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Award Number: DAMD17-98-1-8103

TITLE: Evaluation of DNA Binding Drugs as Inhibitors of ESX, and
ETS Domain Transcription Factor Associated with Breast
Cancer: Effects of ESX/DNA Complex Disruption

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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13. ABSTRACT (Maximum 200 Words)

DNA binding agents, sequence preference and sequence specific, were evaluated for their ability to inhibit ESX binding to the HER2/*neu* promoter and down regulate cancer associated gene expression. Cell-free mobility shift assay results revealed that the sequence specific agents to be far superior to the sequence preference agents at inhibiting transcription factor/DNA complex formation. However, cell-free transcription results did not maintain this order of potency, with each group of agent more equally effective at inhibiting HER2/*neu* regulated expression. Whole-cell analysis, employing techniques such as northern blot analysis and cytotoxicity, revealed no cellular activity by the sequence specific agents. Conversely, for the sequence preference agents the whole-cell studies revealed a general pattern of potency of G/C sequence preference agents compared to A/T preference agents at inhibiting cellular mRNA levels of the targeted gene HER2/*neu* and on cellular growth. In depth analysis has revealed polyamide 22 to be cell permeable and localize to the nucleus. Continued studies are underway to determine the nuclear target of polyamide 22 binding. Examination of the structural changes of polyamides and the resultant effect on cellular localization and biological activity are underway and could influence the continued design of these potential therapeutic agents.

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Annual Summary

Introduction:

Regulation of cancer associated gene expression by anti-cancer agents is our current area of research. Gene expression requires the association of transcription factors both general and gene specific with the promoter region to allow for transcription of that gene. Prevention of transcription factor binding to a gene promoter should decrease gene expression. Our current studies evaluate agents with different modes of DNA binding (minor groove/intercalating) and different sequence preference (GC vs. AT vs. sequence specific) for their ability to target and down regulate gene expression. The target gene, HER2/*neu* is found overexpressed in approximately 30% of all breast cancer cases. Recently an ETS family transcription factor, ESX, has been identified as a possible regulatory transcription factor of the HER2/*neu* gene. Our goal is to assess DNA binding drugs for their ability to interfere with transcription factor (ESX) binding to the target gene's promoter region (HER2/neu). This work has begun to uncover drug DNA binding motifs that can be exploited to develop new drugs or modify existing agents that could target HER2/*neu* promoter expression.

Body:

Binding of basal and gene regulatory transcription factors (TFs) to a gene promoter is facilitated through a DNA binding domain which contains features responsible for sequence and topology recognition thus creating TF/DNA complexes that promote gene expression. Disruption of TF/DNA complexes by DNA binding agents that alter promoter structure are therefore expected to decrease TF function and gene expression. DNA binding agents may also be targeted to particular promoters since they of have sequence preferences (either A/T or G/C sequences) or sequence specific (synthetically designed agents called polyamides), due to available donor and acceptor groups within the DNA recognition sequence. DNA binding agents can be classified also by their mode of binding, either groove binding or intercalating. The minor groove binders (MGBs), such as distamycin, possesses a crescent shape that allows a deep fit into the minor groove and causes it to widen while the major groove simultaneously narrows. This distortion of the helix may contribute to the agent's inhibition of TF/DNA complex formation. Another class of DNA binding agents is the intercalators, which slide the chromophore between the base pairs of DNA and lengthen the helix. This can adversely change the positions of donor/acceptor groups that participate in TF site recognition.

Using a common DNA target for assessment of how DNA binding agents can inhibit TF binding and regulated gene expression in a series of related cell-free and whole-cell studies may reveal which DNA binding motifs are the most effective for disrupting TF function. We have chosen an ETS binding sequence (EBS) on the HER2/*neu* promoter as a model system for evaluating DNA binding drugs. ETS, a large family of transcription factors, binds to the EBS, which contains a GGAA central core. A newly identified ETS transcription factor family member, ESX, is thought to contribute to the regulation of HER2/*neu* expression. The putative ESX binding sequence in the HER2/*neu* promoter, -GAGGAAGT-, lends itself to targeting by both A/T and G/C sequence preference agents as well as different groove binding agents, since ETS factors like ESX contact both DNA grooves.

Agents were first evaluated in a cell-free mobility shift assay to determine their effectiveness in preventing ESX binding to the EBS of HER2/*neu* promoter. A cell-free transcription assay was employed to assess drug inhibition of ESX transactivation. Finally, cell culture studies were used to determine affects of these drugs on intracellular HER2/*neu* mRNA synthesis, as well as, compare their cytotoxic potential. This work has begun to uncover drug DNA binding motifs that can be exploited to continue to develop new drugs or modify existing agents to target HER2/*neu* promoter expression. Some of the DNA binding agents described in the original proposal (nogalamycin, lucanthone, adriamycin, mitoxantrone, and CC10-65) have not been pursued in an effort to conduct more in-depth analysis of fewer agents (Hoechst 33342, distamycin, chromomycin, hedamycin, polyamide **2** and polyamide **22**). In particular, examination of polyamides was expanded, since these agents possess a potential to specifically inhibit gene expression without the accompanying whole-cell cytotoxicity that occurs with more conventional DNA binding agents.

AIM 1: Identification of DNA binding agents that interfere with the binding of ESX to HER/2neu promoter DNA in cell-free assays:

- In accordance with Task 1 of the Statement of Work, months 1-6, I have completed the assessment of DNA binding agents for their ability to prevent ESX/HER2/*neu* complex formation. An assay for quantitative analysis of drug inhibition of transcription factor/DNA complex formation is the mobility shift assay (MSA), which was described in detail in the previous annual report and is in Materials and Methods of the attached manuscript #1. These studies were limited to evaluation of a DNA binding agent's ability to prevent complex formation and will not be evaluated for their ability to disrupt pre-formed complexes (for the same reason listed above for excluding some agents from the study). A series of drug concentration was evaluated. From this data, IC_{50} values (the molar concentration of drug required to inhibit complex formation by 50%) can be obtained to

identify and compare compounds. Agents evaluated are presented in Table 1 with their IC_{50} values for prevention of complex formation.

Table 1: The IC_{50} values for DNA binding agents' ability to prevent ESX/Her2/*neu* complex formation.

Agent	Binding Properties	IC_{50} (μM)
Hoechst 33342	A/T rich minor groove binder	1.400
Distamycin A	A/T rich minor groove binder	0.700
Chromomycin A_3	G/C rich minor groove binder	10.00
Hedamycin	G/C rich intercalator	0.500
Polyamide **2**	Sequence specific minor groove binder	0.010
Polyamide **22**	Sequence specific minor groove binder	0.044

- In accordance with Task 1 of the Statement of Work, months 7-12, DNA footprinting for sequence specific agents, polyamides, are completed at this time with the assistance of Dr. Dervan's group in California. DNA footprint analysis will not be carried out for the sequence preference agents.

AIM 2: Effects of DNA binding agents on ESX regulated expression of HER2/neu:

- In accordance with Task 2 of the Statement of Work, months 13-24, cell-free transcription assays of the agents to be examined have been completed. This cell-free transcription assay was described in detail in previous annual report and is in the Materials and Methods of the attached manuscript #1. A series of drug concentrations were evaluated. From this data, IC_{50} values were obtained and are listed in Table 2.

Table 2: The IC_{50} values for DNA binding agents' ability to prevent transcription in cell-free transcription assay.

Agent	Binding Properties	IC_{50} (μM)
Hoechst 33342	A/T rich minor groove binder	3.0
Distamycin A	A/T rich minor groove binder	3.0
Chromomycin A_3	G/C rich minor groove binder	1.5
Hedamycin	G/C rich intercalator	8.0
Polyamide **2**	Sequence specific minor groove binder	4.5
Polyamide **22**	Sequence specific minor groove binder	17.2

- In accordance with Task 2, months 25-36, at this time this portion of the proposal has been completed for more whole-cell, biologically relevant assays for evaluation of agents as inhibitors of transcription factor binding in whole cells can be undertaken. This is defined in Task 3.

Task 3, to assess DNA binding agents' ability to decrease targeted gene expression in whole-cell assays. These cellular assays included (I) northern analysis for detection of decreased RNA expression in whole cells. Task 3 (II) described in the previous annual report (nuclear run-on assay) has been changed in an effort to concentrate on more biologically relevant cellular uptake and distribution of polyamides in intact cells.

I: Agents tested in both mobility shift and cell-free transcription assays were then assessed for their ability to inhibit gene expression in a cellular environment by northern blot analysis. The northern blot procedure is in the Materials and Methods of the attached manuscript #1. IC_{50} values of the agents examined are included in Table 3.

Table 3: The IC_{50} values for inhibition of cellular HER2/*neu* mRNA expression by Northern blot analysis and cell growth inhibition.

Agent	Northern blots (24 hrs) IC_{50} values (μM)		Cell Growth Inhibition (72 hrs) IC_{50} values (μM)
	HER2/*neu*	GAPDH	
Hoechst 33342	9	9	7.0
Distamycin A	66	57	114
Chromomycin A$_3$	0.04	0.07	0.05
Hedamycin	0.21	0.21	0.10
Polyamide **2**	No effect	No effect	No effect
Polyamide **22**	No effect	No effect	No effect

Cytotoxic evaluations of the drugs were carried out to normalize drug effects on gene expression seen in whole cells. Moreover, a lack of cytotoxicity may indicate that a drug cannot effectively enter whole cells. Such is the case for polyamide **2**, though possessing inhibitory activity in mobility shift and cell-free transcription assays it does not effect gene expression or cell growth. Concern for cellular uptake of these new sequence specific DNA binding agents led us to ask our collaborator to create a fluorescently labeled version of polyamide **2**. This new agent was named polyamide **22**. Assessment of polyamide **22** in cell-free assays was performed due to concern that the fluorescent tag would interfere with the agent's ability to bind to its target sequence. Both mobility shift and cell-free transcription assays showed that polyamide **22** was four-fold less potent but still capable of functioning similar to polyamide **2**.

