Inhibition of transcription factor-DNA complexes and gene expression by a microgonotropen

Christine M. White*, Alexander L. Satz†, Thomas C. Bruice†, and Terry A. Beerman*‡

*Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263; and †Department of Chemistry, University of California, Santa Barbara, CA 93106

Contributed by Thomas C. Bruice, July 19, 2001

Developing minor groove-binding drugs to selectively inhibit transcription factor (TF)/DNA interactions and accompanying gene expression is a current goal in drug development studies. Equipping minor groove-binding agents with positively charged, major groove-contacting side chains yields microgonotropens (MGTs). Previously, we demonstrated that MGTs were superior inhibitors of TF/DNA complexes in cell-free assays compared with ''classical'' groove binders, but MGTs showed limited ability to inhibit gene expression. To determine what chemical characteristics contribute to or improve activity, we evaluate five MGTs for their effectiveness in inhibiting TF complex formation and resultant transcription by using the c-fos serum response element (SRE) as a target. MGT L1 binds DNA via a bisbenzimidazole equipped with a tripyrrole moiety. It is compared with analog L2, which has been functionalized with propylamines on each of the three pyrroles. L2, which binds DNA at subpicomolar concentrations, was at least three orders of magnitude more potent than L1 at inhibiting TF binding to the c-fos SRE in cell-free assays. Unlike L1 and previous MGTs, L2 also inhibited endogenous c-fos expression in NIH 3T3 cells at micromolar levels. Structure/activity relationships suggest that, although the tripyrrole/polyamine functional group of L2 may be largely responsible for its inhibition of TF complexes in cell-free assays, its bisbenzimidazole moiety appears to impart improved cellular uptake and activity. These findings make L2 a promising lead candidate for future, rational MGT design.

Rational design of DNA-binding ligands to inhibit targeted gene expression has widespread implications in understanding the molecular mechanisms of gene regulation and in ultimately developing novel therapeutics. The binding of transcription factors (TFs) to their target sites on gene promoters is essential for proper regulation of transcription. Selectively inhibiting the formation of TF/DNA complexes may therefore potentially decrease expression of a target gene of interest.

Recently, approaches using minor groove-binding agents to inhibit TF/DNA complex formation and transcription in a variety of cell-free systems have been explored (reviewed in ref. 1). The effectiveness of these agents as inhibitors is often closely related to their mode of binding and sequence selectivity, and it appears that many types of TF/DNA complexes can be targeted. For example, the A/T-selective minor groove-binding agent distamycin prevented the TATA binding protein from binding to its A/T-rich target site in the minor groove, but it was unable to inhibit the major groove-binding TF EGR from binding to its G/C-rich sequence (2). However, distamycin could prevent major groove-binding homeodomain peptides from binding to their A/T-rich sites (3).

Most TFs associate with high affinity primarily to their consensus binding sites on the major groove, but they gain increased specificity through simultaneous minor groove contacts (4). This suggests that high-affinity DNA binders capable of contacting both grooves may be more effective inhibitors of TF/DNA complexes than agents that bind solely to a single groove. The microgonotropens (MGTs) were designed with this goal in mind. The first generation of MGTs consisted of minor groove-binding, A/T-selective tripyrrole moieties with protonated polyamine tails attached to the central pyrrole (5). These tails allow MGTs to electrostatically contact the phosphodiester backbone on the major groove side of the helix, endowing them with high DNA-binding affinity (K_a values $\approx 10^{10}$ M^{-1}) and the ability to bend DNA, characteristics that may help them to more effectively compete with TFs for binding to target sites (6, 7). Indeed, initial studies demonstrated that MGTs could be orders of magnitude more potent than distamycin in inhibiting the TF E2F1 from binding to DNA (8). Further exploration into designing agents with similar capabilities led to the development of fluorescent MGTs (9, 10). These agents selectively bind A/T-rich sites in the minor groove via a bisbenzimidazole moiety, but polyamine tails linked in *meta* to the terminal phenyl ring allow electrostatic contact with the phosphodiester backbone. The DNA-binding affinities of these agents are significantly greater than ''classical'' bisbenzimdazoles like Hoechst 33258 (9).

The well defined serum response element (SRE) of the human c-fos promoter was used as a target to evaluate these agents as TF complex inhibitors in increasingly complex assay environments. The binding of a dimer of serum response factor (SRF) to an A/T-rich site and subsequent recruitment of the *ets* factor Elk-1 to a GGA core sequence to form a ternary complex (TC) is required for c-fos transcription (11). Both TFs primarily bind DNA in the major groove but make minor groove contacts (12, 13). This mode of binding and the presence of an A/T-rich site make these TFs potential targets for MGTs. We found that, whereas a bisbenzimidazole possessing a linker without basic side chains was completely ineffective at inhibiting TC formation in cell-free assays, fluorescent MGTs functionalized with polyamine tails of various lengths and degrees of branching could do so at micromolar concentrations and were up to 50 times more potent than the classical bisbenzimidazole Hoechst 33342 (Hoe342). However, although these fluorescent MGTs also inhibited c-fos promoter-driven cell-free transcription, they, like the first generation of MGTs, were inactive in cells (C.M.W. and T.A.B., unpublished data).

