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NETROPSIN IMPROVES SURVIVAL FROM ENDOTOXEMIA BY DISRUPTING HMGA1-BINDING TO THE NOS2 PROMOTER

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SYNOPSIS

The inducible form of nitric oxide synthase (NOS2) plays an important role in sepsis incurred as a result of infection with gram-negative bacteria that elaborate endotoxin. The high mobility group A1 (HMGA1) architectural transcription factor facilitates NOS2 induction by binding a specific AT-rich Oct sequence in the core NOS2 promoter via AT-hook motifs. The small-molecule, minor groove binder (MGB) netropsin selectively targets AT-rich DNA sequences and can interfere with transcription factor binding. Therefore we hypothesized that netropsin would improve survival from murine endotoxemia by attenuating NOS2 induction through interference with HMGA1-DNA binding to the core NOS2 promoter. Netropsin improved survival from endotoxemia in wild type mice, yet not in NOS2-deficient mice, supporting an important role for NOS2 in the beneficial effects of MGB administration. Netropsin significantly attenuated NOS2 promoter activity in macrophage transient transfection studies and the AT-rich HMGA1-DNA binding site was critical for this effect. EMSAs demonstrated that netropsin interferes with HMGA1-NOS2 promoter binding and NMR spectroscopy was undertaken to characterize this disruption. Chemical shift perturbation analysis identified that netropsin effectively competes both HMGA1 DNA-binding AT-hooks from the AT-rich NOS2 promoter sequence. Furthermore, nuclear Overhauser effect spectroscopy (NOESY) data identified direct molecular interactions between netropsin and A/T base pairs within the NOS2 promoter HMGA1 binding site. Finally, we determined a structure of the netropsin/NOS2 promoter Oct site complex from molecular modeling and dynamics calculations. These findings represent important steps toward refined structure-based ligand design of novel compounds for therapeutic benefit that can selectively target key regulatory regions within genes important for the development of critical illness.

Keywords

Netropsin; minor groove binder; nitric oxide synthase; high mobility group A protein; endotoxemia; lipopolysaccharide

INTRODUCTION

Sepsis represents a devastating clinical condition in which systemic infection leads to multi-system organ failure and high mortality [1]. A subset of patients with sepsis suffer from infections with gram negative bacteria that elaborate endotoxin, leading to propagation of a downstream inflammatory cascade, production of pro-inflammatory cytokines, and transcriptional up-regulation of the inducible form of nitric oxide synthase (iNOS, NOS2) that results in production of nitric oxide (NO) and development of refractory hypotension [2]. Complete inhibition of NOS2 induction has not proven beneficial in animal models or human trials of sepsis, and numerous studies have supported a beneficial role for some level of NOS2 expression (e.g., in wound healing and intracellular bacterial killing) [3]. Therefore, the ability to administer a compound that predictably attenuates NOS2 expression *in vivo* represents an important avenue of novel therapeutic investigation in critical illness.

Our group demonstrated an important role for the architectural transcription factor high mobility group A1 (HMGA1) in up-regulating NOS2 expression *in vitro* [4–6]. HMGA1 binds to the AT-rich octamer (Oct) site (base pairs –61 to –54) within the NOS2 promoter and, through formation of a ternary complex, recruits binding of the p50 subunit of NF- κ B to its cognate binding site upstream of the Oct site (base pairs –84 to –75). Optimal induction of NOS2 promoter activity requires HMGA1-DNA binding to the AT-rich region of the core NOS2 promoter. Therefore, the ability to interfere with HMGA1-binding to the NOS2 promoter represents a potential mechanism for targeted regulation of NOS2 expression as a therapeutic strategy to improve survival during endotoxemia.

High mobility group (HMG) proteins are non-histone chromosomal proteins that function as 'architectural transcription factors (TFs)' in organizing the assembly of nucleoprotein-DNA transcriptional complexes for the activation and/or repression of transcription [7,8]. While most transcription factors recognize and bind their cognate DNA promoter sites through the major groove of DNA, HMGA1 (formerly HMG-I/Y) is one of the few TFs that preferentially bind AT-rich sequences through the minor groove [9]. A key feature of the HMGA1 TF is the presence of three DNA-binding domains (DBDs), referred to as AT-hooks, which mediate selective interaction with AT-rich DNA sequences in the minor groove [9]. A single AT-hook preferentially binds a stretch of 4–6 base pairs within an AT-rich DNA sequence. The NMR structures of the second and third AT-hooks of HMGA1 in the presence of DNA identified that the central Arg-Gly-Arg core of each AT-hook is responsible for penetrating and anchoring these DBDs into the minor groove while a pair of Lys and Arg residues at either end of this core mediate electrostatic and hydrophobic contacts with the DNA backbone [10]. The second AT-hook of HMGA1 demonstrates high-affinity DNA-binding through a network of residues C-terminal to the core (Gly-Ser-Lys-Asn-Lys) that interact specifically with the sugar-phosphate backbone of adjacent DNA bases in the minor groove [10].

