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CONTRACTING ORGANIZATION: Health Research, Incorporated
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13. ABSTRACT ***(Maximum 200 Words)***

Disregulated transcription factor (TF)-mediated activation of gene expression may play a key role in oncogenesis, especially in breast cancer. Preventing TF/DNA interactions using small molecule DNA-reactive agents can decrease oncogenic gene expression and potentially halt cancer development. Our goal is to improve DNA-binding drugs' abilities to inhibit specific TF/DNA interactions using the human c-fos promoter's serum response element (SRE) as a target. The targeted TFs form a ternary complex (TC) on the SRE that is required for induction of c-fos by serum. Classical DNA-binding drugs were first evaluated in order to better understand how sequence selectivities and modes of DNA binding relate to drug effectiveness in inhibiting TF/DNA complexes and resultant transcription. The drugs' abilities to prevent TC formation on the SRE *in-vitro* was nogalamycin > Hoechst 33342 > chromomycin. Their potencies in inhibiting cell-free transcription and endogenous c-fos expression in NIH3T3 cells, however, was chromomycin > nogalamycin > Hoechst 33342. The latter order of potency was also obtained for the drugs' cytotoxicity and inhibition of general transcription as measured by [^{3}H]uridine incorporation. These systematic analyses of classical DNA binding drugs should provide insight into how novel DNA-binding drugs (polyamides and microgonotropens) might be modified to yield more specific and potent agents.

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Christine M. White June 6 2000

PI - Signature Date

Christine M. White June 6, 2000

Table of Contents

Manuscript copies and Poster Abstract copies have been attached to the end of this report. Please note that manuscript has not been renumbered.

Introduction:

Disregulated or inappropriate expression of key growth controlling genes are events that can play pivotal roles in oncogenesis. This aberrant gene regulation may be caused by overexpression or abnormal activation of transcription factors (TFs) (1). For example, overexpression of members of the *ets* TF family, such as PEA3 and ESX, has been associated with the development of early stage breast cancer and with the upregulation of Her2/Neu, a receptor tyrosine kinase overexpressed in nearly 30% of breast cancer cases (2, 3). By preventing or disrupting the binding of specific TFs to their target sites on growth-promoting genes, abnormal gene expression can be inhibited and the development or progression of cancer may be halted (4). Classical DNA-binding drugs, such as distamycin, are capable of inhibiting TF/DNA interactions through both steric hindrance and by changing the conformation of the DNA helix (5, 6). However, their use as therapeutics is limited by their lack of specificity. Their non-specific binding to many regions of DNA leads to general inhibition of transcription and substantial cytotoxicity in both normal and cancer cells (7, 8). By improving agents' abilities to distinguish between sequences, selective inhibition of desired target genes might be achieved. Our goal is to evaluate the effectiveness of DNA reactive agents as inhibitors of transcription in order to improve their specificity and potency; a goal that has implications in understanding the molecular regulation of genes as well as the development of therapeutics. In this study, the abilities of DNA-binding drugs to prevent TF/DNA complex formation are being assessed using the serum response element (SRE) of the human c-fos gene as a target. The SRE contains binding sites for a homodimer of serum response factor (SRF) and Elk-1, an *ets* TF (See attached manuscript, Fig. 2A) (9, 10). The binding and regulation of these factors and resultant c-fos transcription have been well characterized (11, 12), making the SRE an appropriate model to study and develop paradigms for drug targeting. The novel, rationally designed agents being analyzed include the polyamides, minor groove binding compounds that can be synthesized to specifically target desired DNA sequences (13), and microgonotropens, A/T rich binding drugs that make contact with both grooves of DNA (14). The effectiveness of these agents is being compared to that of classical, less sequence specific DNA binding drugs in order to gain a better understanding of how drugs' binding characteristics and sequence selectivities relate to their potential as inhibitors of TF binding. Agents were chosen on the basis of their contrasting sequence preferences and modes of DNA binding (See Fig. 1). Their ability to disrupt or prevent SRF and Elk-1 complexes was determined using electrophoretic mobility shift assays (EMSAs). Their inhibition of c-fos promoter-driven cell free transcription was also analyzed. Finally, drug effects on endogenous c-fos expression in whole cells was assessed using Northern blots, but these effects were analyzed in the context of the drugs' other whole cell activities, including their cytotoxicity and inhibition of general RNA synthesis. The systematic study of these agents in increasingly complex assay environments has yielded information which may be useful in strategically altering the structure of the novel agents in order to improve their activity and effectiveness.

Summary of Research and Training:

Materials and Methods/Protocol development:

Assay components were obtained from our collaborators (plasmids encoding SRF and Elk-1 proteins, the SRE sequence, and the human c-fos promoter were provided by Dr. Alfred Nordheim's laboratory), obtained commercially (remaining bioreagents, NIH3T3 cells and plasmids for Northern blot cDNA probes), or synthesized in-house (oligonucleotides for the EMSAs were synthesized at Roswell Park Cancer Institute's Biopolymer facility). Required preparations and purifications were then performed and proper assay conditions were established.

Evaluation of drugs' abilities to inhibit TF/DNA complex formation and resultant transcription was carried out in increasingly complex assay environments. The EMSA, which consists of purified TFs (SRF and Elk-1) and a radiolabeled oligonucleotide containing the SRE, analyzed the effectiveness of drugs to inhibit TF binding to their target sites. In this assay, the components are combined and electrophoresed on a polyacrylamide gel. The free radiolabled DNA migrates through the gel, but when TFs bind, it is retarded in its movement and a shifted complex can be visualized following autoradiography (See manuscript, Fig. 2B). Drug occupation of the TF target site on the DNA prevents the TF from binding, and the amounts of free and shifted DNA can be quantitated from densitometric analysis of the resulting autoradiograms. Percent inhibition of shift due to the drug can then be calculated by comparing drug treated samples to controls.

The cell-free transcription assay quantitated drugs' abilities to inhibit transcription of a plasmid driven by the human c-fos promoter in the presence of nuclear lysate and radiolabeled nucleotides. Prevention of TF binding to the promoter by drugs decreases formation of a radiolabeled transcript of known length, which is electrophoresed, autoradiographed, and quantitated using densitometry, as in the EMSAs. Drug effects on endogenous c-fos expression were also evaluated in NIH3T3 cells using Northern blots. Here, cells are treated with drug for varying times before being induced with serum to upregulate c-fos transcription. Total RNA is then isolated, electrophoresed on a denaturing agarose gel, transferred to a membrane, and probed for c-fos. Other biological analyses included measuring the agents' cytotoxicity using a NIH3T3 colony formation assay. Drug inhibition of general RNA synthesis was determined in NIH3T3 cells by quantitating [^{3}H]uridine incorporation into nucleic acids. The potencies of drugs in each assay were compared by calculating IC_{50} values (the drug concentration needed to inhibit the measured activity by fifty percent).

Detailed protocols are included in the attached manuscript, which describes the evaluation of chromomycin, Hoechst 33342, and nogalamycin (all commercially obtained drugs).

Dr. Thomas Bruice has provided us with several additional compounds. These microgonotropens (MGTs) (See Fig. 2) are novel, logically synthesized agents based on the structure of distamycin or Hoechst 33342. Analyses of these compounds using the c-fos SRE as a target, and comparison of their potencies and effects to those of the classical DNA-binding drugs, is currently underway.

Summary of Findings and Results:

Part 1: Analysis of classical DNA-binding drugs:

Our evaluation of chromomycin, nogalamycin and Hoechst 33342 (see enclosed manuscript) was, to our knowledge, the first study that systematically analyzed the effectiveness of drugs possessing different sequence selectivities and modes of DNA binding to target two different TF binding motifs in increasingly complex assays. Moreover, studying drugs with known mechanisms of action using the c-fos SRE as a target establishes a model system for drug testing and also provides standards to which novel drugs can be compared. The order of drug potency in inhibiting TF/DNA complex formation in EMSAs (nogalamycin > Hoechst 33342 > chromomycin) differed from the order of potency in inhibition of cell-free transcription and endogenous c-fos expression in NIH3T3 cells (chromomycin > nogalamycin > Hoechst 33342). The latter order of potency was also obtained for cytotoxicity and inhibition of RNA synthesis in NIH3T3 cells. Other notable findings from this study include:

• ***The ability of a drug to affect TF/DNA interactions does not depend solely on its sequence preference or mode of DNA binding.*** Surrounding DNA base pairs may influence DNA conformation and

affect the drugs' ability to associate effectively with its target sequence and inhibit TF binding. For example, it was expected that Hoechst 33342, an A/T rich binder, would be a potent inhibitor of SRF complex formation in EMSAs, since SRF binds to an A/T rich sequence. However, the IC_{50} value of Hoechst 33342 in this assay was 20 μM, over thirty times higher than nogalamycin's IC_{50} value of 0.6 μM (See manuscript, Fig. 3C). Hoechst compounds are known to prefer some A/T rich sites over others and the SRF binding site may not have the requisite sequence for optimal Hoechst 33342 binding.

- ***The binding of TFs themselves may influence a drugs' inhibitory ability.*** This was seen by comparing drug inhibition of SRF and Elk-1 complex formation with TC formation in the EMSAs. Nogalamycin's IC_{50} for inhibition of SRF and Elk-1 complexes alone were 0.6 μM and 2.5 μM, respectively, while its IC_{50} for TC formation was 1.25 μM (See manuscript, Fig. 3C), suggesting that the binding of both SRF and Elk-1 together in the TC may change the conformation of DNA to influence drug recognition and binding of its target sequence.
- ***Maintenance of drug potency in the more complicated cell-free transcription system was demonstrated.*** Compared to the EMSAs, drug activity and potency was maintained in the more complex environment of the cell-free transcription assay, suggesting that the presence of additional proteins and DNA of greater length and sequence complexity did not interfere with these agents' effectiveness as transcriptional inhibitors. However, this may be drug and/or target dependent, since previous studies by other groups using different TFs and DNA sequences often reported that drug potency was lower in cell-free transcription assays.
- ***Chromomycin was the most potent agent in inhibiting endogenous c-fos expression in Northern blot assays and also worked the most quickly.*** This was evidenced by its 40% inhibition of c-fos expression after just 40 minutes of drug exposure (See manuscript, Fig. 7). This suggests that chromomycin enters cells relatively quickly and easily.
- ***Interestingly, Hoechst 33342 was more potent in inhibiting cellular c-fos expression after shorter exposure times.*** Compare the IC_{50} values of 5 μM for a one hour exposure with 20 μM for a four hour exposure in manuscript Fig. 8. This suggests Hoechst may be getting broken down or expelled from cells over time.
- ***The agents' effects on gene expression may be associated with their toxicity.*** The similarity in IC_{50} values for the cytotoxicity, RNA synthesis and Northern blot assays following four hour drug exposures (See manuscript, Table 1) suggests that the inhibition of general transcription of these agents is reflected by their toxicity.

In summary, this study furthers the understanding of how drugs' characteristics influence their ability to inhibit transcription. In light of the findings here and those from previous studies (5, 15), it appears that drug effects on transcription are also dependent on the TFs and DNA sequence targeted. This study also underlies the importance of developing sequence specific drugs for future effective therapeutic use. Optimal agents with higher specificity will potentially bind to their target site only, thereby avoiding non-specific DNA interactions that can lead to the cytotoxicity observed in this study. These analyses therefore provide a benchmark to which other drugs, especially novel, more sequence selective agents, can be compared. Additionally, this study demonstrated the utility of the c-fos SRE model in analyzing drugs:

- It allows for drug effects on more than one TF to be analyzed in a single system.
- It provides a systematic and relatively quick way to evaluate drugs in environments of varying complexity.
- The serum inducibility of c-fos and its short mRNA half life of only 30 minutes facilitated the assessment of immediate or short-term drug effects in whole cells.

Part 2: Analysis of the novel MGTs:

The novel MGTs synthesized by the Bruice laboratory (Fig. 2) bind to A/T rich regions in the minor groove of DNA, but make contacts with the phosphodiester backbone of DNA in the major groove using polyamine tails (16). MGT6a is based on the structure of distamycin and has a polypyrrole backbone. Our lab previously demonstrated that this compound was approximately three orders of magnitude more effective

in preventing the association of the E2F TF with its target sequence on the dihydrofolate reductase promoter (17). FMGT-1 and FMGT-2, which had not previously been analyzed for their inhibition of TF complex formation, are based upon the bis-benzimidazole structure of the Hoechst compounds (18). These agents were analyzed using the c-fos SRE as a target in the battery of assays described above. Their potencies were then compared to their parent compounds, distamycin and Hoechst 33342.

• Effects of MGTs on TF/DNA complex formation: The ability of the MGTs and their parent compounds to prevent SRF and TC formation on the SRE was evaluated using EMSAs (Fig. 3). The MGTs were more potent than their parents in preventing complex formation. For SRF complex inhibition, MGT6a was four times more potent than distamycin, while FMGT-1 and FMGT-2 were 25 and 40 times more potent than Hoechst 33342. Likewise, for TC inhibition, MGT6a was 1.3 times more potent, while FMGT-1 and FMGT-2 were about 6 and 10 times more potent than their parent compounds, respectively. Determination of drug effects on Elk-1 complex formation was not possible due to well retention of the drug treated DNA.

• MGTs' inhibition of cell-free transcription: The ability of the MGTs and their parents to inhibit c-fos promoter driven cell-free transcription was evaluated (Fig. 4). The MGTs and their respective parent compounds were equally potent here, as evidenced by their identical IC_{50} values: MGT6a and distamycin both had IC_{50}s of 7 μM, while the FMGTs and Hoechst 33342 all had IC_{50}s of about 17 μM. This loss of potency cannot be easily explained, but may be due to the presence of additional proteins in this assay. Components of the nuclear extract may be interacting non-specifically with the MGTs and thereby preventing their optimal binding. If the polyamine tail is being blocked from interacting with the DNA backbone, binding affinity will be lost, and the MGTs may become more like their parent compounds in that they're able to interact only with the minor groove.

• Cellular effects of the MGTs: To date, none of the MGTs have inhibited endogenous c-fos expression or caused a cytotoxic response in NIH3T3 cells as measured by Northern blot analysis (data not shown). Likewise, cytotoxicity has not been observed at MGT concentrations up to 10 μM in continuous, three day toxicity assays. This suggests that the drugs may not enter or be retained by cells. However, further investigation is required to determine why the MGTs do not exhibit cellular activity.

Part 3: Ongoing and future studies:

• Further analysis of MGTs' properties and characteristics: In order to better understand the lack of cellular activity of MGTs, we have undertaken to assess their uptake into cells. An assay to quantitate uptake was established using Hoechst 33342 and Hoechst 33258 (structures in Fig. 1). These drugs were chosen for preliminary analysis because Hoechst 33342 was able to decrease c-fos expression and cause cytotoxicity in NIH3T3 cells. Hoechst 33258, which differs from Hoechst 33342 only on the basis of its terminal ring substituent, did not show any cellular activity in our studies (data not shown). In the uptake assay, NIH3T3 cells are treated with varying drug concentration for four hours before being lysed in an acidic alcohol solution. The membranes and solid components are pelleted before the fluorescence of the supernatant is quantitated on a spectrofluorophotometer. Quantitation showed that Hoechst 33342 was taken up by cells by about an order of magnitude better than Hoechst 33258 (Fig. 5). The presence of the ethoxy ($-OCH_2CH_3$) group on Hoechst 33342 makes it more lipophilic than Hoechst 33258. Other studies have noted a difference in uptake between the two compounds (19, 20). Addition of the ethoxy group to the Hoechst based FMGTs may improve their activity and we are currently pursuing this prospect in collaboration with Dr. Bruice.

