

# Inhibition of DNA binding of the NF-Y transcription factor by the pyrrolobenzodiazepine-polyamide conjugate GWL-78

Minal Kotecha,<sup>1</sup> Jerome Kluza,<sup>1</sup> Geoff Wells,<sup>2</sup> C. Caroline O'Hare,<sup>1</sup> Claudia Forni,<sup>3</sup> Roberto Mantovani,<sup>3</sup> Philip W. Howard,<sup>2</sup> Peter Morris,<sup>1</sup> David E. Thurston,<sup>2</sup> John A. Hartley,<sup>1</sup> and Daniel Hochhauser<sup>1</sup>

<sup>1</sup>Cancer Research UK Drug-DNA Interactions Research Group, UCL Cancer Institute, University College London; <sup>2</sup>Cancer Research UK Gene Targeted Drug Design Research Group, School of Pharmacy, University of London, London, United Kingdom; and <sup>3</sup>Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Milan, Italy

## Abstract

Many genes involved in cell cycle control have promoters that bind the heterotrimeric transcription factor NF-Y. Several minor-groove binding drugs have been shown to block interactions of transcription factors with cognate DNA-binding sequences. We showed previously that noncovalent minor-groove binding agents block interactions of NF-Y with the promoter of topoisomerase II $\alpha$  (topo II $\alpha$ ). In this study, we investigated the ability of GWL-78, a pyrrolobenzodiazepine-poly(*N*-methylpyrrole) conjugate, to inhibit the binding of NF-Y to DNA. Electrophoretic mobility shift assays showed that GWL-78 could displace NF-Y bound to several CCAAT motifs within promoters of genes involved in cell cycle progression. DNase I footprinting of the topo II $\alpha$  promoter confirmed binding of GWL-78 to AT-rich sequences corresponding to the preferred binding site of NF-Y. Incubation with GWL-78 resulted in displacement of NF-Y binding to DNA. Chromatin immunoprecipitation assays on the topo II $\alpha$  promoter showed that GWL-78 was able to enter the nucleus and interact with specific DNA sequences. Treatment of NIH3T3 cells with GWL-78 resulted in a block of cell cycle progression, which did not involve activation of

p53. Thus, agents such as GWL-78 may be useful in modulating transcription and blocking cellular proliferation. [Mol Cancer Ther 2008;7(5):1319–28]

## Introduction

DNA-interactive drugs are widely used in the treatment of human cancers. There is considerable evidence of preferred DNA sequences to which several of these agents bind. For example, doxorubicin has been shown to bind preferentially to the CAAT quartet sequence and to the alternating polynucleotide d(TATATA)<sub>2</sub> (1). However, the ability of these agents to recognize specific DNA sequences has not been previously considered a significant factor in their anticancer activity. It would be useful therapeutically to develop drugs capable of inhibiting transcription factors. Such agents could be used to modulate chemosensitivity by overcoming resistance mechanisms such as reduced expression of topoisomerase II $\alpha$  (topo II $\alpha$ ; ref. 2).

There is increasing interest in the ability of small molecules to target specific DNA sequences. For example the DNA minor-groove alkylator tallimustine was shown to bind preferentially to 5'-TTTTG-Pu-3' sequences (3). Also, we have shown previously that the bis-benzimidazole Hoechst 33342 binds to inverted CCAAT sequences within the topo II $\alpha$  promoter and thereby blocks binding of the heterotrimeric transcription factor NF-Y (4). NF-Y is a ubiquitously expressed CCAAT-binding protein with roles in cell differentiation and cell cycle progression (5–7). NF-Y cooperates with other transcription factors and regulates promoter activity. It is composed of three subunits, A, B, and C. Both B and C subunits are able to interact with histones, and both A and C subunits are able to bind proteins (8, 9). Abrogation of NF-Y function by expression of a dominant-negative gene results in loss of cell cycle progression (7).

More recently, connections between NF-Y and the p53 pathway were characterized in cells with wild-type p53 and in cells with tumor-derived mutants (10, 11). Importantly, in the latter case, gain-of-function p53 mutants were shown to stimulate growth through binding to the CCAAT/NF-Y complex. Therefore, targeting NF-Y interactions with DNA could be a potential strategy for blocking proliferation of cancer cells. To obtain inhibitors of NF-Y binding to DNA with improved efficacy and potency, we developed a novel series of pyrrolobenzodiazepine (PBD) conjugates, including the molecule GWL-78 (Fig. 1; ref. 18). The PBD series were developed as minor-groove binding covalent molecules. PBDs are antitumor antibiotics derived from *Streptomyces* species and include anthramycin and tomaymycin (12, 13). PBDs interact within the minor groove of DNA by binding covalently to the C2-amino

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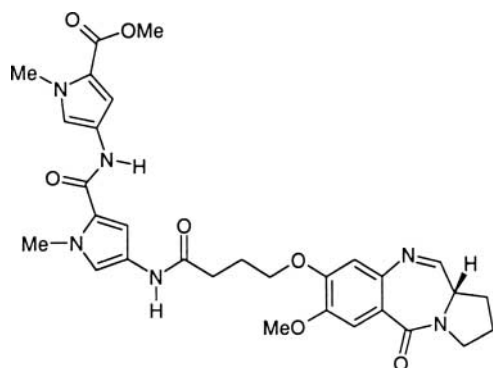
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**Requests for reprints:** Daniel Hochhauser, UCL Cancer Institute, 72 Huntley Street, London WC1E 6RT, United Kingdom.

Phone: 44-20-7679-6006. E-mail: d.hochhauser@ucl.ac.uk

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**Figure 1.** Structure of GWL-78 a PBD-polyamide conjugate.

position of a guanine residue. For example, the pyrrolo[2,1-*c*][1,4]benzodiazepine dimer SJG-136 (NSC 694501) selectively cross-links guanine residues located on opposite strands of DNA and exhibits potent *in vitro* cytotoxicity (12). It spans six nucleotides with a preference for binding to purine-GATC-pyrimidine sequences (14).

The effects of GWL-78 on the binding of NF- $\kappa$ B to DNA were determined using an electrophoretic mobility shift assay (EMSA) on sequences derived from several promoters regulated by NF- $\kappa$ B. The ability of GWL-78 to inhibit NF- $\kappa$ B binding was investigated in cells using a chromatin immunoprecipitation (ChIP) assay. DNase I footprinting experiments were used to show binding to specific sequences within the topo II $\alpha$  promoter. Finally, measurement of topo II $\alpha$  mRNA and protein in drug-treated cells was carried out. These results suggest that PBDs can modulate gene expression by inhibiting specific protein/DNA interactions.

## Materials and Methods

### Cell Lines and Culture Conditions

NIH3T3 cells (obtained from Cancer Research UK London Research Institute) were grown in DMEM (Autogen Bioclear) supplemented with 10% newborn calf serum and 1% glutamine and incubated at 37°C in 5% CO<sub>2</sub>. CaCo2 cells were also obtained from Cancer Research UK London Research Institute and grown in RPMI (Bioclear) supplemented with 10% FCS and 1% glutamine and incubated at 37°C in 5% CO<sub>2</sub>.