MGB drugs represent a class of small molecules, including the antibiotics netropsin, distamycin A, and berenil, that recognize AT-rich sequences within the minor groove of DNA through a sequence- and conformation-dependent mechanism similar to that proposed for the HMGA1 AT-hooks [10]. We and other groups have used distamycin A *in vitro* to disrupt HMGA1-DNA binding and thus prevent the formation of a competent transcriptional complex [4–6,11,12]. In fact, we demonstrated that distamycin A interfered with AT-rich DNA binding to the minor

groove of NOS2 promoter DNA, decreased NOS2 induction, and resulted in intermediate levels of NOS2 expression during murine endotoxemia *in vivo* that proved beneficial for survival and attenuation of hypotension [13]. However, the molecular mechanism of the effect of distamycin A has not been characterized and we note that distamycin A is not water-soluble and is poorly tolerated by humans in its nascent form, although derivatives of this drug have been used as vehicles to target chemotherapeutic agents to DNA [14]. Netropsin is a MGB that avidly binds the HMGA binding sites within promoter elements and is thus capable of effectively competing with HMGAs for DNA binding [11,12,15]. However, to our knowledge, use of netropsin has not been studied *in vivo* during critical illness, and molecular understanding of HMGA1-DNA binding to the core NOS2 promoter has not previously been reported. We therefore set out to determine: (a) whether the beneficial effects of MGBs *in vivo* during endotoxemia extend beyond that of a single agent within this class of drugs and (b) the mechanism of MGB interference with AT-rich DNA binding within a particular region of the NOS2 promoter.

Herein we present molecular and structural data demonstrating that the water-soluble MGB netropsin proves beneficial during murine endotoxemia and interferes with HMGA1-binding to the AT-rich core NOS2 promoter in a sequence- and conformation-specific fashion. We show that netropsin improves murine survival from endotoxemia in wild type mice but not in mice treated with a NOS2-specific inhibitor. In addition, we show that netropsin attenuates LPS-induced NOS2 promoter activity in murine macrophages, and that the AT-rich HMGA1 binding site in the NOS2 promoter is critical for this effect. Furthermore, we provide direct evidence that netropsin disrupts interactions between the high and low affinity DNA-binding AT-hook motifs of HMGA1 and NOS2 Oct site DNA using EMSAs and solution NMR spectroscopy. Additional thermal denaturation curve analyses and two-dimensional NMR spectroscopy data demonstrate that netropsin binds A/T base pairs in the minor groove of the NOS2 Oct site. These data provide the first details for the molecular basis of netropsin's interaction with the AT-rich Oct site within the NOS2 promoter. Importantly, our studies demonstrate the feasibility of correlating biological findings with detailed structure-function data as a means of developing small molecules with therapeutic potential.

EXPERIMENTAL

Mouse model of endotoxemia

Male C57BL/6 wild-type (WT) mice (Charles River Laboratories) 6–8 weeks of age were treated with phosphate buffered saline (vehicle) or with 20 mg/kg s.c. of the NOS2-specific inhibitor 1400W (Cayman Chemical) twice daily. All mice were injected with 40 mg/kg i.p. lipopolysaccharide (LPS; *Escherichia coli* serotype O26:B6, Sigma) and with either 25 mg/kg i.p. netropsin (Calbiochem) or vehicle 30 min prior to LPS injection.

Transient transfections of RAW 264.7 cells and reporter assays

Murine RAW 264.7 macrophages (ATCC) were cultured as described [5]. The generation of the mouse NOS2 luciferase reporter plasmids “NOS2-WT” (–234/+31) and “NOS2-Mut”, which contains a mutated AT-rich Oct site (basepairs –61 to –54, ATGCAAAA to CGTACCCC) have been described previously [6,16]. These plasmids were transiently transfected into RAW 264.7 cells (FuGENE 6 transfection reagent, Roche Applied Science), as described previously [17]. Twelve hrs following transfection of the reporter construct and a β -galactosidase expression vector (to normalize for luciferase activity), the cells were treated with netropsin (10–25 μ M) or vehicle (water), followed by treatment with LPS (50 ng/ml in PBS) 30 min later. Twenty-four hrs following treatment, cells were harvested and assessed for luciferase activity and β -galactosidase, as described previously [17].

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously [5] using a ^{32}P -labeled double-stranded oligonucleotide probe encoding the AT-rich Oct binding site of the murine NOS2 promoter [bases -65 to -50, AGTTATGCAAAATAGC] or control probe containing the Sp1 consensus binding site [ATTCGATCGGGGCGGGGCGAGC]. Either a 43-amino acid synthetic peptide comprised of the second and third AT-hook DNA-binding regions of the HMGA1 protein, HMGA1(2/3) (Peptide Synthesis Core Facility, Tufts University, Boston, MA) or recombinant Sp1 protein generated as described previously [5] from *in vitro* transcription and translation of a Sp1 expression vector were used. Given the small size of the HMGA1(2/3) peptide, EMSA reactions using this peptide were run on a 6% polyacrylamide gel. Netropsin (0.25 μM) or vehicle (water) was incubated with the radiolabeled probe for 1 hr prior to gel electrophoresis.