• Evaluation of the novel polyamides: Dr. Peter Dervan has collaborated with our lab in designing a number of polyamides to target the c-fos SRE. These polyamides are hairpin molecules consisting of repeating units of N-methylpyrrole (Py) and N-methylimidazoles (Im) (See Fig. 6). By pairing these groups across from one another in the drugs' structures, specific base pairs in desired DNA sequences can be targeted. A Py/Py will target A/T or T/A base pairs while Py/Im and Im/Py target G/C and C/G base pairs, respectively (21). These agents bind to DNA in the minor groove and have been shown to be very effective and potent inhibitors of TF/DNA complexes in a variety of systems (22, 23), including binding of the ESX TF to the Her2/Neu promoter (24). Polyamides can be synthesized to recognize up to sixteen base pairs (25).

These agents' DNA-binding characteristics may make them potent inhibitors of TC formation on the SRE. We plan to evaluate these compounds using the assays described above.

Training:

My work on this project has given me the opportunity to develop the assay techniques summarized above. In so doing, I have learned how to perform basic laboratory techniques: growth and use of bacterial and cell cultures, purification of recombinant, tagged proteins as well as DNA oligonucleotides and plasmids, use and application of gel electrophoresis (both SDS-PAGE and agarose gel electrophoresis for proteins, RNA and DNA), proper handling of radioactive and biohazardous waste, and use of lab equipment (microscopes, centrifuges, spectrophotometers, etc.). Additionally, I have become increasingly proficient at data evaluation and presentation. In particular, writing my first manuscript and presenting my first poster on the data obtained in this report has helped hone my writing and public speaking skills.

In addition to the scientific and presentation skills I have obtained, I have also learned how to evaluate data and think more critically. In working with our collaborators, I have learned how to best apply new knowledge to drug development and am now suggesting new strategies for drug design.

Appendices

A) Key Research Accomplishments:

1) Evaluation of DNA-binding drugs' abilities to disrupt TF binding to the c-fos SRE.
   - Obtained and prepared all components required for EMSA analyses of drug effects on: SRF/SRE complexes, Elk-1/E74 complexes and SRF/Elk-1/SRE (ternary complex). Developed optimal running conditions for each complex.
   - Analyzed classical DNA-binding drugs with contrasting sequence selectivities and modes of binding (chromomycin A_3, Hoechst 33342, nogalamycin and distamycin); obtained IC_{50} values for each.
   - Initiated analysis of the novel MGTs provided by Dr. Thomas Bruice's lab (MGT6a, FMGT-1 and FMGT-2); obtained IC_{50} values for each.

2) Evaluation of drugs' abilities to inhibit cell-free, c-fos promoter driven transcription.
   - Prepared necessary components required for this assay and established optimal running conditions.
   - Analyzed the classical DNA-binding drugs listed above and obtained IC_{50} values for each.
   - Analyzed the MGTs listed above and obtained IC_{50} values for each.

3) Evaluation of drugs' effects on endogenous c-fos expression in NIH3T3 cells.
   - Established optimal seeding densities, growing conditions, serum induction durations, and drug exposure times for the NIH3T3 cells.
   - Obtained and prepared the necessary Northern blot assay components.
   - Analyzed the classical DNA-binding drugs listed above using various exposure times and obtained IC_{50} values for each.
   - Initiated analysis of the MGTs in this assay.

4) Evaluation of drugs' other biological effects in NIH3T3 cells.

   Cytotoxicity Assays:
   - Determined optimal plating densities for both 3-day drug exposure assays as well as 10 day colony formation assays
   - Analyzed classical DNA-binding drugs in 10 day colony formation assays and obtained IC_{50} values for each.
   - Assessed the cytotoxicity of chromomycin, Hoechst 33342, Distamycin and the MGTs after 3 days of continuous exposure and obtained IC_{50} values.

   [^{3}H]-Uridine Uptake and Incorporation (RNA Synthesis) Assay:
   - Established assay conditions, then determined classical DNA-binding drugs' effects on general transcription.

Appendices (continued)

B) Reportable Outcomes:

1) Manuscripts:

"Evaluation of the effectiveness of DNA-binding drugs to inhibit transcription using the c-fos serum response element as a target"

Submitted. Manuscript (1 original, 2 copies) has been enclosed.

2) Presentations:

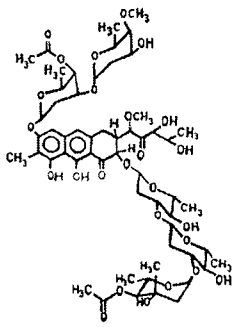
Poster presentation:

"Evaluation of the effectiveness of DNA-binding drugs to inhibit transcription using the c-fos serum response element as a target"

Presented at the Sigma Xi student poster competition at the State University of New York at Buffalo (4/25/00). Poster abstract (1 original, 2 copies) has been enclosed.

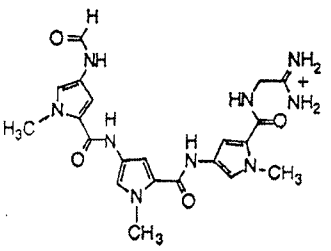
Appendices (continued)

C) Figures



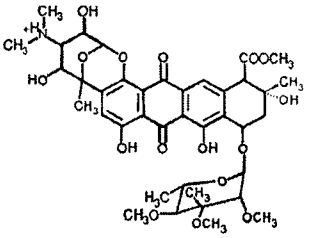
Chromomycin

Binds G/C rich regions in the minor groove.



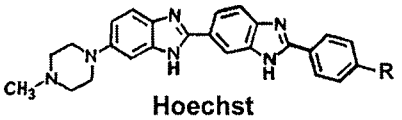
Distamycin

Binds A/T rich regions in the minor groove.



Nogalamycin

Intercalates preferentially into regions of alternating purines and pyrimidines.

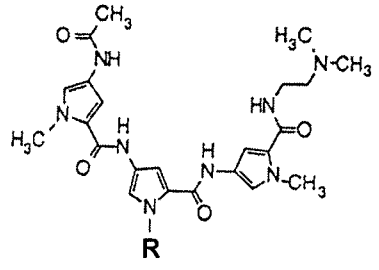


Hoechst

Hoechst 33342 R = $-OCH_2CH_3$
Hoechst 33258 R = -OH

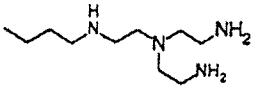
Bind A/T rich regions in the minor groove.

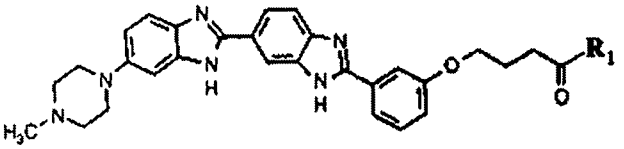
Figure 1: Structures and characteristics of selected classical DNA-binding drugs.



MGT

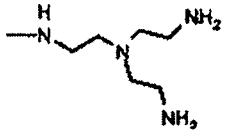
6a R=





FMGT

FMGT-1 R_1 =



FMGT-2 R_1 =

Figure 2: Structures of the microgonotropen (MGT) and fluorescent microgonotropens (FMGTs) provided by Dr. Thomas Bruice's laboratory.

These agents bind to A/T rich regions in the minor groove of DNA, but their polyamine tails make contact with the phosphodiester backbone in the major groove.

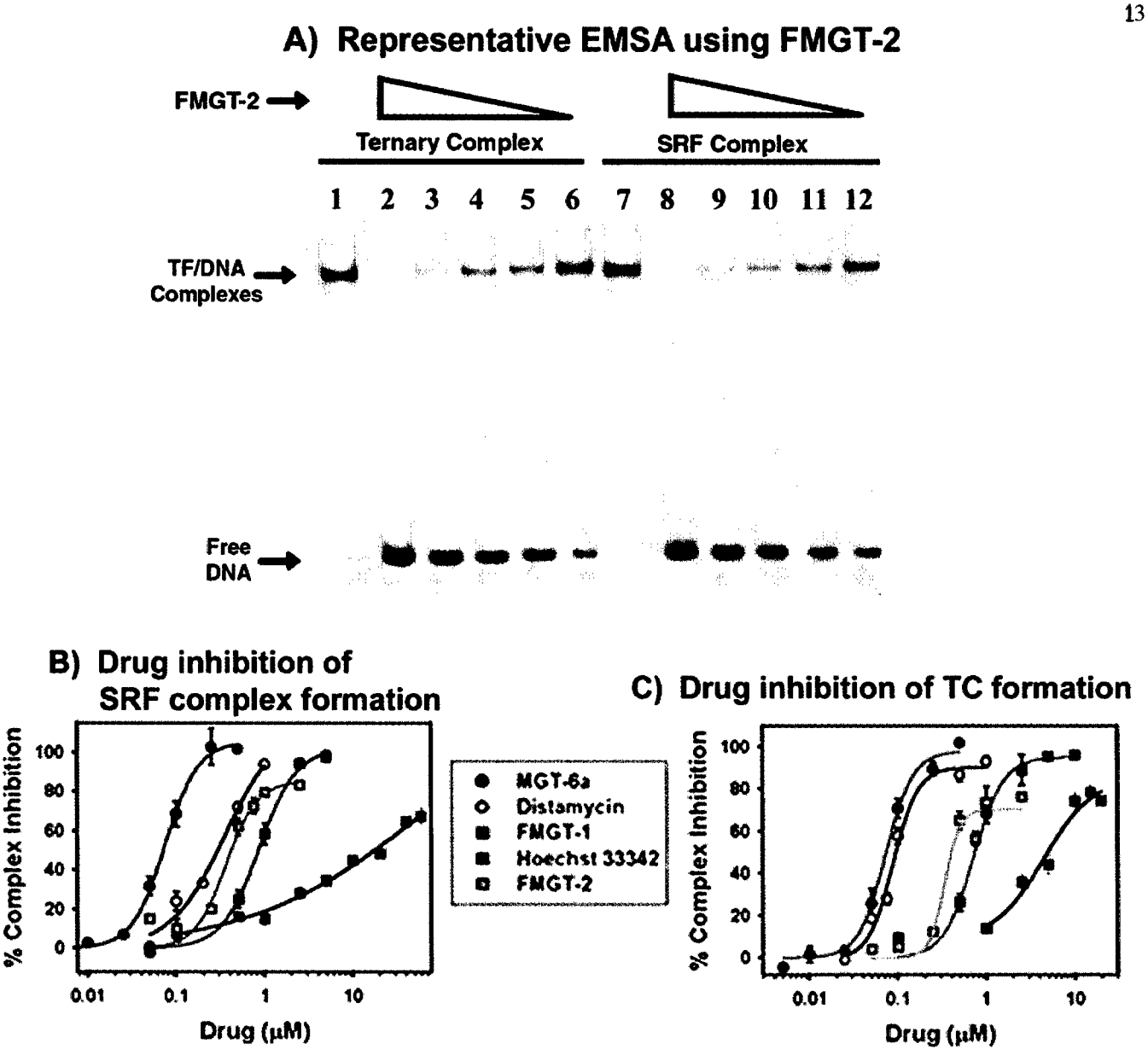


Figure 3: Effects of MGTs on TF/DNA complex formation in EMSAs.

A. FMGT-2 was incubated with radiolabeled SRE for 30 minutes prior to the addition of purified TFs (SRF alone in lanes 7-12, SRF and Elk-1 in lanes 1-6). After an additional 30 minute incubation, the complexes were electrophoresed on a polyacrylamide gel and visualized following autoradiography. *Lanes 1-6*: TC inhibition. *Lane 1*, Control; *Lane 2*, 10 μM; *Lane 3*, 1 μM; *Lane 4-5*, 0.5 μM; *Lane 6*, 0.1 μM. *Lanes 7-12*, SRF complex inhibition. *Lane 7*, Control; *Lane 8*, 10 μM; *Lane 9*, 1 μM, *Lanes 10-11*, 0.5 μM; *Lane 12*, 0.1 μM.

B. and ***C.*** Free and complexed SRE was quantitated by densitometry of the autoradiograms. Percent complex inhibition was calculated by comparing drug treated samples with controls. Curves were plotted for each drug analyzed.

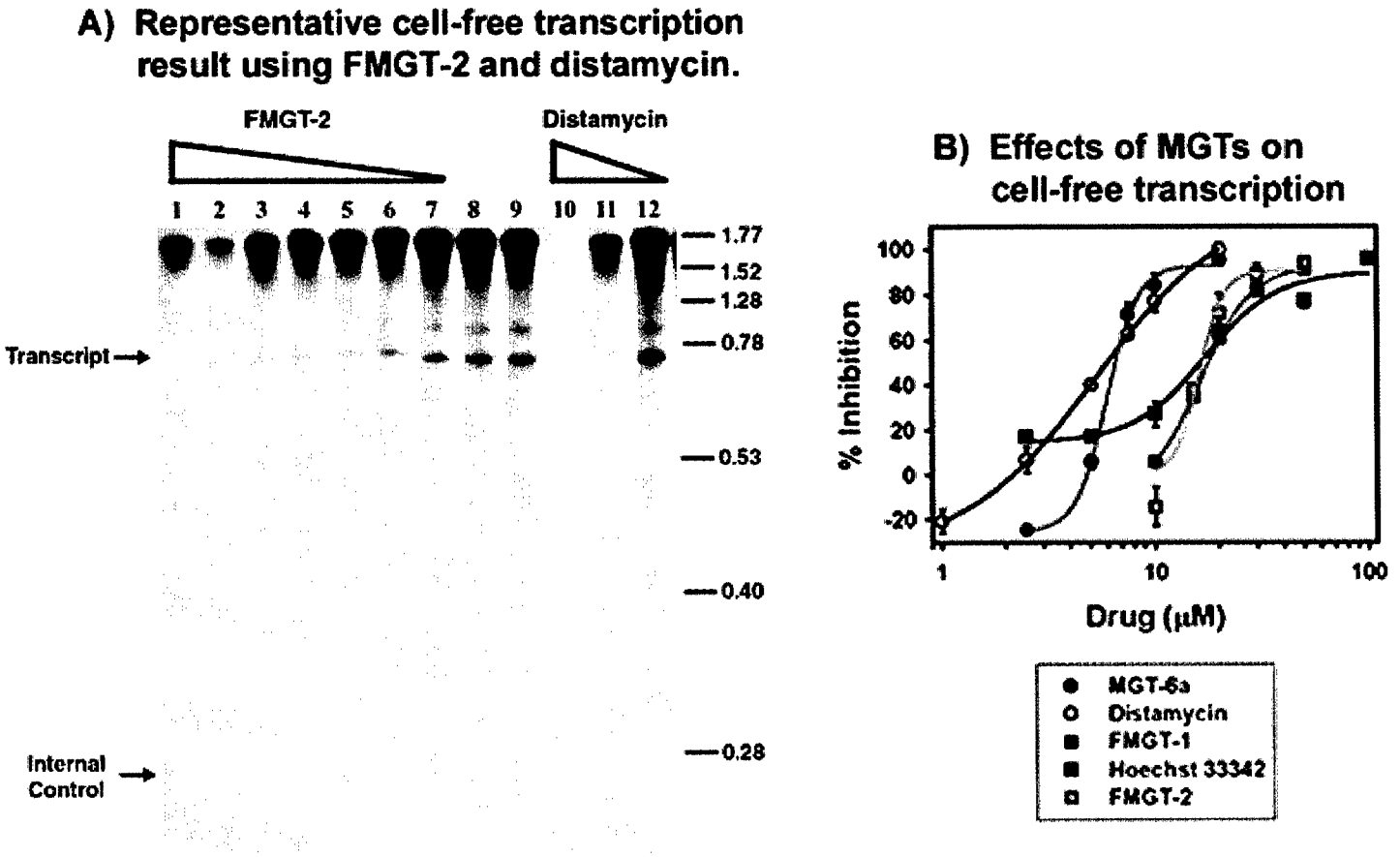


Figure 4: Effects of MGTs on c-fos promoter driven cell-free transcription.