### Serum Starvation and Cell Cycle Synchronization

NIH3T3 cells, 70% confluent, were washed three times with PBS and maintained in DMEM supplemented with 0.1% newborn calf serum for 82 h before replacing the culture medium with DMEM supplemented with 10% newborn calf serum and 0.4 mmol/L mimosine (Sigma) for 14 h. The cells were subsequently rewashed with PBS, released from synchronization, and allowed to progress through the cell cycle by adding complete medium.

### Preparation of Nuclear Extracts

All steps of the preparation of nuclear extracts were done at 4°C in the presence of a protease inhibitor mix

(Complete; Boehringer). Cells were rinsed with ice-cold PBS, scraped from the surface, and collected by centrifugation. The cells were washed with 5 equivolumes of hypotonic buffer containing 10 mmol/L K-HEPES (pH 7.9), 1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, and 0.5 mmol/L DTT (Sigma). Subsequently, the cells were resuspended in 3 equivolumes of hypotonic buffer, incubated on ice for 10 min, and subjected to 20 strokes of a Dounce homogenizer and the nuclei collected by centrifugation. The nuclear pellet was resuspended in 0.5 equivolumes of low-salt buffer containing 20 mmol/L K-HEPES (pH 7.9), 0.2 mmol/L K-EDTA, 25% glycerol, 1.5 mmol/L MgCl<sub>2</sub>, 20 mmol/L KCl, and 0.5 mmol/L DTT. While stirring, 0.5 equivolume of high-salt buffer (as low-salt buffer but containing 1.4 mol/L KCl) was added and the nuclei were extracted for 30 min. Subsequently, the mixture was centrifuged for 30 min at 14,000 rpm in an Eppendorf centrifuge and the supernatant was dialyzed in tubing with a 12-kDa cutoff (Sigma) for 1 h in a 100 times excess of dialysis buffer containing 20 mmol/L K-HEPES (pH 7.9), 0.2 mmol/L K-EDTA, 20% glycerol, 100 mmol/L KCl, and 0.5 mmol/L DTT. The dialyzed fraction was centrifuged for 30 min at 14,000 rpm in an Eppendorf centrifuge and the supernatant was snap frozen in an ethanol dry ice bath and stored at -80°C. The protein concentration of the nuclear extract was assayed using a Bio-Rad micro protein assay kit.

### Electrophoretic Mobility Shift Assay

The oligonucleotides (MWG Biotech) containing inverted CCAAT boxes (ICB; italicized) used in the EMSA assay are topo II $\alpha$  ICB1 sense 5'-CGAGTCAGGGATTGGCTGGTCT-GCTTC-3' and antisense 5'-GAAGCAGACCAGCCAAT-CCCTGACTCG-3', ICB2 sense 5'-GGCAAGCTACGATT-GGTTCTTCTGGACG-3' and antisense 5'-CGTCCAGAAG-AACCAATCGTAGCTTGCC-3', ICB3 sense 5'-CTCCCTAA-CCTGATTGGTTTATTCAAAC-3' and antisense 5'-GTTTG-AATAAACCAATCAGGTTAGGGAG-3', and ICB4 sense 5'-GAGCCCTTCTCATTGGCCAGATTCCCTG-3' and antisense 5'-CAGGGAATCTGGCCAATGAGAAGGGCTC-3'. Oligonucleotides corresponding to *mdr1* sense 5'-GTGGT-GAGGCTGATTGGCTGGGCAGGAA-3' and antisense 5'-TTCCTGCCAGCCAATCAGCCTCACCA-3', *hOGG1* sense 5'-ACCCTGATTCTCATTGGCGCCTCCTACC-TCTCCTCGGATTGGCTACCT-3' and antisense 5'-AGG-TAGCCAATCCGAGGAGGAGGTAGGAGCGCCAAT-GAGAAATCAGGGT-3', *cdc2/cdk1* sense 5'-CGGGCTA-CCCGATTGGTGAATCCGGGGC-3' and antisense 5'-GCC-CCGGATTCACCAATCGGGTAGCCCG-3', *cyclin B1* CCAAT box 1 sense 5'-GACCGGCAGCCGCAATGGG-AAGGGAGTG-3' and antisense 5'-CACTCCCTTCCCATT-GGCGGCTGCCGTC-3', and CCAAT box 2 sense 5'-CCA-CGAACAGCCAATAAGGAGGGAGCAG-3' and antisense 5'-CTGCTCCCTCCTTATTGGCCTGTTCGTGG-3' were also used for EMSA. Oligonucleotides of similar sequence containing mutated ICBs were used as specific competitors, the wild-type ICB sequence replaced by AAACC or GTTT in sense and antisense oligonucleotides, respectively. Sense and antisense oligonucleotides were

annealed in an equimolar ratio. Double-stranded oligonucleotides were 5'-end labeled with T4 kinase (NEB) using [ $\gamma$ - $^{32}$ P]ATP and subsequently purified on Bio-Gel P-6 columns (Bio-Rad). EMSAs were performed as described (15) on 5  $\mu$ g nuclear extract. For supershifts, antibodies against NF-Y-A (IgG fraction; Rocklands) were used and the preincubation on ice was extended for a total of 1.5 h. On addition of  $\sim$ 0.1 ng radiolabeled probe, the incubation was continued for 2 h at room temperature. In competition experiments, radiolabeled probe and competitor were added simultaneously. Subsequently, 0.5  $\mu$ L loading buffer [25 mmol/L Tris-HCl (pH 7.5), 0.02% bromophenol blue, and 10% glycerol] was added and the samples were separated on a 4% polyacrylamide gel in 0.5 $\times$  TBE containing 2.5% glycerol at 4°C. After drying the gels, the radioactive signal was visualized by exposure to Kodak X-Omat-LS film.

#### NF-Y Activation Assay by ELISA

GWL-78 (0.1-5  $\mu$ mol/L) was added in a 96-well plate to which an oligonucleotide containing CCAAT box has been immobilized. Nuclear extract of HT-29 cells were added to each well and incubated for 1 h at room temperature. DNA binding of NF-Y was assessed by TransAM NF-Y Kit (Active Motif). Briefly, samples were incubated with a primary antibody specific to an epitope on the bound form of NF-Y and a horseradish peroxidase-conjugated IgG antibody providing a colorimetric reaction. Absorbance was read on a spectrophotometer at 450 nm with a reference of 655 nm.

#### DNase I Footprinting

A radiolabeled probe of 479 bp corresponding to positions -489 to -10 relative to the transcriptional start site of the topo II $\alpha$  promoter was generated. Antisense oligonucleotide 5'-GTCTGGTTAGGAGAGCTCCACTTG-3' (4 pmol) was 5'-end labeled with T4 kinase (Invitrogen) using [ $\gamma$ - $^{32}$ P]ATP in a 10  $\mu$ L reaction followed by heat inactivation for 20 min at 65°C. Subsequently, 4 pmol sense oligonucleotide (5'-CTGTCCAGAAAGCCGGCACTCAG-3'), 2  $\mu$ L of 10 mmol/L deoxynucleotide triphosphates (Promega), 1 unit Red Hot DNA Polymerase (Abgene), 2  $\mu$ L of 25 mmol/L MgCl<sub>2</sub>, and 4.5  $\mu$ L of 10 $\times$  reaction buffer IV (Abgene) were added (in a final volume of 50  $\mu$ L) and a PCR reaction was performed consisting of 3 min at 95°C and 1 min at 95°C, 1 min at 60°C, and 2 min 72°C for 35 cycles. The product was purified on a Bio-Gel P-6 column (Bio-Rad). DNase I footprint reactions were done with recombinant NF-Y protein in a 50  $\mu$ L reaction in the same buffer as used for EMSA. After preincubation for 30 min at 4°C,  $\sim$ 0.1 ng radiolabeled probe was added and the mixture was incubated at room temperature for another 30 min. Subsequently, 1 unit RQ1 DNase I (Promega) and the solution was made up to 5 mmol/L MgCl<sub>2</sub> and CaCl<sub>2</sub>. Following exactly 3 min of digestion at room temperature, 1 volume stop mix containing 30 mmol/L K-EDTA (pH 8.0), 200 mmol/L NaCl, and 1% SDS was added and samples were purified by phenol-chloroform treatment and alcohol precipitation. The resulting pellets were dried and resuspended in loading buffer [95% formamide,