Competition binding NMR spectroscopy

^{15}N -labeled glycine (^{15}N , 98%; Cambridge Isotope Laboratories) was selectively incorporated at residues Gly11, Gly20, and Gly37 during the chemical synthesis of the HMGA1(2/3) peptide (Peptide Synthesis Core Facility, Tufts University, Boston, MA). NMR samples contained 0.5 mM ^{15}N -G11,G20,G37-HMGA1(2/3) peptide, 10 mM NaPO_4 , pH 5.7, 90% H_2O :10% D_2O . All NMR spectra were acquired on a Varian INOVA 500 MHz spectrometer at 25°C. The carrier frequency was set to the water resonance, which was suppressed using presaturation during the preacquisition delay. 2D ^1H - ^{15}N -heteronuclear single-quantum correlation (HSQC) spectra were acquired with spectral widths of 8,000 Hz and 2,127 Hz for ^1H and ^{15}N , respectively, with 2,048 real data points in t_2 and 256 transients for each of the 380 increments in t_1 [18]. Complementary 16-bp oligonucleotides containing the NOS2 promoter Oct site (5'-AGTTATGCAAAATAGC-3') were annealed following thermal denaturation at 95°C using a cooling rate of 1°C/min. Annealed NOS2 Oct DNA was titrated into the HMGA1(2/3) sample to a ~2:1 stoichiometric equivalence, and netropsin was titrated into the HMGA1(2/3)/DNA complex from a concentrated stock in phosphate buffer. ^1H - ^{15}N resonance assignments for the DNA-bound HMGA1(2/3) peptide were made from 2D ^1H - ^{15}N HSQC spectra of DNA-complexed HMGA1(2/3) peptide with single site ^{15}N -glycine incorporation at either Gly11 or Gly37.

Thermal DNA melting studies

UV DNA melting curves were measured using a Cary 3E UV/visible spectrophotometer (Varian, Inc., Palo Alto, CA) equipped with a thermoelectric temperature controller. Stock solutions of complementary 10-bp oligonucleotides containing the NOS2 Oct site (5'-GCAAAATAGC-3') or mutated-sequence of the Oct site (5'-GCACCCGAGC-3') were annealed as described. Samples of the duplex DNAs (dsDNAs) at concentrations of 50 μM alone or in the presence of netropsin (1:1 molar ratio) were prepared by directly mixing aliquots from each of the stock solutions (dsDNAs and netropsin), followed by incubation for 1 hr at 25°C to ensure equilibration. Samples were heated to 80°C at a rate of 1°C/min while continuously monitoring the absorbance at 260 nm. Data were analyzed with the program Origin (Microcal, Inc., Northampton, MA).

2D ^1H - ^1H nuclear Overhauser effect spectroscopy (NOESY)

Proton assignments for netropsin, NOS2 Oct duplex DNA, or HMGA1(2/3) peptide were determined from 1D and 2D ^1H NMR data recorded at 25°C on a 500 MHz Varian Unity INOVA spectrometer. 2D NOESY spectra were recorded with a mixing times of 50–250 msec. A spectral width of 10,999.4 Hz was used in both dimensions, and a total of 2,048 real data points were acquired in t_2 and 256 time-proportional phase increments (TPPI) were implemented in t_1 . A total of 160 summed transients were collected with a relaxation delay of 1.5 sec. Spectra were processed with gaussian and sine bell functions for apodization in t_2 and

a shifted sine bell function in t_I using the VNMR software processing (Varian, Inc., Palo Alto, CA). All data were zero filled to 4K by 4K real matrices. In the case of the NOS2 Oct DNA/netropsin complex, netropsin was titrated into the 10-bp NOS2 Oct dsDNA sample from a concentrated stock solution.

Netropsin-DNA complex homology structure generation

Initial coordinates for netropsin and NOS2 Oct DNA (5'-GCAAAATAGC-3') were based on the 2.2 Å resolution X-ray crystal structure of the netropsin-DNA (5'-GCAAATTTGC-3') complex (PDB ID:121D) [19] with divergent bps replaced using InsightII software (Accelrys, Inc., San Diego, CA). DISCOVER was used to carry out energy minimization *in vacuo* using 5000 steepest descent steps keeping the entire complex fixed except the newly altered bases and then again by 5000 steepest descent steps without constraints. Further energy minimization refinement from molecular dynamics simulations in explicit solvent were carried out using YASARA (www.yasara.org) with the Yamber3 force field [20,21] and a three-step protocol: 1) a short steepest descent minimization until the maximum atom speed dropped below 2200 m/s; 2) 500 steps of simulated annealing; and 3) a 500 ps molecular dynamics run carried out at 298 K and 1 atm using simulated annealing. Multiple time steps of 2 fs for intramolecular forces and 1 fs for intermolecular forces were used. The time averaged temperature was controlled using the Berendsen thermostat algorithm [22] and atom velocity rescaling. The initial velocities were obtained from a Maxwellian distribution at the desired temperature and rescaled every ~100 steps whenever the average of the last ~100 measured temperatures converged. The system pressure was controlled by rescaling the periodic boundaries according to fluctuations in the time averaged solvent density. Snap shots were taken every 25 ps and trajectories were analyzed and validated iteratively using YASARA-WHATIF Twinset software.