II: As described in the previous annual report, I implemented the use of epifluorescence microscopy in our lab and since then we have acquired our own epifluorescence microscope, CCD camera and image analysis system. This method is described in more detail in the attached manuscript #2. Based upon spectrofluorophotometric analysis of SKBR3 cells treated with **22**, significant uptake occurs within 15 minutes and by four hours about 25 % of the compound is taken up. Microscopic analysis of these cells revealed that by 15 minutes much of the drug was localized within the nucleus and by four hours compound in the cytoplasm was barely detectable. The appearance of the fluorescent signal in the nucleus was not the result of degradation of **2** and the subsequent release of free tag, since the fluorescent tag alone only localizes to the cytoplasm. It was also found that uptake was temperature dependent and that incubations at 4° resulted in no detectable uptake into the cell. Finally, a similar profile of **22** uptake into normal human diploid fibroblasts cells was seen, demonstrating that polyamide permeability into mammalian cells is not limited to the neoplastic SKBR3 cell type.

The observation that treatment with a 10:1 mixture of the non-fluorescent **2** and **22** respectively markedly reduced the fluorescence signal intensity suggests that both agents share a common uptake pathway and that the presence of the fluorescent tag is likely not altering the mechanism for cellular uptake and retention. Studies showing that **2** can chase the fluorescent **22** signal indicate that the parent and fluorescent compound have similar nuclear binding sites. The poor ability of a conventional DNA minor groove binding agent Hoechst 33342 to chase the **22** signal from the nucleus could indicate that the strong DNA binding properties of polyamides are a factor in where these molecules bind and their ability to be retained inside the cell nucleus. Polyamide

22 shows a characteristic pattern of strong signal throughout the nucleus with the exception that almost no fluorescence is observed within the nucleolus region. This compares quite well to the uptake patterns for both Hoechst 33342 and another minor groove binding agent DAPI which are widely used for staining chromatin inside the cell nucleus. In contrast, Hoechst 33258, which differs from Hoechst 33342 by the substitution of an ethoxy group for the hydroxyl group on the phenol ring, shows a very limited amount of uptake within the nucleus. It has been reported that the 33342 Hoechst and DAPI bind almost exclusively to DNA within the nucleus while the 33258 compound can bind to RNA and DNA and is taken up both into the nucleus but even more strongly into the cytoplasm. Similarly, the levels of uptake between the two Hoechst compounds vary, with 33342 being much more efficiently taken up (three fold) by mammalian cells. At this point, it is not known whether other types of polyamides will possess the uptake characteristics and nuclear localization patterns of **22** which is like Hoechst 33342 or that variants will be seen such as those that whose binding is reminiscent of Hoechst 33258 or potentially other patterns of distribution.

Laser scanning confocal microscopic analysis enabled us to more precisely visualize the intranuclear pattern of polyamide **22** fluorescence. The results confirm the extranucleolar localization in the nucleus and the absence of detectable signal in the cytoplasm. Moreover the overall nuclear staining pattern of the chromatin appears as granular-like structures arranged in higher order arrays that often surround the nucleolus, nuclear periphery and in various other regions in the nuclear interior. Further studies of these putative granular-like structures decorated by polyamide **22** are being planned using computer imaging segmentation and three-dimensional computer imaging approaches. Preliminary measurements indicate that the repeating chromatin structures have x-y dimensions of about 0.5 microns. This is in the same size range as the replication/transcription sites detected in mammalian cells. Previous studies have further indicated that replication sites are characteristic features of higher order chromatin domains. Thus the staining patterns of polyamide **22** may be revealing a fundamental aspect of higher order chromatin organization and function in the cell nucleus. The results of these studies are summarized in manuscript #2.

- While these results look promising the fact remains that at this time these agents do not exhibit cellular activity measured by northern blot analysis and cell growth inhibition. Additionally, many of the results summarized in manuscript #2 suggest that polyamide **22** is binding to DNA, studies are underway to further assess the molecular binding target of these agents. These studies have been assigned to Task 4 and are described below.

- Task 4, to indirectly determine the molecular binding target of polyamide **22** using epifluorescence and confocal microscopy. Using immunofluorescence, several co-localization studies are underway including PCNA (proliferating cell nuclear antigen), SC-35 (cellular splicing factor), lamins A,C (nuclear envelope proteins), RPA (replication protein A), propidium iodide (another known DNA binding agent with different fluorescence properties) and BrdU incorporation. Additionally, I will attempt to use flow cytometry to see if a similar cell cycle pattern can be obtained, using polyamide **22** as a DNA dye, in comparison to propidium iodide. Lastly, using a method described by Dr. Ronald Berezney (a co-author of manuscript #2) studies will attempt to determine what nuclear component polyamide **22** may be binding to when using nuclear matrix preparations.

- While determining the molecular binding site of these agents is important, another important area of research concerning these agents is the potential of different polyamide structures resulting in different biological effects. As briefly described in Manuscript #2 (see attached), small changes in structure as seen between Hoechst 33342 and Hoechst 33258, can result in dramatic differences in

their cellular localization and biological activity. Studies to determine the effect of polyamide structure on cellular localization and activity will be assigned to Task 5 and are described below.

- Task 5, to determine if changes in polyamide structure would result in different cellular localization and resulting biological effects. Polyamide **3** is another polyamide designed to bind to the same region of the HER2/*neu* promoter as polyamide **2**, however, this ligand possess a spacer region (β-alanine spacer) which results in a slightly different structure than polyamide **2**. Experiments by others in the lab have revealed that polyamides with β-alanine spacers (such as polyamide 3) **are not** capable of inhibiting cellular uptake of ^{3}H-Uridine while polyamides without these structural changes **are** capable of this cellular activity. Our collaborator, Dr. Peter Dervan, has agreed to send us a fluorescently labeled polyamide **3**, which will be called polyamide **33**. Techniques that were used to assess polyamide **22**'s cellular uptake and localization will also be employed for polyamide **33** assessment. These techniques are described in Task 3 (II) and Manuscript #2, and will include spectrofluorophotometer, epifluorescence and laser scanning confocal microscopy. Assessing the effects of structural changes on polyamide cellular localization and biological activity could play an important role in further developing these sequence specific agents.

Key Research Accomplishments:

> ## Task 1, months 6-12: *COMPLETE*
> - ◆ Drugs to be studied are completed for prevention of complex
> - ◆ Some drugs were not pursued while several new drugs were added
> - ◆ Agents will not be assessed for disruption of pre-formed TF/DNA complexes
> - ◆ **Results**:
>   - ▪ The sequence specific polyamides are by far the most potent inhibitors of complex formation, with polyamide **2** nearly 50X more potent than hedamycin (the most potent sequence preference agent) and polyamide **22** nearly 11X more potent.
>   - ▪ When comparing different modes of binding among the sequence preference DNA binding agents it appears that agents that caused significant helical distortion, via intercalation, were the most potent inhibitors of ESX/HER2/*neu* complex formation.
>   - ▪ When comparing agents with different sequence preference there appears to be a slight increased efficiency of the G/C rich binders* compared to the A/T rich binders for inhibition of TF/DNA complex formation.
>
>   * = chromomycin's activity was probably underestimated due to limitation of optimizing the assay, see manuscript #1 for details

> ## Task 2, months 13-24: *COMPLETE*
> - ◆ Drugs to be studied are completed for testing of inhibition of gene expression is cell-free transcription assay
> - ◆ Some drugs were not pursued while several new drugs were added
> - ◆ **Results**:
>   - ▪ The sequence specific polyamides were equally as effective as the sequence preference agents at inhibiting HER2/*neu* regulated expression.
>   - ▪ When comparing drugs with different modes of binding, the minor groove binding agents appear more effective inhibitors of cell-free expression than the intercalating agent.
>   - ▪ When comparing agents with differences in sequence preference there appears to be a slight increased efficiency of inhibiting cell-free expression by the G/C rich binders over the A/T rich binder, with the exception of hedamycin.

> ## Task 3, months 6-36: *COMPLETE*
> (I) Northern blot analysis and cytotoxicity
> - ◆ Drugs to be studied are completed for testing of inhibition of gene expression (as measured by cellular mRNA levels) in whole-cells as well as cytotoxicity
> - ◆ Some drugs were not pursued while several new drugs were added
> - ◆ Cytotoxicity results correlated well with northern blot analysis results.
> - ◆ **Results**:
>   - ▪ The sequence specific polyamides showed **no** effect on cell growth or gene expression.
>   - ▪ When comparing modes of binding, the agents which cause helical distortion, hedamycin and chromomycin, were among the most potent inhibitors of cellular gene expression. Of note, other factors such as cellular uptake, distribution and stability could play a role in an agent's ability to inhibit gene expression.
>   - ▪ When comparing sequence preference it appears as if the G/C rich binders were more effective inhibitors of cellular mRNA levels than the A/T rich binders, although this may be directly due to the factors mentioned above.

- Chromomycin showed preference for inhibition of HER2/*neu* mRNA over GAPDH mRNA production within 24 hours. Distamycin, below cytotoxic concentrations, after 72-hours showed preferential inhibition of HER2/*neu* mRNA synthesis compared to GAPDH mRNA.

(II) Epifluorescence microscopic analysis of cellular uptake and distribution of polyamide **22**.

◆ **Results:**

- Polyamide **22** is taken up into the nucleus of SKBR3, NIH3T3 and NHDF cells, while avoiding the nucleoli.
- Polyamide **22** accumulates in the nucleus of SKBR3 cells over time.
- Low temperatures (4°C) slow the cellular uptake of polyamide **22** into SKBR3 cells.
- Polyamide **22** in the presence of 10-fold excess polyamide **2** can effectively compete for uptake into the nucleus of SKBR3 cells.
- Pre-treatment of SKBR3 cells with polyamide **22**, followed by treatment of excess polyamide **2**, shows competition for the same binding site.
- Comparison of staining patterns of other known DNA binding agents suggest that polyamide **22** *may* be binding to DNA.

➢ Task 4, months 24-36: *ON GOING*

◆ Epifluorescence and confocal microscopic analysis of molecular binding target of polyamide **22**.

- Co-localization with SC-35: preliminary results suggest that the granular structure of polyamide **22** binding could be the ligand's avoidance of RNA processing sites as evidenced by localization of SC35 (a splicing factor) antibodies in these "open" regions within the nucleus (which are not the nucleoli).
- Co-localization with Lamins A, C: preliminary results suggest very little co-localization of polyamide **22** with this nuclear envelope protein.
- Co-localization with PCNA: preliminary studies are underway
- Co-localization with RPA: preliminary studies are underway
- Co-localization with propidium iodide: preliminary studies are underway
- Co-localization with incorporated BrdU: preliminary studies are underway

◆ Flow cytometry comparison of propidium iodide and polyamide **22**.

- None at this time.

◆ Polyamide 22 binding of nuclear component using nuclear matrix extractions.