Because enhanced inhibition of TF/DNA complexes was observed when simple minor groove-binding agents were equipped with phosphodiester backbone-contacting polyamine tails, we further explored this drug design strategy and structure/activity relationships in an effort to create more potent agents that were also capable of decreasing gene expression. Past studies have shown that DNA-binding drugs lose relative potency as assay conditions become more complex (that is, in cell-free expression assays, where additional proteins and DNA

Abbreviations: TF, transcription factor; MGT, microgonotropen; SRE, serum response element; SRF, serum response factor; TC, ternary complex, dsDNA, double-stranded DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; oligo, oligonucleotide; topo II, topoisomerase II.

‡To whom reprint requests should be addressed. E-mail: Terry.Beerman@Roswellpark.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.191374698

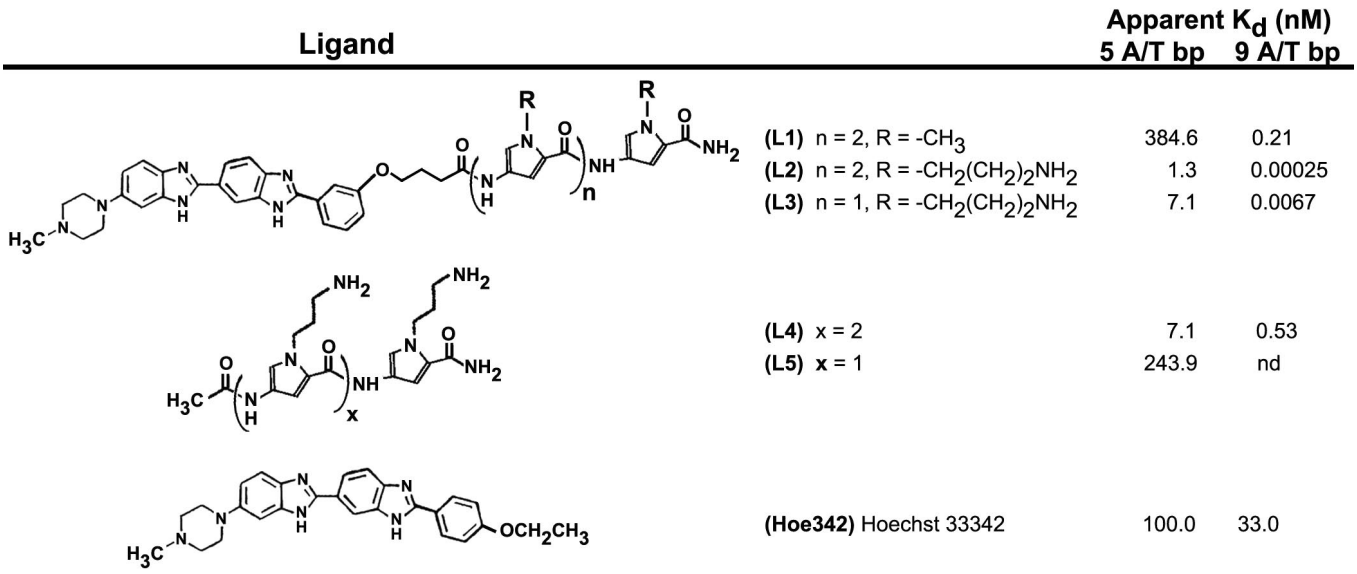

Fig. 1. Chemical structures of the DNA-binding agents used in this study. Previously determined apparent equilibrium dissociation constants (K_d values) were determined from melting curves of ligand:dsDNA complexes using 0.3 μM of the oligos 5′-CGCAAAAACGCACC-3′ and 5′-CGCAAAAAAAAACGC-3′ in 10 mM potassium phosphate buffer, pH 7.0, and 150 mM NaCl in the presence of 0.6 μM ligand. nd, not determined.

are added) (14, 15), and we wished to determine whether such limitations could be overcome by improved rational design.

Recent chemical characterization of the novel tripyrrole-bisbenzimidazole agent **L1** (Fig. 1) showed that it possessed interesting double-stranded DNA (dsDNA) binding characteristics, including recognition of 9 contiguous A/T bp at nanomolar levels and the ability to form antiparallel 2:1 complexes in the minor groove of DNA (16). Because attaching a single polyamine tail to tripyrrole compounds led to orders of magnitude increases in potency in inhibiting TF complexes in cell-free assays, each pyrrole subunit of **L1** was equipped with propylamines to yield **L2**. **L2** was found to have sequence selectivity and discrimination for 9-bp, A/T-rich sites similar to those of **L1**, but it binds dsDNA with a remarkable 1,000-fold higher affinity (16).