20 mmol/L K-EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol]. The sample was heat denatured for 3 min at 95°C and separated on a 6% denaturing polyacrylamide gel (Sequagel; National Diagnostics). The dried gels were exposed to Kodak X-Omat-LS film with intensifying screens at -80°C.

#### Flow Cytometry

Cells were collected for flow cytometric analysis using trypsin. Cells were fixed using a mixture of 70% ethanol (7 mL) and PBS-0.02% sodium azide (1 mL) and analyzed within 1 week of fixation. Cell pellets were washed with PBS-0.02% sodium azide before being resuspended in 50 mL of 1 mg/mL propidium iodide (Sigma), 25  $\mu$ L of 10 mg/ml RNase A, and 925  $\mu$ L PBS-0.02% sodium azide. Subsequently cells were gently mixed and incubated at 4°C for 30 min before analyzing using flow cytometry.

#### Western Blotting

For Western blot analysis, 50  $\mu$ g nuclear extract was denatured by heating for 3 min at 95°C in sample buffer containing 100 mmol/L Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue. Bio-Rad high-range SDS-PAGE molecular weight standards were used as a reference. Proteins were separated on a 7% SDS-polyacrylamide mini gel (Mini Protean II System; Bio-Rad) and subsequently transferred (Trans Blot Cell; Bio-Rad) to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Western blot analysis was performed with the IHIC8 rabbit polyclonal topo II $\alpha$  antibody (kindly provided by Dr. I.D. Hickson) at a 1:5,000 dilution using ECL Western blot detection kit and protocol (Amersham) using 1% blot qualified bovine serum albumin (Promega) as blocking reagents and TBS-0.5% Tween 20 (BDH) as a buffer. The chemiluminescent signal was visualized by exposing the blots to Kodak X-Omat-LS film. cdk2, cyclin B1, and p53 antibodies were all purchased from Abcam and used at dilutions of 1:400, 1:500, and 1:1000, respectively.

#### Reverse Transcription and Real-Time PCR

Reverse transcription was carried out essentially as described in the Promega Protocols and Applications Guide, 3rd Edition, 1996. Real-time PCR was carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Respectively, the forward and reverse topo II $\alpha$  primers used were 5'-ATTGAAGACGCTGCTTCGTTATGGG-3' and 5'-GATGGATAAAATTAATCAGCAAGCCT-3'. The probe sequence (CAGATCAGGACCAAGATGGTCCACATC) used for the reactions was labeled at the 5' end with 6-FAM and TAMRA at the 3' end. The cycling conditions used were 50°C for 2 min and 95°C for 10 min to allow denaturation to occur and 40 cycles of 95°C for 15 s and 58°C for 1 min to amplify the target sequences. Glyceraldehyde-3-phosphate dehydrogenase primer/probe master mix (1.25  $\mu$ L; Applied Biosystems) was used as an internal control in all reactions. The reaction mix was prepared using 12  $\mu$ L of the TaqMan PCR master mix (Applied Biosystems) and 1  $\mu$ mol/L of each primer, 0.2  $\mu$ mol/L probe, and 2.5  $\mu$ L cDNA template in a final volume of 25  $\mu$ L. The results were analyzed using the

mathematical quantification approach described by Pfaffl (16) and ABI User Bulletins 2 and 5 (2001). This is based on the relative expression ratio of the target gene (topo II $\alpha$ ) compared with that of an internal control gene (glyceraldehyde-3-phosphate dehydrogenase). Standard curves were constructed for both internal and reference genes and slopes of these were used to ensure that both primer sets were equally efficient. The threshold cycle values (Ct) and the efficiencies of the reactions were used to compare the relative expression levels of the target gene in various samples. To ease comparison, levels of topo II $\alpha$  RNA in untreated, exponentially growing cells were set at a value of 1 and all test samples were expressed at values relative to this.

#### Chromatin Immunoprecipitation

Immunoprecipitations were carried out essentially as described (17) with a few modifications. Cells were cultured and treated in 150 mm plates and treated with 1% formaldehyde to induce the cross-linking reaction. Treatment with 0.125 mol/L glycine stopped the reaction and cell pellets were stored at  $-20^{\circ}\text{C}$  until analysis. For analysis, cells were resuspended in lysis buffer [5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP-40, 1 $\times$  protease inhibitor cocktail (Sigma)] containing 0.5 mmol/L phenylmethylsulfonyl fluoride. Subsequently, nuclei extracted using a Dounce homogenizer were resuspended in sonication buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 0.1% SDS, 0.5% deoxycholic acid, and 1 $\times$  protease inhibitor cocktail] and sonicated into 500- to 1,500-bp chromatin fragments. The chromatin fragments were stored at  $-80^{\circ}\text{C}$  pending further analysis. Protein G (15  $\mu\text{L}$ ; Kierkegaard Perry Lab) was precleared overnight with 1  $\mu\text{g}/\mu\text{L}$  salmon testis DNA and 1  $\mu\text{g}/\mu\text{L}$  bovine serum albumin in immunoprecipitation buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 0.1% SDS, 0.5% deoxycholic acid, 1 $\times$  protease inhibitor cocktail, 150 mmol/L LiCl]. Chromatin (25-50  $\mu\text{L}$ ) was also precleared by incubating for 2 h with 40  $\mu\text{L}$  protein G slurry in immunoprecipitation buffer at  $4^{\circ}\text{C}$ . The precleared chromatin was placed in pre-siliconated 0.5 mL PCR tubes, up to 8  $\mu\text{g}$  antibody was added (200  $\mu\text{L}$  final volume), and the mixture was incubated overnight at  $4^{\circ}\text{C}$ . Subsequently, 110  $\mu\text{L}$  of the salmon testis DNA-saturated and bovine serum albumin-saturated protein G in immunoprecipitation buffer was added to the chromatin-antibody mixture and the samples were further incubated for 2 h at  $4^{\circ}\text{C}$ . The samples were centrifuged at 4,000 rpm for 2 min and the supernatant was stored at  $-20^{\circ}\text{C}$  as a source of "input DNA." The resin was washed initially at  $4^{\circ}\text{C}$  for 30 min using 300  $\mu\text{L}$  immunoprecipitation buffer. Subsequently, nine more washes were carried out by resuspending the resin in 300  $\mu\text{L}$  immunoprecipitation buffer and centrifuging for 2 min at 4,000 rpm. The bound DNA was then eluted from the resin by adding 100  $\mu\text{L}$  elution buffer (1% SDS, 50 mmol/L  $\text{NaHCO}_3$ , 1.5 ng/ $\mu\text{L}$  salmon testis DNA) and incubating for 1 h at  $37^{\circ}\text{C}$  on a shaker. After centrifugation at 14,000 rpm for 2 min, the supernatant and the input DNA were both incubated overnight at  $65^{\circ}\text{C}$  with 10  $\mu\text{g}$  RNase A and 200 mmol/L NaCl to reverse the cross-