RESULTS

NOS2 is critical for mediating the protective effect of netropsin during murine endotoxemia

WT mice were administered LPS+netropsin or LPS+vehicle and assessed for survival for 90 hrs following injection (Figure 1A). Dose response experiments were performed to determine the dose of LPS that would result in mortality comparable to that reported in the literature [13, 23–27] and an effective dose of netropsin that was not toxic for the animals (data not shown). WT mice exposed to LPS+vehicle exhibited 100% mortality (0% survival) during the first 60 hrs following injection. WT mice injected with LPS+netropsin exhibited a significantly higher survival rate (36% survival) at 70–90 hrs following injection ($p=0.03$ compared with LPS+vehicle). Mice treated with the NOS2-specific inhibitor 1400W were similarly administered LPS+netropsin or LPS+vehicle and assessed for survival for 90 hrs following injection (Figure 1B). 1400W-treated mice exposed to LPS+vehicle exhibited 100% mortality (0% survival) within 50 hrs. However, in contrast to the WT mice in Figure 1A, the 1400W-treated mice receiving LPS+netropsin did not exhibit significant improvement in survival rates ($p=NS$ at all time points compared with LPS+vehicle).

Netropsin attenuates LPS-induced NOS2 promoter activity via the HMGA1 binding site

RAW 264.7 cells were transiently transfected with the NOS2-WT luciferase promoter-reporter plasmid, then harvested for luciferase activity following treatment with; LPS+vehicle, or LPS +netropsin (Figure 2A, left). LPS-induced NOS2 promoter activity was significantly attenuated following the addition of 10 μ M and 25 μ M netropsin (reduced to $79.7 \pm 0.08\%$ and $44.9 \pm 0.08\%$ of the promoter activity observed with LPS+vehicle for 10 μ M and 25 μ M netropsin doses, respectively; $p<0.05$ for LPS+vehicle compared with both LPS+netropsin treatment conditions). We next examined whether the AT-rich HMGA1 binding site in the NOS2 promoter was required for attenuation of LPS-induced NOS2 promoter activity by netropsin.

RAW 264.7 cells were transiently transfected with the NOS2-Mut promoter-reporter construct [6], then harvested for luciferase activity 24 hrs after treatment with LPS+vehicle or LPS+netropsin (Figure 2A, right). In contrast to the NOS2-WT plasmid, no significant reduction in LPS-induced NOS2 promoter activity was observed following addition of 10 or 25 μ M netropsin (p =NS for LPS+vehicle compared with both LPS+netropsin treatment conditions).

Netropsin interferes with HMGA1 binding to the Oct site of the NOS2 promoter

To directly access the effect of netropsin on HMGA1 binding at the Oct site of the NOS2 promoter, an EMSA was performed using a synthetic HMGA1 peptide, HMGA1(2/3) that possesses two DBD AT-hook motifs, DBD2 and DBD3 (Supplementary Material Figure 7). The HMGA1(2/3) peptide was incubated with a radiolabeled probe spanning the AT-rich Oct binding site (base pairs -65 to -50) of the NOS2 promoter (Supplementary Material Figure 7) as well as vehicle or netropsin (0.25 μ M). The reaction mixtures were then subjected to electrophoresis (Figure 2B). HMGA1-DNA binding was observed at baseline, represented by a nucleoprotein complex of increasing intensity, with addition of 100 to 500 ng of HMGA1(2/3) peptide (Lanes 2–3). We observed a significant reduction in HMGA1-DNA binding following the addition of netropsin (Lane 4). Netropsin does not non-specifically reduce transcription factor binding to DNA as indicated by retained binding of Sp1 to its consensus binding site in the presence of netropsin (Lanes 8 vs. 7). As expected, no DNA complex was formed in the absence of protein (Lanes 1, 5) or when empty vector (V) control *in vitro* translation/transcription reaction product was used (Lane 6).

Netropsin competes away HMGA1-DNA binding

To examine netropsin-specific perturbations of the AT-hook-mediated interaction of HMGA1 with the AT-rich Oct DNA site in the NOS2 promoter, we pursued qualitative NMR studies. We monitored the interaction of each HMGA1 AT-hook (DBD2 and DBD3) with NOS2 Oct DNA using a chemically synthesized HMGA1(2/3) peptide in which 15 N-glycine (15 N-glycine, 98%) was selectively incorporated at residues Gly11, Gly20 (in DBD2) and Gly37 (in DBD3) (Supplementary Material Figure 7). Aliquots of annealed 16-bp NOS2 Oct DNA were added incrementally to 15 N-labeled HMGA1(2/3) peptide in the same buffer. The proton chemical shift frequencies in the amide region of these data were moderately degenerate in the absence of DNA suggesting the DBD AT-hook motifs are not well-folded in solution (data not shown). However, in the presence of the NOS2 Oct DNA, these amide proton resonances become well dispersed and demonstrate narrow resonance linewidths (data not shown). These data suggest induction of a structured backbone conformation in the AT-hooks of HMGA1(2/3) as a result of NOS2 Oct DNA binding.

We monitored chemical shift perturbations in the ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) spectrum of ^{15}N -HMGA1(2/3) during the course of NOS2 Oct DNA titration (Figure 3). As noted in the absence of DNA, the backbone amide proton resonance for the three ^{15}N -glycines (11, 20, and 37) of HMGA1(2/3) are degenerate (Figure 3A). Representative spectra from several titration points (Figure 3B–H) demonstrate differential chemical shift perturbations of the backbone amide proton resonances of the labeled glycines following DNA interaction. Under our experimental conditions, one molecule of HMGA1(2/3) binds two molecules of NOS2 Oct DNA. The DBD2 AT-hook binds in the intermediate exchange regime based on the chemical shift perturbation of the backbone amide proton resonance of Gly11 at ratios of DNA:protein that are less than 1:1. At a molar ratio of 1:0.48 this Gly11 crosspeak is shifted to a frequency representative of the bound conformation and does not shift further following the addition of more DNA. In contrast, the backbone amide proton of Gly37 from DBD3 is in fast exchange and its cross peak in the HSQC spectrum only begins to shift from its position in the unbound (free) state at a protein:DNA ratio greater than ~1:0.72, and is in the bound conformation at a ratio of 1:1.2. No chemical shift perturbation is

observed for the remaining backbone amide proton resonance of Gly20. Gly20 is positioned at the C-terminal end of the DBD2 AT-hook where a flexible linker begins that joins the two DBD motifs.