A. A plasmid containing the c-fos promoter was linearized and incubated with FMGT-2 or distamycin for 30 minutes before nuclear lysate from serum induced NIH3T3 cells and radiolabeled nucleotides were added. The resulting 750 base transcript (top arrow) was normalized to a 250 base internal control (bottom arrow), which was added to each sample prior to loading on a denaturing polyacrylamide gel. Results were visualized following autoradiography. *Lanes 1-7*, FMGT-2 treatment. *Lanes 1-2*, 30 μM; *Lanes 3-4*, 20 μM; *Lanes 5-6*, 15 μM; *Lane 7*, 10 μM. *Lanes 8-9*, Controls. *Lanes 10-12*, distamycin treatment. *Lane 10*, 50 μM; *Lane 11*, 10 μM; *Lane 12*, 1 μM. Positions of size markers in a typical RNA ladder, in kilobases, are indicated

B. The transcript was quantitated through densitometry and normalized to internal controls. Percent inhibition of transcript formation was expressed relative to controls. Curves were plotted for each drug analyzed.

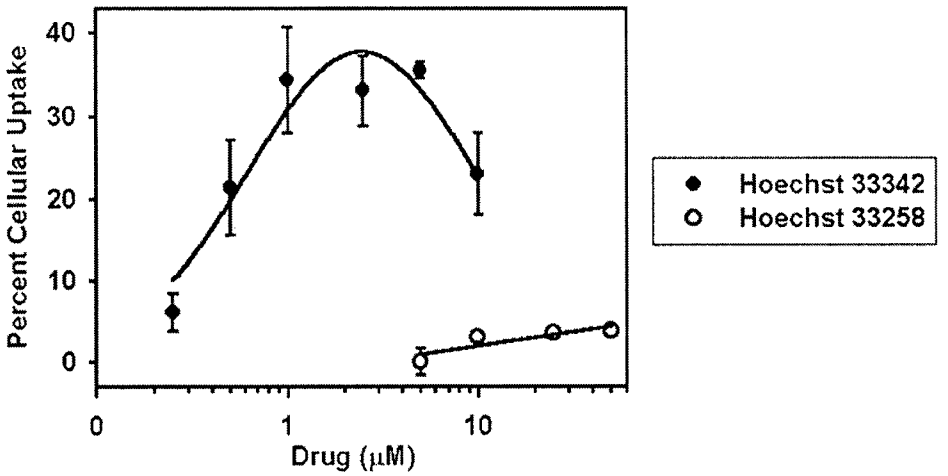


Figure 5: Uptake of Hoechst compounds into NIH3T3 cells.

NIH3T3 cells were treated with drug for four hours. The media was then removed and the cells were scraped into acidified ethanol (0.3 HCl: 96 % EtOH, 1:1). Following an overnight extraction at 4°C, the samples were centrifuged and the supernatant was analyzed on a spectrophotofluorometer (Ex. λ = 335 nM, Em. λ = 470 nM). Drug concentration of each sample was calculated using a standard curve for each Hoechst compound. Percent cellular uptake was calculated by dividing the drug concentration in the sample by the original concentration of the drug treatment.

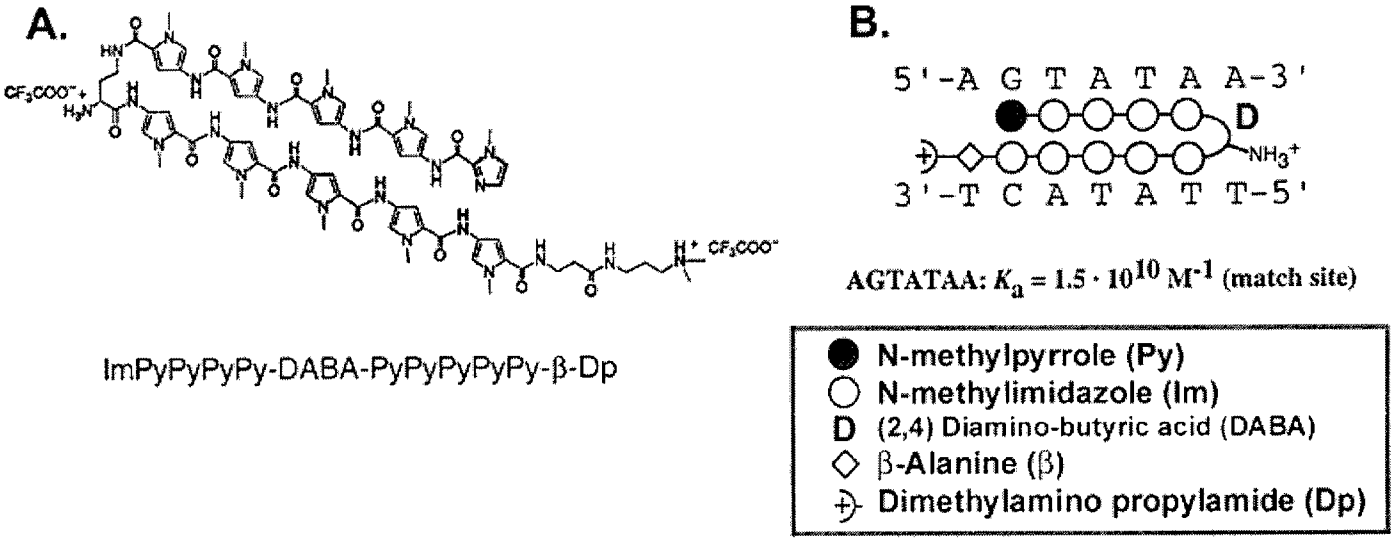


Figure 6: Structure of a representative polyamide.

A. Chemical structure of polyamide 2 (PA-2), designed and synthesized by our collaborator, Dr. Peter Dervan. It is currently being used in our lab to target the Her2/Neu promoter. Similarly designed agents will be used to target the c-fos SRE.

B. Model of PA-2 binding to its target sequence of 5'-ACTATAA-3' on the Her2/Neu promoter. The association constant (K_a), as determined by quantitative DNA footprinting, is given.

Appendices (continued)

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Appendices (continued)

Poster presentation: Sigma Xi student poster competition at the State University of New York at Buffalo (4/25/00)

Abstract:

Previous work has demonstrated that sequence selective DNA-binding drugs can inhibit transcription factors from binding to their target sites on gene promoters. In this study, the potency and effectiveness of DNA-binding drugs to inhibit transcription factor/DNA complex formation and resultant gene expression was assessed using the c-fos promoter's serum response element (SRE) as a target. The classical DNA-binding drugs evaluated included the minor groove binding agents chromomycin A_3 and Hoechst 33342, which bind to G/C and A/T rich regions, respectively, and the intercalating agent nogalamycin, which binds G/C rich sequences in the major groove. The transcription factors targeted, Elk-1 and serum response factor (SRF), form a ternary complex (TC) on the SRE that is necessary and sufficient for induction of c-fos by serum. The drugs' abilities to prevent TC formation on the SRE *in-vitro* was nogalamycin > Hoechst 33342 > chromomycin. Their potencies in inhibiting cell-free transcription and endogenous c-fos expression in NIH3T3 cells, however, was chromomycin > nogalamycin > Hoechst 33342. The latter order of potency was also obtained for the drugs' cytotoxicity and inhibition of general transcription. We subsequently analyzed the potential of novel, rationally designed microgonotropens (MGTs) as inhibitors of transcription. MGTs bind with high affinity to A/T rich sites in the minor groove while making contact with the phosphodiester backbone of DNA through polyamine tails. While these agents proved to be more potent than their classical groove-binding parent compounds in inhibiting transcription factor/DNA complex formation, their potencies in cell-free transcription assays were greatly diminished and they did not exhibit cellular activity. These systematic analyses provide insight into how drug and transcription factor binding characteristics are related to drugs' effectiveness in inhibiting gene expression.

ABSTRACT/SUMMARY:
Previous work has demonstrated that sequence selective DNA-binding drugs can inhibit transcription factors from binding to their target sites on gene promoters. In this study, the potency and effectiveness of DNA-binding drugs to inhibit transcription was assessed using the c-fos promoter's serum response element (SRE) as a target. The drugs chosen for analysis included the minor groove binding agents chromomycin A_3 and Hoechst 33342, which bind to G/C rich and A/T rich regions, respectively, and the intercalating agent nogalamycin, which binds G/C rich sequences in the major groove. The transcription factors targeted, Elk-1 and serum response factor (SRF), form a ternary complex (TC) on the SRE that is necessary and sufficient for induction of c-fos by serum. The drugs' abilities to prevent TC formation on the SRE *in-vitro* was nogalamycin > Hoechst 33342 > chromomycin. Their potencies in inhibiting cell-free transcription and endogenous c-fos expression in NIH3T3 cells, however, was chromomycin > nogalamycin > Hoechst 33342. The latter order of potency was also obtained for the drugs' cytotoxicity and inhibition of general transcription as measured by [^{3}H]uridine incorporation. These systematic analyses provide insight into how drug and transcription factor binding characteristics are related to drugs' effectiveness in inhibiting gene expression.

Title: Evaluation of the effectiveness of DNA-binding drugs to inhibit transcription using the c-fos serum response element as a target

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Running title: Evalulating drugs using the c-fos serum response element

INTRODUCTION:

Drugs that bind to DNA can act as template poisons by inhibiting interactions between cellular proteins and their DNA targets. The activity of RNA polymerases, DNA polymerases, and topoisomerases I and II can all be affected by drug treatment of their DNA templates (1-3). Our studies focus on evaluating DNA reactive agents as inhibitors or disruptors of transcription factor (TF) binding to target sequences in gene promoters and their resultant effects on gene expression. Successful initiation of transcription requires specific binding of TFs with their cognate promoter sequences (Reviewed in 4). Interfering with these specific TF/DNA interactions may therefore lead to decreased expression of a target gene of interest. The ability to selectively prevent TFs from binding to desired DNA targets has implications in the study of the molecular regulation of gene expression and, ultimately, in the development of therapeutics (Reviewed in 5).

DNA-binding agents can be classified according to both their mode of binding as well as their sequence preference. Members of the reversible minor groove binding family (MGBs) such as distamycin, netropsin, and the Hoechst compounds, exhibit preferential binding to A/T tracts at least 4 base pairs long (Reviewed in 6). Their curved structure allows them to interact favorably with base pairs on the floor and wall of the minor groove via hydrogen bonds and van der Waal forces. The sequence selectivity of these agents may be influenced by subtle, sequence dependent variations in DNA structure. The inherent differences in groove width and flexibility that result from neighboring base pair effects are factors that influence these agents' ability to optimally recognize and bind to a particular sequence (Reviewed in 7). This generally holds true for other drugs as well. Anticancer antibiotics, such as chromomycin A_3 and mithramycin, are MGBs that prefer G/C rich elements. Unlike the A/T binding MGBs, which primarily widen the minor groove, chromomycin can unwind the double helix by about 11° and cause more extensive DNA structural alterations (8).

Intercalators, such as nogalamycin, doxorubicin, and hedamycin, possess aromatic moieties that are inserted between base pairs, resulting in unwinding and extension of the DNA helix. Their binding is therefore more disruptive to DNA structure than MGBs. While intercalators generally prefer binding to wider-grooved G/C rich sequences, their binding selectivity may be influenced by surrounding base pairs, as has been demonstrated for nogalamycin (9). Footprinting studies have shown that this agent selectively binds to regions of alternating purines

and pyrimidines, and notably prefers 5'TpG steps (10). Nogalamycin is also somewhat unique because of its extremely slow dissociation kinetics, which likely stem from its characteristic threading mode of intercalation (11).

There has been considerable interest in targeting TFs using each of these drug classes. TFs can be classified into families according to their conserved DNA binding domains. While TFs in each family can recognize and bind to specific consensus sequences, their precise, selective recognition of promoter sites is often dependent on the composition and conformation of neighboring DNA base pairs. Electrophoretic mobility shift assays (EMSAs) using a variety of TF and DNA targets have established the potential of DNA-binding drugs as selective disruptors of target gene function. These previous studies have demonstrated that inhibition of TF/DNA complexes by DNA-binding drugs *in-vitro* is more effective if the TFs and drugs analyzed share binding selectivities and characteristics. For example, the MGB distamycin is a potent inhibitor of TATA box binding protein (TBP) association with its A/T rich target sequence in the minor groove (12). Distamycin's distortion of DNA groove conformation has also been implicated in its ability to disrupt TFs bound to A/T rich sites in the major groove, such as homeodomain peptides (13).

Further investigations have suggested a relationship between the ability of drugs to disrupt TF binding to DNA in the simpler EMSAs and their ability to inhibit cell-free transcription under more complex conditions using a nuclear lysate. Distamycin was an effective inhibitor of both TBP complex formation in EMSAs as well as TBP-driven cell-free transcription (14). Mithramycin and chromomycin inhibited the binding of nuclear factors to G/C rich Sp-1 binding sites on the long terminal repeat (LTR) of the HIV-1 promoter and were also able to inhibit HIV-1 LTR-directed transcription in a cell-free system (15). In such studies, higher drug concentrations were often needed to observe inhibition of transcription. In regards to novel drug development, these studies emphasize the need to maximize drug effectiveness in simpler systems before proceeding with testing in more complex assays or whole cells.

Evaluating DNA reactive compounds and developing paradigms for drug targeting in increasingly complex assay environments will help to improve drug specificity and potency. The c-fos promoter's serum response element (SRE) (Reviewed in 16) has characteristics that make it appropriate for this type of drug evaluation. The c-fos

gene, which has been rigorously studied and characterized due to its importance in growth control, is an immediate-early response gene that is tightly regulated at the level of transcription (17). The SRE is necessary and sufficient for the rapid and transient induction of c-fos by serum (18). Two transcription factors bind to adjacent sites in this promoter sequence and mediate c-fos expression. A homodimer of SRF binds to the CArG box, an A/T rich region (19). SRF binding is required for efficient recruitment of the ternary complex factor Elk-1 to its *ets* motif immediately upstream (20) (See Fig. 2). Like other members of the *ets* family, Elk-1 binds to an invariant GGA core sequence in the major groove of DNA, but makes contacts with the phosphate backbone of unique flanking nucleotides in the minor groove (21). The TC, consisting of SRF and Elk-1, is constitutively bound to the SRE. Activation of this complex and upregulation of transcription are achieved as part of the cellular response to serum. Activation of signal transduction pathways by growth factors results in the phosphorylation of these factors by various kinases, most notably members of the mitogen activated protein kinase (MAPK) family (22,23).

The A/T rich and mixed sequences present in the SRE are well suited for the study of a wide variety of DNA reactive agents. Also, the fact that Elk-1 contacts both grooves makes it an interesting target for drugs that bind in one groove or the other. Moreover, using this sequence as a target allows drug effects on more than one TF to be studied in a single system. Another advantage is that in addition to EMSAs and cell-free transcription assays, use of the c-fos SRE allows drug effects on endogenous c-fos mRNA production to be assessed. The rapid and transient expression of c-fos that follows serum induction provides a facile way of determining immediate or short-term drug effects on transcription in whole cells.

Here, we investigate the effectiveness of drugs as inhibitors of TF/DNA interactions in increasingly complex systems using the c-fos SRE as a target. Representative agents from the drug classes discussed above were chosen based upon their contrasting sequence preferences and modes of DNA binding (drug structures are shown in Fig. 1). The MGBs chromomycin and Hoechst 33342, which don't radically distort DNA, have G/C and A/T rich preferences, respectively. They were compared to nogalamycin, which has less sequence specificity but which causes greater helical distortion. The agents' abilities to affect TF/DNA interactions *in-vitro* were evaluated in EMSAs, a simple system consisting of purified proteins and short oligonucleotides. The cell-free transcription assay, which uses a nuclear lysate to drive transcription from a plasmid, was used to analyze drugs' effects in a more

complicated environment. Finally, effects of these agents on endogenous c-fos expression in whole cells was assessed using Northern blots. Cytotoxicity and RNA synthesis assays also provided insight into how these agents were affecting cells in general.

