articles

molecular pharmaceutics

Intravesical Antisense Therapy for Cystitis Using TAT-Peptide Nucleic Acid Conjugates

Pradeep Tyagi,[†] Rajkumar Banerjee,[‡] Soumitra Basu,[§] Naoki Yoshimura,[†] Michael Chancellor,[†] and Leaf Huang*,[||]

Department of Urology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, Division of Lipid Science & Technology, Indian Institute of Chemical Technology, Hyderabad 50000, India, and Chemistry Department, Kent State University, Kent, Ohio 44242

Received October 25, 2005

Abstract: The present study investigated the potential of intravesical instillation for localized reduction of NGF (nerve growth factor) expression in the urinary bladder. Overexpression of NGF has been linked to the pathogenesis of interstitial cystitis (IC). A minimum free energy algorithm was used to predict suitable regions in mRNA of rat βNGF, which can be targeted for an antisense approach. The candidate antisense oligos were evaluated for their ability to reduce NGF expression in vitro by cotransfecting HEK293 cells with NGF cDNA. A single oligonucleotide ODN sequence was chosen for testing in an acute cystitis model in rat induced by cyclophosphamide. Overexpression of NGF is known to mediate inflammation of bladder in this model. For improved stability, antisense ODN was replaced with antisense peptide nucleic acid (PNA) and its penetration into bladder was facilitated by tethering TAT peptide sequence. Rat bladders were instilled with either antisense or its scrambled control prior to cystitis induction. Cystometrograms performed on rats under urethane anaesthesia exhibited bladder contraction frequency that was significantly decreased in the antisense treated rats than rats treated with the control. NGF immunoreactivity was also decreased in the urothelium of the antisense treated bladders. Our findings demonstrate the feasibility of using TAT-PNA conjugates for intravesical antisense therapy.

Keywords: Intravesical administration; antisense; peptide nucleic acid; TAT peptide; cystitis; nerve growth factor

Introduction

A growing body of evidence in the published literature implicates overexpression of nerve growth factor (NGF), a neurotrophic factor, which is responsible for mediating persistent pain states such as interstitial cystitis (IC), also characterized by increased urinary frequency and detrusor overactivity.[1] NGF belongs to a growth factor family also known as neurotrophins, originally identified by its ability to promote the survival of sensory and sympathetic neurons during development.[2] A 13.6 kDa natural protein, NGF is composed of two α and two β subunits, and the biologically active β subunit is proteolytically cleaved from a precurosor

* Corresponding author: Leaf Huang, Ph.D., Fred N. Eshelman Distinguished Professor and Chair, Division of Drug Delivery and Disposition, UNC School of Pharmacy, 2316 Kerr Hall CB# 7360, Chapel Hill, NC 27599-7360. Tel: (919) 843-0736. Fax: (919) 966-0197. E-mail: leafh+@email.unc.edu.

[†] University of Pittsburgh.

[‡] Indian Institute of Chemical Technology.

[§] Kent State University.

[||] University of North Carolina.

(1) Cruz, F. *Urology* **2004**, *63*, 65−73.

(2) Whittemore, S. R.; Ebendal, T.; Larkfors, L.; Olson, L.; Seiger, A.; Stromberg, I.; Persson, H. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 817−821.

10.1021/mp050093x CCC: $33.50 **© 2006 American Chemical Society**
Published on Web 03/30/2006

called prepro NGF, which is translated from the 3′ exon of NGF mRNA.[1]

Adenoviral mediated transfection of NGF gene in rat bladder was able to transiently increase NGF expression in rat bladder, which was responsible for sensitizing afferent pathways and bladder overactivity in the absence of any inflammation.[3] Microarray analysis done on rodent models of IC also implicated overexpression of βNGF gene as a primary cause of the disorder.[4] Moreover, increased NGF followed the increase in the level of NGF mRNA in response to chemically induced bladder inflammation in rats.[5] NGF can either activate mast cells via its trkA receptor or cause their degranulation to produce hyperalgesia through sensitization of nociceptive sensory neurons as well as increase their proliferation.[5,6] Elevated levels of NGF protein were also found in the bladder of IC patients.[7]

Most groups have demonstrated bladder urothelium to be the primary source of NGF in neurogenic inflammation of murine bladder and in patients with idiopathic sensory urgency.[7,8] In a study done on patients with idiopathic detrusor instability, NGF was determined to be produced by bladder smooth muscle.[9] Neutralization of NGF by antibody has been demonstrated to be effective in blocking bladder afferent sensitization in spinal cord injured rats.[10] Recombinant antibody against NGF is effective in reducing the severity of IC in patients. Although short-term therapeutic use of monoclonal antibody in humans appears to be relatively safe, there are anecdotal accounts of antibodies used in disorders unrelated to IC causing severe side effects in a few patients.[11] Moreover, development of anti-antibody responses cannot be ruled out, which can diminish efficacy on chronic use for even "humanized" antibodies.[12]

In the present study, we explore an alternative approach for downregulation of NGF expression by using an antisense approach. Antisense based therapy has begun to gain acceptance from clinicians, and this technology has emerged as a major tool in deciphering function of new genes in the post human genome project era. Peptide nucleic acids (PNAs) have been used for their antisense effect in various studies, because they form stable duplexes with the target mRNA and arrest translation.[13,14] PNA was chosen in this study owing to its superior binding properties, and higher stability in biological media over a wide pH range, compared to traditional oligos and ribozymes.[15,16] PNAs offer an exciting option for silencing gene expression in mammalian cells, if strategies can be developed to improve their poor intracellular delivery into mammalian cells.[17] Nonselective cellular uptake of macromolecules including oligonucleotides has been reported to be facilitated by a protein transduction domain (PTD) of the TAT protein of human immunodeficiency virus.[17] Moreover, an 11-mer (YGRKKRRQRRR) cell penetrating peptide derived from its PTD can even penetrate the blood−brain barrier and most other tissues.[18] The PNA molecule cannot by itself cross the cell membrane, and in a recent study on cultured cells, a short length TAT peptide was used to enhance the penetration.[19] In the present study we use the same peptide to deliver PNA across the rat urothelium, after intravesical instillation, for blocking NGF overexpression.