To examine netropsin interference with HMGA1 AT-hook binding to NOS2 Oct DNA, we added netropsin to the ^{15}N -HMGA1(2/3)/NOS2 Oct DNA complex and observed this sample using NMR spectroscopy. When netropsin was present at a concentration equivalent to the amount of NOS2 Oct DNA it effectively competed away DNA-bound HMGA1(2/3) (Figure 3I). In the presence of netropsin, the backbone amide proton resonances of ^{15}N -labeled Gly11 and Gly37 shift from their unique DNA-bound positions back to degenerate resonance frequencies nearly identical to the unbound conformation of HMGA1(2/3). These data demonstrate the ability of netropsin to competitively inhibit the binding of both the high affinity DBD2 and lower affinity DBD3 AT-hooks of HMGA1 to NOS2 Oct DNA.

Netropsin directly binds the AT-rich Oct site from the NOS2 core promoter

To validate targeting the Oct site within the NOS2 promoter with the MGB netropsin, we sought evidence of a direct interaction between netropsin and a 10-bp core fragment of the Oct site from the NOS2 promoter (5'-GCAAAATAGC-3'). For these data we used UV spectroscopy thermal melting experiments. In the absence of netropsin, the melting temperature (T_m) of unbound NOS2 Oct DNA, *i.e.* the temperature at which 50% of the duplex strands denature, was measured to be 47.7°C (Figure 4). In the presence of an equal molar amount of netropsin, the T_m of the NOS2 Oct DNA increased to 60.7°C. This shift to a higher T_m in the presence of netropsin supports that netropsin directly complexes with NOS2 Oct DNA and forms a small-molecule/DNA complex that renders the DNA more resistant to the thermal denaturation. In contrast, netropsin did not significantly affect the T_m of mutated Oct-site DNA (5'-GCACCCGAGC-3'). This finding demonstrates that netropsin's interference with HMGA1 binding to the NOS2 promoter is through direct and specific interaction at the core sequence of the Oct site, argues against non-specific effects on HMGA1 protein, and validates targeting the Oct site within the NOS2 promoter with the MGB netropsin.

Molecular interactions between netropsin and NOS2 Oct DNA

To elucidate molecular interaction between netropsin and NOS2 Oct DNA we applied NMR methods. 1D proton (^1H) NMR spectra of netropsin were collected in the presence and absence of 10-bp NOS2 Oct DNA (5'-GCAAAATAGC-3') and used for netropsin resonance assignments along with previously reported NMR data [28,29]. We monitored chemical shift perturbations of the non-exchangeable netropsin resonances as a result of DNA titration in 2D nuclear Overhauser effect spectroscopy (NOESY) data. In the presence of NOS2 Oct DNA, the CH5 and CH11 ^1H resonances of netropsin (refer to Supplementary Material Figure 8) were conformationally perturbed as noted by their enhanced spectral resolution and downfield chemical shift perturbation (data not shown). We also monitored the chemical shifts for the imino proton resonances of Watson-Crick A-T and G-C base pairs in NOS2 Oct DNA as a function of netropsin. We observed significant downfield chemical shift (δ) perturbation and line broadening of several A-T base paired imino protons ($\delta > 13.5$ ppm [30]), and significantly less chemical shift perturbation and/or no peak broadening of G-C base paired imino protons ($\delta < 13.5$ ppm [30]) (Supplementary Material Figure 9). Thus, the association of netropsin with the NOS2 Oct site changes the environment of proton groups on the concave face of netropsin and perturbs the environment of specifically the imino protons in A-T base pairs.

In addition, we examined proton-proton nuclear Overhauser effect crosspeak correlations (NOEs) between netropsin and NOS2 Oct DNA in NOESY spectra. Peak assignments of non-exchangeable DNA base and sugar H1' protons were made based on sequential H8 or H6 to H1' NOE connectivities and sequential adenine H2 to H2 NOE connectivities, as well as NOEs