We now analyze the ability of these agents to inhibit TF/DNA complex formation on the c-fos SRE and subsequent gene expression in increasingly complex assays. Moreover, the activities of **L2** analogs are also evaluated to better determine how MGT structure and activity relate. These compounds include **L3**, which is similar to **L2** but possesses one less pyrrole subunit, and **L4** and **L5**, the pyrrole/polyamine moieties of **L2** and **L3**, respectively. A representative classical bisbenzimidazole, Hoe342, is used as a control minor groove binder. Besides comparing their potencies in inhibiting TF/DNA complex formation and transcription in cell-free assays, we explored how selective these agents were for inhibiting DNA template-related activities by using a topoisomerase II (topo II) assay. Their abilities to inhibit endogenous c-fos expression in whole cells and to cause cell death were also evaluated. Our studies reveal that equipping bisbenzimidazoles with phosphodiester backbone-contacting functional groups leads to increased inhibitory effectiveness by orders of magnitude in cell-free assays and also results in the first demonstration of MGT cellular activity. Importantly, the potency and cellular activity of **L2** make it a promising lead compound for future drug design.

Methods

DNA-Binding Ligands. MGTs were synthesized as described (16) and reconstituted in 25% DMSO. Hoe342 (Aldrich) was prepared in ddH_2O. Drug stocks were stored at −20°C and were diluted into ddH_2O before use.

Cell Culture. NIH 3T3 cells were maintained as described (14). HeLa cells were maintained in suspension cultures in Joklik-modified MEM (S-MEM) with 5% FBS and 5% horse serum at 37°C.

Electrophoretic Mobility Shift Assay (EMSA). The 24-bp oligonucleotides (oligos) derived from the human c-fos SRE (5′-ACACAGGATGTCCATATTAGGACA-3′) and the *Drosophila* E74 promoter (5′-GATACCGGAAGTCCATATTAGGAC-3′) were synthesized, purified, and end-labeled as previously described (14). Purification of 6-histidine-tagged SRF and Elk-1 following expression in bacteria, the assay reaction conditions, and subsequent autoradiogram analysis have also been described in detail (14). In brief, drug and oligo were allowed to incubate 30 min before addition of purified TFs and subsequent electrophoresis on a polyacrylamide gel.

Cell-Free Transcription. HeLa nuclear extracts were prepared following published protocol and stored at −80°C until use (14). The reaction was carried out and the results analyzed as previously described (14, 17). In brief, a linearized plasmid containing the full-length c-fos promoter was incubated with drug for 30 min at 30°C before the addition of 15 μg of nuclear extract and a subsequent 15-min incubation. After a 60-min incubation in the presence of [α-^{32}P]CTP, the resulting 750-base transcript was purified and electrophoresed on a denaturing polyacrylamide gel. A radiolabeled 250-base T3 transcript of pGEM4z (Promega) was used as an internal control. Normalization of the c-fos transcript to the internal control allowed accurate comparisons between samples.

Topo II Activity Assay. The isolation of HeLa nuclei and the subsequent assay reactions were as described (18, 19). Briefly, HeLa cells radiolabeled for 24 h with [^{14}C]thymidine were lysed in the presence of detergent and nuclei were pelleted by centrifugation. Nuclei were incubated with 20 μM drug for 10 min at 37°C before the addition of 20 μM VM-26, a topo II/DNA

crosslinking agent. After 15 min at 37°C, the topo II/DNA crosslinks were precipitated by hot K^+/SDS. The washed pellets were hydrolyzed in perchloric acid before precipitated counts were measured by scintillation counting.

Northern Blot Assay. Following published protocol (14), NIH 3T3 cells were treated with drugs for 16 h under starvation conditions (growth media with 0.5% serum) before c-fos expression was induced by raising serum concentration to 15%. Total RNA was isolated, electrophoresed on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled cDNA probes to c-fos and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Densitometric analysis of autoradiographs allowed comparison between drug-treated samples and controls and calculation of percent inhibition of expression.

Cytotoxicity Assay. A total of 1×10^4 NIH 3T3 cells were seeded into 24-well plates and allowed to grow 24 h before being exposed to drug in triplicate. Controls received solvent only. Cells were incubated under normal growth conditions for 72 h. The medium was removed, and cells were rinsed once with PBS before being trypsinized and counted with a particle counter (Coulter). Drug-treated samples were compared with controls to calculate percent control growth.

Results

Design of the Novel DNA-Binding Ligands. To explore whether adding basic side chains to novel minor groove binders by using a rational design approach would improve their ability to inhibit TF/DNA interactions, **L1** was used as a starting compound (Fig. 1). Previously determined K_d values show that **L1** binds with high affinity to A/T-rich sites and can distinguish between 5 and 9 A/T bp. Adding propylamine tails to each of **L1**'s pyrroles yields **L2**, which has vastly improved binding affinity and greater sequence selectivity. The effect of side chain length on inhibitory activity was assessed by synthesizing **L3**, which has one less pyrrole than **L2**. The contribution of the pyrrole/propylamine tails to activity was explored by using the analogs **L4** and **L5**, which correspond to the basic side chains of **L2** and **L3**, respectively. Hoe342, a well studied classical bisbenzimidazole chemically and structurally distinct from the MGTs, was used as a control and as a reference compound to which the former agents were compared.