Material and Methods

Design of Antisense Sequence. We utilized a minimum free energy algorithm available on the Internet to predict RNA secondary structure (using *m*fold server with *m*fold version 3.1[20] of the 3′ exon of rat βNGF mRNA;[21] the predicted secondary structure estimation of local folding and possible unpaired regions such as loops and bulges is shown in Figure 1a,b). The following sequences were selected for in vitro evaluation. Sequence K-TAACGATAGACACATGCC was complementary to the region close to the adjoining 5′ intron from nt 347−364. Similar was the case for sequence Q-GCCCGAGACGCCTCCCGA from 427 to 444, sequence B-TACACCTTCTGACCCAC from 661 to 678, sequence T-TGGAGGTCCGTCGTCGGA from 939 to 956, and sequence A-AGTACGTCAGGAATATTA from 1097 to

(3) Lamb, K.; Gebhart, G. F.; Bielefeldt, K. *J. Pain* **2004**, *5*, 150−156.
(4) Saban, M. R.; Nguyen, N. B.; Hammond, T. G.; Saban, R. *Am. J. Pathol.* **2002**, *160*, 2095−2110.
(5) Dmitrieva, N.; Shelton, D.; Rice, A. S.; McMahon, S. B. *Neuroscience* **1997**, *78*, 449−459.
(6) Dines, K. C.; Powell, H. C. *J. Neuropathol. Exp. Neurol.* **1997**, *56*, 627−640.
(7) Lowe, E. M.; Anand, P.; Terenghi, G.; Williams-Chestnut, R. E.; Sinicropi, D. V.; Osborne, J. L. *Br. J. Urol.* **1997**, *79*, 572−577.
(8) Bjorling, D. E.; Wang, Z. Y. *Urology* **2001**, *57*, 40−46.
(9) Tanner, R.; Chambers, P.; Khadra, M. H.; Gillespie, J. I. *BJU Int.* **2000**, *85*, 1115−1119.
(10) Seki, S.; Sasaki, K.; Igawa, Y.; Nishizawa, O.; Chancellor, M. B.; De Groat, W. C.; Yoshimura, N. *J. Urol.* **2004**, *171*, 478−482.
(11) de Bono, J. S.; Rha, S. Y.; Stephenson, J.; Schultes, B. C.; Monroe, P.; Eckhardt, G. S.; Hammond, L. A.; Whiteside, T. L.; Nicodemus, C. F.; Cermak, J. M.; Rowinsky, E. K.; Tolcher, A. W. *Ann. Oncol.* **2004**, *15*, 1825−1833.
(12) Sharkey, R. M.; Brenner, A.; Burton, J.; Hajjar, G.; Toder, S. P.; Alavi, A.; Matthies, A.; Tsai, D. E.; Schuster, S. J.; Stadtmauer, E. A.; Czuczman, M. S.; Lamonica, D.; Kraeber-Bodere, F.; Mahe, B.; Chatal, J. F.; Rogatko, A.; Mardirrosian, G.; Goldenberg, D. M. *J. Nucl. Med.* **2003**, *44*, 2000−2018.
(13) Marin, V. L.; Roy, S.; Armitage, B. A. *Expert. Opin. Biol. Ther.* **2004**, *4*, 337−348.
(14) Nielsen, P. E. *Methods Mol. Biol.* **2005**, *288*, 343−358.
(15) Pooga, M.; Land, T.; Bartfai, T.; Langel, U. *Biomol. Eng.* **2001**, *17*, 183−192.
(16) Ray, A.; Norden, B. *FASEB J.* **2000**, *14*, 1041−1060.
(17) de Koning, M. C.; van der Marel, G. A.; Overhand, M. *Curr. Opin. Chem. Biol.* **2003**, *7*, 734−740.
(18) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. *Science* **1999**, *285*, 1569−1572.
(19) Koppelhus, U.; Awasthi, S. K.; Zachar, V.; Holst, H. U.; Ebbesen, P.; Nielsen, P. E. *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 51−63.
(20) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406−3415.
(21) Whittemore, S. R.; Friedman, P. L.; Larhammar, D.; Persson, H.; Gonzalez-Carvajal, M.; Holets, V. R. *J. Neurosci. Res.* **1988**, *20*, 403−410.