links. Following this, the DNA was precipitated with 99% ethanol at  $-20^{\circ}\text{C}$ . The pellets were collected by centrifugation at 13,000 rpm for 30 min, washed with 70% ethanol, and air-dried. The protein was removed from the DNA by resuspending the pellets in 40  $\mu\text{g}$  proteinase K, 25  $\mu\text{L}$  proteinase K buffer [1.25% SDS, 50 mmol/L Tris (pH 7.5), 25 mmol/L EDTA], and 100  $\mu\text{L}$  TE (pH 7.5) and incubating at  $42^{\circ}\text{C}$  for 2 h. Digested protein was removed with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA was precipitated at  $-20^{\circ}\text{C}$  overnight with 30  $\mu\text{L}$  of 3 mol/L sodium acetate, 1  $\mu\text{L}$  of 5 mg/mL tRNA, and 750  $\mu\text{L}$  of 99% ethanol. The sample DNA pellets were resuspended in 60  $\mu\text{L}$  sterile water and the input DNA in 200  $\mu\text{L}$ . The DNA was then used for PCR using 2  $\mu\text{L}$  DNA/sample.

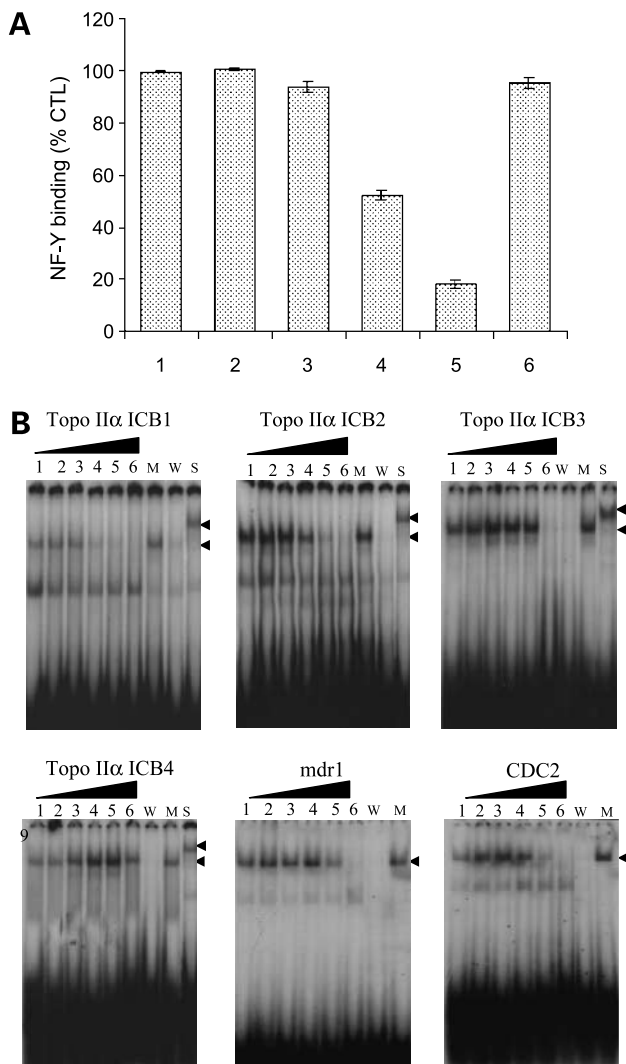
## Results

### GWL-78 Can Inhibit Protein Binding to the Inverted CCAAT Motif

The structure and chemical properties of GWL-78 have been described (18). DNA footprinting and *in vitro* transcription stop assay results suggest that it binds preferentially to the consensus motif 5'-XGXWz ( $z = 3 \pm 1$ , W = A or T, X = any base but preferably a purine) and that robust sequence-selective blockade of transcription occurs at sites corresponding approximately to its DNA footprints. GWL-78 has also been shown to have good cellular/nuclear penetration properties, and a degree of correlation between cytotoxicity and DNA-binding affinity has been observed (18).

We first investigated the effect of GWL-78 on NF-Y binding to an oligonucleotide containing a CCAAT motif immobilized on a 96-well plate. The immobilized oligonucleotides were incubated with GWL-78 with subsequent addition of HT-29 nuclear extracts. Relative DNA-binding activity of NF-Y is shown in Fig. 2A. To monitor the specificity of the interaction, we used competitive DNA. In this positive control, NF-Y binding was reduced by >80% (lane 5), whereas incubation with a nonspecific oligonucleotide had no effect on NF-Y binding (column 6). Following treatment with GWL-78, there was 48% inhibition of the NF-Y activity (column 4) compared with the control sample (column 1). This showed the ability of GWL-78 to inhibit NF-Y binding to the CCAAT motif.

The topo II $\alpha$  promoter contains five inverted CCAAT motifs that have been shown to be involved in control of transcriptional expression at confluence, p53-induced down-regulation, and response to heat shock-induced up-regulation (19-21). We performed EMSA assays on ICB motifs within the topo II $\alpha$  promoter. Using radiolabeled probes of ICB1 to ICB4, GWL-78 was able to inhibit binding of NF-Y to the core motif. There were significant differences in the ability of GWL-78 to inhibit binding to the different ICBs. For ICB1, abolition of binding was seen at 1 and 5  $\mu\text{mol}/\text{L}$  with ICB2 (Fig. 2) and 10  $\mu\text{mol}/\text{L}$  with ICB3 and was not observed at 50  $\mu\text{mol}/\text{L}$  with ICB4. A supershift obtained using antibodies to NF-Y-B confirmed the involvement of NF-Y in the complexes binding to ICBs



**Figure 2.** **A**, inhibition of NF-Y DNA binding by GWL-78. Oligonucleotides containing the CCAAT motif were incubated with control buffer (column 1), increasing concentrations (0.1, 1, and 5  $\mu\text{mol/L}$ ) of GWL-78 (columns 2-4), cDNA (column 5), or nonspecific DNA (column 6) for 2 h followed by incubation with HT-29 nuclear extracts. The DNA binding of NF-Y is measured by ELISA after incubation with a primary antibody specific to an epitope on the bound form of NF-Y. After a colorimetric reaction, absorbance at 450 nm was recorded using an automatic microplate reader.  $A_{450}$  proportional to NF-Y binding was measured for GWL-78-treated samples and values obtained were compared with  $A_{450}$  obtained from untreated nuclear extract (control). Data expressed as mean  $\pm$  SE percent reduction. **B**, inhibition of DNA binding by GWL-78 EMSA assays were carried out using oligonucleotides containing the ICB sequences present on the promoters of topo II $\alpha$  (**A-D**), *mdr1* (**E**), *cdc2* (**F**), cyclin B1 (**G** and **H**), E2F1 (**I**), and *cdc25* (**J** and **K**). In all cases, oligonucleotides were incubated with GWL-78 for 24 h before incubation with NIH3T3 cell extracts. Lanes 1 to 6, 0, 0.5, 1, 5, 10, and 50  $\mu\text{mol/L}$  GWL-78; single arrow, band corresponding to NF-Y bound oligonucleotide; lanes 7 and 8, mutated (M) and unlabeled (S) wild-type oligonucleotides. Supershifts (S) using an anti-NF-Y-A antibody (top arrow) indicate that the observed shift is caused by NF-Y binding.

within the topo II $\alpha$  promoter. The addition of GWL-78 following incubation of nuclear extract with radiolabeled probe showed that GWL-78 is able to displace protein bound to DNA.