MATERIALS AND METHODS:

Drugs – Stocks of 5 mM chromomycin A_3 (Sigma, St. Louis, MO) and 5 mM nogalamycin (Pharmacia Upjohn Corporation, Peapack, NJ) were prepared in dimethyl sulfoxide. A 20 mM stock of Hoechst 33342 (Aldrich Chemical Co., Milwaukee, WI) was prepared in distilled water. All drugs were stored in the dark at –20°C and diluted into water immediately before use.

Oligonucleotides –Two 24-mer oligonucleotides and their complementary strands were synthesized by the Biopolymer facility at Roswell Park Cancer Institute (Buffalo, NY) and purified on a Poly-Pak column. The first oligo (5'-ACACAGGATGTCCATATTAGGACA -3'), designated "SRE", contained the -301 to -324 sequence of the human c-fos promoter SRE. The second oligo (5'-GATACCGGAAGTCCATATTA-GGAC-3'), designated "E74", was similar, but contained the high affinity *ets* binding site from the E74 *Drosophila* promoter (underlined), based on the consensus sequence published by Urness, et al. (24). Oligos were reannealed according to Lee, et al. (25). These double stranded oligos were 5'-end labeled with [γ-^{32}P]ATP (10mCi/ml) and T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA) according to manufacturer's instructions. Unincorporated nucleotides were removed using a Sephadex G-25 microspin column (Amersham Pharmacia Biotech., Piscataway, NJ).

Protein Purification: pILASRF, a plasmid encoding the SRF protein with an N-terminal His-tag, was developed in the Nordheim laboratory (Institut fuer Zellbiologie, Universitaet Tuebingen, Tuebingen, Germany). Expression of this protein and its purification was achieved following the protocol by Heidenreich, et al. (26), but with two changes. First, the bacterial pellet was resuspended in PBS and lysed using 3 freeze/thaw cycles. The lysate was sonicated and pelleted at 10,000 x g for 25 minutes at 4°C before being combined with Ni-NTA beads (Qiagen, Inc., Valencia, CA). Secondly, after transfer to a column, the beads were initially rinsed four times with 2 column volumes of PBS. pAS278, a plasmid encoding full-length Elk-1 with a C-terminal His-tag (27), was generously

provided by Dr. Andrew Sharrocks (University of Newcastle, Newcastle upon Tyne, England). Following expression in BL21-pLysS bacteria, the protein was purified under native conditions using Ni-NTA beads, following manufacturer's instructions (Qiagen).

Electrophoretic Mobility Shift Assays – In general, experiments were performed as follows: drug, radioactively end-labeled oligo and binding buffer were combined and allowed to incubate at room temperature for 30 minutes. Purified transcription factors were diluted into binding buffer. Following addition of purified protein(s), the reactions were allowed to incubate an additional 30 minutes at room temperature before being electrophoresed on a polyacrylamide gel. These incubation times were based on timecourse experiments that established 30 minutes as sufficient time to achieve equilibrium of complex formation.

Specifically, for the Elk-1 EMSAs, a binding buffer containing 25 mM Hepes-KOH, pH 7.9, 10 mM MgC_2, 10 mM EDTA, 10 mM spermidine, 10 mM dithiolthreotol, 7.5 μg/ul bovine serum albumin, and 20% glycerol was used. 10 ng purified Elk-1 was added to 1 nM ^{32}P end -labeled E74 oligo in these reactions. For the SRF EMSAs, the binding buffer contained 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% milk, 10 mM DTT and 5% glycerol. 25 ng purified SRF was added to 1 nM ^{32}P-end -labeled SRE in these reactions. For ternary complex formation, the SRF binding buffer was used. 25 ng SRF and 6.25 ng Elk-1 were added to 1 nM ^{32}P-end -labeled SRE. In samples containing chromomycin, Mg^{2+} was added to a final concentration of 10 mM in the binding reactions.

For all EMSAs, a 5% native polyacrylamide gel was pre-run at room temperature at 200 V in 0.5x TBE buffer (44.6 mM Tris base, 44.5 mM boric acid, and 10 mM EDTA). Reactions were loaded onto the gel and electrophoresed for a maximum of 2 hours. Adequate separation of free and complexed DNA on the Elk-1 and SRF MSAs was achieved by as little as 30 minutes of electrophoresis. Dried gels were exposed to Kodak Biomax Scientific Imaging film. Quantitation of free and complexed DNA was carried out by scanning the resulting autoradiogram on a computing laser densitometer (Molecular Dynamics, Sunnydale, CA) and analyzing the results with the manufacturer's ImageQuant program. Fifty percent inhibition of complex formation by the drugs (IC_{50}) was calculated by comparing drug treated samples to controls.

Where specified, purified proteins were added to the oligo before drug. These "reverse" experiments were electrophoresed as detailed above.

Cell Culture – Murine NIH3T3 fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/ml) and sodium pyruvate (110 mg/ml) and supplemented with 10% calf serum. Cells were maintained at 37°C and 5% CO_2.

NIH3T3 Nuclear Lysate Preparation for Cell-Free Transcription – NIH3T3 cells were grown in 175 cm^2 flasks until approximately 60% confluent before being starved overnight in starvation media (DMEM containing 0.5% calf serum). In general, a minimum of 10 flasks was needed to see adequate lysate activity. Cells were induced by adding induction media (DMEM with 15% calf serum) for 30 minutes, rinsed with room temperature PBS, and scraped into ice cold PBS. Nuclear lysates were then prepared essentially as described by Blake, et al. (28). All centrifugations were performed at 4°C. In brief, cells were pelleted by centrifugation, washed, and repelleted in 5 pellet volumes of Buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [Hepes]/KOH, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiolthreotol [DTT]), then allowed to swell on ice in the same volume of Buffer A for approximately 20 minutes. Cells were dounced on ice until 95% cell lysis was achieved, then centrifuged in a J2-HS Beckman centrifuge until 31,000 x g was reached. As soon as this speed was obtained, the centrifuge was turned off and the rotor was allowed to come to a halt. The pelleted nuclei were resuspended in Buffer C (20 mM Hepes/KOH, pH 7.9, 20% glycerol, 0.2 mM EDTA, 2 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 2 mM DTT, and 1 mM phenylmethanesulfonyl fluoride [PMSF]) to a final minimum concentration of $5x10^8$ nuclei/ml. An equal volume of Buffer C + NaCl (same composition as buffer C, but with 0.75 M NaCl) was then added dropwise, with swirling. The lysate was rocked at 4°C for 30 minutes, then centrifuged in a Beckman L5-50 ultracentrifuge at 214,000 x g for 45 minutes. Supernatants were pooled, loaded into Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) with a 10,000 molecular weight cut off, and dialyzed against 500 volumes of buffer D (20 mM Hepes/KOH, pH 7.9, 100 mM KCL, 20% glycerol, 0.2 mM EDTA, 0.2 mM EDTA, 12.5 mM $MgCl_2$, 2 mM DTT, and 1 mM PMSF) at 4°C for 3 hours, with a buffer change after the first 1.5 hours. Dialyzed extract was cleared by a 15 minute spin in the J2-HS centrifuge at 31,000 x g. Aliquots of the resulting supernatant were immediately frozen on dry ice and stored at –80°C.

Cell-Free Transcription Assay – The template, pFosLuc19, containing a human c-fos promoter fragment (-711 to –3) upstream of a luciferase reporter gene, was developed in the Nordheim laboratory. Digestion of this plasmid with Sph I prior to use in the assay yields a transcript approximately 750 bases in length. For drug studies, 0.5 μg of this plasmid was combined with drug and 5 ul of Buffer D for a total volume of 9 ul, and allowed to incubate 30 minutes at 30°C. Approximately 15 μg NIH3T3 nuclear lysate was added, the total volume was brought to 19 ul with Buffer D, and the reaction was allowed to incubate for 15 minutes at 30°C. The subsequent reaction and transcript purification steps were carried out as described by Chiang, et al. (29). A T3 transcript of pGEM4z (Promega, Madison, WI), 250 bases long, was used as an internal control. Quantitation following autoradiography was as described for the EMSAs, and the visualized transcripts were normalized to the internal controls.

Whole Cell Drug Treatment and Northern Blot Analysis – For typical drug treatments prior to Northern blot analysis, 2.5 x 10^5 NIH3T3 cells were plated in 60 mm dishes and allowed to grow for 48 hours until approximately 60% confluent. Growth media was removed, the cells were rinsed with PBS, and starvation media (DMEM containing 0.5% calf serum) was added. Appropriate dilutions of drugs were made into sterile water and 20 ul of these dilutions were added directly to the plates. Solvent controls in which no drug was added were also prepared. Cells were generally starved for 16 hours at the growth conditions detailed above. Induction of c-fos was accomplished by adding calf serum directly to the plates to a final concentration of 15%, followed by incubation at 37°C for 30 minutes.

RNA Isolation/Northern Blot Analysis – Following serum induction of NIH3T3 cells, total RNA was isolated using TRIzol (GIBCO BRL, Grand Island, NY). In brief, 20 μg of total RNA was loaded onto 1.5% denaturing agarose gel (2.2 M formaldehyde, 40 mM MOPS, pH 7.0, 10 mM sodium acetate, and 10 mM EDTA) and electrophoresed in 1x MOPS buffer (40 mM MOPS, 10 mM sodium acetate, and 10 mM EDTA) at 80 V for 4.5 hours. The gel was rinsed in ddH_2O and the RNA was transferred to GeneScreen (NEN Life Science Products, Boston, MA) overnight. Following UV crosslinking of the RNA, the membrane was pre-hybridized for one hour at 60° in pre-hybe buffer (0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% bovine serum albumin [BSA]). A plasmid containing 4.8 kb of the murine c-fos coding sequence (pGEM4z-Fos) was obtained from Loftstrand Labs, Ltd. (Gaithersburg, MD) and linearized with Hind III before being radioactively labeled using a DecaPrime II kit (Ambion Inc., Austin,

Texas) and [α-^{32}P]dCTP (10 mCi/ml) for use as a probe. A phagemid containing the coding sequence for human glyceraldehyde 3-phosphate dehydrogenase (G3) (American Type Culture Collection) was also linearized with Hind III and similarly labeled. Hybridization with the radiolabeled probes was overnight at 60°C. Membranes were washed twice with wash buffer A (40 mM sodium phosphate, pH 7.2, 5% SDS, 1 mM EDTA, and 0.5% BSA) and twice with wash buffer B (20 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA) at 60°C (each wash was for 20 minutes). The blot was exposed in a phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA) and scanned with a Molecular Dynamics phosphorimager.

Cytotoxicity Assay: Colony Formation using NIH3T3 Cells – 1 x 10^5 NIH3T3 cells were plated in 35 mm dishes and allowed to grow for 48 hours until approximately 50% confluent. Growth media was removed and 1 ml of fresh media containing the desired drug concentration was added. Following a 4 hour drug exposure, the cells were trypsinized, serially diluted, and replated into 60 mm dishes. The total number of cells plated per dish ranged from 1 x 10^4 to 1 x 10^2. Cells were incubated at normal growth conditions for 10 days. The media was then removed and the cells were stained using 2 ml of methylene blue staining solution (7 mg/ml methylene blue in 70% EtOH) per dish for 30 minutes. Following removal of the staining solution, the dishes were rinsed in lukewarm water and air dried. Colonies, designated as groups of 50 or more cells, were counted under a stereo microscope. Plating efficiencies were calculated by dividing the number of colonies by the total number of cells plated. Relative plating efficiencies for the drug treatments were then calculated by dividing the plating efficiencies of the drug treated samples by the plating efficiencies of the controls.

RNA Synthesis ([^{3}H]Uridine Incorporation Assay) – 6.6 x 10^5 NIH3T3 cells were plated in 60 mm dishes and allowed to grow for 48 hours until approximately 85% confluent. Drugs were diluted appropriately and added directly to the growth media for a 4 hour exposure under normal growth conditions. 2 μCi of [5-^{3}H]uridine (15 mCi/mmol) and unlabeled uridine to a final concentration of 50 μM were added to each dish. Following a 30 minute pulse, the cells were rinsed with cold PBS and then each dish was scraped into 1 ml of ice cold 0.5 M perchloric acid (PCA) to begin precipitation of nucleic acids. 0.5 ml of the resuspension was transferred to pre-chilled eppendorf tubes, 1 ml of cold 0.5 M PCA was added, and the tubes were incubated on ice for 30 minutes. The samples were pelleted at 2800 rpm at 4°C in a Sorvall RT6000 centrifuge (Kendro Lab Products, Newtown, CT)

and washed 2 times in 0.4 M PCA before 0.5 ml 0.5 M PCA was added. The tubes were then heated to 70°C for 1 hour and the counts in 0.5 ml were measured on an LS 1800 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

RESULTS SECTION:

c-fos Components. The c-fos SRE sequence targeted in this study is shown in Fig. 2A. This sequence is located approximately 300 bp upstream of the c-fos transcription initiation site. Binding sites for the ternary complex factor Elk-1 and the homodimer of SRF are indicated. The TC is formed when binding of SRF to the CArG box recruits Elk-1 to the *ets* motif immediately upstream. SRF and Elk-1 were expressed as 6-Histidine tagged proteins in bacteria and purified for use in the EMSAs as described in detail in the "Materials and Methods" section. While the SRE can be used to study drug effects on SRF binding and ternary complex formation on the SRE, it cannot be used to assess drug effects on Elk-1 binding alone, since this protein cannot bind to the SRE without SRF present (30). However, it can bind to the high affinity *ets* motif in the *Drosophila* E74 promoter (31). Therefore, we made use of this promoter to study drugs' influence on Elk-1 association with DNA. Recombinant proteins were combined with 24 bp radiolabeled oligonucleotides containing the targeted promoter regions, and the complexes were electrophoresed on a polyacrylamide gel. Free SRE and E74 probe was successfully shifted by the addition of SRF or Elk-1 alone (Fig. 2B, compare lanes 1 and 3 with lanes 2 and 4). In lanes 5 and 6, the gel was electrophoresed longer in order to maximize the difference in shift evident when SRF and Elk-1 were combined to form the TC (lane 6) as compared to SRF alone (lane 5). Under these conditions, the free probe ran off the gel. In subsequent experiments, where the complexes were electrophoresed under standard conditions as in Fig. 2B (lanes 1-4), we noted that the TC was still distinguishable from the SRF complex on the basis of a slight difference in mobility (See Fig. 3A, compare lanes 8 and 10 with lane 9).

Inhibition of TF binding to the SRE in EMSAs. The ability of drugs to prevent TF binding to their specific promoter sites was assessed using the components of the SRE system. Three drugs were chosen on the basis of their different binding properties. The structures of chromomycin and Hoechst 33342, minor groove binding agents with G/C and A/T preferences, respectively, and nogalamycin, an intercalating drug that prefers G/C rich regions, are shown in Fig. 1. When incubated with the radiolabeled probes prior to protein addition, these drugs prevented TF

binding in a dose dependent manner. Representative results are shown in Fig. 3A for chromomycin. As the amount of chromomycin added to the E74 promoter decreases in lanes 3 – 7, the amount of Elk-1 complexed with the probe increases back to control levels (lanes 1 and 2, no drug addition). Chromomycin also yields a dose dependent inhibition of the TC as seen in lanes 11 – 15, as compared to controls in lanes 8 and 10. Similar inhibition was also achieved for SRF complex formation (data not shown).

Quantitation of the free and shifted DNA in drug treated samples, and comparison to non-drug treated controls allowed percent inhibition of complex formation to be calculated. Representative dose response curves for chromomycin (Fig. 3B) show that this agent exhibits different potencies on the three TF complexes analyzed. The Elk-1 complex is by far the most sensitive to this drug, exhibiting a steep dose response curve that plateaus by 10 μM. Formation of the SRF complex is less sensitive and yields a more gently sloping curve that starts to level off around 30 μM. When Elk-1 and SRF are combined to form the TC, the dose response curve falls between those obtained for the individual factors, but retains the rather steep increase of the Elk-1 curve.