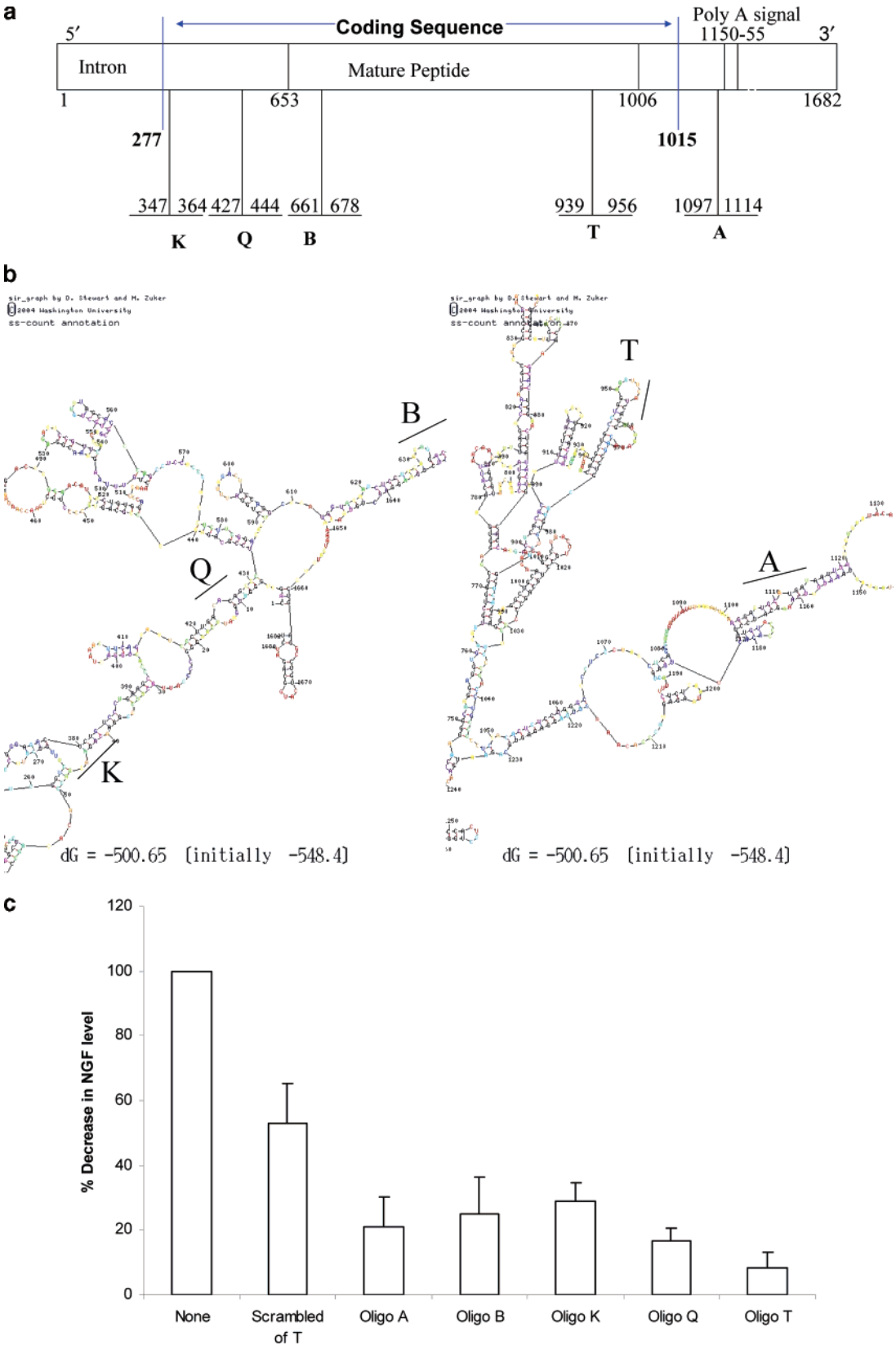

Figure 1. (a) Schematic diagram of 3′ exon of rat NGF gene and the regions selected for designing antisense oligos. (b) Folded structure of NGF mRNA as predicted by minimum free energy algorithm and local folding of selected regions. (c) The decrease in NGF expression measured by ELISA in the cell media of HEK293 cotransfected with NGF cDNA and antisense phosphorothioate oligos.

1114 in the 3′ UTR. A randomly scrambled control of identical length was also used in all the experiments.

Identification of Active Antisense Oligonucleotide. The activity in the panel of candidate oligos having theoretical binding affinity to discrete sites in the 3′ exon of NGF mRNA was evaluated in Griptite HEK293 cells (Invitrogen) transfected with cDNA coding for rat βNGF. The rat βNGF cDNA was a gift from Dr. William Goins, University of Pittsburgh. Griptite HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 5% nonessential amino acids. Cells were plated overnight in 24 well plates. Phosphorothioate (PS) oligonucleotides with different sequences were procured from Integrated DNA Technology Inc. Cationic liposomes composed of DOTAP (1,2-dioleoyl-3-trimethyl-ammonium-propane) were used as transfection reagent for cotransfecting antisense oligos (500 nM) and cDNA coding for rat βNGF (1 μg) for a transient production of NGF. Forty eight hours after transfection, NGF secreted into the cell medium was measured by ELISA. The isolated culture medium samples were stored at −20 °C until assay. The samples were assayed in triplicate in an antigen capture ELISA Emax Immuno-Assay System (Promega, Madison, WI) according to the manufacturer's instructions. ELISA plates were read at 450 nm on an Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT).

Synthesis of TAT-PNA. The protein transduction domain (PTD) from the HIV-1 TAT protein, residue 47−57, GGGGYGRKKRRQRRR-COOH, known as TAT peptide, was synthesized at the University of Pittsburgh Peptide Synthesis Facility. The 11-mer peptide flanked by four glycine units had its 9-fluorenylmethyloxycarbonyl (Fmoc) protection intact on its N-terminal and was not cleaved from the synthetic resin. The TAT peptide bound to the resin was extended by Fmoc solid-phase synthesis of PNA by an Expedite 8909 instrument (Applied Biosystems, Farmington, MA). The first round of coupling linked the C-terminal residue of the PNA to the N-terminal of TAT peptide. The synthesized TAT-PNA conjugates were cleaved from the resin after synthesis, and PNA conjugates showed as a single peak in reverse phase HPLC after purification as expected. Synthesis of full length TAT-PNA conjugate was confirmed by analyzing the HPLC purified compound by MALDI-TOF. The obtained mass numbers were nearly equal to the theoretical mass numbers.

Rat Bladder Uptake of TAT-PNA Postinstillation. To label the PNA, TAT-PNA was chemically coupled to fluorescent probe rhodamine isothiocyanate (TRITC, Molecular Probes) before the TAT-PNA conjugate was cleaved from the resin. To determine the bladder uptake of TAT-PNA conjugates, Sprague−Dawley rats (150−200 g) were anesthetized by halothane inhalation and instilled with 30 μM of either labeled PNA or TAT-PNA-rhodamine conjugate in a volume of 0.2 mL through PE-50 plastic tubing (Clay-Adams, Parsippany, NJ) inserted into the rat urethra. The bladder outlet was tied with a suture thread for 1 h and 3 h after instillation, the bladders were drained with 0.9% saline, and animals were allowed to recover from the anesthesia. Twenty-four hours later, animals were sacrificed and bladders were isolated and cryopreserved. Six micrometer thick bladder sections were prepared by cryostat for confocal microscopy (Olympus, Fluoview). Sections were counterstained with sytox green dye.

Efficacy Evaluation of TAT-PNA Conjugates. Rats were injected with cyclophosphamide (CYP) (100 mg/kg ip) to induce acute cystits. Under halothane anesthesia, either saline or TAT-PNA conjugates with selected antisense sequence including its scrambled control were instilled into the bladder at the concentration of 100 μM, 30 min prior to injection of cyclophosphamide. The volume for intravesical instillation was 0.5 mL in each animal. Two hours after cyclophosphamide injection, rats were injected with urethane (1.2 g/kg sc) to perform open transurethral cystometry. PE-50 tubing (Clay-Adams, Parsippany, NJ) was inserted into the bladder through the urethra. The catheter system was filled with 0.9% w/v saline, and cystometrogram (CMG) was performed by filling the bladder with a constant infusion (0.04 mL/min) of saline by a syringe pump. The gradual rise in the internal pressure of bladder (intravesical pressure) caused by this slow filling was monitored by a pressure transducer connected to a sidearm of the filling catheter. The frequency of reflex bladder contractions per minute was recorded. Measurements in each animal represented the average of 3−5 bladder contractions. All animal experimental protocol was approved by the institutional animal care and use committee (IACUC) of University of Pittsburgh.