Effect of the Agents on TC Formation in EMSAs. The ability of these agents to inhibit TF/DNA complex formation was first analyzed by using EMSAs, a system consisting of purified TFs and a radiolabeled 24-bp oligo. In a representative gel (Fig. 2*A*), SRF and Elk-1 added to the SRE forms the TC, visible as a "shifted" complex (lanes 1 and 2, top arrow). Incubating the oligo with DNA-binding ligand before adding TFs inhibits TC formation in a dose-dependent manner, as shown for both **L5** and **L4** (lanes 3–5 and 6–9, respectively). Quantitation of the percent complexed DNA in each lane and subsequent comparison of ligand-treated samples to controls allows calculation of percent inhibition of complex formation. Plotting these values against ligand concentration yields inhibition curves for each agent (Fig. 2*B*). The most potent agent, **L2**, inhibited TC formation completely by 0.25 μM, while two orders of magnitude more **L1**, the least potent compound, did not even attain 20% inhibition. Compared with **L5**, which also performed poorly and was less effective than Hoe342, **L4** was about 50-fold more potent. To note, the bisbenzimidazoles **L2** and **L3** were 8 and 91 times more effective, respectively, than their polyamide/propylamine analogs **L4** and **L5** in this assay. Results are summarized and potencies of the agents are compared in Table 1, which lists IC_{50} values (the ligand concentration needed to inhibit the observed activity by 50%).

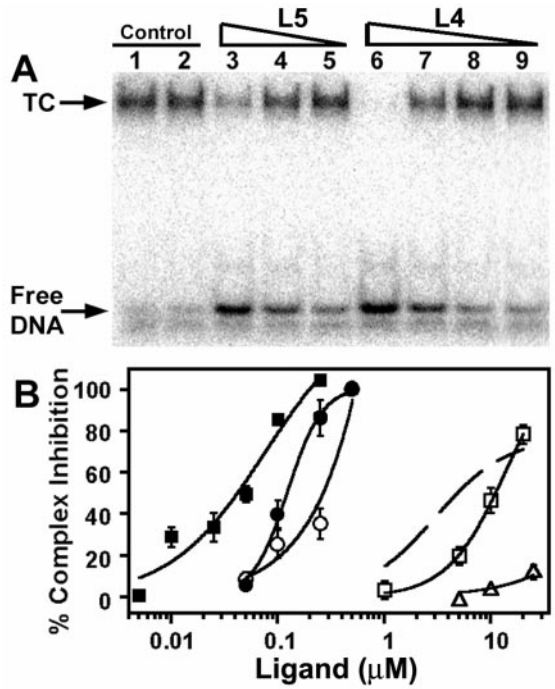

Fig. 2. Effect of agents on complex formation in EMSAs. (*A*) Representative EMSA showing drug inhibition of TC formation on the SRE. DNA-binding agent and oligo were co-incubated 30 min at room temperature before the addition of purified TFs, followed by another 30-min incubation and electrophoresis on a polyacrylamide gel. Lanes: 1 and 2, controls; 3, 4, and 5, 20 μM, 10 μM, and 5 μM **L5**, respectively; 6, 7, 8, and 9, 0.5 μM, 0.25 μM, 0.1 μM, and 0.05 μM **L4**, respectively. (*B*) Inhibition curves for drug effects on TC formation. Quantitation of autoradiographs as in *A* allowed percent inhibition to be plotted against drug concentration for: **L1** (△), **L2** (■), **L3** (●), **L4** (○), **L5** (□), and Hoe342 (– – –). Results are from three experiments (mean value ± SE).

Inhibition of Cell-Free Transcription. In general, although minor groove-binding agents may successfully inhibit TF/DNA interactions in simple EMSAs, many are known to lose relative potency as assay conditions become more complex and are less effective as inhibitors of gene expression (14, 17, 20). To determine whether the five MGTs exhibited similar characteristics, their ability to inhibit cell-free expression in the presence of additional DNA and proteins was analyzed next. In the cell-free transcription assay, a linearized plasmid containing the full-length human c-fos promoter is co-incubated with DNA-binding ligand before a nuclear lysate and radiolabeled nucleotides are added. The resulting transcript is isolated and electro-

Table 1. Comparison of agent potencies on inhibiting TF complexes on the c-fos promoter

	IC_{50} values, μM		
Compound	EMSA: TC formation	Cell-free transcription	N. blot: c-fos expression
L1	>10	nd	*
L2	0.041	0.62	9
L3	0.12	3.15	*
L4	0.32	4.8	*
L5	10.9	27.3	*
Hoe342	4.8	16.8	*

IC_{50} values were obtained from inhibition curves as in Figs. 2*B* and 3*B*. N., Northern; nd, not determined.
*No inhibition was noted at 10 μM.

