p16\(^{Ink4a}\), p19\(^{Arf}\), and p27\(^{Kip1}\) by immunoblotting (left and middle panels) or p16\(^{Ink4a}\) and p19\(^{Arf}\) mRNAs by RT-PCR (right panel). (B) Cells described in (A) were analyzed for expression of positive regulators of cell-cycle progression by immunoblotting. (C) Reduced levels of anti-apoptotic proteins in cells treated with TAT-NPM\(\Delta C\), as examined by immunoblot analysis of cell extracts described in (A). (D) TAT-NPM\(\Delta C\) suppresses expression of inflammatory genes. Expression of genes encoding inflammatory genes was examined in BSA, TAT-NPM, and TAT-NPM\(\Delta C\)-treated cells stimulated with TNF-\(\alpha\) (10 ng/ml) for 30 min. Cells were collected, total RNA was prepared, and gene expression was analyzed by real-time PCR and normalized to GAPDH mRNA. Results are means ± SD of three independent experiments.

**Figure 4. TAT-NPM\(\Delta C\) inhibits NF-\(\kappa B\) activation.** (A) Effect of TAT-NPM\(\Delta C\) on TNF-\(\alpha\)-induced nuclear translocation of NF-\(\kappa B\). HEK293T cells were first treated with BSA, TAT-NPM, or TAT-NPM\(\Delta C\) (30 \(\mu\)g/ml each) for 30 min then stimulated with TNF-\(\alpha\) (10 ng/ml) for 30 min. Nuclear localization of the NF-\(\kappa B\) subunit p65 in the nuclear extracts was analyzed by immunoblot analysis of the NF-\(\kappa B\) subunit p65. (B) Effect of TAT-NPM\(\Delta C\) on TNF-\(\alpha\)-induced DNA-binding activity of NF-kB. HEK293T cells were first treated with BSA, TAT-NPM, or TAT-NPM\(\Delta C\) (30 \(\mu\)g/ml each) for 30 min then stimulated with TNF-\(\alpha\) (10 ng/ml) for the
indicated time. Nuclear extracts were then prepared and DNA-binding activity of NF-κB was measured by transcription factor ELISA assays. Data are presented as fold activation relative to the DNA-binding activity in BSA-treated cells without TNF-α stimulation. (C) TAT-NPMΔC prolongs TNF-α-induced nuclear accumulation of NF-κB. HEK293T cells were first treated with BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each) for 30 min then stimulated with TNF-α (10 ng/ml) for the indicated time. Nuclear localization of the NF-κB subunit p65 in the nuclear extracts was analyzed by immunoblot analysis of the NF-κB subunit p65. (D) TAT-NPMΔC inhibits NF-κB transcriptional activity. HEK293T cells transfected with a 3×κB-Luc plasmid were treated with BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each) for 30 min then stimulated with TNF-α (10 ng/ml) for another 30 min. Results of triplicate experiments are shown with mean and standard deviation.

Figure 5. TAT-NPMΔC suppresses proliferation and induces apoptosis in Fancc-/- pre-leukemic stem cells. (A) Clonogenic assay. WT BM cells or Fancc-/- pre-leukemic cells cultured in semi-solid medium containing 30 μg/ml of the indicated proteins were analyzed for colony-forming efficiency of hematopoietic progenitors. Data represents the mean ± SD of three experiments. (B) Bone marrow transplantation assay. 1×10^6 WT BM cells or Fancc-/- pre-leukemic cells (CD45.2+) were transplanted, along with 1 x 10^6 competitor cells from B6.BoyJ mice (CD45.1+), into lethally irradiated recipient (B6.BoyJ) mice, which after 10 days were injected i.p. with the indicated proteins (10 mg/kg in 0.5
ml of PBS and 10% glycerol) twice a week for 4 weeks. Engraftment was evaluated at 4 weeks post-transplantation. Data represent mean ± SD of three independent experiments, each with 6 animals (total 18 mice). (C) TAT-NPMΔC induces growth arrest and apoptosis in colony-forming and repopulating Fancc−/− cells. Bone marrow cells from colonies described in (A) or bone marrow cells from recipient mice described in (B) were stained with propidium iodide (PI) followed by analysis for cell cycle distribution. Shown are representative flow cytometric presentations of three colony assays or 3-4 recipients in each treatment.