Histopathological Analysis. After cystometry, whole bladders were harvested from the animals, fixed in 10% buffered formalin, and cryopreserved. Tissue blocks were sectioned (20 μm thickness) for hematoxylin and eosin (H&E) staining and immunohistochemistry for NGF. The polyclonal antibody against NGF was raised in goat (Sigma Biochemicals), and bound antibody in the tissue sections was detected with anti-rabbit antibody labeled with Cy3 (Jackson Immunomedics).

Statistical Analysis. Quantitative data are expressed throughout this paper as means ± standard error. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA, followed by post hoc comparisons with Newman Keuls test, according to Graph Pad prism v. 3.0 (GraphPad Software, San Diego, CA). Differences among groups were considered significant if $p < 0.05$.

Results

Selecting the Antisense Sequence. The minimum free energy algorithm helped to predict the secondary structure of rat βNGF mRNA (using *m*fold server with *m*fold version 3.1).[22] The sequence elements were then chosen and evaluated by in vitro screening using a transient transfection assay (Figure 1a). We checked for dose dependent decrease in NGF expression with oligos, and concentration of 500 nM was

(22) Mathews, D. H.; Sabina, J.; Zuker, M.; Turner, D. H. *J. Mol. Biol.* **1999**, *288*, 911−940.

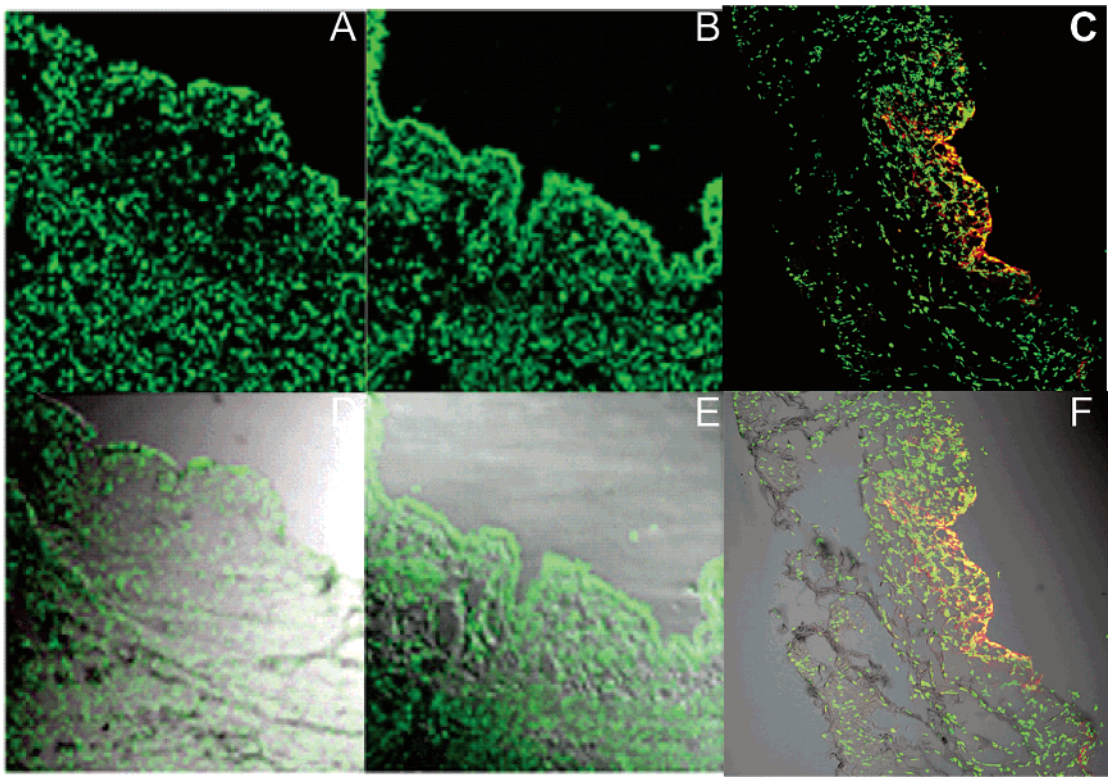

Figure 2. Photomicrogaphs of rat bladder taken with confocal microscopy after instillation of rhodamine labeled PNA alone or covalently tethered with TAT peptide. Cryopreserved rat bladder sectioned into 6 μm thick sections were counterstained with sytox green dye to stain nuclei. Panels A and B show absence of any red fluorescence and represent untreated rat bladder and bladder instilled with free PNA conjugated with rhodamine, respectively. Red fluorescence of rhodamine is seen overlapping with green fluorescence in panel C, which represents bladder instilled with rhodamine labeled PNA conjugated with the TAT peptide. Panels D, E, and F are overlay images of panels A, B, and C with their respective bright field images. Magnification is 20$\times$ in all sections.

found most effective. The oligos with sequence T, Q, and A were able to decrease the NGF expression in transfected cells (Figure 1b), and sequence T was identified to be the most effective in blocking the NGF expression with minimal effect on cell viability measured by MTT assay (data not shown). The decrease in NGF protein was sequence specific as scrambled control did not show activity.

Bladder Uptake. The confocal images of 6 μm bladder sections taken from rat were able to localize rhodamine fluorescence alongside the fluorescence from nuclear counterstain, 24 h after instillation of TAT-PNA conjugates (Figure 2). Colocalization of green fluorescence from nuclear counterstain with the red fluorescence from the rhodamine probe used in our experiment indicates that PNA covalently tethered to the 11-mer cell penetrating peptide could successfully penetrate into the cells of the urothelium. The penetration of TAT-PNA into the top cellular layers of urothelium is evident from the overlap yellow color seen in panels C and F of Figure 2 is due to the merge of the red fluorescence of rhodamine with green fluorescence of sytox counterstain. Bladders treated with PNA alone without the tethered TAT did not show any rhodamine fluorescence (panels B and E, Figure 2) indicating TAT was required for successful delivery of PNA to the cells in the urothelium.