- Preliminary results suggest that polyamide **22** binding is mostly lost following the full nuclear matrix extraction method. Staining with dapi after extraction shows co-localization of polyamide **22** and dapi.
- Studies are underway to assess where along the nuclear matrix extraction pathway the polyamide **22** binding is lost.

➢ Task 5, months 24-36: *ON GOING*

◆ Polyamide **33** was obtained (08/00) from Dr. Peter Dervan.

- Preliminary results obtained by epifluorescence microscopy suggest that polyamide **33** localizes to the nucleus; however, the localization pattern of polyamide **33** is different from that of polyamide **22**.

Reportable Outcomes:

> Manuscripts
>> ♦ #1: Studies of the sequence preference agents will be submitted to Cancer Research (09/00) entitled "DNA binding drugs as inhibitors of ETS regulated expression of HER2/*neu*".
>>
>> ♦ #2: Studies of the cellular uptake and distribution of polyamides is in progress and will also be submitted to Cancer Research in the near future, entitled, "Polyamide **22**, a Sequence Specific DNA Binding Ligand is Sequestered in the Cell Nucleus Following Uptake in SKB3 Human Breast Cancer Cells".

> Grants Obtained
>> ♦ A four-year grant from NIH was awarded to Dr. Terry Beerman for the study of polyamides designed to bind to the Elk-1 (an ETS transcription factor family member) and SRF binding sites on the c-fos promoter. A preliminary draft version of manuscript #2 was submitted with the grant proposal as supplemental material to directly address whole-cell uptake of polyamides.

> Poster and/or Platform Presentations
>> ♦ Pharmaceutical Sciences Day at the State University of New York at Buffalo, March 2000.
>>
>> ♦ Sigma Xi Poster Competition at the State University of New York at Buffalo, April 2000.
>>
>> ♦ Era of Hope Meeting in Atlanta, June 2000.

> Job Offers
>> ♦ I have been offered and have accepted a post-doctoral position with Dr. Ronald Berezney at the State University of New York at Buffalo, Department of Biological Sciences. I will be studying the three-dimensional effects on the functional genome following treatment with DNA binding agents.

> Advanced training course:
>> ♦ I was selected for an alternate position in an international, highly competitive course offered by the Marine Biological Laboratory of Woods Hole entitled "Optical Microscopy and Imaging in Biomedical Sciences."

Conclusions:

While cell-free assays are informative studies to determine if DNA binding agents (either sequence preference or sequence specific) are performing as intended, these assays are not predictive of drug activity in whole-cells and thus continue to point to the need for continued use and development of biologically relevant whole-cell assays. Analysis of agents in whole-cells suggest the G/C sequence preference agents are highly active and effective inhibitors of cellular gene expression and growth. These results also point to the possible role of other factors such as cellular uptake and stability on drug activity. Like wise, this analysis suggest that the ability of a drug to create helical distortions, regardless of mode of binding, plays an important role on biological activity as seen with chromomycin and hedamycin.

The studies of the sequence specific polyamides also point to the importance of whole-cell analysis since the cell-free studies did not predict the lack of cellular activity by these agents. Further analysis has revealed that these sequence specific agents are capable of cellular uptake. Additionally, these agents localize to the nucleus of the cell while avoiding sites of RNA (nucleoli and splicing factor sites) suggesting that these agents could be reaching their designed target, DNA.

Finally, analysis of the effects on activity due to structural changes of polyamides has begun to reveal that slight modification in structure could have significant effects on biological activity and that these results will ultimately effect the further design and development of the potential therapeutic agents.

References:

See references sited in the attached manuscript drafts.

Appendices:

A. Manuscript draft to be submitted 09/00 to Cancer Research:
"DNA binding drugs as inhibitors of ETS regulated expression of HER2/*neu*"

B. Manuscript draft to be submitted at a later date to Cancer Research:
"Polyamide **22**, a Sequence Specific DNA Binding Ligand is Sequestered in the Cell Nucleus Following Uptake in SKB3 Human Breast Cancer Cells".

C. Copy of award letter to Dr. Terry Beerman from NIH.

D. Copy of platform presentation abstract given at Pharmaceutical Sciences Day at the State University of New York at Buffalo, March 2000.

E. Copy of poster abstract presented at Sigma Xi Poster Competition at the State University of New York at Buffalo, April 2000.

F. Copy of poster abstract presented at Department of Defense Era of Hope Meeting in Atlanta, June 2000.

G. Copy of Dr. Ronald Berezney's letter offering post-doctoral position in his laboratory at the State University of New York at Buffalo, Department of Biological Sciences.

H. Copy of Admissions letter from the Marine Biological Laboratory at Woods Hole regarding a position in "Optical Microscopy and Imaging in Biomedical Sciences" course.

Appendix A:

Manuscript draft to be submitted 09/00 to Cancer Research:

"DNA binding drugs as inhibitors of ETS regulated expression of HER2/*neu*"

Title: DNA binding drugs as inhibitors of ETS regulated expression of HER2/*neu*[1].

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Running Title: DNA binding drug effects on gene expression.

Key Words: DNA binding drugs   Drug binding motifs

Transcription factor   Binding

Regulated expression

Footnotes:

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[3] The abbreviations use are: TF, transcription factor; MGB, minor groove binder; EBS, ETS binding sequence;

EMSA, electrophoretic mobility shift assay

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Abstract:

Four drugs with different DNA binding modes (minor groove or intercalating) and sequence preferences (A/T or G/C) were examined for their ability to inhibit ESX binding to the HER2/*neu* promoter and ESX regulated expression of HER2/*neu*. ESX, a member of the ETS family of transcription factors, binds to sequences in the major groove of DNA with a GGAA central core, called the ETS binding sequence, as well as contacting the minor groove. Based upon mobility shift assays, Hoechst 33342, distamycin and hedamycin prevented ESX/DNA complex formation by 50% (IC_{50} value) at concentrations ~1.0 μM, while chromomycin was considerably less active. In contrast, in cell-free transcription experiments, chromomycin was among the most potent (IC_{50} ~1.5 μM) inhibitors of HER2/*neu* expression along with distamycin and Hoechst 33342, while hedamycin was the least effective. Drug treatment of SKBR3 cells, a human breast adenocarcinoma cell line which overexpresses both HER2/*neu* and ESX, revealed hedamycin and chromomycin to be highly potent (IC_{50} in the 40-210 nM range) inhibitors of HER2/*neu* mRNA levels while Hoechst 33342 and distamycin were relatively weak inhibitors (IC_{50} ~9 μM and ~60 μM, respectively). Under some treatment conditions, distamycin and chromomycin showed a preference for inhibition of HER2/*neu* mRNA production when compared to a housekeeping gene (GAPDH), while Hoechst 33342 and hedamycin were almost always equally inhibitory to both. The most cytotoxic agents, chromomycin and hedamycin, were also the strongest inhibitors of HER2/*neu* gene expression in cells. The effectiveness in preventing TF/DNA complex formation and gene expression appears to vary with a drug's mode of binding and sequence preference, however, other factors may play a more important role in drug inhibition of gene expression in whole-cells.

Introduction:

Binding of basal and gene regulatory transcription factors (TFs) to a gene promoter is facilitated through a DNA binding domain which contains features responsible for sequence and topology recognition thus creating TF/DNA complexes that promote gene expression (1, 2). Disruption of TF/DNA complexes by DNA binding agents that alter promoter structure are therefore expected to decrease TF function and gene expression (3-6). DNA binding agents may also be targeted to particular promoters since they prefer either A/T or G/C sequences, due to available donor and acceptor groups (hydrogens or electrons) within the DNA recognition sequence. For example, one class of A/T sequence preferring drugs includes the DNA minor groove binders (MGBs), such as distamycin, which possesses a crescent shape that allows a deep fit into the minor groove and causes it to widen while the major groove simultaneously narrows (7). This distortion of the helix may contribute to the agent's inhibition of TF/DNA complex formation (8-10). Another class of DNA

binding agents is the intercalators, which slide their chromophores between the base pairs of DNA and lengthen the helix. This can adversely change the positions of donor/acceptor groups that participate in TF site recognition (11-13).

Studies of MGBs whose binding motifs (sequence and groove preference) are similar to those of TFs are capable of directly competing with TFs for docking sites on DNA and consequently preventing TF/DNA complex formation. Distamycin's effective inhibition of TBP binding is an example of direct competition since both TF and drug bind to A/T rich regions in the DNA minor groove (8, 14-16). A MGB with a similar sequence but a different groove binding preference than the TF, can also be an effective inhibitor of TF/DNA complex formation. For instance, mithramycin, a MGB with G/C sequence preference, inhibits Sp1 function on the SV40 promoter and decreases Sp1 regulated c-*myc* expression although Sp1 binds G/C regions in the major groove (9, 10). MGBs with similar groove but different sequence binding preference than the TF can also inhibit TF/DNA complex formation as exemplified by chromomycin's (a G/C preference MGB) effective inhibition of TBP binding (15). Generally, MGBs with neither sequence preference nor groove binding preference relative to the TF are weak inhibitors of TF/DNA complex formation, as demonstrated by distamycin's limited ability to inhibit EGR1 from binding to its G/C major groove binding site, even at very high drug concentrations (15).

Intercalators, which generally show less sequence preference than MGBs, can also be used to target TF binding sites. An example of an intercalator with similar sequence preference to a TF, is hedamycin (G/C preference) which prevents EGR1 and E2F-1 from binding to their consensus G/C binding sites (15, 17). However, sequence preference binding may be less stringent for intercalators because these agents often favor the wider G/C or mixed base regions, which provide more space for intercalation (18). The significant disruption of DNA structure caused by intercalators that also form DNA adducts may promote disruption of TF/DNA complexes regardless of TF sequence preference, as typified by hedamycin's effective inhibition of TBP/DNA complexes (8, 15). In contrast, a reversible G/C preference intercalator such as ethidium bromide is a weak inhibitor of both TBP/ and EGR-1/DNA complex formation, indicating that intercalation alone is not always sufficient to disrupt TF/DNA complex formation (8, 15).