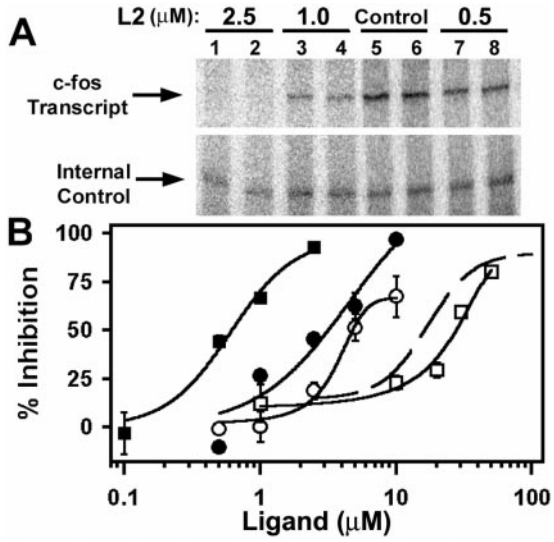

Fig. 3. Effect of agents on c-fos promoter-driven cell-free transcription. (*A*) Representative autoradiograph of assay results using **L2**. A linearized plasmid containing the full-length human c-fos promoter was co-incubated with DNA-binding agent before nuclear lysate, and a mix of radiolabeled nucleotides were added. Analysis of the purified transcript (upper arrow) was as described in the text. A radiolabeled, 250-base transcript (lower arrow) served as a loading control. **L2** concentrations are indicated. (*B*) Effect of agents on cell-free transcription. Normalizing transcripts to internal controls allowed percent inhibition to be calculated and plotted against drug concentration for: **L2** (■), **L3** (●), **L4** (○), **L5** (□), and Hoe342 (– – –). Results are from three experiments (mean value ± SE).

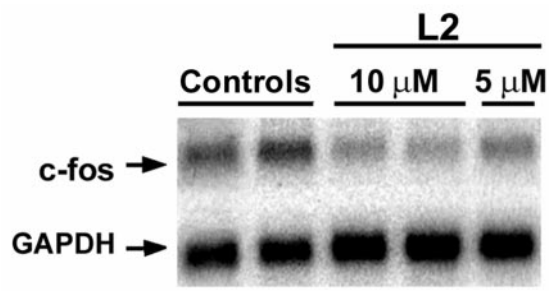

Fig. 4. Effects of **L2** in NIH 3T3 cells. (*A*) **L2** inhibits endogenous c-fos expression. NIH 3T3 cells were exposed to **L2** for 16 h in the presence of low (0.5%) serum. Expression of c-fos was induced by raising serum concentration to 15%. Total RNA was isolated 30 min later and processed as described in the text. Hybridization to radiolabeled cDNA probes for c-fos and GAPDH followed. **L2** concentrations are indicated.

phoresed on a denaturing polyacrylamide gel, where it is identified on the basis of its known size. In the absence of DNA-binding ligand, a high-intensity transcript is produced (Fig. 3*A*, lanes 5 and 6, upper arrow). Treating the plasmid with **L2** results in a dose-dependent decrease in transcript formation (lanes 1–4 and 7–8). After normalizing the transcripts to internal controls (lower arrow), percent inhibition was calculated and inhibition curves were plotted (Fig. 3*B*). Because **L1** exhibited such poor activity in the EMSAs, it was not analyzed in this assay. The inhibition curve profiles for the remaining agents were similar to the EMSAs: **L2** was most potent while **L5** exhibited the lowest potency. However, the gap between the least and most effective agents has narrowed appreciably. Overall, higher concentrations of the agents were needed to inhibit transcription as compared with the EMSAs, with the highest increase (≈26 fold) noted for **L3**. Notably, **L2** was significantly more effective than the other agents tested. Its potency exceeded that of its pyrrole/propylamine analog **L5** by 44-fold (Table 1).

Effect of the Agents on Cellular Gene Expression. Because the potency of **L2** in the cell-free transcription assay was promising, we analyzed this and the other compounds more rigorously by evaluating their effects on endogenous c-fos expression in NIH 3T3 cells. The dependence of c-fos transcription on the formation of a functional TC, its serum inducibility, and its 30-min mRNA half-life (21, 22) facilitated this analysis. Evaluation of the agents showed that only **L2** inhibited c-fos expression in NIH 3T3 cells after a 16-h exposure (Fig. 4 and Table 1). At 10 μM, **L2** inhibited c-fos expression by about 60%. The absolute level of GAPDH mRNA was unchanged. The remaining agents, at concentrations up to 10 μM for 16 h, failed to inhibit c-fos transcription here.

Collateral Effects of the Agents. Because the DNA-binding ligands proved to be effective inhibitors of transcription in cell-free assays and because we were interested in characterizing the biological activity of these agents more fully, we wished to explore whether other activities (unrelated to c-fos expression) were affected by their association with DNA. As an enzyme that maintains DNA topology, topo II induces a doublestrand break, becomes covalently attached to the free DNA ends, and passes the strands through the gap before resealing them (23). Its activity can be measured by trapping the covalent topo II/DNA complex in its intermediate form through the use of drugs such as VM-26, followed by precipitation and quantitation of the complexed DNA (see *Methods*). Minor groove-binding drugs have been found to inhibit the catalytic activity of topo II in this assay (18). The percent inhibition of topo II activity in isolated nuclei obtained at 20 μM for each agent are presented in Table 2. It was noted that the effectiveness of the agents did not correlate to their potencies in the EMSAs or cell-free transcription assays. For example, **L3** inhibited topo II less than would be expected based on its potency in the latter assays. Conversely, **L5**, which was the least potent compound in the previous cell-free evaluations, inhibited topo II activity better than Hoe342 and roughly as well as **L3**.