The drug concentration needed to inhibit complex formation by 50% (IC_{50}) was determined from dose response curves plotted for each agent analyzed and used to compare the drugs' effectiveness in preventing TF binding (Fig. 3C). Hoechst 33342 exhibited a trend similar to that observed for chromomycin: it was not particularly potent in preventing the SRF complex from forming, but was much more effective in inhibiting TC formation. Notably, Hoechst 33342 was approximately twice as potent as chromomycin in preventing TC formation. Unfortunately, the effects of Hoechst 33342 on Elk-1 complexes could not be determined, since binding of this drug to the E74 probe alone resulted in its retention in the well of the gel and excessive smearing, making quantitation impossible. Of the three drugs tested, nogalamycin was by far the most potent agent – IC_{50}s for all complexes fell well below 5 μM. Its inhibition profile differed from chromomycin in that SRF, rather than Elk-1, was the most sensitive target. Like chromomycin, however, the IC_{50} for TC inhibition fell between the IC_{50}s for the individual factors' complexes. In addition to its higher potency and unique inhibition profile in comparison to the MGBs, nogalamycin possessed another distinct characteristic. Reverse assays, in which each of the drugs was added after the TC was already formed on the SRE, demonstrated that only nogalamycin required significantly higher concentrations to disrupt complex formation (data not shown).

Drug Effects on c-fos Promoter-Driven Cell-Free Transcription. Upon observing different potencies among the drugs in the EMSAs, we wished to determine if similar levels of effectiveness could be maintained in a more complex, cell-free environment containing additional nuclear proteins and larger amounts of DNA with greater sequence complexity. The cell-free transcription assay makes use of a linearized plasmid containing the c-fos promoter. Upon addition of nuclear lysate from serum-induced NIH3T3 cells and the proper mix of nucleotides, the c-fos promoter drives the production of a transcript of a known length of 750 bp (Fig. 4A, control lanes 3 and 8 marked by asterisks, top arrow). Pre-incubation of the plasmid with drug before nuclear lysate addition results in a dose dependent inhibition of transcript production, as is seen for representative results following chromomycin treatment (lanes 1, 2, and 4 – 7). At 7.5 μM chromomycin (lanes 1 and 2), transcript appearance is abolished. At lower drug concentrations (5 and 2.5 μM in lanes 4-5 and 6-7, respectively), the intensity of the transcript is diminshed, but there is no change in transcript size. The lack of detectable shorter transcripts suggests that transcriptional elongation is not being affected by the drug treatment. Quantitation of the bands followed by normalization to a 250 bp internal standard (Fig. 4A, bottom arrow), and comparison to controls, yields percent inhibition of transcription. Dose response curves, as seen in the representative graph for chromomycin in Fig. 4B, were plotted for each drug treatment. The IC_{50}s for this assay were then used to compare the drugs in Fig. 4C. Chromomycin and nogalamycin showed a level of potency that was only about 3 times higher than Hoechst 33342. The trend evident in the EMSAs, where an unusually high level of chromomycin was required to inhibit TC formation, therefore did not hold true in the cell-free transcription assays. While the IC_{50}s for nogalamycin and Hoechst 33342 increased approximately fourfold from inhibition of the TC to inhibition of cell-free transcription, the respective IC_{50}s for chromomycin's inhibition actually decreased by a factor of 2.5.

Use of Northern Blots to Measure c-fos mRNA Induction. After observing that the drugs' potencies in the EMSAs and cell-free transcription assays were comparable, we next wished to assess the drugs' effectiveness in inhibiting c-fos expression in whole cells using Northern blots. The serum inducibility of the c-fos gene and its quick mRNA turnover (17) are advantageous in analyzing drug effects on endogenous c-fos transcription because complications arising from pre-existing levels of c-fos mRNA are minimized. Optimal conditions established for c-fos induction in NIH3T3 cells are shown in Fig. 5A. Unsynchronized, logarithmically growing cells have

undetectable levels of c-fos mRNA (Fig. 5A, lane 1). Inducing these cells with 15% serum for 30 minutes results in its detectable upregulation (lane 2). Starving the cells overnight (16 hours) in media containing 0.5% serum downregulates c-fos (lane 3), and a subsequent 30 minute induction of these cells with serum results in optimal expression (lane 4), as determined in time course studies (data not shown). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3) is used in these blots as a loading control.

Analysis of Drug Effects on c-fos Expression in Whole Cells. For drug treatments, cells were starved for 16 hours, exposed to varying drug concentrations for 1 hour, and subsequently induced with serum. Chromomycin, as shown in Fig. 5B, was capable of decreasing absolute c-fos mRNA levels in a dose dependent manner (lanes 4–9), as compared to non drug treated controls (lanes 1-3). An approximately 30% decrease in c-fos expression is noted at only 0.5 μM (lanes 6 and 7), and the message is almost completely eliminated with a 1 μM treatment (lanes 4 and 5). There is no significant effect on G3 mRNA after a one hour treatment at any drug concentration. In addition, as was noted in the cell-free transcription assay, there were no detectable levels of shorter transcripts following drug exposure.

Nogalamycin and Hoechst 33342 were analyzed in the above manner to see if they were also capable of inhibiting c-fos expression. Quantitation of the resulting hybridized signals allowed dose-response curves to be plotted (Fig. 6). From these curves, it is evident that the drugs differ in their effectiveness: a 1 hour exposure to 1 μM of chromomycin results in 80% inhibition of c-fos expression, compared to no substantial inhibition for nogalamycin or Hoechst 33342 at the same concentration (Compare panels A to B and C). At 2.5 μM of Hoechst 33342 or nogalamycin, expression is inhibited approximately 40%, compared to greater than 95% inhibition for chromomycin. Chromomycin is therefore about an order of magnitude more potent than nogalamycin or Hoechst 33342 in inhibiting c-fos expression after a 1 hour treatment. This is in contrast to the results obtained in the cell-free transcription assay, where chromomycin and nogalamycin exhibited similar potencies that were only 3 times greater than Hoechst 33342.

Chromomycin's Effects on c-fos Expression Over A Shorter Time Course. After observing very effective inhibition of c-fos expression following exposure of cells to low levels of chromomycin after only 1 hour, we

wished to further characterize this drug's effect during shorter exposures. NIH3T3 cells were therefore starved for 16 hours in low serum media before being exposed to 1 μM chromomycin for various times (zero to 60 minutes). For the zero time point, drug was added to cells immediately before serum induction. Cells were then induced for 30 minutes before RNA isolation as discussed above. A concentration of 1 μM was chosen for this time course because of its substantial effect on c-fos expression (~80% inhibition after a 1 hour exposure). As seen in Fig. 7, there is a slight induction of c-fos mRNA after a 15 minute exposure to chromomycin, but inhibition begins by 30 minutes and increases fairly rapidly to reach nearly 80% after an hour.

Drugs' Abilities to Inhibit c-fos Expression Over Time. Clearly, chromomycin is a rapid inhibitor of c-fos expression, but is this effect maintained over time to result in a continued decrease in expression? Longer exposures of 4 and 16 hours were carried out to determine if each drug was able to maintain its effectiveness in the cellular environment. Like the 1 hour time points, the 4 hour drug exposures followed a 16 hour overnight starvation period. However, for 16 hour treatments, cells were starved and exposed to drug concurrently. The IC_{50} values were then used to compare the time course results as well as to compare drugs to one another. For chromomycin (Fig. 8A), there was a continued increase in drug potency over time. That is, less drug was needed to obtain 50% inhibition of c-fos expression following a 16 hour exposure compared to a 1 hour exposure. Interestingly, Hoechst 33342 yielded a pattern of inhibition that was completely opposite. While a 16 hour exposure to Hoechst 33342 yielded no consistent inhibition (not shown), the drug was more effective at shorter time points, with the IC_{50} dropping 4 fold between 4 hours and 1 hour (Fig. 8B). The pattern exhibited by nogalamycin (Fig. 8C) was similar to that obtained for chromomycin. However, 5 times more drug was needed to obtain an equivalent level of inhibition at 1 hour as compared to 4 hours, after which more time did not result in any substantial further increases in drug effectiveness.

Drugs' Effects on Cell Survival and RNA Synthesis. The Northern blot results demonstrated that the drugs chosen are capable of affecting endogenous c-fos expression in a dose and time dependent manner. However, the complexity of the cellular environment necessitates that these results be interpreted in the context of the agents' other biological activities and whole cell effects. Therefore, to further characterize the drugs' effects in whole cells, NIH3T3 cells were exposed to a range of drug concentrations for 4 hours before being plated in a colony formation assay. The colonies formed after 10 days of growth were counted and used to calculate relative plating efficiencies

for each drug treatment as compared to non-drug treated controls (Fig. 9). Chromomycin was found to be the most toxic agent, causing 50% cell death at only 0.125 μM. The amount of Hoechst 33342 required to obtain the same level of toxicity (4.5 μM) was over an order of magnitude greater. Nogalamycin's toxicity fell between these values, with an IC_{50} of 1 μM. The cytotoxicity of these compounds may be reflected by their ability to shut down general transcription in cells, since they are capable of binding to many regions on DNA. To get a sense of how general transcription was being affected, RNA synthesis in NIH3T3 cells, as quantitated by [^{3}H]uridine incorporation, was measured following 4 hour drug exposures (data not shown). The IC_{50}s from this assay are summarized in Table 1, along with IC_{50}s for the 4 hour drug treatments in the cytotoxicity and Northern blot assays. The order of potency for drugs in the cellular assays was chromomycin > nogalamycin > Hoechst 33342. For all agents, similar drug concentrations were needed to achieve equivalent levels of activity in each assay.

DISCUSSION SECTION:

This study has compared and contrasted the ability of various classes of DNA-binding agents to inhibit TF/DNA interactions and resultant gene expression using a defined target gene promoter sequence, the c-fos SRE. To our knowledge, this is the first study that systematically analyzes the effectiveness of drugs possessing different sequence selectivities and modes of DNA binding to target two different TF binding motifs in increasingly complex assays. The drugs' abilities to inhibit TF binding to target sequences in EMSAs were compared to their potencies in inhibiting cell-free transcription as well as their abilities to inhibit cellular gene expression. The drugs selected for analysis, chromomycin, nogalamycin and Hoechst 33342, exhibited different potencies in each assay. Interesting differences were also noted when the drugs' effectiveness was compared between assays. These variations may stem from many factors including the DNA binding characteristics of the compounds as well as their overall stability in cells.

In the EMSAs, the overall order of decreasing potency for inhibiting SRF and Elk-1 complexes was nogalamycin > Hoechst 33342 > chromomycin. Potential binding sites for nogalamycin, consisting of alternating purines and pyrimidines, are located near the *ets* motif of the Elk-1 binding site and in the CArG box of the SRF binding site. As an intercalator that makes contacts in both the major and minor grooves (32), and in comparison to the MGBs, nogalamycin dissociates from DNA very slowly (11), favoring more effective inhibition of TF binding under

equilibrium conditions. It was anticipated that since SRF is required for Elk-1 binding, the IC_{50}s for inhibition of TC and SRF binding would be equivalent. However, two times more nogalamycin was needed to inhibit TC formation by 50% as compared to SRF binding. Structural studies and circular permutation analyses have shown that binding of a homodimer of SRF bends DNA 72° (33), while DNA bound to the TC has increased flexibility and is bent approximately 50^0 (34). This alteration in DNA conformation upon Elk-1 binding and its effects on neighboring DNA structure may therefore influence a drug's ability to optimally recognize and bind its target sequences.

In contrast to nogalamycin, chromomycin was more potent in inhibiting TC formation as compared to SRF binding but exhibited the highest potency in inhibiting Elk-1 complexes. While the E74 oligo used in the EMSAs contains four contiguous G/C base pairs in the *ets* motif that may be an appropriate chromomycin binding site (5'-CCGG-3'), the SRE lacks such a sequence. However, this drug was still able to prevent SRF and TC binding with lower potency, suggesting that if a consensus sequence is not available, the drug will associate with other sequences by default. Similar results have been obtained using chromomycin to inhibit TBP association with its A/T rich binding site (35). The higher concentration of chromomycin required to inhibit TC formation as compared to Elk-1 complex formation may be due to SRF's stabilization of Elk-1 binding. Analogous results were seen in previous evaluations of drug effects on TBP binding. When TBP's binding was stabilized by addition of TFIIA, higher concentrations of distamycin were needed to disrupt complex formation (12).

Analysis of Hoechst 33342's inhibition of Elk-1 complexes was limited by well retention of the oligo when complexed to drug, regardless of whether protein was present (data not shown). This effect has been seen previously in our lab with other drug/oligonucleotide combinations. Despite its A/T preference, Hoechst 33342 was not a potent inhibitor of SRF binding to the A/T rich CArG box on the SRE. Sequence selection studies have demonstrated that other bisbenzimidazole based drugs, such as Hoechst 33258, greatly prefer some A/T sites over others, and surrounding sequences appear to influence the drug's affinity and optimal binding ability (36). In particular, the presence of 5'TpA steps greatly decreases the affinity of this Hoechst dye for DNA (37). The two 5'TpA steps present in the CArG box may therefore be contributing to the low potency observed in the SRF

complex analysis. Given its high IC_{50} value, it's possible that Hoechst 33342 is binding to the target oligo by default, much like chromomycin.

When EMSA analyses using the TC were carried out, drugs never solely inhibited Elk-1 binding, which would have resulted in the appearance of the SRF complex. This suggests that these agents are generally disruptive and that they apparently alter the conformation of the DNA through bending or groove widening so that neither TF is able to bind. This is supported by previous studies that investigated long-range effects of drug binding on DNA. For example, the MGB distamycin can alter DNA allosterically up to 100 bp away from its binding site (38). Furthermore, the alterations in local DNA structure following binding of distamycin or the intercalator actinomycin produce changes in DNase I cleavage patterns at flanking sites (39).

In reverse EMSAs, where drug was added to pre-formed TF/DNA complexes, only nogalamycin required higher concentrations to achieve equivalent levels of TC inhibition. The equilibrium conditions required to inhibit complex formation were therefore altered, since more drug was needed to disrupt the TC if it was added after the proteins were bound. Dissociation studies carried out for the TC showed that this complex was stably bound for over two hours under assay conditions (data not shown). SRF and Elk-1 primarily contact the SRE in the major groove. Since nogalamycin binds in a similar manner, the drug's association with DNA may be hindered by the presence of TFs at or near its binding site. In contrast, drugs such as chromomycin or Hoechst 33342 may be more effective in inhibiting TF binding to the major groove in reverse assays because they can approach DNA from the opposite, minor groove (35).

Drug activity was maintained in a more complex milieu of nuclear proteins and plasmid DNA in the cell-free transcription assay. IC_{50}s obtained in this assay were not substantially different from the EMSA IC_{50}s for TC inhibition but the order of potency was chromomycin > nogalamycin > Hoechst 33342. As noted in Fig. 4C, there was only a fourfold decrease in potency for nogalamycin and Hoechst 33342 in this assay compared to the EMSA results. In contrast, chromomycin was about 2.5 times more effective in inhibiting transcription than in preventing TC formation. Overall, the presence of a additional proteins and a higher amount of DNA with greater sequence complexity do not seem to greatly interfere with the ability of these drugs to inhibit cell-free transcription. This is in

contrast to previous studies in which the MGB distamycin was used to inhibit the TF E2F from binding to the dihydrofolate reductaste (DHFR) promoter and to inhibit DHFR promoter-driven cell-free transcription (29). Here, 200 times more drug was needed to inhibit transcription as compared to inhibition of E2F/DHFR promoter complex formation. However, studies carried out using mithramycin (which is chemically related to chromomycin) demonstrated that similar levels of drug were needed to inhibit both TF complex formation and cell-free transcription from the c-myc promoter (40). The ability of any given drug to inhibit TF complex formation and to maintain an equivalent level of activity in a cell-free environment may therefore be dependent on the particular TFs studied and the DNA sequence used as a target. The results obtained here may also stem from the drugs' effects on other sequences in the c-fos promoter. By interfering with the binding of other TFs, recruitment of a functional RNA polymerase complex may be inhibited and levels of transcription will drop. The TFs bound to the c-fos promoter may be more sensitive to chromomycin than the other drugs analyzed. This may explain chromomycin's greater potency in inhibiting cell-free transcription and endogenous c-fos expression, as discussed below. None of the drugs tested resulted in the detectable production of shorter transcripts, which suggests that these agents are acting on the level of transcription initiation, rather than elongation. This is supported by previous work where transcriptional elongation inhibition was not observed except at high drug concentrations in some systems (14,41).