The NF-Y transcription factor binds to CCAAT motifs within a wide variety of eukaryotic promoters. To investigate whether GWL-78 could inhibit NF-Y binding, we did EMSA experiments using radiolabeled oligonucleotides containing NF-Y binding sites from several promoters regulated by NF-Y (5). These included *mdr1*, *cdc2/cdk1* (cyclin-dependent kinase), E2F1, and cyclin B1 (Fig. 2). EMSA experiments showed that GWL-78 was able to inhibit protein binding to cognate NF-Y binding sites within these promoters at concentrations varying from 1 to 50  $\mu\text{mol/L}$ . Binding of GWL-78 to the ICB in *mdr1* was abolished at 50  $\mu\text{mol/L}$  and in *cdc2* at 10  $\mu\text{mol/L}$ . The two CCAAT boxes in cyclin B1 differed significantly with binding being abolished at 50  $\mu\text{mol/L}$  at CCAAT box 1 and at 5  $\mu\text{mol/L}$  at CCAAT box 2. In the case of the E2F1 sequence, binding was abolished at 10  $\mu\text{mol/L}$ . The two CCAAT boxes of *cdc25* did not differ with binding being abolished at 50  $\mu\text{mol/L}$  at both ICB1 and ICB2. Supershift experiments using an antibody to NF-Y-B confirmed that the transcription factor NF-Y was involved in binding to these sites as shown in previous studies.

#### **In vitro** Binding of GWL-78 to the Topo II $\alpha$ Promoter

To determine the pattern of binding of GWL-78 to specific sequences within the context of the topo II $\alpha$  promoter, *in vitro* DNase I footprinting was performed. A radiolabeled probe containing the topo II $\alpha$  promoter was incubated with varying concentrations of GWL-78 (Fig. 3A). There was a detectable footprint over the ICB2 sequence at 2.5  $\mu\text{mol/L}$ . Addition of recombinant NF-Y to DNA following incubation with GWL-78 showed that it was able to inhibit NF-Y binding to sequences within the topo II $\alpha$  promoter at 2.5  $\mu\text{mol/L}$  (Fig. 3B). The pattern of the footprint over the ICBs was altered with increasing concentration of drug resulting in a pattern similar to that obtained with drug and DNA alone. This pattern was observed whether the radiolabeled probe was incubated with drug followed by recombinant NF-Y or probe incubated with recombinant NF-Y followed by GWL-78. Therefore, GWL-78 is able to both block the interaction of NF-Y and DNA and displace NF-Y from DNA following binding.

#### **Demonstration of In vivo** Effects of GWL-78 by ChIP Assay

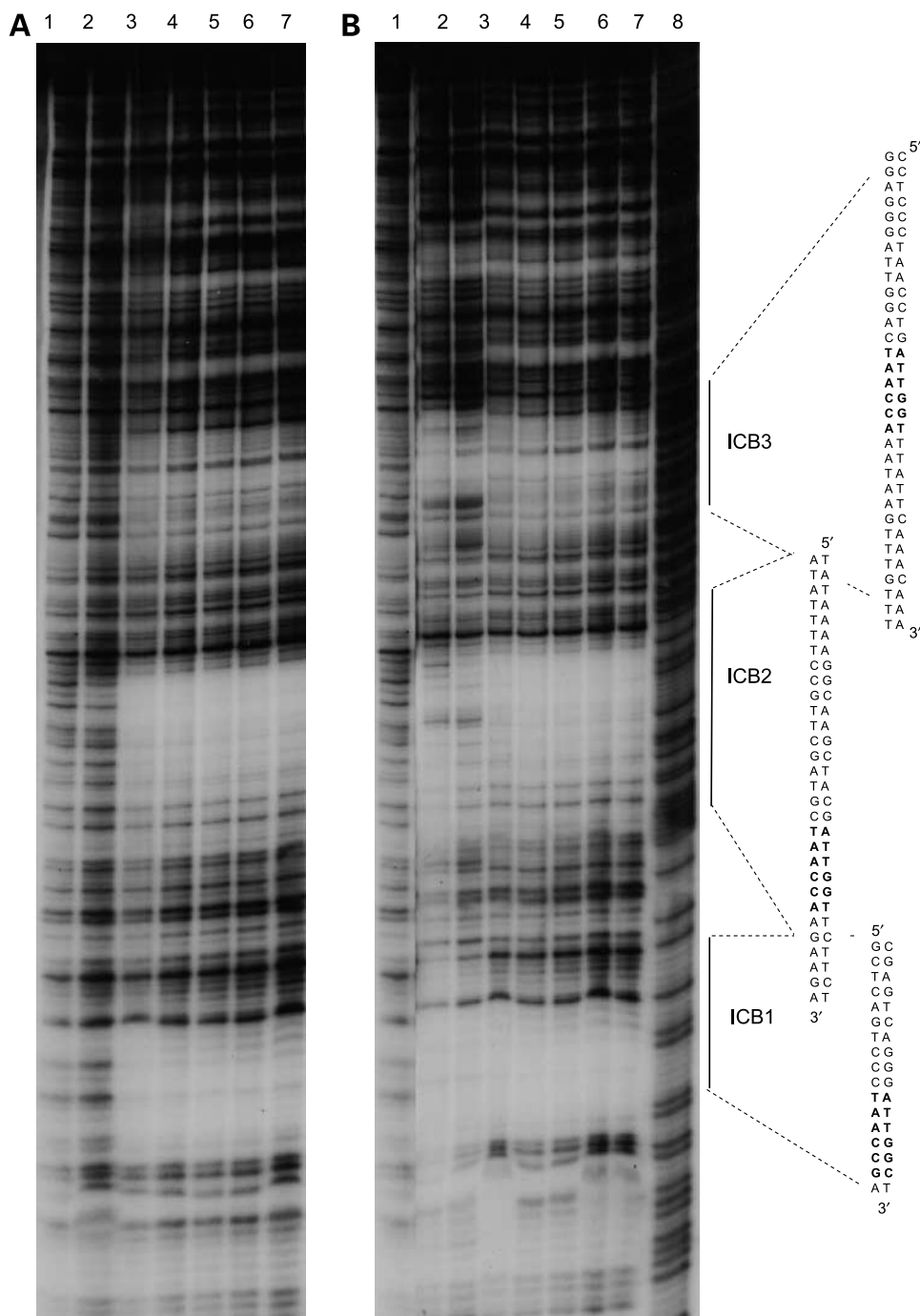
The EMSA experiments demonstrate the ability of GWL-78 to bind to inverted CCAAT sequences *in vitro*, but it was important to determine if these effects could be shown in intact cells. To assess the cellular effects of GWL-78 on the interaction of NF-Y with DNA, ChIP assays were called out on promoters examined in Fig. 2 with antibody to NF-Y-B of the NF-Y heterotrimer. To investigate whether GWL-78 could inhibit binding of NF-Y to the topo II $\alpha$  promoter, NIH3T3 cells were treated with increasing doses of GWL-78 (0.1, 0.3, and 1  $\mu\text{mol/L}$ ), and extracts were immunoprecipitated with antibodies to NF-Y-B. Binding of NF-Y to the topo II $\alpha$  promoter was clearly shown by the ChIP assay, and inhibition of NF-Y binding by GWL-78 was observed at the lowest concentration of the drug and essentially complete at 1  $\mu\text{mol/L}$  (Fig. 4). In parallel, we analyzed binding to other promoters whose CCAAT boxes were