In Vivo Efficacy. The activity of sequence T was further confirmed in vivo by evaluating the efficacy of antisense TAT-PNA having exactly the same sequence as oligo T in cyclophosphamide induced cystitis in rat. Different mechanisms underlie the antisense effect of PS and PNA, but if both of them have the same sequence of bases, they can be expected to bind to the same region of target mRNA as both of them obey the Watson−Crick base pairing rules for binding to the mRNA. Cystometrograms (CMG) performed on rats was one of the outcome measures for the efficacy. The slow filling of bladder during CMG gradually raises the internal pressure of bladder, which is known as intravesical pressure, and micturition contractions are elicited. These reflex contractions are triggered by afferent activity of the bladder wall. It is suggested that hyperexcitability of afferent bladder reflexes is partly mediated by NGF during inflammation induced by cyclophosphamide.[23] Injection of cyclophosphamide raises the frequency of micturition contractions by irritation of bladder through its metabolite acrolien. Pretreatment of antisense PNA conjugated with TAT was effective in attenuating the bladder hyperreflexia induced by cyclophosphamide (Figure 3a). The bladder contraction frequency in the antisense

(23) Vizzard, M. A. *Exp. Neurol.* **2000**, *161*, 273−284.

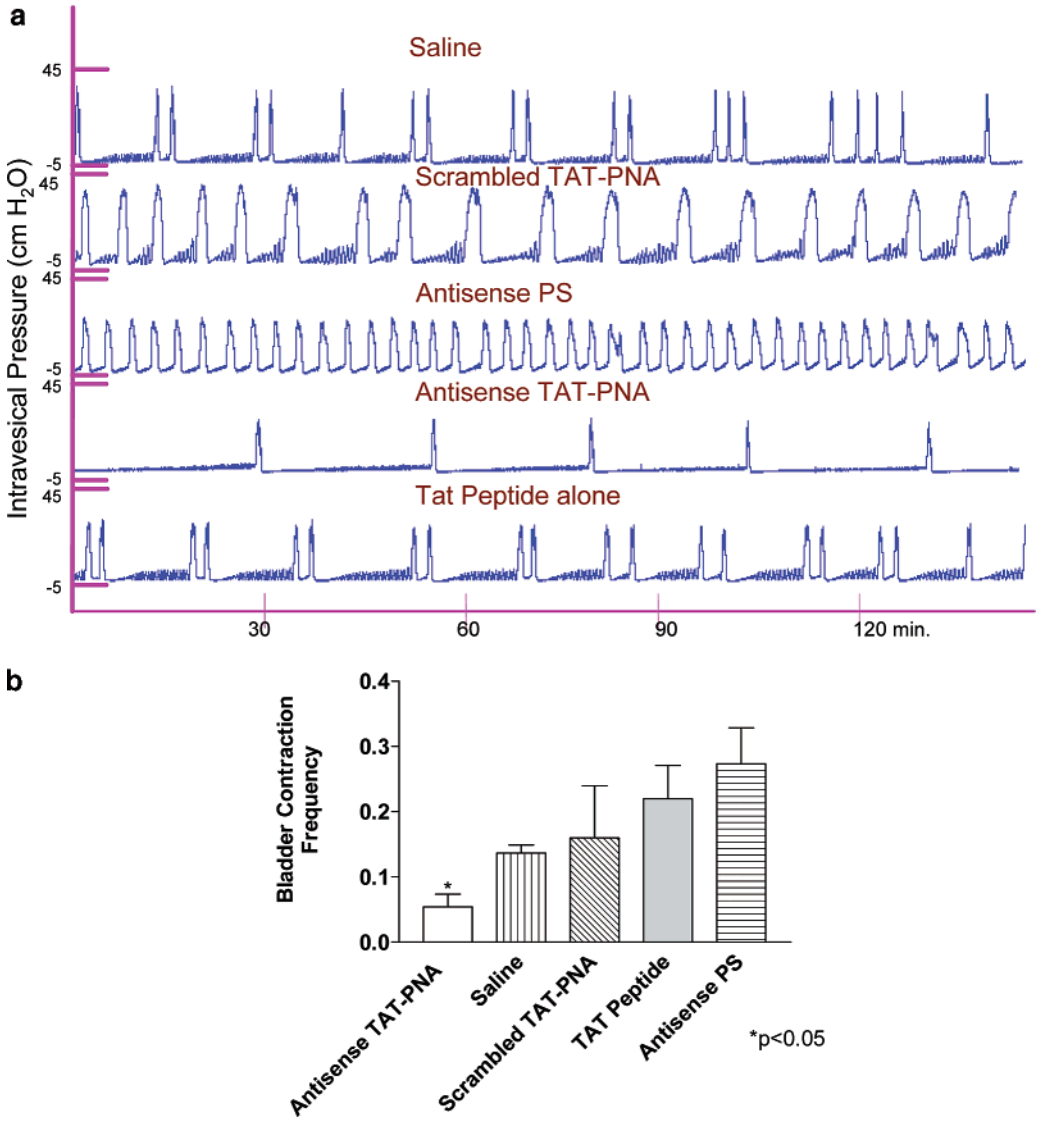

Figure 3. (a) Effects of intraperitoneal injection of cyclophosphamide (100 mg/kg) on cystometrograms in rats instilled with either saline, TAT-PNA of a scrambled sequence, or antisense sequence against rat βNGF mRNA. Phosphorothioate oligonucleotide of the antisense sequence served as a control. Note that in rats instilled with antisense TAT-PNA, the cyclophosphamide induced bladder hyperactivity was suppressed. (b) Bladder contraction frequency of rats under urethane anesthesia after instillation of either saline or TAT-PNA or PS oligo with either antisense or scrambled sequence. The bladder contraction frequency (BCF) was significantly decreased in rats instilled with antisense TAT-PNA compared to saline and other controls (*$p < 0.05$, $n = 5$). The rats instilled with TAT peptide alone and scrambled control showed BCF slightly higher than rats instilled with saline, but the difference was not statistically significant.

treatment group was significantly reduced compared to that for rats instilled with other treatments such as scrambled sequence or saline or TAT peptide alone (Figure 3b). Rats instilled with TAT peptide alone and scrambled TAT-PNA sequence showed a slightly higher bladder contraction frequency than rats in the group instilled with saline, but the difference was not statistically significant. The antisense mediated effect of PNA is supported by the observed phenotypic effects of PNA combined with the lack of activity in the scrambled PNA sequence and in the free peptide itself. The higher bladder contraction frequency observed in rats instilled with phosphorothioate oligos is probably due to nonspecific interaction of polyanionic oligos leading to aggravation of bladder irritation caused by CYP injection.