The serum inducibility of the c-fos promoter and the rapid turnover of its mRNA facilitated assessment of immediate or short-term drug effects on gene expression in NIH3T3 cells. In Northern blots, the order of the drugs' potency in inhibiting endogenous c-fos transcription following serum induction was chromomycin > nogalamycin > Hoechst 33342. The time course study, which demonstrated an approximately 40% inhibition after only 40 minutes of treatment with 1 μM chromomycin, demonstrates the fast-acting nature of this drug and suggests that it is able to enter cells quickly and effectively. These rapid effects, in addition to the cell-free transcription data discussed above, suggests that this drug is acting on the level of DNA by inhibiting TF association with the c-fos promoter. Higher concentrations of chromomycin were needed at shorter time points in order to achieve an equivalent level of inhibition. This drug's effects may therefore depend, at least in part, on accumulation within the cell. The fact that the drug still exhibits inhibitory effects after 16 hours in cells suggests that it's relatively stable. Similar results were obtained in studies using mithramycin and chromomycin to inhibit the expression of a stably transfected c-myc gene

in NIH3T3 cells (41). Here, expression of an exogenous gene was very effectively inhibited after a 24 hour exposure to 1 μM of either drug.

Nogalamycin's pattern of inhibition of gene expression was similar to that obtained using chromomycin. Hoechst 33342, however, was more effective at shorter time points as evidenced by a lower IC_{50} for one hour exposures. The inhibition seen following 16 hour exposures, although variable, was marginal at best (data not shown). Hoechst 33342 may therefore become unstable or inactivated in these cells over time. Because there are undetectable levels of c-fos mRNA prior to serum induction and because shorter transcripts cannot be detected, the presence of drug is likely preventing the initiation of transcription. No decrease in absolute G3 mRNA levels was noted after any drug treatment. In short-term drug exposures, this may be due to G3's longer mRNA half life (8 hours for G3 compared to 30 minutes for c-fos) (42). In addition, the lack of detectable shorter G3 transcripts following drug treatment of cells is again consistent with the drugs' inhibition of transcription initiation. If the drugs were causing transcript degradation or inhibition of transcription elongation, partial transcripts or smearing of the RNA samples would have been visible.

The IC_{50}s for drug inhibition of c-fos expression as measured in the Northerns were less than the IC_{50}s calculated for the cell-free transcription assay. The largest difference of an order of magnitude was obtained for chromomycin. The process of transcription is far more complex on an endogenous promoter, where many other proteins and long-range changes in DNA conformation come into play to provide an intricately regulated cellular response. These drugs' abilities to bind to many other regions on the c-fos promoter is likely contributing to their increased potency in the whole cell environment. The complexity of the drugs' effects is further evidenced by the results obtained in the other whole cell analyses. The similarity in IC_{50}s for the cytotoxicity, RNA synthesis and Northern blot assays following 4 hour drug exposures suggests that the toxicity of these agents is reflected by their inhibition of general transcription.

The non-specific effects due to drug association with many sequences in the genome and the resultant toxic effects could potentially be avoided by developing more specific DNA-binding compounds. Designing more effective and potent DNA-binding drugs with the goal of disrupting specific TF/DNA complexes is an essential step in developing

potentially therapeutic compounds. Structural studies of drugs bound to their DNA targets has led to a better understanding of the key chemical groups needed for appropriate and strong drug binding. For example, structural studies have elucidated the antiparallel binding mode of two distamycin molecules in the minor groove of DNA and similar studies have helped to explain the chemical basis for this agent's A/T sequence preference (43,44). This knowledge subsequently led to improved rational design of sequence selective agents as well as advances in designing novel, sequence specific, lexitropic drugs, such as Dervan's polyamides (45). Polyamides synthesized to target specific TF binding sites, such as those for Ets-1, have proven successful in inhibiting TF/DNA complex formation in EMSAs as well as cell-free transcription (46). Similar results have been obtained for Bruice's microgonotropens, high affinity MGBs that preferentially bind to A/T rich sites, but make contact with the major groove via polyamine tails. These characteristics made these agents extraordinarily potent inhibitors of E2F binding to its mixed A/T and G/C target sequence in the major groove (47). Analysis of the effectiveness of these novel compounds in inhibiting transcription and comparisons to the sequence selectivity and potency of the drugs described here will be carried out using the c-fos SRE.

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FIGURE LEGENDS:

FIG. 1. **The structures of chromomycin A_3, nogalamycin, and Hoechst 33342.**

FIG. 2. **Components of the c-fos promoter.** *A.* TFs bound to the human c-fos promoter's SRE. Binding of the homodimer of SRF to the A/T rich CArG box is required for the recruitment of Elk-1. Elk-1 binds to the *ets* motif, which contains an invariant GGA core. Together, these TFs make up the ternary complex (TC). *B.* The binding of SRF and Elk-1 to radiolabeled probes in EMSAs. 1 nM of ^{32}P-labeled oligonucleotides was incubated with purified proteins for 30 minutes at room temperature before being electrophoresed on a 4% polyacrylamide gel and autoradiographed. The 24 bp oligonucleotides contained sequences from the c-fos SRE or the Drosophila E74 promoter. *Lane 1*, free SRE probe; *lanes 2 and 5*, SRE plus SRF protein; *lane 3*, free E74 probe; *lane 4*, E74 plus Elk-1 protein; *lane 6*, SRE plus SRF and Elk-1 forms the TC. In lanes 5 and 6, the gel was electrophoresed longer to maximize shift differences. The free SRE probe ran off the gel under these conditions.

FIG. 3. **Effect of drugs on preventing complex formation in EMSAs.** *A.* Chromomycin's effect on Elk-1 and SRF complex formation. Increasing amounts of drug were incubated with the probes for 30 minutes before addition of purified proteins and electrophoresis as described in Fig. 2. *Lanes 1-7* contain Elk-1 complexes. *Lanes 1-2*, Elk-1 complex controls, no drug; *lanes 3-7* contain 50, 25, 10, 5 and 1 μM chromomycin respectively. *Lanes 8 and 10-15* contain TC bound to the SRE. *Lanes 8 and 10*, TC controls, no drug; *lane 9*, SRF complex control, no drug; *lanes 11-15* contain 100, 50, 25, 10 and 1 μM chromomycin respectively. *B.* Quantitation of chromomycin's inhibition of complex formation. Autoradiographs, as shown in *A*, were scanned on a densitometer and quantitated using the manufacturer's software (ImageQuant). Percent probe shifted in each drug treatment was then compared to non-drug treated controls to yield percent complex inhibition for SRF(O), Elk-1(■) and TC (Δ). Results are the mean of four experiments (mean value ± standard error). *C.* Comparison of drugs' potency in preventing complex formation in the EMSAs. Drug concentrations needed to prevent each complex formation by 50% (IC_{50}s) were calculated using graphs as shown in *B.* The higher the IC_{50}, the less potent the drug.

FIG. 4. **Effect of drugs on cell-free c-fos promoter-driven transcription** *A.* Chromomycin's effect on cell-free transcription. All incubations are at 30°C. Sph I-linearlized pFosLuc, a plasmid containing the human c-fos promoter upstream of a luciferase gene, was incubated with varying concentrations of drug for 30 minutes. Nuclear lysate prepared from serum induced NIH3T3 cells was then added and allowed to incubate for 15 minutes before the addition of a mix of nucleotides and [^{32}P]CTP. After a 1 hour incubation, the reaction was stopped, internal control was added, and the RNA transcripts were phenol extracted, ethanol precipitated, re-suspended in formamide loading buffer, and electrophoresed on a 4% denaturing polyacrylamide gel. *Top arrow*: the expected pFosLuc transcript at approximately 750 bases; *lower arrow*, internal control: a T3 transcript from pGEM4z at approximately 250 bases. *Lanes 1-2, 4-5, and 6-7* contain 7.5, 5, and 2.5 μM chromomycin, respectively. *Lanes 3 and 8*, marked by asterisks, are controls, with no drug treatment. Positions of size markers in a typical RNA ladder, in kilobases, are indicated. *B.* Quantitation of chromomycin's inhibition of cell-free transcription. As in Fig. 3B, quantitation of scanned autoradiographs and comparison to controls yielded percent inhibition of transcription. Results are the mean of three experiments (mean ± standard error). *C.* Comparison of drugs' effectiveness as inhibitors of cell-free transcription. IC_{50}s for each agent were calculated from graphs as shown in *B.*

FIG. 5. **Representative Northern blot results.** *A.* Characteristics of c-fos expression in NIH3T3 cells. Following various treatments, total RNA was isolated and 20 μg was run on formaldehyde containing agarose gels, transferred to a nylon membrane, and hybridized with radiolabeled probes for c-fos and G3. The treatments were as follows: *Lanes 1-2*, normally growing cells, *lanes 3-4*, 16 hour starvation in 0.5% serum, *lanes 2 and 4*, induced cells by raising serum concentration to 15% for 30 minutes *B.* Representative results on c-fos expression following exposure of NIH3T3 cells to chromomycin. After cells were starved for 16 hours, drug was added for 1 hour, then cells were induced for 30 minutes as in *A*. *Lanes 1-3*, controls, no drug treatment; *lanes 4-5, 6-7, and 8-9* were exposed to 1, 0.5, and 0.25 μM chromomycin, respectively.

FIG. 6. **Quantitation of drugs' effects on endogenous c-fos expression in NIH3T3 cells following 1 hour exposures.** Cells were starved for 16 hours before being exposed to a range of drug concentrations, in μM, for one hour and induced with 15% serum for 30 minutes as described in Fig. 5. The RNA isolated was analyzed in Northern blot assays through hybridization to radiolabeled c-fos and G3 probes. The blots were visualized following their exposure to a phosphorimaging screen and scanned using a phosphorimager (Molecular Dynamics).

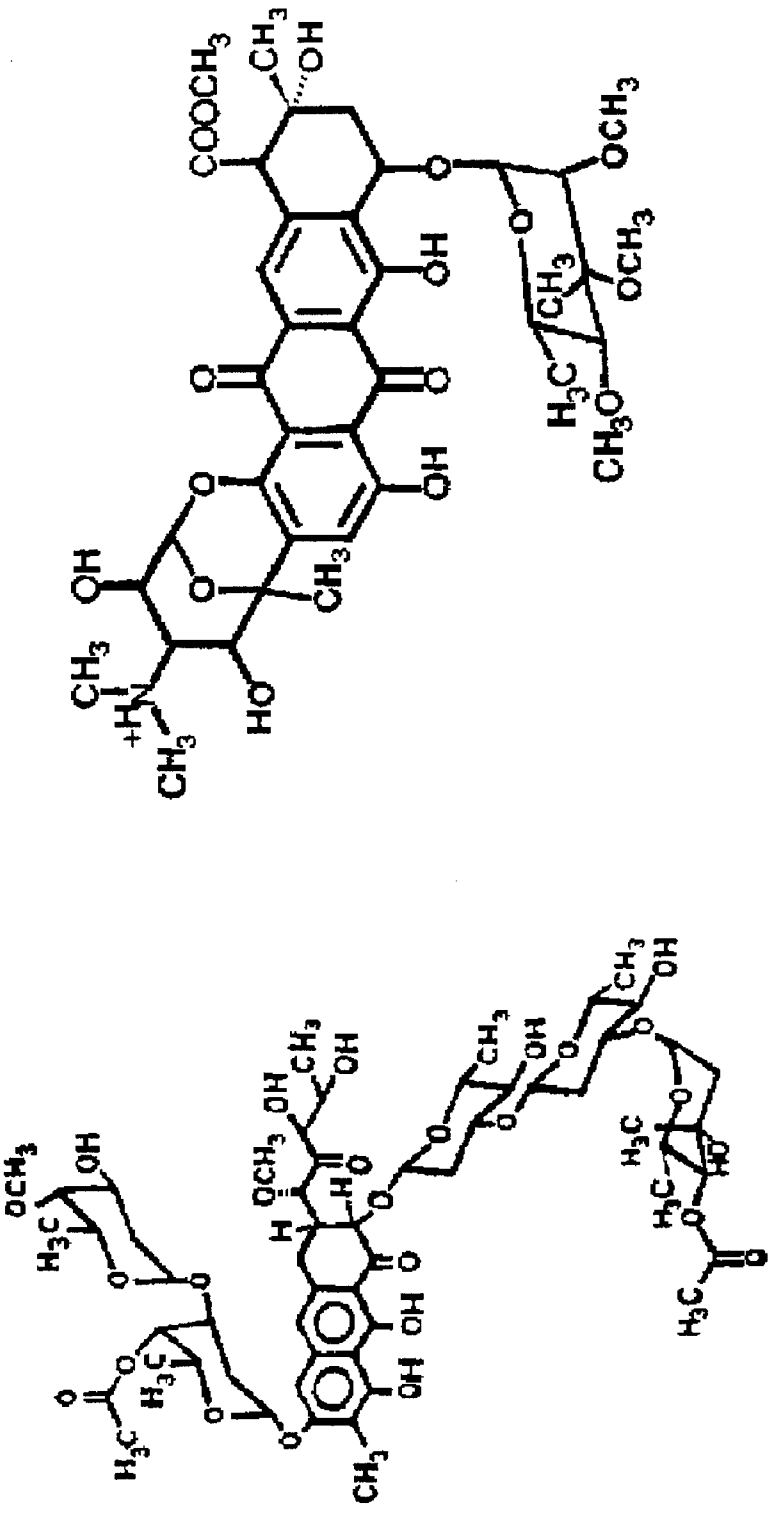
Quantitation of the bands and comparison of the drug treated lanes to controls yielded percent inhibition for each drug: *A*, chromomycin, *B*, nogalamycin, and *C*, Hoechst 33342. Results are the mean of five experiments (mean ± standard error). The IC_{50}s calculated from these graphs are presented in Fig. 8.

FIG. 7. **Chromomycin's inhibition of endogenous c-fos expression in NIH3T3 cells over time.** Cells were starved for 16 hours before being exposed to 1 μM chromomycin for various lengths of time. They were then induced with 15% serum for 30 minutes. Analysis using Northern blots and quantitation was as previously described in Fig. 6. Results are the mean of three experiments (mean ± standard error).

FIG. 8. **Effect of time on drug inhibition of endogenous c-fos expression.** One hour drug exposures (gray bars) following 16 hour starvations of NIH3T3 cells were performed as described in Fig. 5. The 4 hour exposures (white bars) were also performed after the cells were starved 16 hours. For the 16 hour exposures, (black bars), cells were starved and exposed to drug simultaneously. Northern blot analysis following hybridization to c-fos and G3 probes and subsequent quantitation yielded IC_{50} values. These values are plotted for each time point for *A*, chromomycin; *B*, Hoechst 33342, and *C*, nogalamycin.