Using a common DNA target for assessment of how DNA binding agents can inhibit TF binding and regulated gene expression in a series of related cell-free and whole-cell studies may reveal which DNA binding motifs are the most effective for disrupting TF function. We have chosen an ETS binding sequence (EBS) on the HER2/*neu* promoter as a model system for evaluating DNA binding drugs. HER2/*neu* encodes a growth

factor receptor whose overexpression is found in ~30% of breast cancers (19-22). ETS, a large family of transcription factors, binds to the EBS, which contains a GGAA central core (reviewed in 23). A newly identified ETS transcription factor family member, ESX, is thought to contribute to the regulation of HER2/*neu* expression (24, 25). The putative ESX binding sequence in the HER2/*neu* promoter, -GAGGAAGT-, lends itself to targeting by both A/T and G/C sequence preference agents as well as different groove binding agents, since Ets factors like ESX contact both DNA grooves.

In this study, agents with differences in sequence preference and binding modes were chosen for evaluation. These include A/T sequence preference minor groove binding agents (distamycin and Hoechst 33342), a G/C sequence preference minor groove binding agent (chromomycin A_3) and a G/C sequence preference intercalator (hedamycin). Drugs were first evaluated in a cell-free mobility shift assay to determine their effectiveness in preventing ESX binding to the EBS of HER2/*neu* promoter. A cell-free transcription assay was employed to assess drug inhibition of ESX transactivation. Finally, cell culture studies were used to determine affects of these drugs on intracellular HER2/*neu* mRNA synthesis, as well as, compare their cytotoxic potential.

Materials & Methods:

Drugs:

Hedamycin, supplied by the National Cancer Institute, was prepared by dissolving in 1/10 volume 0.1 N HCl, adding 8/10 volume ddH_2O, and neutralizing with 1/10 volume 0.1 N NaOH. Stocks of distamycin A (Sigma, St. Louis, MO) and Hoechst 33342 (Aldrich, Chemical Co., Milwaukee, WI) were prepared in ddH_2O. Chromomycin A_3 (Sigma, St. Louis, MO) was prepared in DMSO.

Cell Culture:

The HER2/*neu* amplified and overexpressing SKBR-3 cells (human breast adenocarcinoma) were purchased from ATCC (Rockville, MD) and grown in McCoy's 5a medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum. On average cells were sub-cultured after reaching ~80% confluence (1×10^6 cells/ml) by re-suspending in media and plating at 3×10^5 cells/ml.

Electrophoretic Mobility Shift Assay (EMSA):

Bacterial-expressed ESX protein was prepared as previously described (24). Briefly, a full-length ESX cDNA was cloned into a pRSET His-tag expression plasmid (NheI-HindIII; Invitrogen) and expressed in IPTG-induced BL21[DE3] pLysS competent bacteria (Stratagene, La Jolla, CA). His-tagged ESX protein was purified

from the bacteria by Ni-chelate affinity chromatography, as recommended by the manufacturer (Qiagen Inc., Valencia, CA). A 34-mer oligonucleotide (TA5-34 oligo), 5'-GGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAAT-3' containing the EBS (underlined sequence), derived from the HER2/neu gene promoter and its complementary strand were synthesized by the Biopolymer facility (RPCI, Buffalo, NY). The oligonucleotide was purified, annealed and end-labeled with [γ-^{32}P]-ATP using T4-polynuclotide kinase (New England BioLabs, Beverly, MA), as described previously (8, 26). For optimization of EMSA conditions, full-length ESX protein was titrated in the presence of ^{32}P-end labeled TA5-34 oligonucleotide (1 nM) in binding buffer (30 mM KCl, 5% glycerol, 25 mM Tris (pH 7.5), 0.1% NP-40, 0.1 mg/ml bovine serum albumin and 1 mM dithiothreitol) and maximal ESX/DNA complex formation (~90%) was achieved at 40 ng of ESX protein. The 30-minute incubation time was chosen based on the observation that this was sufficient time at room temperature to achieve equilibrium between ESX and the oligo. Following complex formation, samples were electrophoresed for 60 minutes at 200 volts on a 4% polyacrylamide gel using 0.5x TBE buffer. Gels were dried and either exposed to Kodak Biomax Scientific Imaging film or PhosphorImaging screen. A Molecular Dynamics densitometer was used for quantitation of EMSA transcription factor/DNA complexes on film, or with a Molecular Dynamics PhosphorImager screens and ImageQuant software.

A DNA binding agent's ability to disrupt EMSA quantitated ESX/DNA complexes was assessed by 30-minute pre-incubation of the oligonucleotide with drug, prior to the 30-minute incubation of the probe with the ESX protein. Percent inhibition of complex formation by drug was determined by comparing the mean EMSA signal of drug treated samples to that generated by 4 non-drug treated control conditions, and IC$_{50}$ values (amount of drug needed to inhibit 50% of complex formation) for all agents were determined.

Cell-free transcription assay:

CsCl-purified plasmid DNA (RO6), containing an insert DNA fragment from the HER2/neu promoter fragment in the reporter gene expression construct pCDNA3-Luc (Invitrogen, Carlsbad, CA), was linearized with restriction enzyme, SphI (New England BioLabs, Beverly, MA) (27). Nuclear extract from SKBR-3 cells, which overexpress both HER2/neu and ESX, was produced as described previously (24, 26).

Radiolabeled transcripts were generated by incubation of DNA template and SKBR3 nuclear extract in labeling cocktail (containing [α-^{32}P]-CTP (800Ci/mmole; NEN, Boston, MA) for 60-minutes. Samples were extracted and electrophoresed under conditions described by Chiang, et al (17, 26). A PhosphorImager screen was used to visualize the ^{32}P signal from dried gels and the signal was quantified using a Molecular Dynamics computing laser densitometer. T3 transcript (250 bases; Promega Co., Madison, WI) was used as an internal

loading control for each sample. An RNA ladder (Gibco BRL, Grand Island, NY) of 1.77 – 0.155 base pairs was dephosphorylated, end-labeled and purified as described previously and was used to verify the band of interest based on an expected HER2/*neu* transcript size of ~760 base pairs (26). Nuclear extract was titrated in the presence of a constant amount of plasmid DNA (1 μg) to obtain the optimal signal for the 760 base pair HER2/*neu* transcript.

Inhibition of ESX regulated gene expression was assessed by incubation of the drug with the DNA template in reaction buffer for 30 minutes at 30°C prior to addition of the SKBR3 nuclear extract and labeling cocktail. ImageQuant signal intensity of the HER2/*neu* reporter gene transcript (luc) was normalized to the internal loading control signal and the drug vs. the mean of 4 control treatment values was determined for each agent in addition to IC_{50} values for all agents studied.

Northern Blot Analysis:

SKBR3 cells were plated in 60 mm dishes at a sub-confluent density of 5 x 10^5 cells/dish. 72 hours after plating, the cells entered log phase and the media was changed followed by drug addition at the indicated concentrations. At the times indicated after drug treatment, total RNA was harvested from the cells using TRIzol Reagent (GIBCO, Grand Island, NY), as recommended by the manufacturer. Equal amounts (based upon absorbances at 260nm) of total RNA from each sample was electrophoresed for 4.5 hours at 80 volts on a 1.5% agarose-formaldehyde gels (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 10 mM EDTA and 2.2 M formaldehyde) using 1X MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 10 mM EDTA) and then transferred overnight to a nylon membrane (Genescreen, NEN Life Science Products, Boston MA). The membrane was UV cross-linked (CL-1000 series, UVP Inc.) and cut in half, just above the 18S rRNA band. Each half of the membrane was placed in separate hybridization bottles with pre-hybridization buffer (0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% bovine serum albumin) at 60°C for 60 minutes. The membranes were hybridized overnight with random prime (DecaPrime II, Ambion, Austin, TX) labeled [α-^{32}P]-CTP HER2/*neu* cDNA (pBluescript, 1.5Kb *EcoRI* fragment) for the top half of the membrane and GAPDH cDNA (American Type Culture Collection, pBluescript SK-, 1.2Kb *EcoRI* fragment) for the bottom half of the membrane. The hybridized membrane was washed twice for 20 minutes at 60°C with wash buffer A containing 40 mM sodium phosphate, pH 7.2, 5% SDS, 1 mM EDTA, and 0.5 % bovine serum albumin, followed by two additional washes for 20 minutes at 60°C with wash buffer B containing 20 mM sodium phosphate, pH 7.2, 1% SDS and 1 mM EDTA. Blots were exposed to a PhosphoImaging Screen and the ^{32}P signal of both HER2/*neu* and GAPDH transcript signals were quantitated by a Molecular Dynamics computing laser densitometer and ImageQuant software program. The assessment of drug inhibition of gene expression was determined by

dividing the signal of the mRNA bands from drug treated samples by the average mRNA signal generated by 4 non-drug treated control samples.

Results:

DNA binding agent effects on ESX/DNA complex formation with the HER2/*neu* promoter.

DNA binding agents with varying modes of binding and sequence preferences (structures presented in Figure 1A) were evaluated for their ability to prevent ESX binding to the HER2/*neu* promoter. An oligonucleotide probe (Figure 1B) containing the EBS consensus binding site from the HER2/*neu* promoter was incubated with purified ESX (30 minutes at room temperature) and reactions were resolved on a native polyacrylamide gel. A representative mobility shift assay is presented in Figure 2. Binding of ESX to the probe resulted in a slower migrating ESX/DNA probe complex (lanes 2, 8 and 14) compared to free oligo (lane 1). A DNA binding agents' effectiveness in preventing ESX binding to DNA was assessed by pre-incubating the drug with the DNA probe prior to the addition of ESX. In Figure 2, Hoechst 33342, an A/T preference MGB, prevented ESX/DNA complex formation in a concentration dependent manner. Almost complete inhibition of ESX/DNA complexes was seen at a concentration of 2.0 μM (lanes 3 and 9) while detectable inhibition occurred at a concentration as low as 0.75 μM (lanes 5 and 11). Inhibition of complex formation by 50% (IC_{50}) required a concentration of 1.4 μM.

Results from each of the DNA binding agents tested are shown in Figure 3 and the IC_{50} values are provided in Table 1. Comparison of the IC_{50} values of the two A/T preference MGBs, distamycin and Hoechst 33342, indicated distamycin was ~2 fold more potent (0.7 μM and 1.4 μM, respectively) in preventing complex formation. Detectable inhibition by distamycin was seen at a concentration of 0.5 μM with almost complete inhibition of the complex at 2.5 μM (Figure 3B). The G/C preference intercalator, hedamycin, had the lowest IC_{50} value (0.5 μM) for inhibition of ESX/DNA complex formation while close to complete inhibition was observed at 1.0 μM (Figure 3D). The evaluation of chromomycin was complicated by the fact that 12 mM Mg^{+2} is needed for optimal chromomycin binding to DNA and at these levels the ESX/DNA complex was reduced in the absence of drug. A maximal Mg^{+2} concentration of 5 mM was chosen which retained ~60% ESX/DNA complex compared to controls without Mg^{+2} (data not shown). These sub-optimal concentrations of Mg^{+2}, likely contributed to the relatively high IC_{50} value of 5 μM for ESX/DNA complex inhibition. In fact, chromomycin binding to DNA was so inefficient, it could not completely inhibit the ESX/DNA complex even up to 20 μM, the highest concentration tested (Figure 3C).