from CH3 thymine protons to H2 adenine protons. Assignments were supplemented with assessments of H8 or H6 to H2' and H2'' NOE connectivities, as well as observance of upfield CH3 resonances from thymines. Several NOE crosspeaks between DNA base protons and netropsin protons were identified in the NOESY data (Figure 5). For example, the H2 protons of adenine bases A4 and A5 (numbered 5'-G₁C₂A₃A₄A₅A₆T₇A₈G₉C₁₀-3') exhibit NOEs to the netropsin concave face pyrrole CH11 proton (peaks 1 and 2, respectively) and the H2 protons of adenine bases A6 and A5 exhibit strong NOEs to the netropsin concave face pyrrole CH5 proton (peaks 3 and 4, respectively). Further inspection of the netropsin CH11 pyrrole proton frequencies reveals NOEs to the H1' protons of thymine bases T17 and T16 (complementary strand numbered 5'-G₁₁C₁₂T₁₃A₁₄T₁₅T₁₆T₁₇T₁₈G₁₉C₂₀-3'), respectively (peaks 5 and 6). Similarly, we observed additional crosspeaks reflecting intramolecular interaction between the H2 proton of A6 and the non-equivalent CH₂ methylene protons of position 2 in netropsin (data not shown). We did not observe NOE interactions between convex pyrrole CH7 or CH13 protons of netropsin and H2 protons of adenine bases or H1' protons of thymine bases. These NOE data support that the netropsin concave face pyrrole protons CH5 and CH11 and A/T base protons within the NOS2 Oct DNA are positioned ~3–5 Å from one another within the netropsin/DNA complex indicating netropsin binding in the NOS2 Oct minor-groove AT-track and its binding mode. These NOEs establish points of intermolecular contact between netropsin and NOS2 Oct DNA and suggest that the concave face pyrrole CH5 proton of netropsin lies close to the H2 proton of bases A5 and A6, while the pyrrole CH11 proton of netropsin is juxtaposed with the H2 proton of base A5 and H1' protons of bases T16 and T17.

Molecular modeling of the netropsin/NOS2 Oct DNA complex

Computational examination of netropsin binding to the minor groove of NOS2 Oct site was carried out using molecular modeling and dynamics simulation (refer to Experimental Methods). Assessments of the lowest energy complex structures indicate that hydrogen-bonds, Van der Waals interactions, and electrostatic forces play important roles in stabilizing this netropsin/DNA complex. Consistent with experimentally determined crystal structures of netropsin/AT-rich DNA complexes [19,31,32], netropsin is positioned squarely in the 6 base pair 5'-AAAATA-3' region of the minor groove NOS2 Oct site, interacting with 6 bases on one strand and 4 bases on the other (Figure 6). The NH amide groups of netropsin form bifurcated hydrogen-bonds to adenine base N3 atoms and thymine base O2 atoms on opposite DNA strands in the AAAT core (Supplementary Material Figure 10). Several stabilizing Van der Waals interactions between O4' atoms of the DNA sugars and netropsin atoms are observed especially at the guanidinium and amidinium ends of netropsin (Supplementary Material Figure 10). The narrow width of the minor groove increases the extent of Van der Waals interactions between netropsin and atoms along the floor and walls of the DNA minor groove. Electrostatic interactions between the negatively charged groups of the AT-stretch minor groove and the positively charged end groups of netropsin may also play a role in complex formation and the sequence selectivity of netropsin. This netropsin/NOS2 Oct complex structure is consistent with our biochemical and NMR data, as well as the wealth of structural data available for netropsin interaction with AT-tracks, and provides a powerful tool to understand the molecular basis for netropsin interaction at this AT-rich minor groove site as well as netropsin's ability to effectively compete for DNA binding with the AT-hooks of the HMGA1 transcription factor. These are the first structural details describing the mechanism of interference in HMGA1 activity within the NOS2 promoter at the Oct site using the MGB netropsin.

DISCUSSION

In this study, we demonstrate that: (a) the water-soluble MGB drug netropsin improves survival from murine endotoxemia; (b) NOS2 expression plays an important role in this protective

effect; and (c) netropsin modulates NOS2 expression by disrupting HMGA1-DNA binding to the AT-rich core NOS2 promoter in a sequence-specific fashion. These findings highlight two important new concepts. First, these results, in combination with our previous *in vivo* findings using the MGB distamycin A, suggest that MGBs as a class of drugs hold promise for targeted therapeutics in controlling gene expression during critical illness. Second, our data represents a novel correlative study, in which NMR was used to explore the molecular basis of an observed effect in a murine model of critical illness. Thus, this work provides an important proof of concept in supporting the synergy of *in vivo* animal model data with biophysical studies for structure-based design of molecules that alter transcriptional regulation of key gene targets.

While MGB drugs and their derivatives have been explored extensively as chemotherapeutic agents [33,34], their toxicity profile and concern for lack of binding specificity has limited broader application of the agents to other disease processes. We previously demonstrated that another MGB, distamycin A, similarly improved murine survival from endotoxemia through attenuating NOS2 induction [13]. Although distamycin A has long been used by our group and others as a tool to interfere with HMGA1-DNA binding *in vitro*, we found that distamycin A also disrupted the binding of another transcription factor, IRF1, to an AT-rich upstream site in the NOS2 promoter. Moreover, distamycin A has been used as a tool to study the regulation of other genes by HMGA1 as well, suggesting that MGB drugs can have effects on additional genes, although it is also interesting to speculate that families of genes with similar regulatory regions might be targeted for therapeutic benefit [35]. Previous work using MGBs suggests that AT-rich DNA sequence alone does not predict binding and, rather, that DNA conformation plays a key role in predicting protein-DNA binding [36]. Thus, the sequence- and conformational-dependent effects of distamycin A at more than one TF binding site within more than one gene highlight the importance of reliable *in vivo* and *in vitro* readouts of MGB activity and mechanism of action, such as those described here. The current study demonstrates use of NMR to gain an increased molecular understanding of interactions between a MGB agent and an AT-rich DNA sequence that has biologic relevance to human critical illness. This information provides an important framework for extension of our work to the development of novel small molecule therapeutic agents that can alter gene transcription during disease.