Effect on Bladder NGF Levels. The presence of NGF in urothelium was detected by immunofluorescence. The bright red fluorescence of Cy3 was visible in deeper layers of rat bladder in all treatment groups, indicating lack of change in expression of NGF in deeper layers (Figure 4a). The red fluorescence was seen in the urothelium (indicated by arrow) of all sections except in the urothelium (panel D) of bladder sections isolated from rat bladder instilled with TAT-PNA of the antisense sequence, indicating effective downregulation

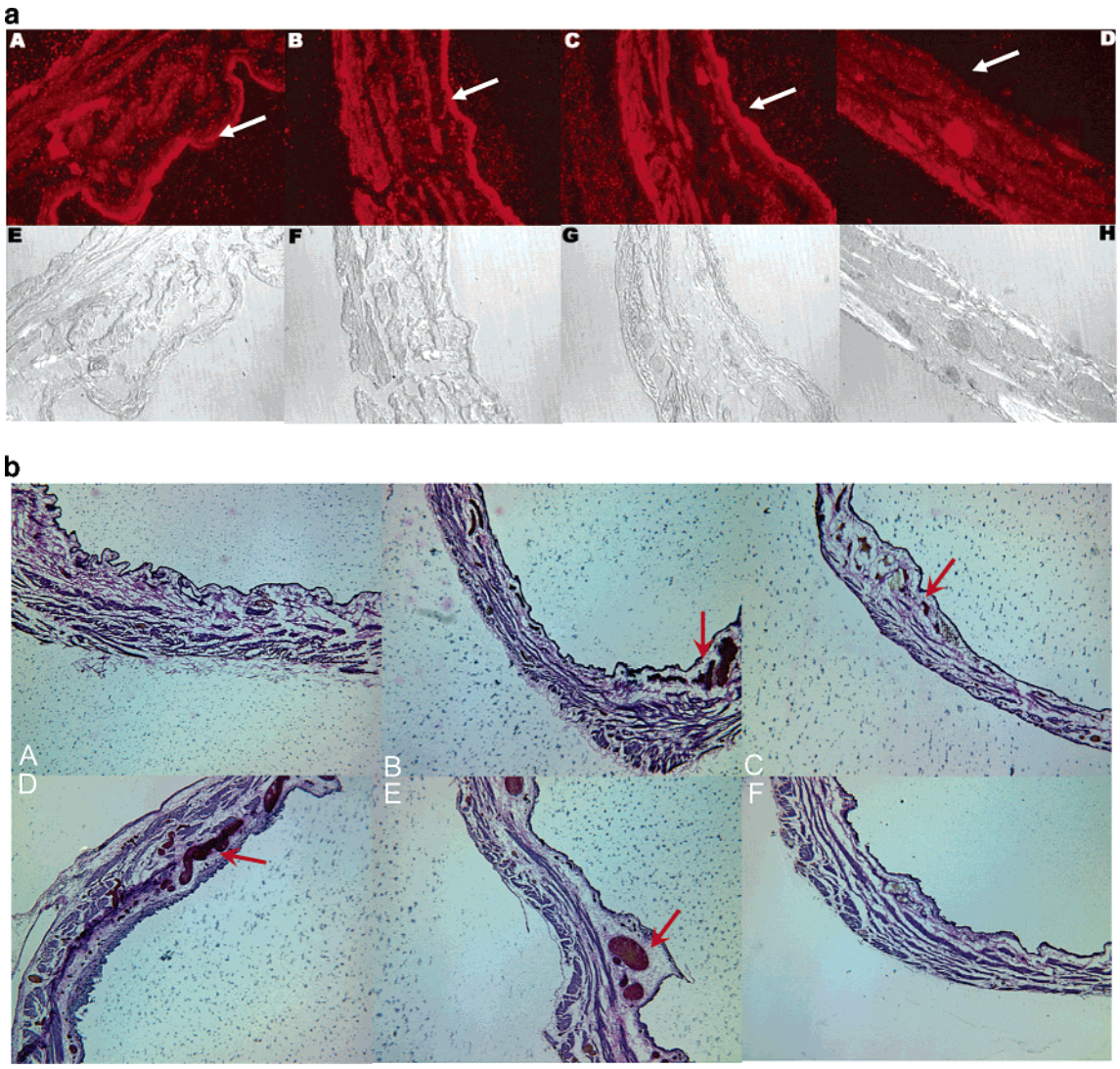

Figure 4. (a) Effects of TAT-PNA antisense against βNGF mRNA on immunoreactivity of βNGF in rat urothelium. Twenty micrometer thick rat bladder sections were incubated overnight at 4 °C with anti-NGF antibody, and then the bound primary antibody was localized with secondary antibody labeled with Cy3. Panel A is rat instilled with saline, panel B is rat instilled with phosphothioate oligo, and panel C is rat instilled with TAT-PNA of scrambled sequence. Bladder instilled with TAT-PNA of the antisense sequence is shown in panel D. The decreased immunoreactivity of βNGF is apparent in panel D from the decrease (shown by white arrows) in the intensity of Cy3 stain in the urothelium of rat instilled with TAT-PNA of the antisense sequence. Magnification in all of the sections was 20×. (b) Bladder histology of untreated (panel A) and cyclophosphamide treated rat bladders instilled with the following: panel B, saline; panel C, phosphothioate oligo; panels D, E, and F, rat instilled with TAT peptide alone, with a scrambled or antisense TAT-PNA. The submucosal bleeding spots seen in rats treated with cyclophosphamide are indicated by red arrows, and antisense treatment could afford near normal histology of the rat bladder.

of NGF by TAT-PNA antisense. The activity was specific, because the bladder treated with TAT-PNA of a scrambled sequence showed a high level of NGF in the urothelium (panel C).