Because the agents demonstrated an ability to inhibit topo II activity in nuclei and because **L2** could decrease endogenous gene expression, we wondered whether they might affect cell survival. Representative results are shown for **L2** in Fig. 5. We found that **L2** decreased NIH 3T3 viability after a 3-day continuous exposure. Over 80% cell death was effected by 10 μM. Concentrations needed to kill 50% of the cells (LD_{50} values) are presented in Table 2. Analysis of the other agents revealed that

Table 2. Collateral effects of the agents

Compound	Topo II % inhibition*	Cytotoxicity LD_{50}†, μM
L1	49.9	7.0
L2	95.1	6.6
L3	59.6	>10
L4	91.0	‡
L5	61.9	‡
Hoe342	49.3	3.4

*Percent inhibition of topo II activity in HeLa nuclei at 20 μM drug. Nuclei from [^{14}C]thymidine-labeled HeLa cells were treated with 20 μM DNA-binding agent for 10 min at 37°C. The crosslinking agent VM-26 was added to a final concentration of 20 μM and reactions were incubated 15 min. DNA-protein crosslinks were quantitated following sodium dodecyl sulfate precipitation. Results are from three experiments.

†LD_{50} values were calculated from dose-response curves as shown in Fig. 5.

‡No effect at 10 μM.

BIOCHEMISTRY

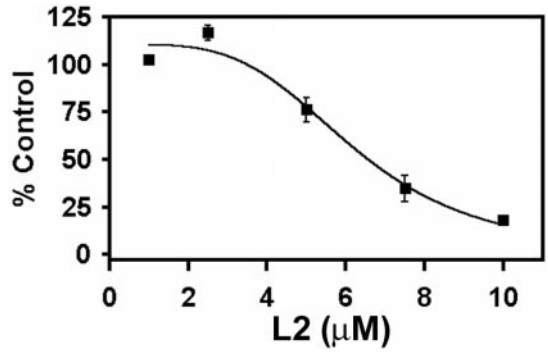

Fig. 5. **L2** decreases cell viability. NIH 3T3 cells were continuously exposed to **L2** under normal growth conditions for 3 days before being counted as described in the text. Percent control growth was calculated by comparing drug treated samples to controls. Results are from three experiments containing triplicate samples (mean value ± SE).

L3 was also able to kill cells, but required higher concentrations to achieve the same effects as the former compound. After 3 days, only 40% of the cells were killed by 10 μM **L3**. While Hoe342 was about 2-fold more potent than **L2**, the polypyrrole MGTs **L4** and **L5** were ineffective up to 10 μM. Interestingly, although **L1** was unable to decrease endogenous c-fos expression, it was approximately as cytotoxic as **L2.**

Discussion

Much effort has been applied toward developing DNA-reactive agents that bind longer sequences with high affinity and greater selectivity. Designing such agents that inhibit DNA-related functions but still maintain cellular permeability can be a formidable task. We have demonstrated that these goals can be achieved by using a unique strategy of equipping minor groove-binding ligands with functional groups that allow for electrostatic contact with the phosphodiester backbone. When **L1**, chosen for its ability to preferentially bind long A/T sites at nanomolar concentrations, was functionalized with propylamines to yield **L2**, its ability to inhibit TF complexes was enhanced by at least 1,000-fold. It is the first MGT to maintain significantly improved potency relative to a classical minor groove binder in the cell-free transcription assay and is also the first MGT to inhibit endogenous gene expression in whole cells.

The structure/activity relationships apparent from our studies provide some insight into how MGT design plays a role in determining inhibitory profiles. Compounds lacking basic side chains (**L1** and Hoe342) were poor inhibitors of TC formation in EMSAs. Although equipping a bisbenzimidazole with a tripyrrole improves its binding affinity and ability to distinguish between A/T-rich sites of different lengths, it appears that this alone is not enough to create an ideal TF complex inhibitor. The polyamine tails on each of **L2**'s pyrroles apparently not only endow it with a capability to bind dsDNA at subpicomolar levels (25), but also impart a vastly improved TF inhibitory potency compared with **L1**. Although the SRE contains only six contiguous A/T bp rather than the optimal nine, this site was sufficient for **L2** binding. TFs that bind longer A/T-rich sites may prove to be even better targets. Inhibitory potency, like DNA binding affinity, correlated with pyrrole/polyamine side chain length, because **L3** and **L5** were less potent than their longer analogs **L2** and **L4**, respectively.

Although all of the agents are A/T selective and can potentially disrupt SRF binding and subsequent TC formation, their potencies in the cell-free assays may depend on their intrinsic chemistry and how well they bind to the SRE sequence provided. For example, the small size of **L5** may prevent it from providing adequate steric hindrance or from altering DNA conformation enough to effectively inhibit TF binding. The increased length of **L4** may improve these characteristics and make it nearly as effective as the bisbenzimidazoles **L2** or **L3**. The latter agents, with their potential to provide more extensive distortion over a longer sequence, may overcome the limitations of the classical minor groove binders and even the polypyrrole-based MGTs, making them more effective and potent transcriptional inhibitors.