We chose to use the MGB netropsin in our current study based upon on the *in vivo* biological activity of this compound and its notable affinity for HMGA1-DNA binding sites relative to the other MGB drugs [12]. Moreover, in contrast to distamycin A, netropsin is water-soluble, which removes the need for formulation studies and/or the addition of excipients prior to administration. Examination of the structures of netropsin and distamycin A reveals that these small-molecules structurally mimic the conformation of the highly conserved Pro-Arg-Gly-Arg-Pro core of the HMGA1 DBD AT-hooks (Supplementary Material Figure 8). In addition, all of these compounds reversibly bind to the minor groove of AT-rich sequences with high selectivity. Distamycin A is the tris-*N*-methylpyrrole analogue of netropsin and is distinguished from netropsin by the presence of a single cationic guanidinium group, and a more extended DNA-binding site, as observed in a co-crystal structure with the sequence d(CGCAAATTTGCG)₂ [37]. Thus, the high affinity of netropsin for minor groove DNA sites in the NOS2 promoter reflects a greater similarity between the molecular structures of netropsin and the AT-hook motifs of HMGA1 [10,12], as both contain two five membered rings that are important for presentation of a narrow planar structure and two guanidinium groups important in hydrogen bonding to bases and Van der Waals interactions with the narrow minor groove wall (Supplementary Material, Figure 11). Studies on the DNA binding behavior of MGBs, such as netropsin, have afforded unique insight into the mechanisms whereby these small-molecules recognize DNA and thus target the DNA, which may have useful biological and possibly therapeutic effects. One impetus for studies of this sort is to provide feasibility data allowing one to determine if there might be alternative/novel motifs that are capable of

recognizing and thus disrupting TF-DNA interactions in the context of therapeutic development.

Recent years have witnessed an exciting explosion of *in vitro* minor groove small molecule design, primarily with the goal of chemotherapeutic development. For example, a biosensor-surface plasmon resonance (SPR) method was used to monitor HMGA2 binding to immobilized DNA sequences selected by SELEX (systematic evolution of ligands by exponential enrichment) [15]. While HMGA2 is a member of the HMGA family of architectural transcription factors with properties similar to HMGA1, HMGA2 is a unique gene from HMGA1 and has distinct gene regulatory functions [7,8]. Biosensor-SPR demonstrated that netropsin interfered with HMGA2 binding to an AT-rich DNA sequence *in vitro*, although no effect on a specific target gene or organism *in vivo* was demonstrated. Based on the known binding characteristics of HMGA proteins and an analysis of the promoter structures of genes that are known to be positively regulated by these proteins *in vivo*, others have proposed that HMGA protein AT-hooks read specific DNA 'bar codes' [38]. If so, it should be possible to create MGB compounds that are also capable of recognizing this 'bar code' and thus competitively inhibit HMGA binding to genes of interest. A number of recent studies have addressed targeting minor groove DNA through the use of molecular docking approaches [13,39–42]. In addition, a very recent study has documented important proof-of-concept data describing how one might explore ligand-DNA complexes using computer aided drug discovery approaches including pharmacophore modeling [43]. Pharmacophore modeling methods are able to reproduce the experimentally determined preferred binding sites of minor groove ligands. Furthermore, significant advances have been made in the design and synthesis of small, minor groove binding polyamide molecules, called lexitropsins, that bind with high affinity to predetermined DNA sequences in the human genome [31,34,44,45]. These compounds are based on an understanding of the structural basis of polyamide/minor groove DNA interactions provided by the Dervan laboratory, which resulted in the first rationally designed polypyrrole-polyamide compounds that bound specifically to AT-sequences with affinities and specificities comparable to native DNA-binding proteins [14,36,46–49]. Triple helix forming oligonucleotides (tplexes), synthetic zinc fingers, and hairpin polyamides are other sequence selective representatives of molecules directly targeting DNA [50].

In summary, these results are the first to demonstrate the *in vivo* effect of a MGB agent in a murine model of critical illness that correlates biologic function with sequence-specific inhibition of protein-DNA binding. Moreover, we provide new information regarding the interaction of netropsin with a biologically relevant AT-rich target within the NOS2 promoter. The escalating abundance of genome sequence information is fueling efforts aimed at the design of compounds capable of modulating the expression of selected genes that have predictable biologic effects. Growing information about regulatory regions of genes suggests such "designer" drug design is becoming increasingly feasible, especially in critical illness during which dysregulated gene expression plays a key pathophysiologic role. Moreover, new treatment approaches are desperately needed in the area of critical care medicine, as decades of research have failed to produce any effective targeted treatments for sepsis [1]. The combination of *in vivo* models of human disease and *in vitro* assessment of functional DNA-protein binding to target genes will be critical for guiding the rational development of therapeutics in complex disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Abbreviations used:

MGB	minor groove binder
LPS	lipopolysaccharide
IP	intraperitoneally
VSM	vascular smooth muscle
NO	nitric oxide
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
AT	adenine thymine
HMGA	high mobility group A
DBD	DNA-binding domain
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
HSQC	heteronuclear single quantum correlation spectroscopy
WT	wildtype
EMSA	electromobility shift assay

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Figure 1. Netropsin improves survival from endotoxemia in WT mice, but not in WT mice treated with a NOS2-specific inhibitor, 1400W

(A) WT mice were injected with LPS 40 mg/kg + netropsin 25 mg/kg IP (■ WT LPS+netropsin, n=11) or LPS 40 mg/kg + vehicle IP (□ WT LPS, n=10). Mice were evaluated for survival every 10 hrs following injection (*p<0.05 compared with WT LPS). (B) 1400W-treated WT mice were subjected to the same experimental protocol as outlined for the WT animals (n=6 in each treatment group, p=NS between groups of 1400W-treated WT animals at each time point).