Effect on Bladder Histology. H&E staining of bladder sections revealed submucosal bleeding in most groups following cyclophosphamide injection (Figure 4b). The number of bleeding spots appearing red in the submucosa of bladder sections (marked by arrows) were similar in rats instilled with either saline, TAT peptide alone, or TAT-PNA of a scrambled sequence (panels B, D, and E, respectively). Bladders instilled with phosphorothioate oligo with the antisense sequence were slightly better with respect to less areas of bleeding spots. The histology of rat bladder instilled with antisense TAT-PNA (panel F) appeared similar to the histology of untreated bladder (panel A). This result indicates that bladders instilled with antisense TAT-PNA were able to resist the inflammatory changes induced by cyclophosphamide. Such resistance activity appeared to be specific for the antisense effect, as it was absent in bladders instilled with the scrambled TAT-PNA.

Discussion

PNA is a second generation of antisense agents first reported by Nielsen et al.[24] In a recent study, intraperitoneal administration of a 12-mer antisense PNA against a subunit of glutamate receptor in transgenic mice with familial amyotrophic lateral sclerosis improved their performance on rotarod and survival rate.[25] The present study is the first to report the delivery of TAT-PNA conjugates by an intravesical route for blocking βNGF overexpression in the bladder. Systemic administration of any drug or an agent blocking NGF expression is bound to have adverse effects on long-term use as NGF signaling subserves multiple neuroprotective and repair functions in both the central and peripheral nervous systems.[26] Local delivery at the disease site could be a viable approach for blocking NGF expression with reduced toxicity. Indeed, the therapeutic advantage of drug administration by an intravesical route is underlined by high local drug concentrations with minimal systemic exposure.

In our study, the process of selecting a biologically active antisense sequence against βNGF was done in two steps: first, the most favorable target site was selected with the aid of an algorithm followed by a second and more focused, experimental procedure to identify the effective antisense sequence positioned within the predicted accessible target sites in mRNA.[27,28] Traditional PS oligonucleotides were used for determining antisense sequence against βNGF in an in vitro screening assay using transiently transfected HEK 293 cells. NGF secreted into the cell media by transfected cells was measured by ELISA. Surprisingly, the selected T sequence was located near the 3′-UTR of the βNGF mRNA, and it has been previously reported that AU nucleotide-rich sequence present around that region affects βNGF mRNA stability.[29] The sequence of T oligo, which was effective in reducing NGF expression with minimal effect on cell viablity, was selected for in vivo experiments. The scrambled PNAs used in our experiments as a control for nonspecific antisense effects have also been used in other studies.[30] PNA was selected to substitute for PS oligos in evaluating the therapeutic efficacy in cyclophosphamide cystitis.

The charged phosphothioester backbone in PS oligo is replaced by the uncharged *N*-(2-aminoethyl-glycine) units in PNA.[13] It has been shown that PS oligo and PNA can bind to the same region of target mRNA and then operate by a different mechanism for showing an antisense effect. The absence of a repetitive charged backbone avoids the intrastrand repulsion during hybridization to their target mRNA. The affinity of association obeys Watson−Crick hydrogen bonding rules and is independent of salt concentration.[31] Not only does the achiral polyamide backbone provide resistance against nucleases and proteases[31] but also nonspecific interactions arising from PNA binding to proteins that normally recognize polyanions are prevented by the neutral backbone.[32] However, antisense PNA uses a steric blocking of RNA translation or processing to decrease protein expression.[33] Unlike the first generation antisense agents, PNA-RNA hybrids are not substrates for RNase H.[15,34] The problem of poor cellular uptake of PNA has been resolved by conjugating cell penetrating peptides to inhibit gene expression in primary and transformed human cells.[15,33,35] This approach is uniquely suitable for PNA, as most of the penetrating peptides are highly positively charged, which can form an intramolecular complex with the highly negatively charged oligos. In our study we examined the in vivo potential of tethering TAT peptide to aid translocation of PNA across the urothelium. Red fluorescence of rhodamine tagged to PNA was only visible in the rat bladder section administered the TAT peptide-PNA conjugates. It has been previously reported that conjugation with cell penetrating peptides such as TAT enhances delivery of PS oligonucleotides without interfering with their base pairing function,[36] but this observation was obtained from the study of physical interaction, and effect on physiological activity was not determined. The exact mechanism of TAT transduction is unknown, but recently, convincing biochemical and genetic findings have established that the full-length TAT protein was internalized in cells via binding with the ubiquitous heparan sulfate proteoglycans.[37,38] The presence of the glycoaminoglycan layer at the apical surface of urothelium in rats and humans has been extensively demonstrated.[39−41]

(24) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500.
(25) Rembach, A.; Turner, B. J.; Bruce, S.; Cheah, I. K.; Scott, R. L.; Lopes, E. C.; Zagami, C. J.; Beart, P. M.; Cheung, N. S.; Langford, S. J.; Cheema, S. S. *J. Neurosci. Res.* **2004**, *77*, 573−582.
(26) Sofroniew, M. V.; Howe, C. L.; Mobley, W. C. *Annu. Rev. Neurosci.* **2001**, *24*, 1217−1281.
(27) Mathews, D. H.; Burkard, M. E.; Freier, S. M.; Wyatt, J. R.; Turner, D. H. *RNA* **1999**, *5*, 1458−1469.
(28) Scherr, M.; Rossi, J. J.; Sczakiel, G.; Patzel, V. *Nucleic Acids Res.* **2000**, *28*, 2455−2461.
(29) Tang, B.; Wang, M.; Wise, B. C. *Brain Res. Mol. Brain Res.* **1997**, *46*, 118−126.
(30) Mayhood, T.; Kaushik, N.; Pandey, P. K.; Kashanchi, F.; Deng, L.; Pandey, V. N. *Biochemistry* **2000**, *39*, 11532−11539.
(31) Demidov, V. V.; Potaman, V. N.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchard, O.; Sonnichsen, S. H.; Nielsen, P. E. *Biochem. Pharmacol.* **1994**, *48*, 1310−1313.
(32) Hamilton, S. E.; Simmons, C. G.; Kathiriya, I. S.; Corey, D. R. *Chem. Biol.* **1999**, *6*, 343−351.
(33) Pooga, M.; Soomets, U.; Hallbrink, M.; Valkna, A.; Saar, K.; Rezaei, K.; Kahl, U.; Hao, J. X.; Xu, X. J.; Wiesenfeld-Hallin, Z.; Hokfelt, T.; Bartfai, T.; Langel, U. *Nat. Biotechnol.* **1998**, *16*, 857−861.
(34) Knudsen, H.; Nielsen, P. E. *Nucleic Acids Res* **1996**, *24*, 494−500.
(35) Kaihatsu, K.; Huffman, K. E.; Corey, D. R. *Biochemistry* **2004**, *43*, 14340−14347.
(36) Astriab-Fisher, A.; Sergueev, D.; Fisher, M.; Shaw, B. R.; Juliano, R. L. *Pharm. Res.* **2002**, *19*, 744−754.
(37) Tyagi, M.; Rusnati, M.; Presta, M.; Giacca, M. *J. Biol. Chem.* **2001**, *276*, 3254−3261.
(38) Vives, E.; Richard, J. P.; Rispal, C.; Lebleu, B. *Curr. Protein Pept. Sci.* **2003**, *4*, 125−132.
(39) de Deus, J. M.; Girao, M. J.; Sartori, M. G.; Baracat, E. C.; Rodrigues de Lima, G.; Nader, H. B.; Dietrich, C. P. *Am. J. Obstet. Gynecol.* **2003**, *189*, 1654−1659.