Effects of DNA binding agents on cell-free expression of HER2/*neu*.

The agents studied in MSAs were next tested as inhibitors of ESX regulated cell-free expression from the HER2/*neu* promoter. This assay provides a more complex environment for drug evaluation compared to MSAs since it includes nuclear components and it uses a relatively large plasmid DNA target that contains the HER2/*neu* promoter. Briefly, plasmid was incubated for 1 hour with nuclear extracts from SKBR-3 cells and a labeling cocktail containing $[\alpha\text{-}^{32}P]$-CTP. The radio-labeled transcripts were resolved on a denaturing polyacrylamide gel along with an RNA ladder to identify the major HER2/*neu* band (~760 base pairs) of interest.

DNA binding agents were incubated with the DNA template prior to transcript formation to assess drug effectiveness as an inhibitor of cell-free expression from the HER2/*neu* promoter. Figure 4, is a representative result, showing the concentration dependent inhibition of HER2/*neu* transcript formation by Hoechst 33342. About 95% inhibition of HER2/*neu* transcript formation was observed at 10 μM (lanes 2 and 8) while detectable inhibition is seen at a concentration as low as 2.5 μM (lanes 4 and 10). The IC_{50} value is 3.0 μM.

Results of cell-free transcription assays performed for each agent are presented in Figure 5 and IC_{50} values are listed in Table 1. Similar to the MSA studies both A/T sequence preference MGBs, distamycin and Hoechst 33342, showed similar IC_{50} values of 3.0 μM (Figure 5A, B). Detectable inhibition by distamycin was seen at a concentration of 2.5 μM with complete inhibition by 10 μM. Contrary to being the most effective agent in the MSA results, hedamycin (Figure 5D) was the least potent inhibitor of cell-free transcription requiring 25 μM to reach ~90% inhibition. While optimal drug-DNA binding conditions could not be achieved in the MSAs for chromomycin, the cell-free transcription conditions allowed for more effective chromomycin-DNA binding. Under these assay conditions, chromomycin was the most potent HER2/*neu* transcript inhibitor with detectable inhibition by 1.0 μM, an IC_{50} of 1.5 μM and maximal inhibition by 5.0 μM (Figure 5C).

In addition to a concentration dependent decrease in the major HER2/*neu* transcript (760 base pair), we noted a concentration dependent increase in partial transcript production in some drug treated samples suggesting an effect on transcript elongation. In Figure 4, an increase in Hoechst 33342 concentration (from lanes 5 to 2) decreased the major 760 base pair band and was accompanied by an increase of partial transcripts located below the 0.280 base pair marker (lane 13) and just above the internal control located in each lane. These partial transcripts were observed for all MGBs but were not detected for the intercalating drug, hedamycin (data not shown).

Effects of DNA binding agents on HER2/*neu* cellular mRNA levels in SKBR3 cells.

Cell-free assays, such as MSA and cell-free transcription, are valuable tools for studying DNA binding agents that bind to a common ESX DNA binding site, and determining their effectiveness at inhibiting ESX binding to DNA and subsequent effects on ESX-regulated transcription. However, testing of these agents within the complexity of the whole cell is needed to determine their effectiveness as inhibitors of ESX function on the endogenous HER2/*neu* promoter. Northern blot analysis of cells treated 24 hours with each DNA binding agent was used to determine their ability to inhibit ESX regulated expression of HER2/*neu* mRNA. SKBR-3 cells, which overexpress HER2/*neu*, were used in these studies. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), a housekeeping gene with comparable mRNA half-life as HER2/*neu* mRNA (~8h), was used to measure drug inhibition of general transcription compared to the gene of interest (28, 29). Figure 6 is a representative northern blot showing concentration-dependent inhibition of HER2/*neu* mRNA production by Hoechst 33342. Detectable inhibition was seen at 5.0 μM (lane 4) while significant inhibition (~60%) was noted at 10 μM (lane 3) and a resultant IC_{50} of 9.0 μM. In comparison, GAPDH mRNA was also inhibited to comparable levels as HER2/*neu* following Hoechst 33342 treatment (Figure 6). Northern blots were performed for each of the other drugs and the inhibition data is shown in Figure 7[4] while IC_{50} values are presented in Table 2.

Contrary to the findings for inhibition of transcription under cell-free conditions where both A/T sequence preference MGBs were quite effective, high concentrations of Hoechst 33342 and distamycin were needed to inhibit cellular HER2/*neu* mRNA levels (IC_{50}s of 9.0 μM and 60 μM, respectively). On the other hand, hedamycin, which was the least effective inhibitor of cell-free transcription, was a very effective inhibitor of cellular HER2/*neu* mRNA levels (IC_{50} = 0.21 μM). Chromomycin, as in the cell-free expression analysis, was also found to be the most effective inhibitor (IC_{50}=0.04 μM). Comparison of inhibitory activity on HER2/*neu* mRNA levels (■) to GAPDH mRNA levels (◪) revealed that most agents tested generally inhibited both genes to the same extent (Figure7A, B and D). Chromomycin, on the other hand, displayed stronger inhibition of HER2/*neu* mRNA levels compared to GAPDH mRNA (see Figure 7C and Table 2).

Time dependent differential inhibition of cellular mRNA levels by distamycin.

After observing a concentration dependent inhibition of HER2/*neu* mRNA for all agents, we next wanted to determine their time-dependent effects on inhibition of mRNA levels since cellular uptake and stability may affect drug action. The standard assay used a 24-hour time point since short-term drug evaluation

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[4] some data points are missing error bars due to cytotoxic limitations encountered in RNA recovery from drug treated samples.

was limited by the relatively long half-life of HER2/*neu* and GAPDH mRNA, ~8 hours, thus making it difficult to detect transcript inhibition due to pre-existing HER2/*neu* transcripts.

Studies of the time-dependent effects on mRNA inhibition were restricted to the least cytotoxic agent, distamycin (see Table 2), since long-term treatment by the other drugs resulted in a significant decrease of recoverable total RNA. Northern blot analysis was performed on total RNA harvested from SKBR-3 cells treated with distamycin at 50 μM for 24, 48, and 72 hours. Results depicted in Figure 8 show there was a time-dependent increase of HER2/*neu* mRNA inhibition by distamycin. Moreover, HER2/*neu* and GAPDH mRNA production were inhibited to almost equal levels up to the 48-hour time point. However, at 72 hours post-treatment, distamycin preferentially inhibited HER2/*neu* mRNA production over GAPDH.

Effects of DNA binding agents on SKBR3 cells growth.

While all agents tested in northern blot analysis inhibited HER2/*neu* mRNA, differences of over two orders of magnitude were observed in drug effectiveness. These differences could be an indication of drug inhibition of many cellular processes, as well as cytotoxicity, rather than a more specific effect on HER2/*neu* transcription. To address this point each agent was evaluated for the ability to inhibit SKBR3 cell growth over a 72-hour continuous exposure. Cell growth inhibition was determined by comparing the cell count of drug treated samples with the cell count of non-drug treated controls and the IC_{50} values are presented in Table 2. Hedamycin and chromomycin had IC_{50} values in the sub-micromolar concentrations while Hoechst 33342's IC_{50} value was found in the low micromolar concentration range. Distamycin was clearly the least active inhibitor, requiring an IC_{50} concentration in the hundred-micromolar range to inhibit cell growth. Agents that were most potent at inhibiting mRNA production, chromomycin and hedamycin, were also the most potent inhibitors of cell growth. Similarly, for Hoechst 33342 and distamycin, there was a correlation between effectiveness of mRNA inhibition and the ability to inhibit cell growth, with Hoechst 33342 being more effective than distamycin in both assays.

Discussion:

Previous studies have shown that some DNA binding agents can inhibit TF/DNA complexes and gene regulation although it is not generally known how a drug's DNA binding motif relates to its effectiveness as a TF inhibitor (9, 10, 16, 17, 26, 30-32). This study analyzed DNA binding agents with different binding modes and sequence preference on a common DNA target in related cell-free and whole-cell studies to determine how each drug functions as a TF inhibitor. The model system employed for drug evaluation was ESX and the HER2/*neu* promoter, which offers a TF binding site with both A/T- and G/C- regions and contacts both the

DNA major and minor grooves (see Figure 1B and 23, 24). Over-expression of HER2/*neu* has been associated with poor prognosis of breast cancer patients (33), so that disruption of ESX binding to and regulation of the HER2/*neu* promoter is not only an excellent model but a potential therapeutic target for development of drugs that selectively down-regulate its expression.

Based upon the simplest of the cell-free assays, the MSA, all agents examined inhibited ESX/HER2/*neu* promoter complex formation in a dose dependent manner with an overall order of potency: hedamycin = distamycin > Hoechst 33342 >> chromomycin. Hedamycin and distamycin, while being equally potent inhibitors of ESX binding, possess very different DNA binding modes and sequence preferences. Binding of hedamycin to the oligo was anticipated to potently inhibit ESX/DNA complex formation since intercalation and placement of its sugar moieties in the minor groove would create significant distortion of the DNA with loss of ESX's recognition binding site on both DNA grooves (34-38). Additionally, DNA binding agents such as hedamycin, which can form covalent bonds, have been shown to be significantly more effective inhibitors of TF/DNA complex formation than agents that bind reversibly (8, 15).

Reversible DNA binding drugs like distamycin and Hoechst 33342 may also effectively inhibit ESX/HER2/*neu* promoter complex formation by directly competing with ESX's minor groove contacts within its DNA binding domain. Distamycin's slightly increased efficiency compared to Hoechst 33342, may be due to distamycin's capacity to bind as a side-by-side dimer in regions with at least five A/T base pairs resulting in further narrowing of the major groove thus interfering with ESX binding to its major groove contacts (34, 38, 39). Chromomycin, also a minor groove binder, was the least effective inhibitor of ESX/HER2/*neu* promoter complexes, in contrast to its effects observed on many other types of TF/DNA complexes, such as EGR1 and TBP, where it was found to be a potent inhibitor at concentrations $\leq$ 1 μM (15). Since chromomycin was studied under sub-optimal conditions due to the limitations of maintaining the ESX/DNA complex in the presence of the high Mg^{+2} concentrations which are required for strong drug binding to DNA, its potency as an ESX/ HER2/*neu* promoter complex inhibitor was most likely underestimated.