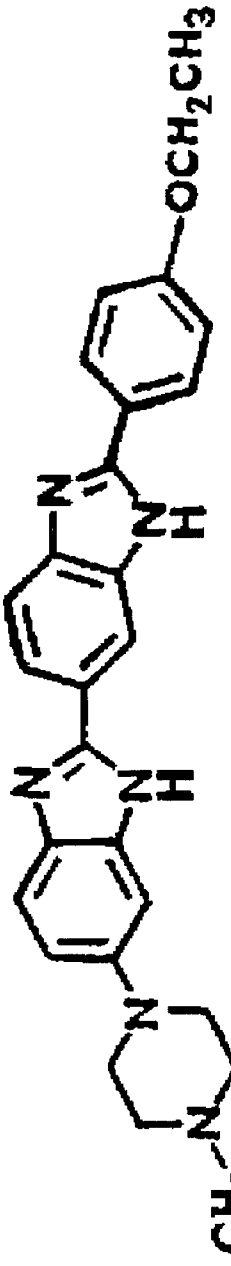
FIG. 9. **Cytotoxic effect of drugs on NIH3T3 cells.** Cells were exposed to a range of drug concentrations for four hours before being serially diluted, replated, and allowed to grow undisturbed for 10 days. The cells were then stained and fixed in a solution of methylene blue and ethanol. Groups of more than 50 cells were deemed colonies. Colonies were counted using a stereo microscope. Plating efficiencies were calculated by dividing the number of colonies by the number of cells plated. Relative plating efficiencies were then calculated by dividing the plating efficiency of each drug treatment by the control plating efficiency. The results shown for chromomycin (Δ), nogalamycin (□), and Hoechst 33342 (O) are the mean of three experiments (mean ± standard error).

TABLE 1. **IC_{50} values for selected whole cell assays.** Concentrations of drug, in μM, needed to inhibit the measured activity by 50%. The values for each assay, as described in the "Materials and Methods" section, were obtained after exposing NIH3T3 cells to drug for four hours. The higher the IC_{50} value, the less potent the drug.



Chromomycin

Nogalamycin



Hoechst 33342

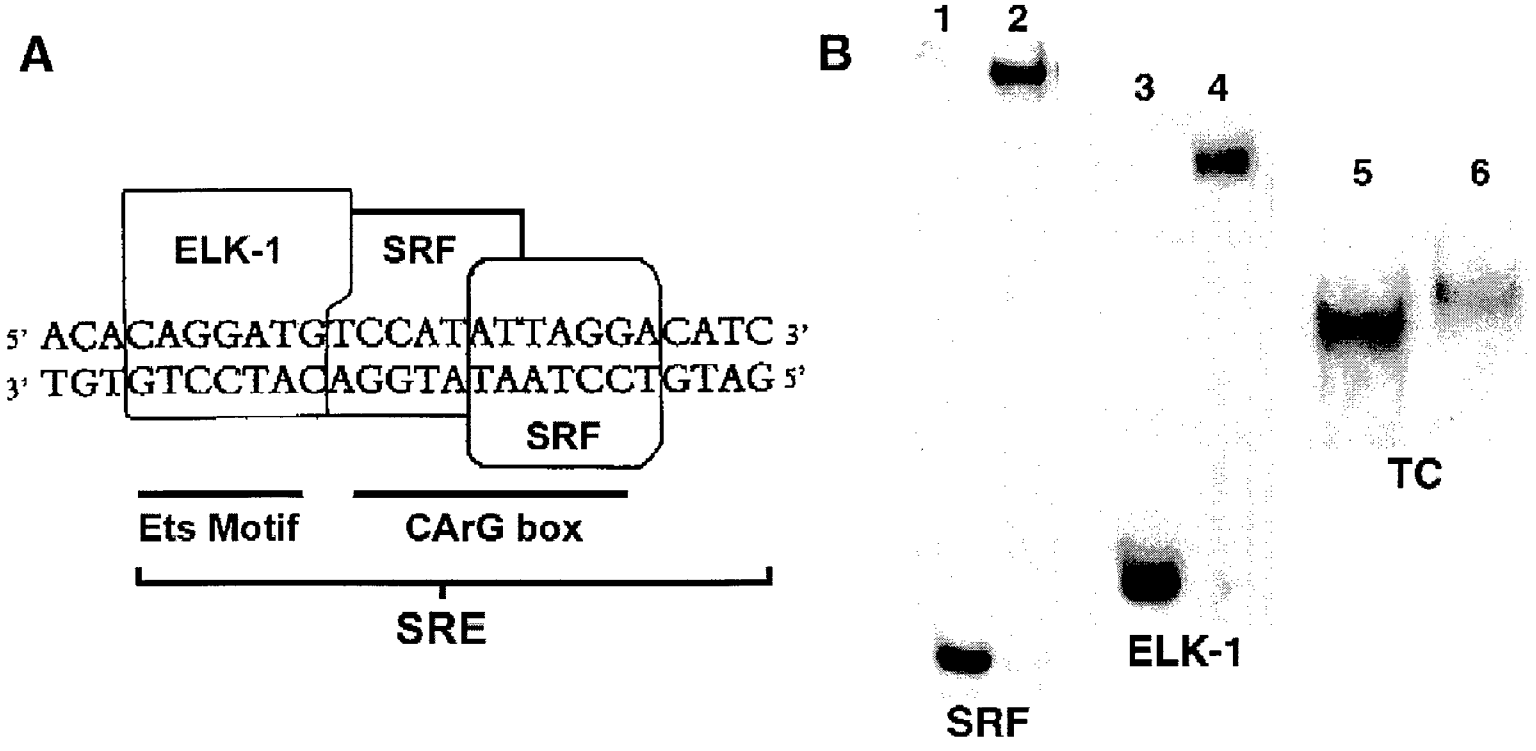
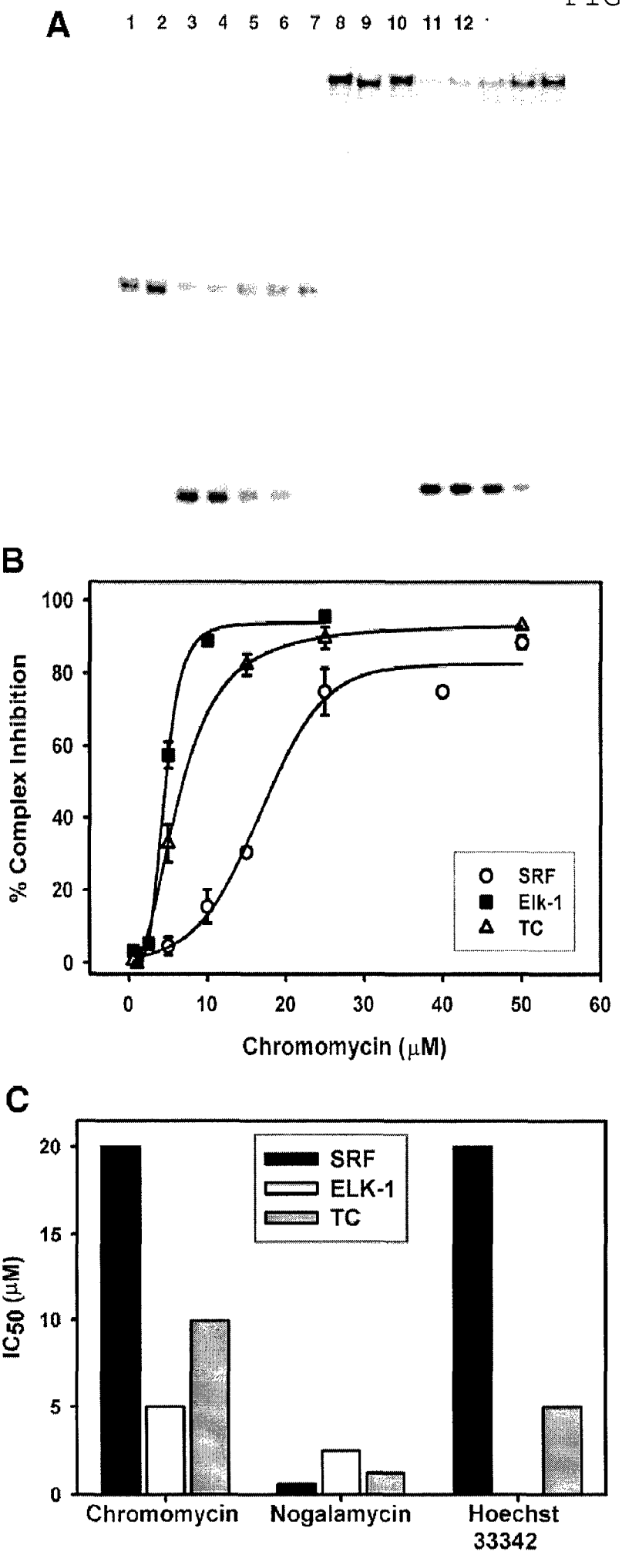
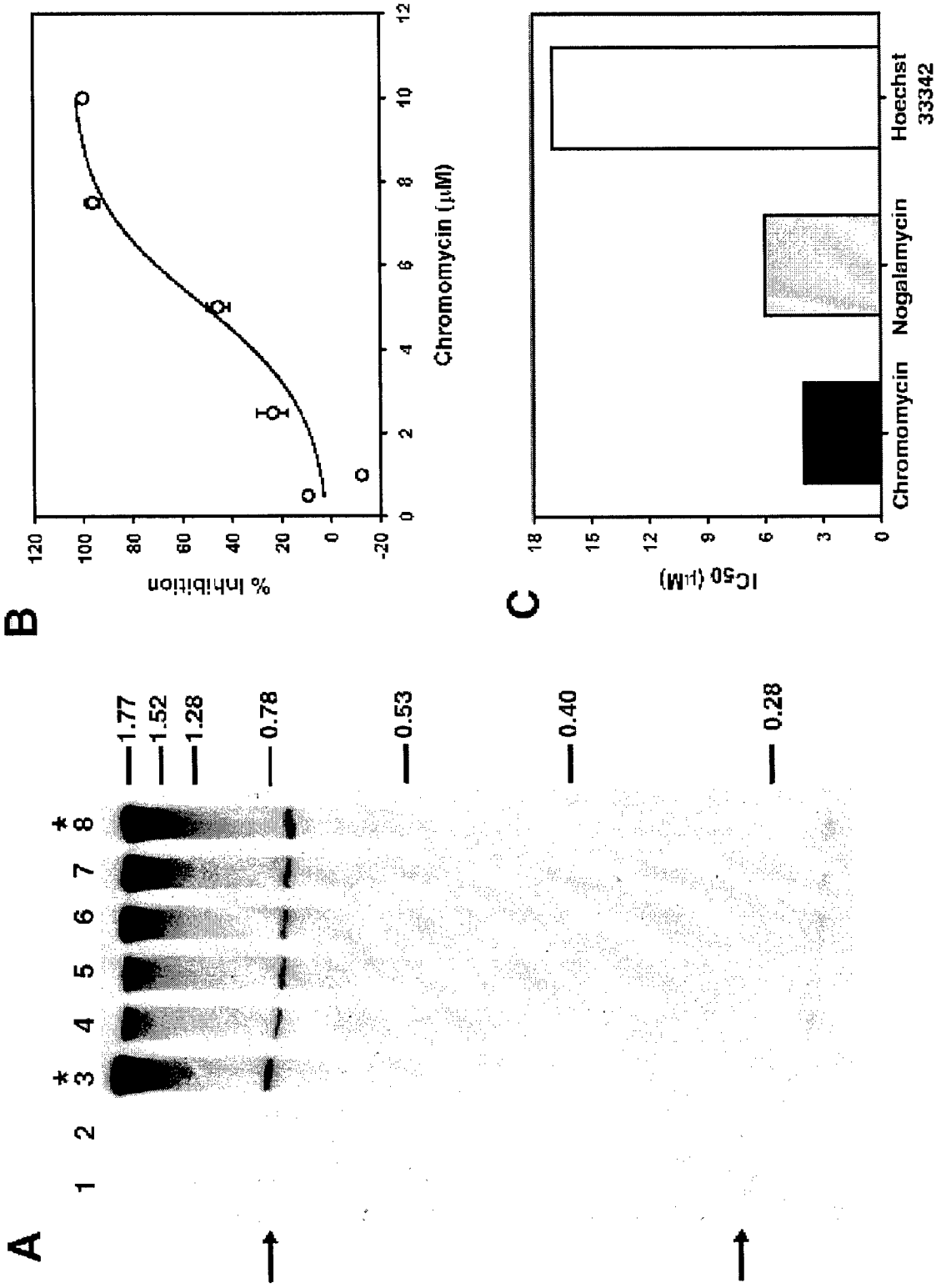
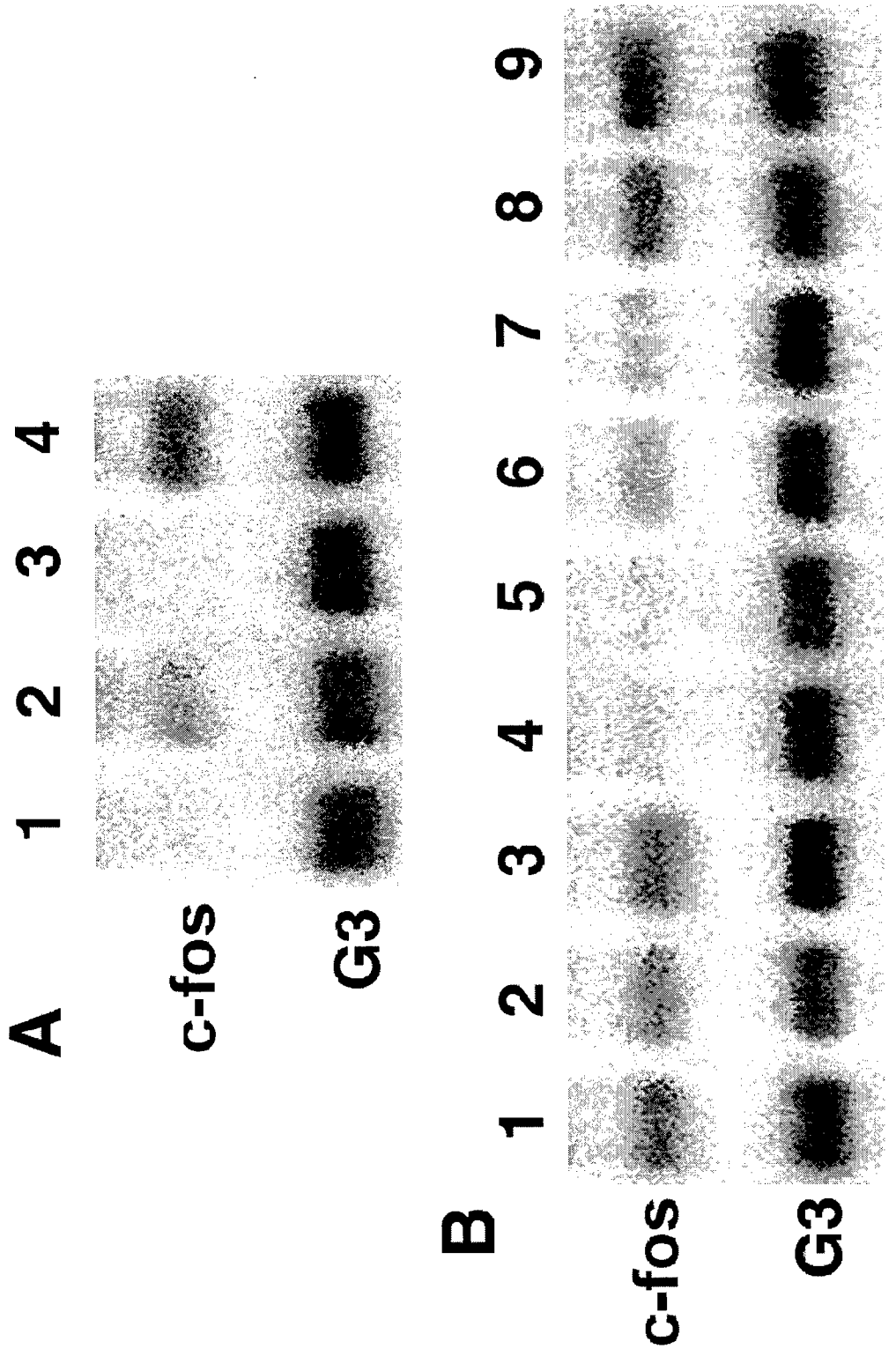
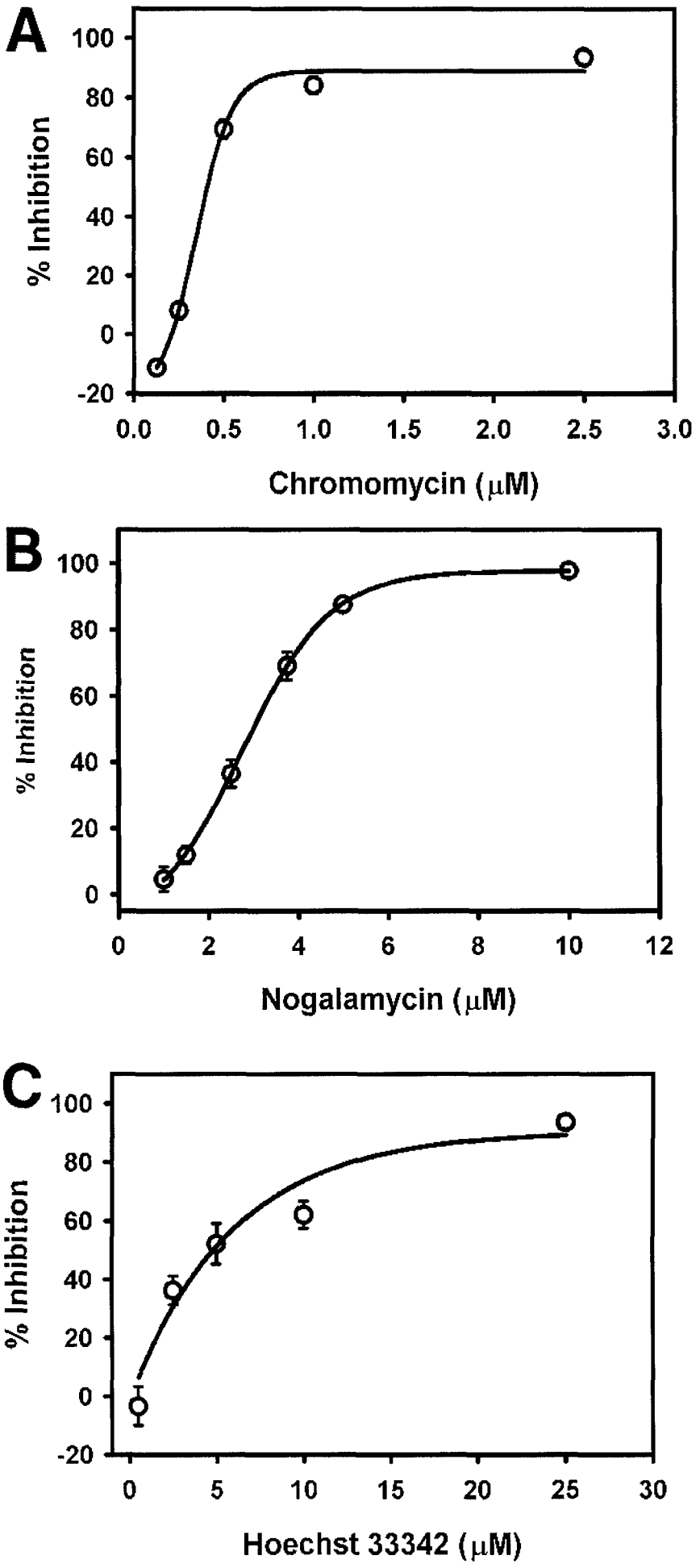