Whether the action of TAT-PNA in the bladder is facilitated by the glycosaminoglycans is presently unknown. Studies on cultured cells have shown that TAT delivered "cargo" is destined to endosomes.[42] However, these studies may not shed any light on the trafficking of TAT-PNA inside the bladder as cells from urothelium were not part of studies done earlier.

A well-established model of cystitis was used to evaluate the in vivo efficacy of TAT-PNA conjugates, and bladder reflex activity was recorded by CMG, which is a suitable index for integrated neuronal response to inflammation induced by CYP. The physiological response of animals treated with antisense was evaluated by CMG, 4 h after injection of CYP. Concentration of acrolein, an irritant CYP metabolite, is at its peak around that time, and gene expression of other mediators has been shown to reach its peak around 4 h after CYP injection in rats.[43] Rats instilled with antisense PNA showed a significantly reduced bladder contraction frequency compared to rats instilled with saline alone or other controls. Rats instilled with phosphorothioate oligo exhibited significantly increased bladder contraction frequency compared to saline. It is possible that nonspecific interaction of charged oligos could aggravate the irritation of urothelium caused by acrolein produced from CYP.[44] It was recently reported that full length TAT protein can upregulate Id1 expression and inhibit the nerve growth factor (NGF) induced neuronal differentiation of PC12 cells.[45] It is highly unlikely that a peptide having only the PTD of the TAT protein will have such deleterious effects. Moreover, such potential concerns for systemic toxicity from TAT peptide argue for local delivery of TAT-PNA conjugates. However, instillation of TAT peptide alone in CYP treated rats produced a physiological response similar to rat instilled with saline, indicating that the observed pharmacological effect of TAT-PNA antisense was not due to TAT, but resulted from the antisense effect of PNA. The slightly higher mean bladder contraction frequency of rats instilled with TAT peptide alone may be related to the different cellular entry pathways for unconjugated and conjugated TAT peptide.[46]

Immunohistochemistry of the bladder sections cryopreserved after CMG showed a decrease in immunoreactive NGF only in the urothelium of rat bladder following instillation with TAT-PNA conjugates. This technique has been used in many studies to demonstrate depletion of NGF in bladder of rodents after instillation of LPS or IC urine.[47,48] However, in our study, when whole bladders were used for measuring total NGF by ELISA, we failed to show any difference between the treatment groups (data not shown). It is possible that preformed NGF residing in tissue layers lying below urothelium contributes to the bulk of the total NGF content of the bladder. The majority of bladder NGF resides in the smooth muscle region, and it is possible that reduction of NGF in uroethelium at 6 h after instillation of antisense PNA did not make a measurable difference in the total NGF of bladder. The apparent less homogeneous uptake of PNA conjugate into the urothelium (Figure 2) may be explained by the substantial time and concentration differences in the bladder uptake studies (24 h) and NGF immunostaining in Figure 4a (6 h).

Nevertheless, our study was able to demonstrate that modulating the NGF content in the urothelium can bring about changes in the physiological response of the bladder. Besides, histological studies performed on bladders also corroborated with the therapeutic benefit accrued from treating cyclophosphamide injected bladders with antisense PNA.

Our studies show that intravesical instillation of TAT-PNA conjugates is a feasible approach for determining and modulating molecular determinants of interstitial cystitis.

Acknowledgment. This work was partially supported by NIH Grant DK 068556.

MP050093X

(40) Poggi, M. M.; Johnstone, P. A.; Conner, R. J. *Urol. Oncol.* **2000**, *5*, 234−237.
(41) Sun, Y.; Chai, T. C. *BJU Int.* **2002**, *90*, 381−385.
(42) Kawamura, K. S.; Sung, M.; Bolewska-Pedyczak, E.; Gariepy, J. *Biochemistry* **2006**, *45*, 1116−1127.
(43) Saban, M. R.; Hellmich, H.; Nguyen, N. B.; Winston, J.; Hammond, T. G.; Saban, R. *Physiol. Genomics* **2001**, *5*, 147−160.
(44) Tzan, C. J.; Berg, J. R.; Lewis, S. A. *Am. J. Physiol.* **1993**, *265*, C1637−1647.
(45) Bergonzini, V.; Delbue, S.; Wang, J. Y.; Reiss, K.; Prisco, M.; Amini, S.; Khalili, K.; Peruzzi, F. *Oncogene* **2004**, *23*, 7701−7711.
(46) Richard, J. P.; Melikov, K.; Brooks, H.; Prevot, P.; Lebleu, B.; Chernomordik, L. V. *J. Biol. Chem.* **2005**, *280*, 15300−15306.
(47) Baykara, M.; Erdogru, T.; Gulkesen, K. H.; Sargin, C. F.; Savas, M.; Ates, M. *Urol. Int.* **2003**, *71*, 66−72.
(48) Bjorling, D. E.; Jacobsen, H. E.; Blum, J. R.; Shih, A.; Beckman, M.; Wang, Z. Y.; Uehling, D. T. *BJU Int.* **2001**, *87*, 697−702.