Although the simplicity of the MSA provides an initial means to relate inhibition of ESX/DNA complex formation to drug binding motifs, the assay is limited by the need for a minimal length of DNA and purified ESX, conditions which do not well represent the complexity of a nuclear environment. Cell-free transcription assays maintain an exogenous DNA source (albeit larger and in higher concentration) in a cellular milieu that includes functional transcriptional machinery and endogenous ESX, and allows for evaluation of the agents' ability to inhibit ESX function. While all the tested drugs showed an ability to inhibit cell-free transcription, in

contrast to the EMSA findings, the order of potency of the G/C preference agents was switched, with chromomycin being the most potent and hedamycin the least: chromomycin > distamycin = Hoechst 33342 > hedamycin (Table1).

As noted, sub-optimal Mg^{+2} concentrations were used to evaluate chromomycin binding in MSAs, whereas 7.5 mM Mg^{+2} concentrations were used in the cell-free transcription assays. This increased Mg^{+2} concentration most likely enhances chromomycin's binding to DNA and perhaps improves its ability to inhibit ESX-regulated expression. Many MGBs bind to DNA without creating significant distortion of the helix, however, the binding of chromomycin as a Mg^{+2}-coordinated head-to-tail dimer results in major distortion and opening of the minor groove which would likely contribute to chromomycin's potent inhibition of cell-free transcription (34, 38, 40). The effectiveness of distamycin and Hoechst 33342 as inhibitors of cell-free transcription is in good agreement with their ability to block ESX/DNA complex formation in the EMSA analysis. Also as depicted in Figure 1B, there is a TBP consensus-binding site that overlaps the 3'-end of the ESX binding site on the HER2/*neu* promoter and recent studies suggest interactions between these two proteins (41). This TBP binding site is also a potential binding site for both distamycin and Hoechst 33342 so that blocking TBP binding would contribute to a decrease in HER2/*neu* transcript formation. Of note was the appearance of partial HER2/*neu* transcripts caused by treatment with all of the MGBs, suggesting these agents might also bind to site(s) downstream of ESX and prevent the movement of processive enzymes along the DNA, perhaps by distortion of the DNA grooves. Hedamycin was a relatively weak inhibitor of ESX regulated HER2/*neu* transcript formation. It also has limited sequence preference so that it may bind to sites on the plasmid DNA distant from the transcription start site so that the helical distortion may have less influence on cell-free transcription, at least at moderate concentrations.

Northern blot analysis of each agent revealed a concentration dependent inhibition of cellular HER2/*neu* mRNA levels with the following potency order: chromomycin > hedamycin >> Hoechst 33342 >> distamycin. Similar to cell-free transcription results, in whole-cells chromomycin was the most potent inhibitor of HER2/*neu* mRNA production. Studies of mithramycin, a structurally similar G/C sequence preference MGB, which showed the agent's effective disruption of Sp1 binding to the SV40 promoter and prevention of c-myc transcription initiation, suggest that the DNA conformational changes induced upon drug binding also disrupted DNA/protein interactions (9, 10). Like mithramycin, chromomycin binds to DNA as a dimer, creating significant helical distortions that presumably contributes to its potent inhibition of TF binding and function (31, 34, 38, 40). Hedamycin was also an effective inhibitor of cellular HER2/*neu* mRNA levels. The significant helical distortion created by drug intercalation and the ability of this agent to form DNA adducts, likely

contributed to hedamycin's potency since these covalent modifications could hinder the movement of processing enzymes (42). Additionally, studies have shown that alterations in DNA structure caused by TBP binding, promoted pluramycin binding to the 3'-side of the TATA box, hence, it is likely that hedamycin binding at these same sites could interfere with binding of many cellular DNA factors that influence transcription (43).

Hoechst 33342 and distamycin, natural A/T sequence preference MGBs that are known to cause minimal distortion of the DNA helix, were the least effective inhibitors of cellular HER2/*neu* expression. While similar inhibitory activity was anticipated for these agents as seen in the EMSA and cell-free transcription assays, Hoechst 33342, however, proved to be 6 fold more effective than distamycin at inhibiting cellular mRNA levels. Differences in additional factors such as cellular uptake and drug stability between these two drugs could contribute to disparities in potency (44, 45). Additionally, these results could suggest that agents, which create helical distortion upon DNA binding, regardless of the binding mode (intercalation vs. groove), are more effective inhibitors of transcription than are agents that create more local distortion of the DNA. On the other hand, lucanthone, an A/T preference intercalating agent which creates significant DNA distortions, showed weak inhibition of complex formation of either A/T or G/C sequence preference TF in MSAs (Welch and Beerman, unpublished data and 46).

Northern blot results also revealed a preferential inhibition of HER2/*neu* mRNA over GAPDH mRNA by chromomycin within 24 hours while all the other agents tested, equally inhibited both messages. The differences in sensitivity between these two transcripts to chromomycin might be attributed to the number of available drug binding sites found on the endogenous promoters of HER2/*neu* and GAPDH. For instance, an increase of chromomycin binding on the HER2/*neu* promoter could possibly result in greater DNA distortion and decreased transcription. Furthermore, the location of chromomycin binding sites, within close proximity to a basal and/or gene regulatory transcription factor binding site, may contribute to its inhibitory effects by significantly narrowing the major groove and thus interfering with the binding or movement of transcriptional machinery (47-50). Studies with mithramycin have also shown selective inhibition of gene expression (9, 10, 31). Time course studies beyond 48 hours for distamycin showed that HER2/*neu* mRNA was also preferentially inhibited compared to GAPDH mRNA. Previous whole-cell studies employing distamycin showed selective inhibition of gene expression including estrogen receptor isoforms and muscle-specific genes (30, 32). Additionally, cell-free studies indicate higher concentrations of distamycin are required to inhibit the binding of major groove binding regulatory proteins compared to minor groove binding proteins such as TBP (8, 15). It is possible that the complex of proteins involved in HER2/*neu* regulation, in addition to ESX, may contain more

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minor groove binding proteins that could be disrupted by distamycin compared to the proteins that regulate GAPDH expression. This differential inhibition which was not detected until the 72-hour time point, suggests the possibility of slower cellular uptake and drug accumulation of distamycin compared to chromomycin (51-53).

Investigation of drug cytotoxicity on SKBR3 cell growth revealed the same order of potency as observed for northern blot analysis: chromomycin > hedamycin >> Hoechst 33342 >> distamycin. The similarity of these results suggest that the observed inhibition of cellular mRNA levels may be due more so to cytotoxicity, rather than a more specific inhibition of HER2/*neu* transcription since most agents tested showed almost equally inhibitory effects on HER2/*neu* and GAPDH mRNA levels. Additionally, analysis of some drug concentrations or time points were limited due to the inability to obtain enough total RNA from treated cells, suggesting that these agents were having detrimental effects on all cellular processes prior to specific gene inhibition. These results support the use of cytotoxicity as a good predictive indicator of highly active agents. On the other hand, these results did not predict the observed preferential inhibition by the least cytotoxic agent distamycin when cells were treated below cytotoxic concentrations for longer periods of time.

This study revealed that DNA binding motifs could play a role in a drug's effectiveness as an inhibitor of TF binding to DNA. Certainly, the data demonstrates that sequence preference and DNA binding motifs of a drug influence their ability to interfere with transcription factor/DNA complexes and associated gene expression, however, it was less clear how drug DNA binding motif translates into effectiveness as an inhibitor of gene expression in cells. The A/T sequence preference MGBs, Hoechst 33342 and distamycin which as expected produced similar results in the cell-free evaluation, showed a significant difference as inhibitors of cellular expression which correlated with cytotoxicity results. In fact for each agent, inhibition of expression related the best to cytotoxicity, suggesting that in whole-cell studies additional factors such as cellular uptake and stability may contribute more to their level of activity than their mode of DNA binding. This study points out the importance of whole-cell analysis of drugs since cell-free studies were not always predictive of results observed in intact cells. Most importantly, there is a need for further development of drugs that cannot only inhibit gene expression in cells, but can do so in a manner that is based upon a DNA targeting strategy rather than being a consequence of a general activity in cells. Recent studies have found that specially designed sequence specific minor groove binding agents, polyamides, which bind DNA as side-by-side dimers, are extremely effective inhibitors of ESX/DNA complex formation and cell-free expression (26, 38, 54-56). Currently, studies are underway to develop these agents as specific inhibitors of HER2/*neu* expression in cells without the accompanying whole-cell cytotoxicity that occurs with more conventional DNA binding agents.

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Table 1: IC50 values for each agent tested in cell-free assays. The DNA binding agents are listed below with their respective DNA binding properties and IC_{50} values for inhibition of ESX/DNA complex formation in mobility shift assays and HER2/*neu* transcript formation in cell-free transcription assays.

Table 2: IC_{50} values for each agent tested in whole-cell assays. Each agent was evaluated for ability to inhibit HER2/*neu* and GAPDH mRNA production following a 24-hour drug exposure using northern blot analysis. Comparing the cell count of samples following a 72-hour continuous drug exposure to the cell count of non-drug treated controls yielded cell growth inhibition values.

Table 1

Drug	Binding Properties	MSA IC$_{50}$ values (μM)	Cell-free transcription IC$_{50}$ values (μM)
Hoechst 33342	A/T rich minor groove binder	1.4	3.0
Distamycin A	A/T rich minor groove binder	0.7	3.0
Chromomycin A$_3$	G/C rich minor groove binder	10.0	1.5
Hedamycin	G/C rich intercalator	0.5	8.0

Table 2

Drug	Northern blots (24 hrs) IC$_{50}$ values (μM)		Cell Growth Inhibition (72 hrs) IC$_{50}$ values (μM)
	HER2/*neu*	GAPDH	
Hoechst 33342	9	9	7.0
Distamycin A	66	57	114
Chromomycin A$_3$	0.04	0.07	0.05
Hedamycin	0.21	0.21	0.10

Figure 1: *A,* structures of the DNA binding agents. *B,* partial HER2/*neu* promoter sequence containing the ESX binding site (bold) and the EBS core sequence (underline). There is also a TBP binding site overlapping the 3'-end of the ESX binding site.