**Figure 6. TAT-NPMΔC delays leukemia development.** (A) 1×10⁶ Fancc−/− pre-leukemic cells (along with 1×10⁶ competitive cells) were injected i.v. into lethally irradiated recipients, which after 10 days were injected i.p. with the indicated proteins (10 mg/kg in 0.5 ml of PBS and 10% glycerol) twice a week for up to 12 weeks. Survival of recipient mice was quantified by Kaplan-Meier analysis. Experiments were performed two times, each with 3 recipient mice (total 6 mice per group). (B) 2×10⁵ WT BM cells or Fancc−/− leukemic cells were treated with BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each) for 30 min. Cells were then washed and cultured for 24 h in normal growth medium supplemented with 10 μM BrdU, and level of BrdU incorporation was determined by flow cytometry. Data represent mean ± SD of three experiments. (C) TAT-NPMΔC inhibits leukemic cell proliferation. Tissue sections of spleen and liver from recipient mice treated with the indicated proteins for 4 weeks were stained with ki-67 and
counterstained with H&E. Magnification: 40×. (D) TAT-NPMΔC induces apoptosis in leukemic mice. Tissue sections of bone marrow from recipient mice treated with the indicated proteins for 4 weeks were analyzed by TUNEL staining (green). Magnification: 40×.

**Figure 7. TAT-NPMΔC forms complexes with endogenous NPM and p65 and abrogates NF-κB activation in Fancc-/ pre-leukemic cells.** (A) Up-regulation of NPM in Fancc-/ pre-leukemic cells. Whole cell extracts of freshly-isolated bone marrow cells from WT and Fancc-/ mice or Fancc-/ pre-leukemic cells were analyzed for NPM protein levels by immunoblotting. Data with two mice from each group are shown. (B) Nuclear association of TAT-NPM fusions with endogenous NPM in Fancc-/ pre-leukemic cells. Fancc-/ pre-leukemic cells were treated with BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each) for 30 min. Nuclear extracts were prepared and used for immunoprecipitation (IP) with an anti-NPM antibody. The immuno-complexes were then analyzed by Western blotting with antibodies against 6×Histine (top panel), NPM (middle panel) or actin (bottom panel). Input controls (10%) are shown in left lane of each group. (C) Nuclear association of NPMΔC with endogenous NPM in Fancc-/ pre-leukemic cells. Fancc-/ pre-leukemic cells were infected with vector (V), WT NPM (M), or NPMΔC (C) retroviruses. Nuclear extracts were prepared and used for immunoprecipitation (IP) with an anti-FLAG antibody. The immuno-complexes were then analyzed by Western blotting with antibodies against FLAG (top panel), NPM (middle panel) or actin (bottom panel). Input controls (10%) are
shown in left lanes. (D) TAT-NPMΔC does not associate with p19ARF. Fancc--/-- pre-leukemic cells were treated with BSA (B), TAT-NPM (M), or TAT-NPMΔC (C) (30 μg/ml each) for 30 min. Whole cell extracts were prepared and used for immunoprecipitation (IP) with an anti-His antibody. The immuno-complexes were then analyzed by Western blotting with antibodies against p19ARF (top panel), 6×Histine (middle panel) or actin (bottom panel). Input controls (10%) are shown in left lane of each group. (E) TAT-NPMΔC inhibits NF-κB activity in Fancc--/-- leukemic mice. 1×10^6 Fancc--/-- pre-leukemic cells (along with 1×10^6 competitive cells) were injected i.v. into lethally irradiated recipients, which after 10 days were injected i.p. with the indicated proteins (10 mg/kg in 0.5 ml of PBS and 10% glycerol) twice a week for 4 and 12 weeks. Nuclear extracts were then prepared from BM cells of the recipient mice 24 hrs after the last injection and DNA-binding activity of NF-κB was measured by transcription factor ELISA assays. The results are the means ± SD of two independent experiments, each with three recipient mice from each group. Statistical significance (p<0.05) between BSA or TAT-NPM and TAT-NPMΔC. (F) Kinetics of apoptosis induced by TAT-NPMΔC other NF-κB inhibitors in Fancc--/-- leukemic cells. Fancc--/-- pre-leukemic cells were treated with TAT-NPMΔC (30 μg/ml), Sodium salicylate (5 mM), Emodin 3 μg/ml), Parthenolide (2 μM), or BAY11072 (0.2 μM) for the indicated time. Apoptosis was determined by flow cytometry for the percentage of cells with active caspase 3. The results are presented as the means ± SD of two experiments. There is no statistically significant difference between TAT-NPMΔC and other tested NF-κB inhibitors. (G) Increased binding of TAT-NPMΔC and p65 to NF-κB-responsive
promoters in Fancc/- pre-leukemic cells. Chromatin immunoprecipitation assays. Fancc/- pre-leukemic cells were treated with TNF-α for the indicated time in the presence of BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each). Recruitment of TAT-NPMΔC or the p65 NF-κB subunit to the IL-6 and MIP-2 promoters was assessed by immunoprecipitation and PCR amplification of the promoter sequences. (H) Down-regulation of pro-inflammatory and anti-apoptotic genes in TAT-NPMΔC-treated Fancc/- pre-leukemic cells. Fancc/- pre-leukemic cells were treated with TNF-α for the indicated time in the presence of BSA, TAT-NPM, or TAT-NPMΔC (30 μg/ml each). RNA was isolated, and gene expression was quantified by real-time PCR and normalized to the level of GAPDH mRNA.
Figures