Figure 2. Netropsin attenuates induction of NOS2 promoter activity through the AT-rich Oct binding site in LPS-treated mouse macrophages

Netropsin interferes with HMG1(2/3)-binding to the NOS2 promoter. (A) RAW 264.7 mouse macrophages were transfected with 0.5 μg of the NOS2 luciferase WT promoter-reporter construct (NOS2-WT, left panel) or 0.5 μg of the NOS2-Oct mutant promoter-reporter construct (NOS2-Mut, right panel), then treated with LPS+vehicle, or LPS+netropsin. Cells were harvested for luciferase activity, which was normalized for β-galactosidase, then compared with LPS-vehicle cells (normalized to 100%) to determine % reduction with netropsin treatment (* $p < 0.05$ compared with LPS+vehicle-treated cells). This experiment was repeated a minimum of three separate times, with each condition performed in duplicate during each experiment. (B) EMSA was performed using HMG1(2/3) peptide and a radiolabeled probe spanning the NOS2 promoter Oct site; or recombinant Sp1 protein generated by in vitro transcription/translation (TNT) or empty vector (V) subjected to TNT reaction and a radiolabeled probe containing the consensus Sp1 binding site. 100–500 ng of HMG1(2/3) was incubated with radiolabeled probe and either vehicle (Lanes 2–3) or netropsin (0.25 μM) (Lane 4). Equivalent amounts of empty vector (V) or Sp1 protein were incubated with radiolabeled probe and either vehicle (Lanes 6–7) or netropsin (0.25 μM) (Lane 8). Reaction mixtures were subjected to electrophoresis. As a control for each probe, reaction mixtures were incubated without the addition of protein (Lanes 1, 5). This experiment was repeated three

separate times. (*nucleoprotein complex represents HMGA1-DNA binding; **nucleoprotein complex represents Sp1-DNA binding)

Figure 3. Chemical shift mapping of selectively ^{15}N -labeled glycines in HMGA1(2/3) upon NOS2 DNA binding and addition of netropsin

^1H - ^{15}N HSQC spectra illustrating the chemical shift perturbations of the amide backbone resonances of Gly11 and Gly37 in ^{15}N -G11,G20,G37-HMGA1(2/3) upon the addition of increasing amounts of a 16-bp DNA sequence containing the Oct site from the NOS2 promoter (5'-AGTTATGCAAATAGC-3'). Molar equivalents of NOS2 Oct DNA added are as follows: A, 0; B, 0.04; C, 0.24; D, 0.32; E, 0.4; F, 0.48; G, 0.72; H, 1.2. I, addition of netropsin to the HMGA1(2/3)/NOS2 Oct DNA complex (peptide:DNA:netropsin molar ratio 1:1.2:1.2).

Figure 4. Thermal UV melting curves

Thermal denaturation curves of the 10-bp NOS2 Oct DNA duplex (5'-GCAAAATAGC-3') (●) and the complex with netropsin (■), and the 10-bp Oct-mutant DNA duplex (5'-GCACCCGAGC-3') alone (○) and with netropsin (□). All solutions were in 10 mM Tris, pH 7, 50 mM KCl, and 0.5 mM EDTA. The absorbance values have been normalized to allow for a visual comparison of the curves.

Figure 5. Two dimensional nuclear Overhauser spectroscopy data

A comparison of regions from the NOESY spectrum of NOS2 Oct DNA alone (A) to the 1:1 NOS2 Oct DNA:netropsin complex at 25°C (B). Sweep width in both dimensions is 10999.2 Hz, pulse width 6.2 μ sec, recycle delay 1.5 sec, number of complex points in f2 2048, number of increments in f1 256, number of transients 128. Observed intermolecular NOE crosspeaks are: 1) NtCH11-A4 H2; 2) NtCH11-A5 H2; 3) NtCH5-A6 H2; 4) NtCH5-A5 H2; 5) NtCH11-T17 H1'; 6) NtCH11-T16 H1'; denoting netropsin (Nt) proton-DNA base proton correlations. Numbering based on the 5'-G₁C₂A₃A₄A₅A₆T₇A₈G₉C₁₀-3' sequence and its complementary strand 5'-G₁₁C₁₂T₁₃A₁₄T₁₅T₁₆T₁₇T₁₈G₁₉C₂₀-3'.

Figure 6. Model of the NOS2 Oct DNA-netropsin complex

Space filling model of the lowest-energy structure resulting from molecular dynamics simulations of netropsin with NOS2 Oct DNA (5'-GCAAAATAGC-3') emphasizing the relative orientation of netropsin within the DNA minor groove. Left, showing both DNA strands in space filling model; and right, showing one of the two strands in stick rendering.