analyzed *in vitro*. *cdc2* showed a decrease at the lower doses and abolition at 1 mmol/L. *E2F1* and *mdr1* were affected only at the highest dose, whereas *cdc25c* showed only modest inhibition of NF-Y binding. As a control, we analyzed the CCAAT box in the NF-Y-A promoter, whose binding to NF-Y was not affected by GWL-78, and the CCAAT-less promoter nucleolin, which does not contain an ICB. Therefore, GWL-78 is able to enter the nucleus and inhibit transcription factor interaction with DNA with a

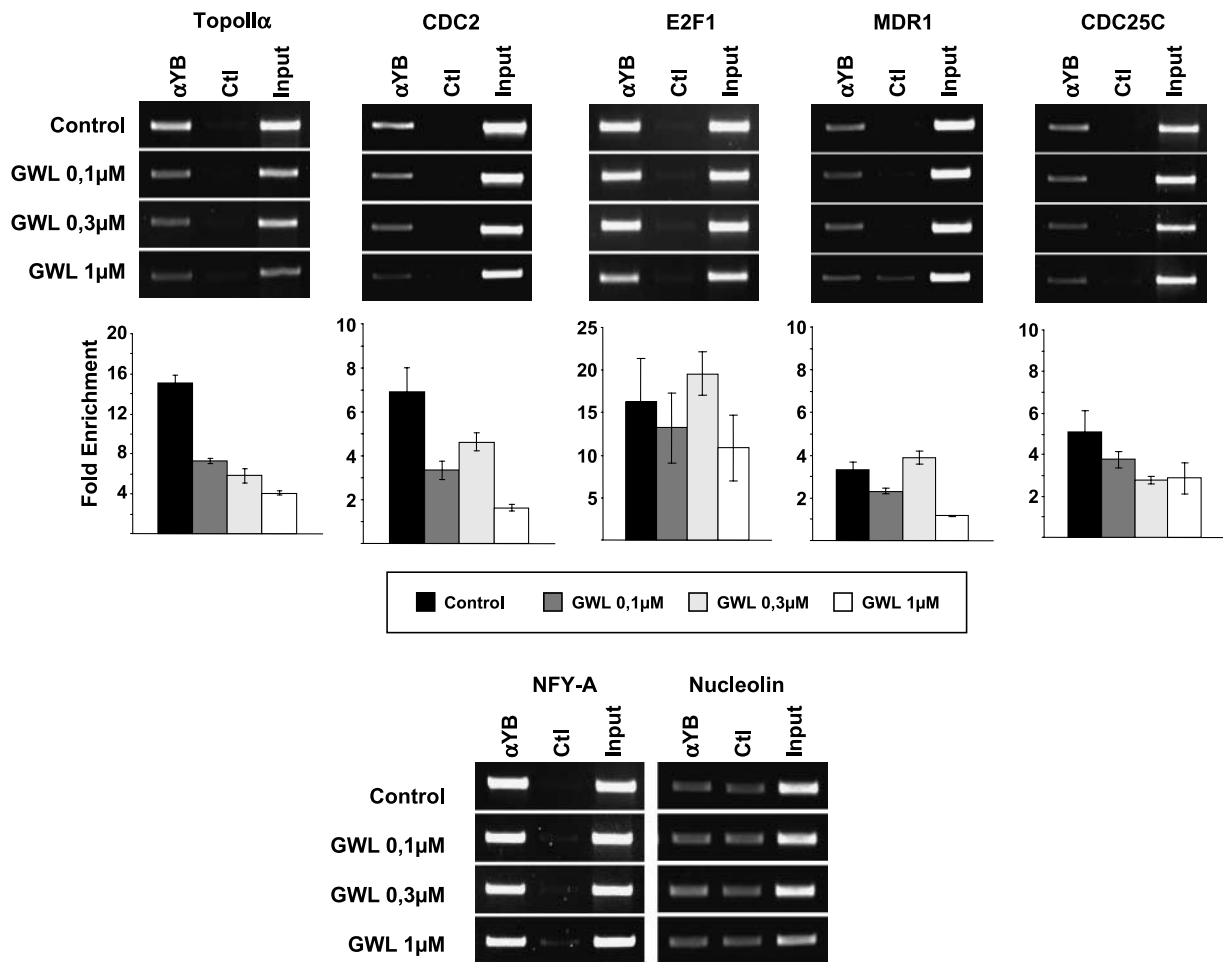
selectivity mirroring the *in vitro* binding inhibition data. Topo II $\alpha$  appears to be the most sensitive promoter in this assay.

**GWL-78 Induction of Cell Cycle Arrest**

Having shown that GWL-78 blocks NF-Y binding in cells, we then investigated the biological effects of cellular exposure. NF-Y has been shown to be a critical effector of cell cycle progression, and transfection of a dominant-negative NF-Y gene blocks mouse fibroblast cells in the



**Figure 3.** A and B, DNase I footprinting of the topo II $\alpha$  promoter with GWL-78. Experiments were performed in the presence of GWL-78 alone (A) and in combination with recombinant NF-Y protein (B). A, lane 1, untreated, DNase I-digested probe; lanes 2 to 7, probe incubated with 0.1, 2.5, 5, 7.5, 10, and 12.5  $\mu$ mol/L GWL-78, respectively, before digestion. B, lane 1, untreated digested probe; lane 2, probe incubated with recombinant NF-Y before digestion; lanes 3 to 8, samples incubated with 0.1, 2.5, 5, 7.5, 10, and 12.5  $\mu$ mol/L GWL-78 for 24 h before incubation with recombinant NF-Y protein; lane 9, GA lane. Thick vertical lines, ICBs. Examples of GWL-78 preferred binding sites emboldened (18).



**Figure 4.** Effects of GWL-78 on promoters using ChIP assay. NIH3T3 cells were treated for 24 h with 1 μmol/L GWL-78 and ChIP with control (*Flag*) and anti-NF-Y-B antibodies. The results on CCAAT promoters for topo IIα, E2F1, mdr1, cdc25c, NF-Y-A, and nucleolin, are also shown. Analyses were done with semiquantitative PCR and the data were plotted after scanning of several amplifications in the linear range. Enrichments of the treated samples over the control *Flag* material were shown as percentage of enrichments of untreated controls: 14-fold (topo IIα), 7-fold (cdc2), 16-fold (E2F1), 4-fold (mdr1), and 5-fold (cdc25c). Enrichment of NF-Y-A was >10-fold. No enrichment was observed on the CCAAT-less nucleolin.