Figure 1

A

B

C

[Diagrams and images related to protein expression and analysis]
Figure 2

A

WT

Fancc-/-

B

WT

Fancc-/-

C

WT

Fancc-/-
Figure 3

A

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D

Relative mRNA levels

IL-1β  | IL-6  | MIP-2 | Cox-2

BSA    | TAT-NPM | TAT-NPM/AC
Figure 5

A

Colony counts (10^4 cells)

WT Pre-Leuk

BSA TAT-NPM TAT-NPMΔC

B

Donor-derived cells (%)

WT Pre-Leuk

BSA TAT-NPM TAT-NPMΔC

C

BM cells from colonies

BSA TAT-NPM TAT-NPMΔC

BM cells from recipient mice

Channels
Figure 7

A

WT    Fancc/-    Pre-Leuk

NPM

β-actin

B

BSA    TAT-NPM    TAT-NPMΔC

IP (α-NPM)

NPM

NPMΔC

β-actin

C

kDa: 40- 25- 20-

Input: V M C

IP: FLAG

V M C

WB: α-FLAG

NPM

NPMΔC

Western: α-NPM

β-actin

D

kDa: 40- 25- 20-

Input: B M C

IP: α-His

B M C

WB: α-His

Western: α-p19ARF

NPM

NPMΔC

β-actin

E

Relative DNA-binding activity

4 weeks  12 weeks

BSA    TAT-NPM    TAT-NPMΔC

F

% Apoptosis

4 hr  12 hr  24 hr

PBS

BAY11072

Emodin

Parthenolide

Salicylate

TAT-NPMΔC
**G**

### 6×His ChIP:

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- IL-6 prom
- MIP-2 prom
- MIP-2 exon
- GAPDH

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**H**

### IL-6

- BSA
- TAT-NPM
- TAT-NPMΔC

### MIP-2

- BSA
- TAT-NPM
- TAT-NPMΔC

### Bcl-XL

- BSA
- TAT-NPM
- TAT-NPMΔC

### cIAP-2

- BSA
- TAT-NPM
- TAT-NPMΔC

Relative mRNA level vs. TNF-α (min)
TAT-mediated intracellular delivery of NPM-derived peptide induces apoptosis in leukemic cells and suppresses leukemogenesis in mice

Yun Zhou, Wei Du, Tara Koretsky, Grover C Bagby and Qishen Pang