The EMSA trends noted above were also apparent in the cell-free transcription assay, where a more complex environment provides a more rigorous assessment of an agent's ability to inhibit TF/DNA interactions. While the order of potency was the same as that observed in the EMSAs, drastic drops in effectiveness were noted (Table 1). Loss of DNA-binding drug potency in this assay has been observed before (14, 15) and is expected, because the increased amount and length of DNA provide a greater number of potential drug binding sites. More DNA-binding proteins may also interfere with drug binding to the SRE. The ability of **L3** to inhibit transcription was especially hindered, because a 26-fold higher concentration was required compared with the EMSAs. In notable contrast to the other compounds, **L2** inhibited transcription by 50% at submicromolar levels, an order of magnitude improvement over **L5** or Hoe342. Overall, the results suggest that conjugating pyrrole/polyamine constructs to bisbenzimidazoles not only enhances effectiveness in EMSAs, but also proportionately maintains potency in the presence of additional proteins and more DNA with greater sequence complexity.

Although **L2** was designed to bind DNA, its effect on another template-related activity, topo II, was significantly less than its ability to decrease gene expression, suggesting that its collateral damage effects are minimal. We noted that higher drug concentrations were needed in the topo II activity assays, which may be partially attributable to increased amounts of DNA in the nuclear environment. The most effective agents in this assay, **L2** and **L5**, both possessed tripyrrole/propylamine functional groups, but there was little correlation between potencies in the cell-free assays and the ability to inhibit topo II activity in nuclei. This suggests that topo II inhibition may rely on ligand characteristics that are different from those required for TF complex inhibition. Because topo II is a processive enzyme, it may not be affected by the agents in the same way that TFs are. At 20 μM Hoe342, both cell-free transcription and topo II activity are inhibited nearly equally. In contrast, 2 μM **L2** completely inhibited cell-free transcription but had no effect on topo II (data not shown). This observation suggests that **L2** might be more selective for inhibiting TF complexes compared with other DNA-template related activities. Because different Hoechst analogs inhibited topo II activity with varying degrees of potency in previous studies (18), it is reasonable to postulate that additional modification of the fluorescent MGTs may eventually yield compounds with improved selectivity for inhibiting other desired DNA template functions.

The superior potency of **L2** was further demonstrated when it was found to be the only agent with the ability to inhibit endogenous gene expression. A 16-h exposure to 10 μM **L2** was sufficient to inhibit c-fos transcription in NIH 3T3 cells by over 50%. Serum-starved cells have undetectable levels of c-fos mRNA and transcription is up-regulated only upon serum induction. Therefore, any decrease in c-fos mRNA is likely attributable to direct drug effects on transcription. The inhibition of c-fos transcription suggests that **L2** is cell-permeable and can localize to the nucleus. There was no effect on total GAPDH mRNA levels. The longer half-life of GAPDH mRNA [8 h, compared with 30 min for c-fos (24)] may only partially account for this observation. If **L2** was capable of down-regulating GAPDH immediately upon drug treatment, there should have been a detectable decrease in its mRNA. This observation has

been made for other DNA-binding drugs, suggesting that GAPDH may be relatively insensitive to down-regulation by such agents (14). The other MGTs may have no effect on gene expression because of lower bioavailability or because their potency in the whole cell environment is too weak. Additionally, binding of the agents to other A/T-rich sites in the genome may lower the effective concentration available to inhibit TF complex formation on the SRE. This nonspecific binding is likely contributing to the lower potency of **L2** in whole cells, because an order of magnitude more drug is required to inhibit endogenous c-fos transcription compared with cell-free assays (24).

The overall activity profile of **L2** is therefore consistent with its binding to DNA in whole cells, an event that affects gene regulation and might contribute to its cytotoxicity. Although **L3** was unable to decrease c-fos expression in NIH 3T3 cells after 16 h, it effected cell death after 3 days of continuous exposure, suggesting that it may take longer to accumulate to bioreactive levels in cells, or that its cytotoxic mechanism of action may not depend solely on its ability to bind DNA. This latter possibility may also apply to **L1**, which was cytotoxic, but unable to inhibit c-fos gene expression.

Earlier MGT studies demonstrated that rationally designed agents with the potential to contact both grooves are more potent inhibitors of TF/DNA interactions than agents that interact solely with a single groove (8). The current study corroborates these findings but also surpasses them, because the rationale used resulted in agents that inhibited TF complex formation in EMSAs as well as cell-free transcription assays and also decreased gene expression in whole cells. Specifically, combining the pyrrole/polyamine-based structure of the MGTs with a bisbenzimidazole moiety yielded agents (**L2**, **L3**, and **L4**) that were significantly more potent TF complex inhibitors than the classical minor groove-binder Hoe342. It is of interest to note that our work suggests that the polypyrrole/polyamine and bisbenzimidazole moieties may contribute separately to MGT activity characteristics. The tripyrrole/propylamine functional group was essential for potent inhibition of TC formation, because **L4** was an effective inhibitor and **L1** was not. Furthermore, this expands the possibilities for MGT design, because it has now been demonstrated that equipping each pyrrole with a polyamine does not compromise the agent's inhibitory activity in cell-free assays. A bisbenzimidazole attached to **L4** to yield **L2** was only slightly more potent than the former in the cell-free assays, but inhibited gene expression in whole cells. Thus, it appears that the bisbenzimidazole moiety is essential for cellular activity. The combination of bisbenzimidazole and tripyrrole/propylamine therefore seems particularly well suited for inhibiting TF/DNA contacts in both cell-free assays and cellular assays. In contrast, the *p*-OH bisbenzimidazole Hoechst 33258 shows little cellular activity, suggesting that the nature of terminal ring substituents may have profound effects on the cellular uptake and/or biological effects of these compounds.