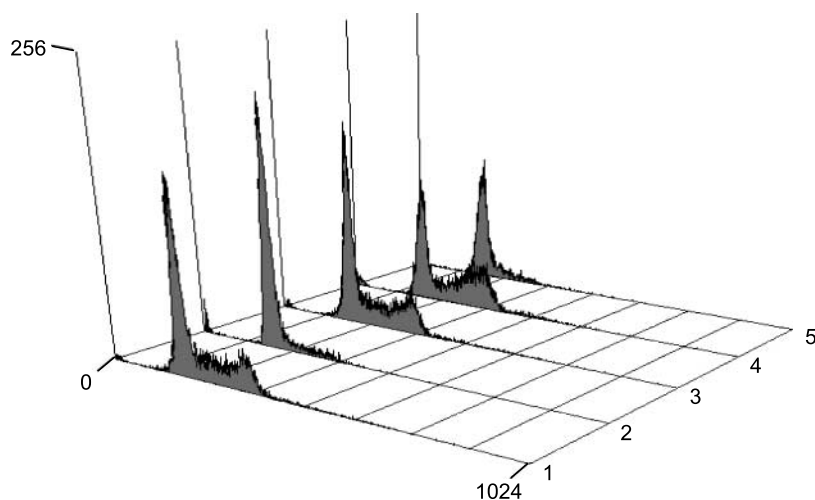
S phase of the cell cycle (7). To determine if GWL-78 could inhibit NF-Y function by blocking DNA-protein interactions, the effects of GWL-78 on cell cycle progression were investigated.

NIH3T3 cells were serum starved and treated with 0.4 mmol/L mimosine to induce G<sub>1</sub> arrest. Release of cells by addition of fresh medium containing 10% serum resulted in progression through the cell cycle as shown by flow cytometric analysis (Fig. 5). Following incubation with 1 μmol/L GWL-78, there was G<sub>1</sub> arrest at 24 h. Lower doses of GWL-78 (0.1 and 0.5 μmol/L) had a minimal effect on cell cycle progression, and cells progressed through the cell cycle in a normal or slightly more rapid manner in NIH3T3 cells. Figure 5 indicates the percentage of cells in each phase of the cell cycle.

Cells were synchronized by serum starvation and mimosine treatment before release in the presence of GWL-78. Mimosine treatment coupled with serum starva-

tion arrested the cells at the G<sub>1</sub> phase of the cell cycle and caused the levels of topo IIα protein to fall in NIH3T3 cells (Fig. 6A). Subsequent release from cell cycle arrest caused the levels of topo IIα protein to rise as the cells progressed through the cell cycle. In NIH3T3 cells treated with 0.1 μmol/L GWL-78, levels of protein increased as the cells progressed through the cell cycle. Treatment with 0.5 μmol/L GWL-78 on release from synchronization also resulted in an increase in the levels of topo IIα, although levels did not return to those observed in untreated and exponentially growing cells. In contrast, cells treated with 1 μmol/L GWL-78 did not progress through the cell cycle. Consequently, the levels of topo IIα did not increase in these cells when released from cell cycle arrest.

Levels of cdk2 and cyclin B1 protein were also examined. The levels of both proteins fell on serum starvation and increased in untreated cells on release from synchronization to levels similar to those of normally cycling cells. On



**Figure 5.** Effect of GWL-78 on cell cycle progression. Synchronized NIH3T3 cells were treated with GWL-78 for 24 h after release from synchronization and analyzed by flow cytometry. Cells were synchronized by serum starvation and treated with 0.4 mmol/L mimosine for 14 h before release into the cell cycle. 1, exponentially growing cells; 2, synchronized cells; 3, synchronized but untreated cells allowed to cycle normally and used as controls; 4 and 5, cells treated with 0.1 and 1  $\mu$ mol/L GWL-78, respectively. Cells were stained with propidium iodide and the percentages of total cells in the different phases of the cell cycle were calculated (see Table).

NIH3T3 cells	G1	S	G2
Exponential	45.9 +/- 1.6	46 +/- 0.6	6.1 +/- 2
Serum starved	70.5 +/- 2.7	25.7 +/- 0.9	2.7 +/- 0.4
0	14.4 +/- 1.9	47.3 +/- 1.5	35.7 +/- 0.8
0.1	10.4 +/- 0.9	45.5 +/- 2.3	38.8 +/- 1.3
1	68.7 +/- 1.9	25.1 +/- 1.1	3.1 +/- 0.8

treatment with both 0.1 and 0.5  $\mu$ mol/L GWL-78 cdk2 levels also increased although not to the levels observed in untreated cells. Cyclin B1 only increased when 0.1  $\mu$ mol/L GWL-78 treatments were carried out. No increase in either protein was observed at 1  $\mu$ mol/L GWL-78 concentration (Fig. 6B).

It was possible that the biological effects of GWL-78 were secondary to induction of p53 and consequent cell cycle arrest rather than being specifically due to the effects of the drug on blockade of protein/DNA interactions. We assessed induction of p53 following incubation of cells with GWL-78. As shown in Fig. 6C, there was no induction of p53 in cells following GWL-78 treatment at a dose that caused cell cycle arrest (1  $\mu$ mol/L). In contrast, exposure to both 50 and 100  $\mu$ mol/L etoposide resulted in induction of p53. Therefore, it is likely that the biological effects of GWL-78 in these experiments are not due to nonspecific effects through DNA damage-induced induction of p53.

## Discussion

The heterotrimeric transcription factor NF-Y has been implicated in a variety of cellular processes, including cell cycle progression and differentiation. A study using transfection of a dominant-negative NF-Y expression plasmid showed that NF-Y is essential for cell cycle progression (7). NF-Y has been shown to exert both stimulatory and inhibitory effects on gene expression. For example, we showed the ability of the bis-benzimidazole Hoechst 33342 to block NF-Y binding to the topo II $\alpha$

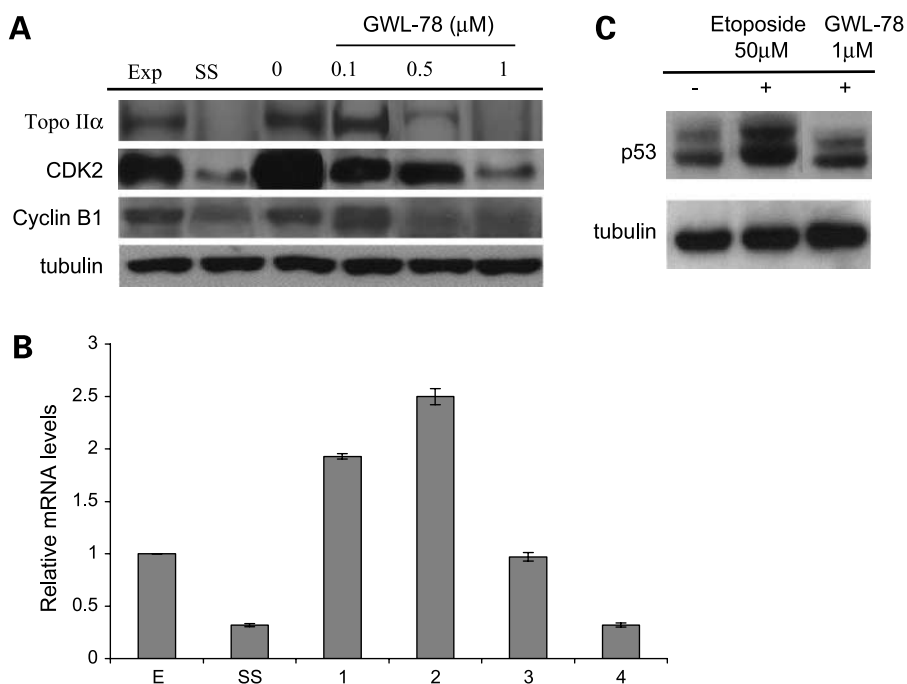
promoter and thereby increase cellular expression of topo II $\alpha$  in confluent cells (4). In confluent cells, NF-Y has an inhibitory effect and abrogation of NF-Y binding to the promoter results in de-repression and thereby increased levels of topo II. In contrast, in cycling cells, topo II $\alpha$  is critical for cell cycle progression; inhibition of NF-Y results in decreased topo II $\alpha$  expression. Several other agents have been shown to inhibit NF-Y/DNA interactions, including the minor-groove binder distamycin (22).