Figure 2: Inhibition of ESX binding to the HER/*neu* promoter by Hoechst 33342. Hoechst 33342 was incubated with ^{32}P-labeled oligonucleotide containing a portion of the HER2/*neu* promoter followed by incubation of purified bacterially expressed ESX protein. Samples were electrophoresed on 4% native polyacrylamide gels, dried, exposed to film and inhibition of ESX/DNA complex formation quantitated by densitometric analysis. The following is a representative MSA demonstrating the concentration dependent ability of Hoechst 33342 to prevent ESX binding to the HER2/*neu* promoter. *Lane 1* is non-complexed DNA control, *lanes 2, 8, and 14* are non-drug treated control ESX/DNA complexes, *Lanes 3-7* are Hoechst 33342 treatments at 2.0, 1.0, 0.75, 0.50, and 0.1 μM, respectively. *Lanes 9-13* are duplicate samples of *Lanes 3-7*.

Figure 3: Prevention of ESX binding to the HER2/*neu* promoter by each DNA binding agent. MSAs were performed for each agent and the percent inhibition of complex formation was determined by comparing the shift of drug treated samples to the average shift of 3 non-drug treated controls. The percent inhibition of complex formation was averaged from a minimum of 3 separate experiments and plotted against drug concentration (μM). *Bars,* SD. The IC_{50} values, that is the amount of drug required to inhibit complex formation by 50%, are summarized in Table 1. *A,* Hoechst 33342, A/T sequence preference MGB. *B,* distamycin, A/T sequence preference MGB. *C,* chromomycin, G/C sequence preference MGB. *D,* hedamycin, G/C sequence preference intercalator.

Figure 4: Inhibition of cell-free transcription from the HER2/*neu* promoter by Hoechst 33342. Hoechst 33342 was incubated with linearized plasmid DNA containing the HER2/*neu* promoter followed by incubation with SKBR3 nuclear extract and labeling cocktail, as described in Methods. Samples were loaded onto a 7 M-urea polyacrylamide gel, electrophoresed, dried, and exposed to Phosphor Imaging Screen with ^{32}P signal quantitated by ImageQuant software. Normalization for equal loading was based on an internal control (IC). The following is a representative cell-free transcription showing the concentration dependent inhibition of HER2/*neu* transcript formation by Hoechst 33342. *Lanes 1, 6, 7, 12* are non-drug treated controls. *Lanes 2-5* are Hoechst 33342 treatment at 10.0, 5.0, 2.5 and 1.0 μM, respectively. *Lanes 8-11* are duplicate samples of

Lanes 2-5. *Lane 13* is a standard(s) RNA ladder to verify the band of interest based on transcript size, ~760 base pairs, as noted on the scale to the right.

Figure 5: Inhibition of transcript expression off the HER2/*neu* promoter by each DNA binding agent.
For each DNA binding agent, cell-free transcription assays were performed and the percent inhibition of transcript formation was determined by comparing the normalized HER2/*neu* transcript signal of drug treated samples to the average normalized HER2/*neu* transcript signal of 4 non-drug treated controls. The percent inhibition of transcript formation was averaged from 3-4 experiments and plotted against drug concentration (µM). *Bars*, SD. *A*, Hoechst 33342. *B*, distamycin. *C*, chromomycin. *D*, hedamycin.

Figure 6: Inhibition of HER2/*neu* cellular mRNA levels in SKBR3 cells by Hoechst 33342. SKBR-3 cells were exposed to Hoechst 33342 for 24 hours at the indicated concentrations followed by harvesting total RNA. Samples were electrophoresed on a formaldehyde-agarose gel, transferred to nylon membrane and probed with GAPDH and HER2/*neu* ^{32}P-labeled cDNAs. The following is a representative northern blot demonstrating concentration dependent decrease in (kb) HER2/*neu* and (kb) GAPDH mRNA signals produced by Hoechst 33342. *Lanes 1, 2, 7, 8* are non-drug treated control, *Lanes 3-6* are Hoechst 33342 treatments at 10, 5.0, 2.5 and 1.0 µM, respectively.

Figure 7: Drug inhibition of mRNA levels in SKBR3 cells. Northern blot analysis on cells treated 24 hours by each agent was performed and the percent inhibition of mRNA production determined for both HER2/*neu* (■) and GAPDH (▨). Comparison of the drug treated HER2/*neu* signal to the average HER2/*neu* signal of 4 non-drug treated controls yielded percent inhibition of mRNA production. The results were averaged from a minimum of 2 experiments with duplicate samples and plotted against drug concentration. GAPDH mRNA was used in this study as a measure of general transcription versus HER2/*neu* mRNA as the drug-targeted site of transcription. Equal loading was verified by visual inspection on a UV light box. *A*, Hoechst 33342. *B*, distamycin. *C*, chromomycin. *D*, hedamycin.

Figure 8: Time-dependent inhibition of HER2/*neu* and GAPDH cellular mRNA levels by distamycin.
Northern blot analysis was performed after continuous 50 µM distamycin treatment of SKBR3 cells for 24, 48 and 72 hours. Comparison of distamycin treated mRNA signal to the average signal generated by 4 non-drug

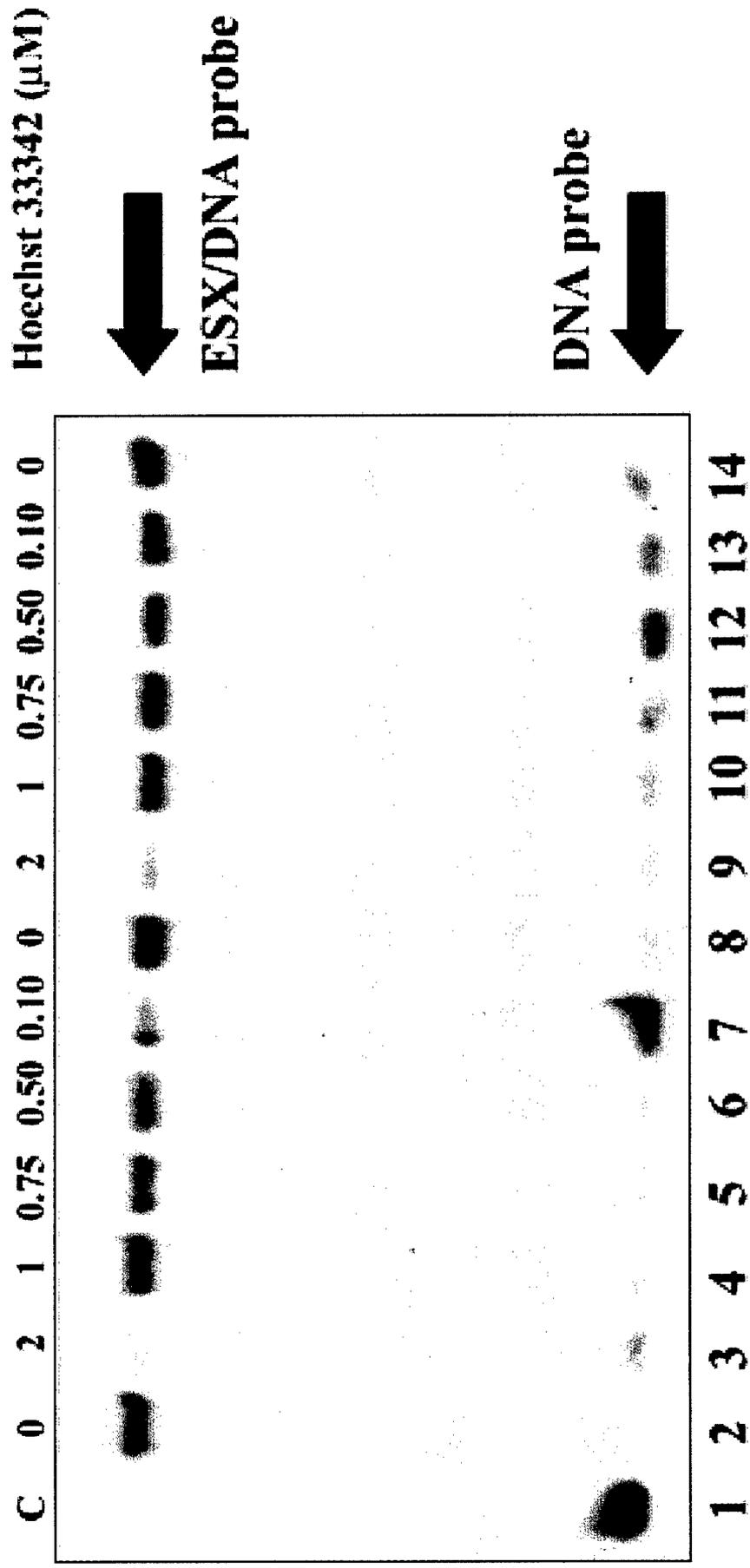treated controls yielded percent inhibition HER2/*neu* (■) and GAPDH (◪) mRNA production. The results of 6 separate studies were plotted against time. *Bars*, SD.

A.

Distamycin A

Hedamycin

Hoechst 33342

Chromomycin A₃

B.