The enhanced potency observed when minor groove-binding agents are equipped with constructs capable of electrostatically interacting with the phosphodiester backbone has implications for future drug design. Logical construction of a bisbenzimidazole functionalized with a tripyrrole/propylamine tail yielded a MGT, **L2**, with significantly improved ability to inhibit TF complex formation in cell-free assays and to decrease gene expression in the whole cell environment. Similar stimulation of activity may therefore be seen by endowing other minor groove-binding compounds with the ability to contact the DNA backbone. The correlations among pyrrole/polyamine subunit number, polyamine tail length, and potency can be investigated further to determine whether additional increases in subunit number and/or modified polyamine tails will create even more powerful and selective agents.

This work was supported by National Cancer Institute Grant CA16056 (to T.A.B.), American Cancer Society Grant DHP 158 (to T.A.B.), U.S. Army Medical Research Grant BC980100 (to C.M.W), and National Institutes of Health Grant 5R37DK0917136 (to T.C.B.)

1. Wemmer, D. E. & Dervan, P. B. (1997) *Curr. Opin. Struct. Biol.* **7,** 355–361.
2. Chiang, S. Y., Welch, J. J., Rauscher, F. J., 3rd, & Beerman, T. A. (1996) *J. Biol. Chem.* **271,** 23999–24004.
3. Dorn, A., Affolter, M., Muller, M., Gehring, W. J. & Leupin, W. (1992) *EMBO J.* **11,** 279–286.
4. Schleif, R. (1988) *Science* **241,** 1182–1187.
5. Bruice, T. C., Houng, Y. M., Gong, X. H. & Lopez, V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1700–1704.
6. Browne, K. A., Gong, X. H. & Bruice, T. C. (1993) *J. Am. Chem. Soc.* **115,** 7072–7079.
7. Gong, X. H., Browne, K. A., Groppe, J. C., Blasko, A., Houng, Y. M. & Bruice, T. C. (1993) *J. Am. Chem. Soc.* **115,** 7061–7071.
8. Chiang, S. Y., Bruice, T. C., Azizkhan, J. C., Gawron, L. & Beerman, T. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2811–2816.
9. Satz, A. L. & Bruice, T. C. (1999) *Bioorg. Med. Chem. Lett.* **9,** 3261–3266.
10. Satz, A. L. & Bruice, T. C. (2000) *Bioorg. Med. Chem.* **8,** 1871–1880.
11. Treisman, R. (1992) *Trends Biochem. Sci.* **17,** 423–426.
12. Huang, K., Louis, J. M., Donaldson, L., Lim, F. L., Sharrocks, A. D. & Clore, G. M. (2000) *EMBO J.* **19,** 2615–2628.
13. Mo, Y., Vaessen, B., Johnston, K. & Marmorstein, R. (2000) *Nat. Struct. Biol.* **7,** 292–297.
14. White, C. M., Heidenreich, O., Nordheim, A. & Beerman, T. A. (2000) *Biochemistry* **39,** 12262–12273.
15. Bellorini, M., Moncollin, V., D'Incalci, M., Mongelli, N. & Mantovani, R. (1995) *Nucleic Acids Res.* **23,** 1657–1663.
16. Satz, A. L. & Bruice, T. C. (2001) *J. Am. Chem. Soc.* **123,** 2469–2477.
17. Chiang, S. Y., Azizkhan, J. C. & Beerman, T. A. (1998) *Biochemistry* **37,** 3109–3115.
18. Beerman, T. A., McHugh, M. M., Sigmund, R., Lown, J. W., Rao, K. E. & Bathini, Y. (1992) *Biochim. Biophys. Acta* **1131,** 53–61.
19. Woynarowski, J. M., Sigmund, R. D. & Beerman, T. A. (1988) *Biochim. Biophys. Acta* **950,** 21–29.
20. Chiang, S. Y., Burli, R. W., Benz, C. C., Gawron, L., Scott, G. K., Dervan, P. B. & Beerman, T. A. (2000) *J. Biol. Chem*. **275,** 24246–24254.
21. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311,** 433–438.
22. Treisman, R. (1985) *Cell* **42,** 889–902.
23. Burden, D. A. & Osheroff, N. (1998) *Biochim. Biophys. Acta* **1400,** 139–154.
24. Dani, C., Piechaczyk, M., Audigier, Y., El Sabouty, S., Cathala, G., Marty, L., Fort, P., Blanchard, J. M. & Jeanteur, P. (1984) *Eur. J. Biochem.* **145,** 299–304.
25. Satz, A. L. & Bruice, T. C. (2001) *Bioorg. Med. Chem*., in press.