A
ELK-1
SRF
5' ACACAGGATGTCCATATTAGGACATC 3'
3' TGTGTCCTACAGGTATAATCCTGTAG 5'
SRF
Ets Motif
CArG box
SRE
B
1
2
3
4
5
6
TC
ELK-1
SRF

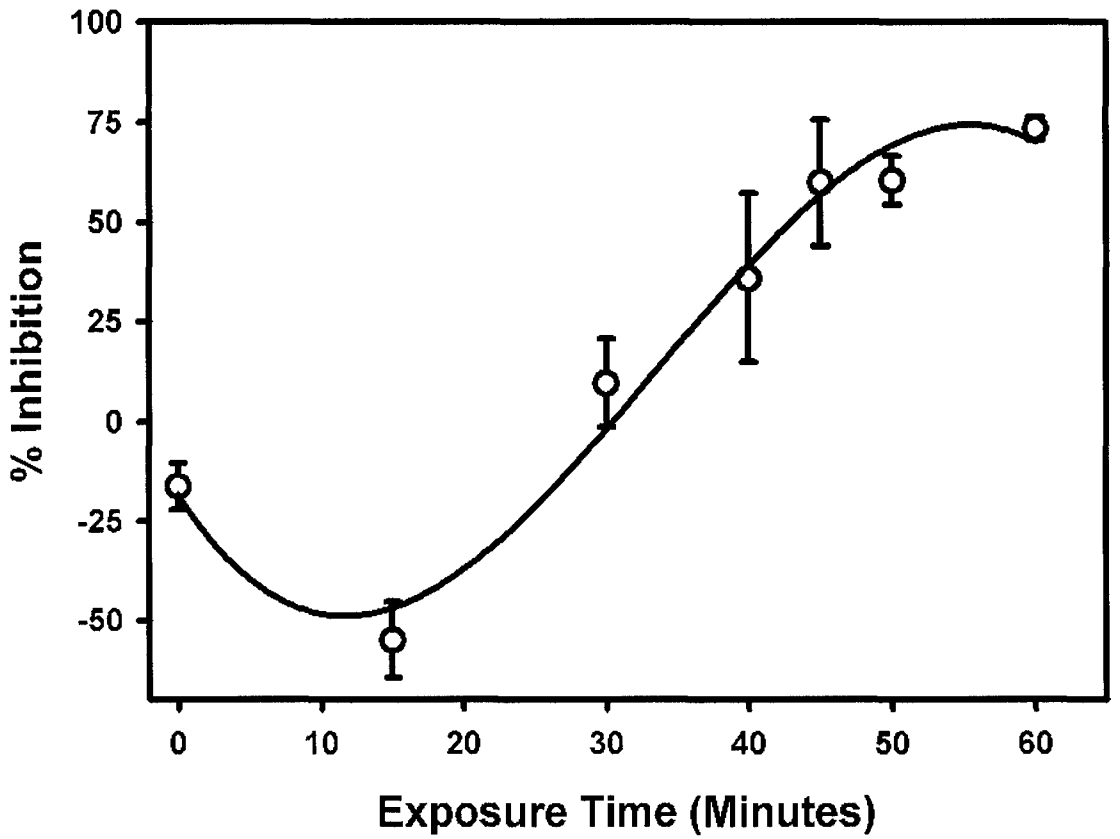
FIGURE 2

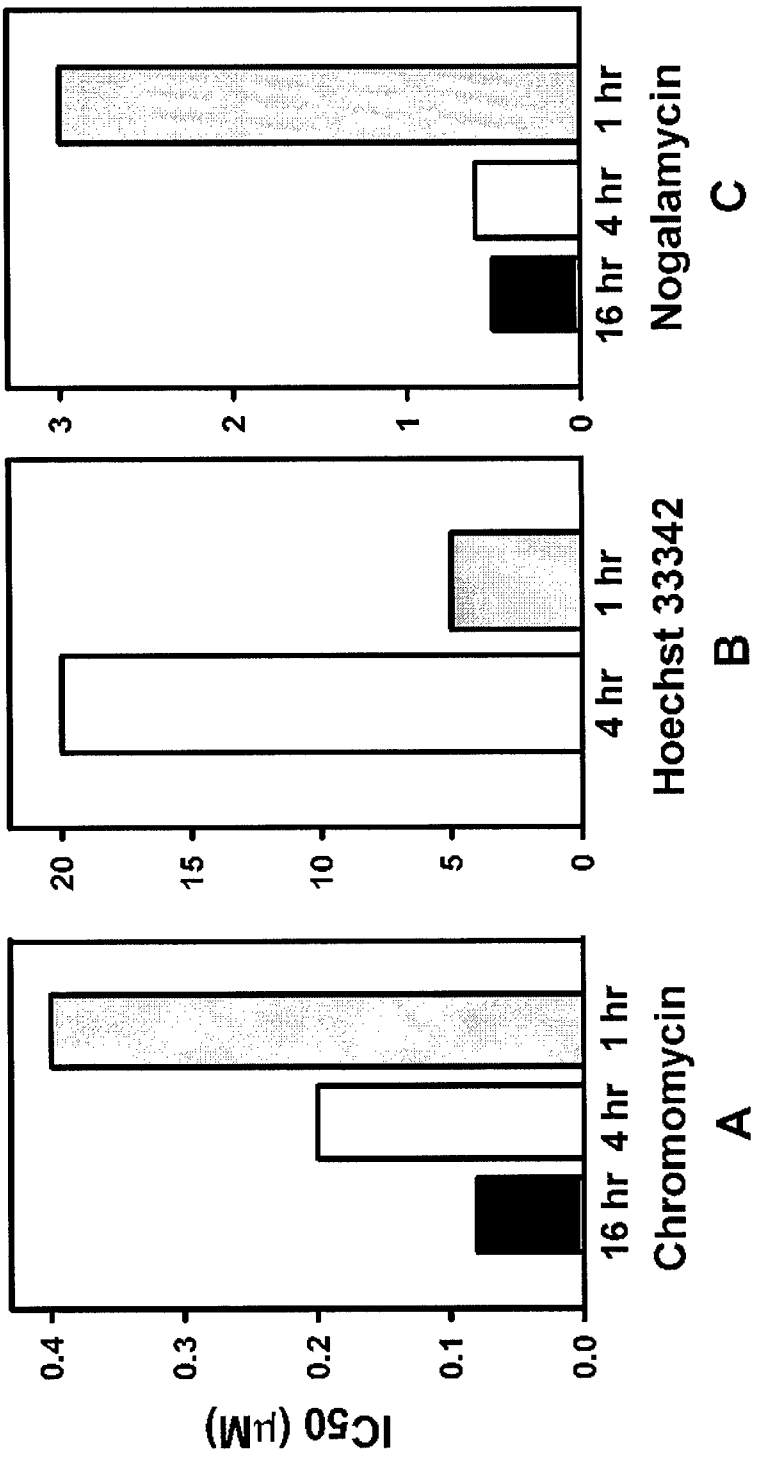

A
1 2 3 4 5 6 7 8 9 10 11 12
B
% Complex Inhibition
100
80
60
40
20
0
0 10 20 30 40 50 60
Chromomycin (μM)
SRF
Elk-1
TC
C
IC50 (μM)
20
15
10
5
0
SRF
ELK-1
TC
Chromomycin
Nogalamycin
Hoechst 33342

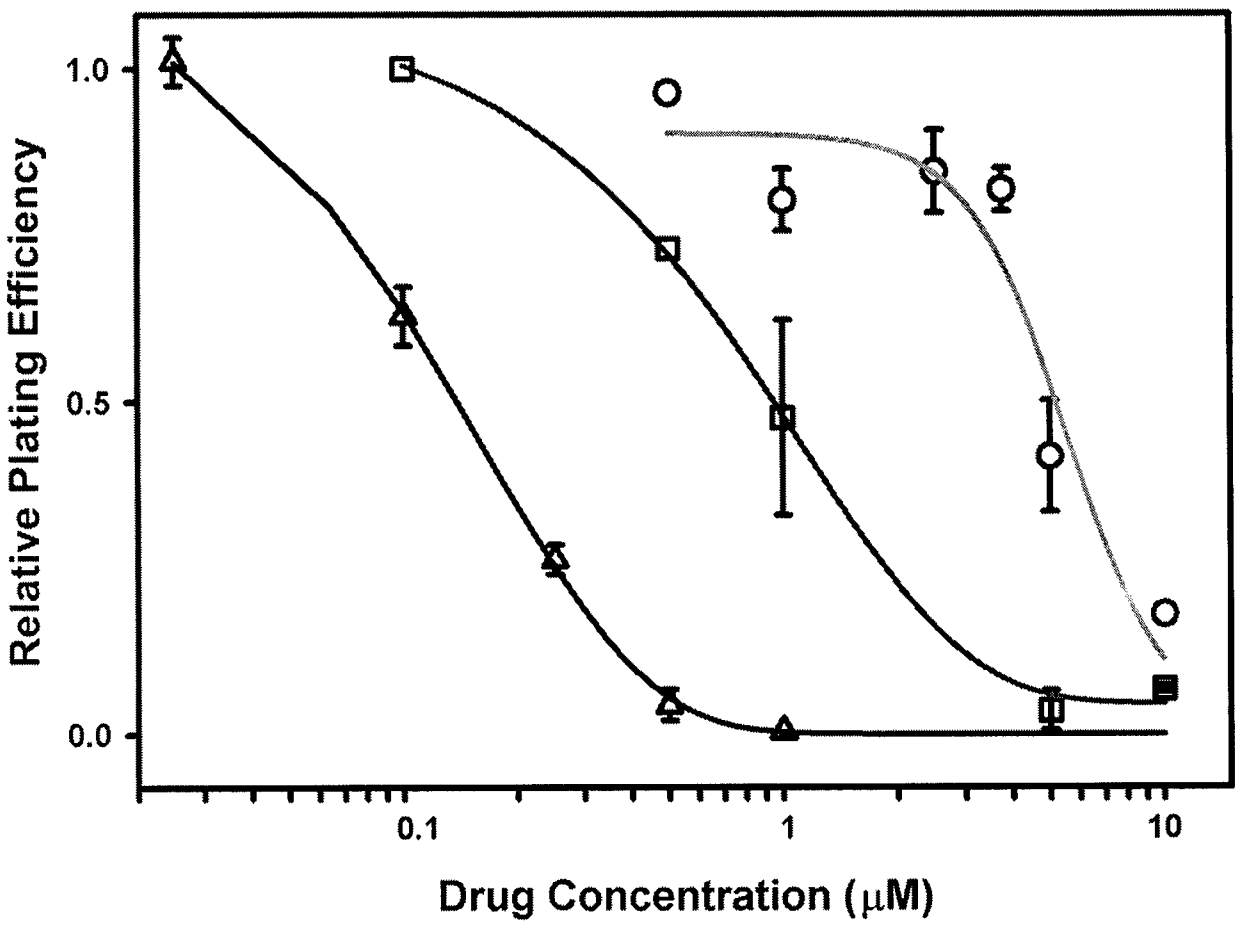

A
1
2
*3
4
5
6
7
*8
1.77
1.52
1.28
0.78
0.53
0.40
0.28
B
% Inhibition
Chromomycin (μM)
C
IC50 (μM)
Chromomycin
Nogalamycin
Hoechst 33342


A
1 2 3 4
c-fos
G3
B
1 2 3 4 5 6 7 8 9
c-fos
G3


A
100
80
60
40
20
0
-20
% Inhibition
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Chromomycin (μM)
B
100
80
60
40
20
0
% Inhibition
0
2
4
6
8
10
12
Nogalamycin (μM)
C
100
80
60
40
20
0
-20
% Inhibition
0
5
10
15
20
25
30
Hoechst 33342 (μM)


100
75
50
25
0
-25
-50
% Inhibition
0
10
20
30
40
50
60
Exposure Time (Minutes)


IC50 (μM)
0.0
0.1
0.2
0.3
0.4
16 hr 4 hr 1 hr
Chromomycin
A
0
5
10
15
20
4 hr 1 hr
Hoechst 33342
B
0
1
2
3
16 hr 4 hr 1 hr
Nogalamycin
C


1.0
0.5
0.0
Relative Plating Efficiency
0.1
1
10
Drug Concentration (μM)

Drug	Cytotox.	N.Blot	RNA Synthesis
Chromomycin	0.125	0.2	0.1
Nogalamycin	1	0.6	0.5
Hoechst 33342	4.5	20	7.5

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

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