Ideally, one could envisage altering gene expression by using sequence-selective DNA-binding agents to interfere with transcription factor-DNA interactions. In this scenario, human genomic DNA would be the intended target and it is unclear whether the DNA-binding drugs described thus far would be sufficiently selective for a specific sequence to avoid being quenched by the large mass of genomic DNA. This is exemplified by the example of NF-Y, which has 56,000 potential CCAAT box binding sites on chromosome 21 alone, yet ChIP on chip experiments only detected some 1,000 sites *in vivo*.<sup>4</sup> Some of this selectivity is certainly due to the inaccessibility of large parts of the genome through compaction in heterochromatin. Some could be regulated by local, promoter-specific hindrance or preference for flanking sequences and recruitment by fellow transcription factors could be critical factors.

<sup>4</sup> Ceribelli et al., submitted for publication.

**Figure 6.** Expression of cell cycle-regulated genes after GWL-78 incubation.

**A**, Western blots of NIH3T3 cells synchronized by serum starvation and 0.4 mmol/L mimosine treatment (SS) before treatment with GWL-78 at 0.1, 0.5, and 1  $\mu\text{mol/L}$  concentrations. Row 1, topo II $\alpha$  protein of 170 kDa detected using a rabbit anti-topo II $\alpha$  specific antibody, rows 2 and 3, proteins detected using cdk2 and cyclin B1 antibodies, respectively; row 4, loading controls. Samples obtained 24 h after release into the cell cycle in the presence of GWL-78 are shown. **B**, real-time PCR analysis of mRNA obtained from NIH3T3 cells synchronized and treated with GWL-78. The level of topo II $\alpha$  mRNA in exponentially cycling cells was set at a value of 1 (E). Columns 1 to 4, levels of mRNA present in cells on release from synchronization in the presence of 0, 0.1, 0.5, and 1  $\mu\text{mol/L}$  GWL-78, respectively. **C**, Western blots of NIH3T3 cells treated with 1  $\mu\text{mol/L}$  GWL-78 for 24 h and probed with antibodies to p53 were compared with cells treated with 50  $\mu\text{mol/L}$  etoposide. Bottom, loading controls.



In this study, we have investigated the ability of the PBD-poly(*N*-methylpyrrole) conjugate GWL-78 to block NF-Y binding to DNA. We have shown that following exposure of cells to GWL-78, there is inhibition of cell cycle progression. Interestingly, lower doses of GWL-78 appear to induce more rapid traverse through the cell cycle. The mechanism for this differential effect of GWL-78 is unclear. It could be due to the differential effects of GWL-78 on different promoters depending on the concentration of drug.

Our study suggests that the effects of GWL-78 on gene expression are mediated by a transcriptional block following cellular exposure to drug. Interestingly, this does not appear to involve induction of p53. The data from our study, including EMSA and DNase I footprinting assays, suggest that GWL-78 blocks NF-Y interaction with cognate binding sequences within DNA. Additionally, the DNase I footprint analysis shows that GWL-78 is able to displace NF-Y already bound to the promoter. The result of the ChIP assay confirms the inhibitory effects of GWL-78 in cells. Thus, the effects of GWL-78 are comparable with those found in a study where expression of a dominant-negative NF-Y construct blocked cell cycle progression (7). Most importantly, the effects observed *in vivo* are a good match with inhibition data. In terms of preference for CCAAT boxes, topo II $\alpha$  > cdc2 > mdrl = E2F1 > cdc25. Remarkably, the NF-Y-A promoter, which has four CCAAT boxes, is refractory to GWL-78 treatment. This indicates that NF-Y disappearance from promoters is not due to interference with NF-Y-A production and emphasizes the sequence selectivity and promoter preference of the compound. In some of the EMSA studies (e.g., cyclin B1), there was an appearance of increased protein binding at lower drug

concentrations, which could suggest conformational changes in DNA resulting in higher-affinity NF-Y binding. Interestingly, the ChIP assay shows increased NF-Y binding to the E2F1 and mdrl promoters at lower drug concentrations. However, the inhibition of NF-Y binding to the topo II $\alpha$  promoter is found even at low concentrations of GWL-78. There is no direct correlation between results of EMSA and ChIP assays. *In vivo* binding shown in the ChIP assay is dependent on several factors not affecting EMSA, including cell line, intracellular transport mechanisms, and presence of motifs modulating NF-Y binding such as SP1-binding sites (23).

These results suggest that GWL-78 may be exerting its biological effects by binding selectively to key DNA sequences, especially those associated with the ICBs, and leading to transcription block. Several potential binding sites, which fit the consensus motif for GWL-78 as reported by Wells et al. (18) (that is, 5'-XGXWz, where z = 3  $\pm$  1, W = A or T, X = any base but preferably a purine), are evident in the DNA sequences relating to ICB1 to ICB3. One binding site example from each ICB is highlighted in bold in the sequence labels of the footprints in Fig. 3, although there are many more.

We recently showed that a polyamide directed against the binding site of NF-Y in the topo II $\alpha$  promoter was able to inhibit NF-Y binding and alter gene expression in confluent cells (2). Other studies have shown that polyamides can modulate gene expression *in vivo* (24). An important difference with GWL-78 is the apparent progression of cell cycle found with lower concentrations. NF-Y has been shown to have pleiotropic transcriptional effects on different genes with both activation and repression of promoters. It is possible that GWL-78 may

affect a differing population of promoters binding NF-Y at different concentrations. Given the large number of promoters with consensus NF-Y binding sites, it is likely that it is the pattern of promoter repression that determines the effects of GWL-78 at a given dose. To determine such patterns, we are currently using a ChIP on chip strategy.

In conclusion, by all criteria used here (EMSA, DNase I footprint, ChIP, and expression analysis), the PBD-polyamide conjugate GWL-78 shows the ability to inhibit specific DNA-protein interactions. Its cellular penetration and consequent modulation of gene transcription suggest that such agents can be exploited to target the cellular transcription machinery. The balance between the cytotoxicity caused by agents of this type and their transcriptional effects will be a critical factor in their therapeutic application and will be the subject of future investigation.

### Disclosure of Potential Conflicts of Interest

P.W. Howard, D.E. Thurston, and J.A. Hartley: equity interest in Spirogen Ltd., owner of GWL-78; D. Hochhauser: consultancy fees from Spirogen Ltd. The other authors reported no potential conflicts of interest.

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