ESX

TBP

5'-GGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAAT-3'

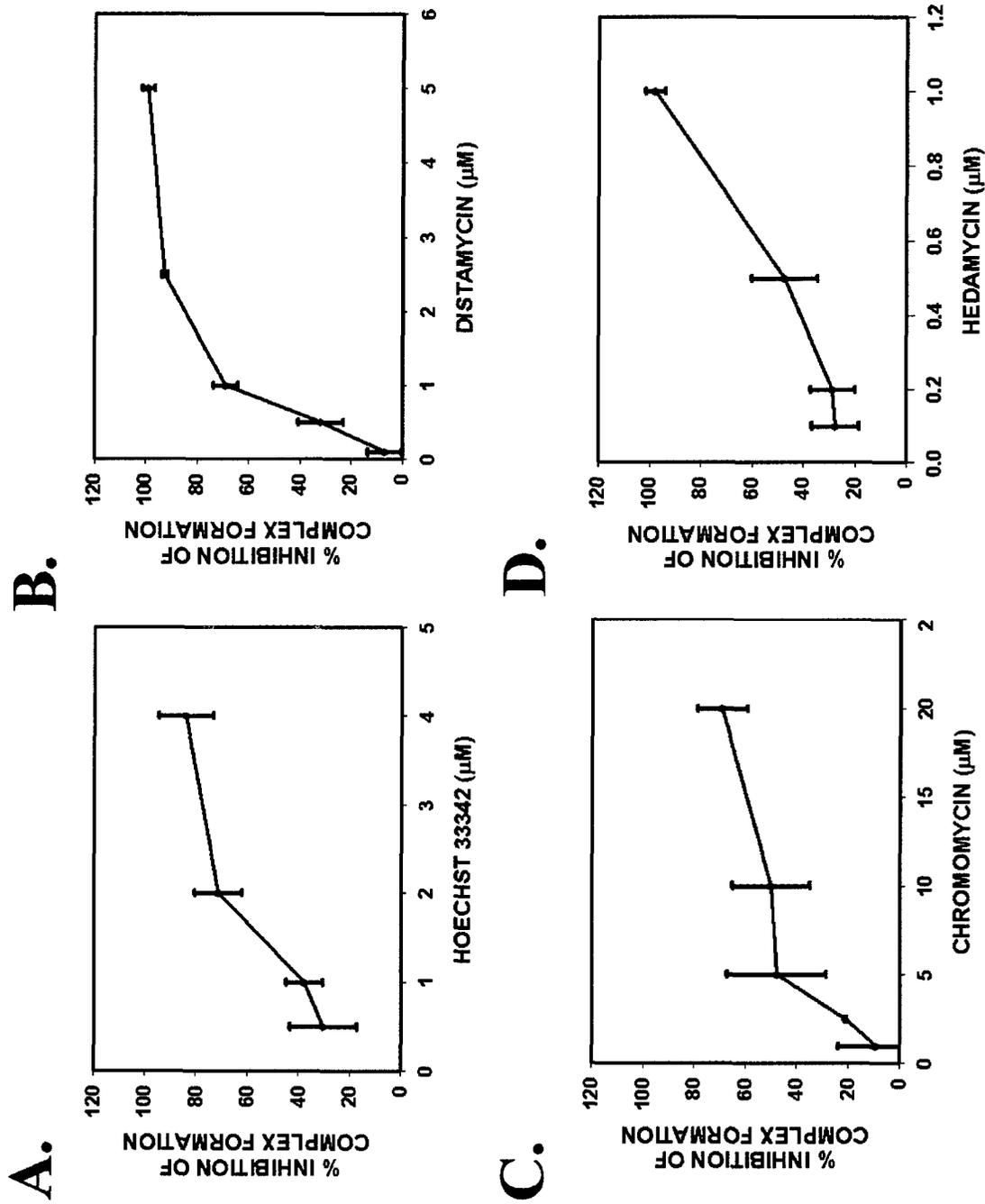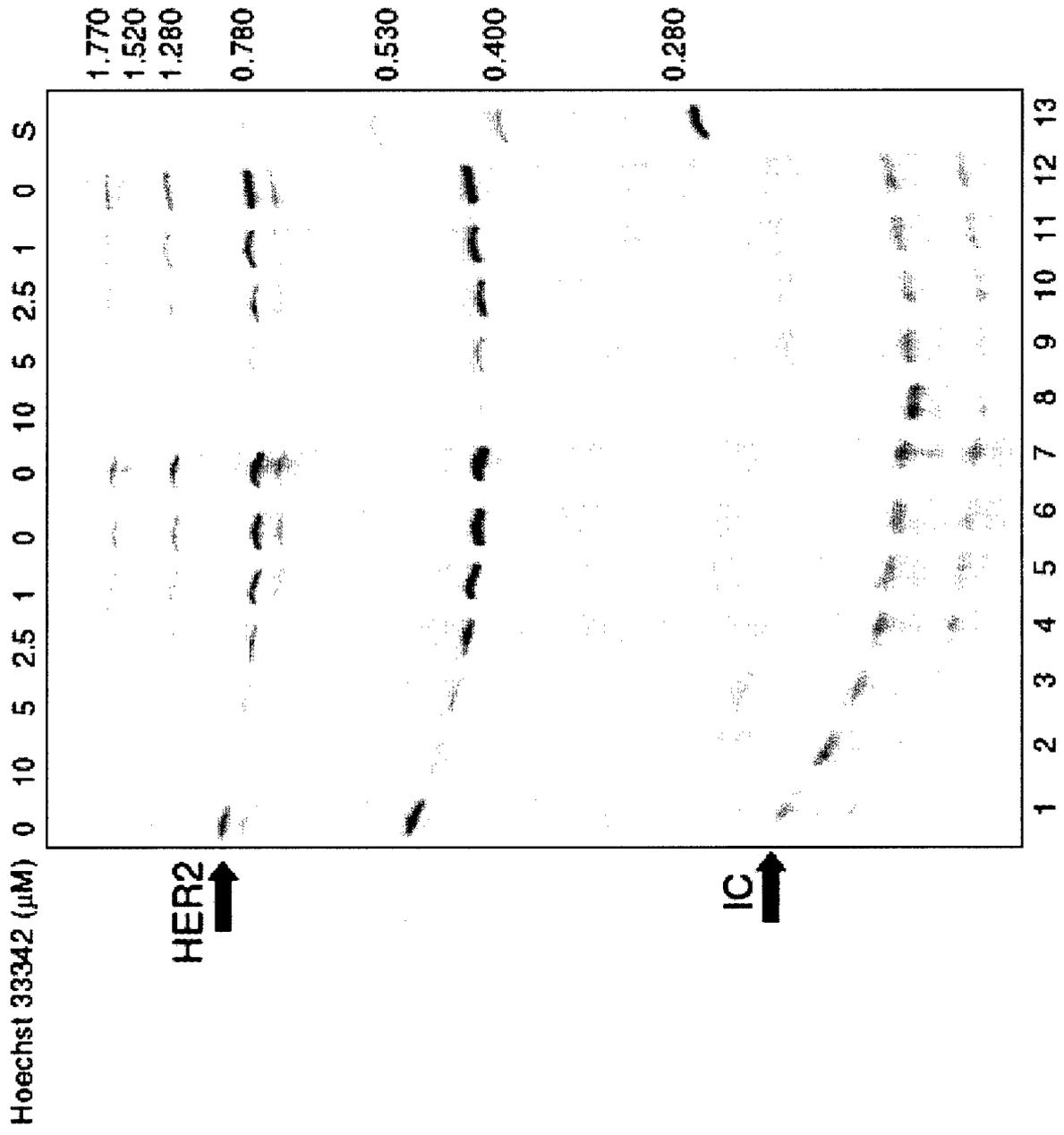MINOR GROOVE   MAJOR GROOVE   MINOR GROOVE

Figure 2

Figure 3

Figure 4

Figure 4

Figure 5

Figure 6

Hoechst 33342 (μM)

0	0	10	5	2.5	1.0	0	0

HER2

GAPDH

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
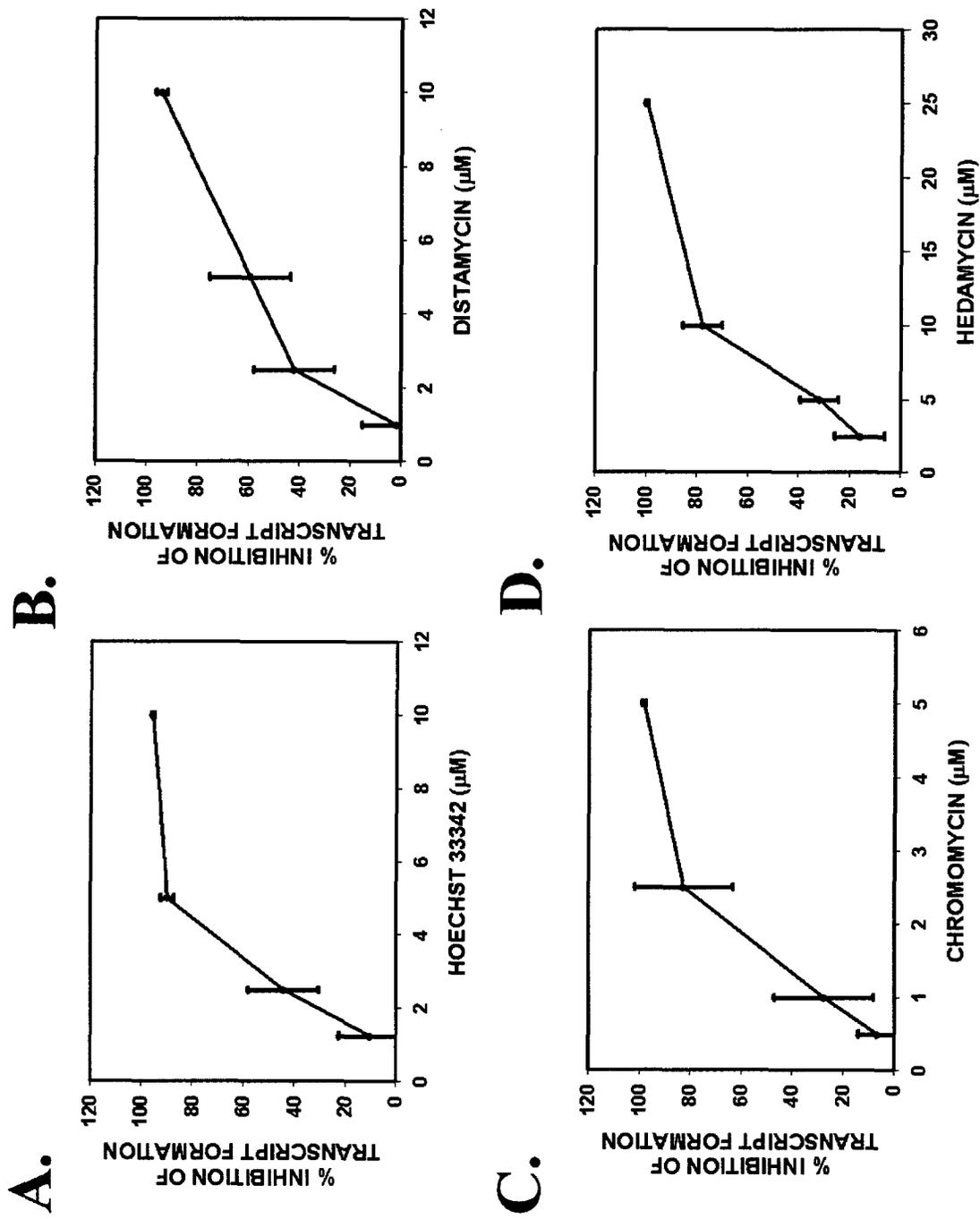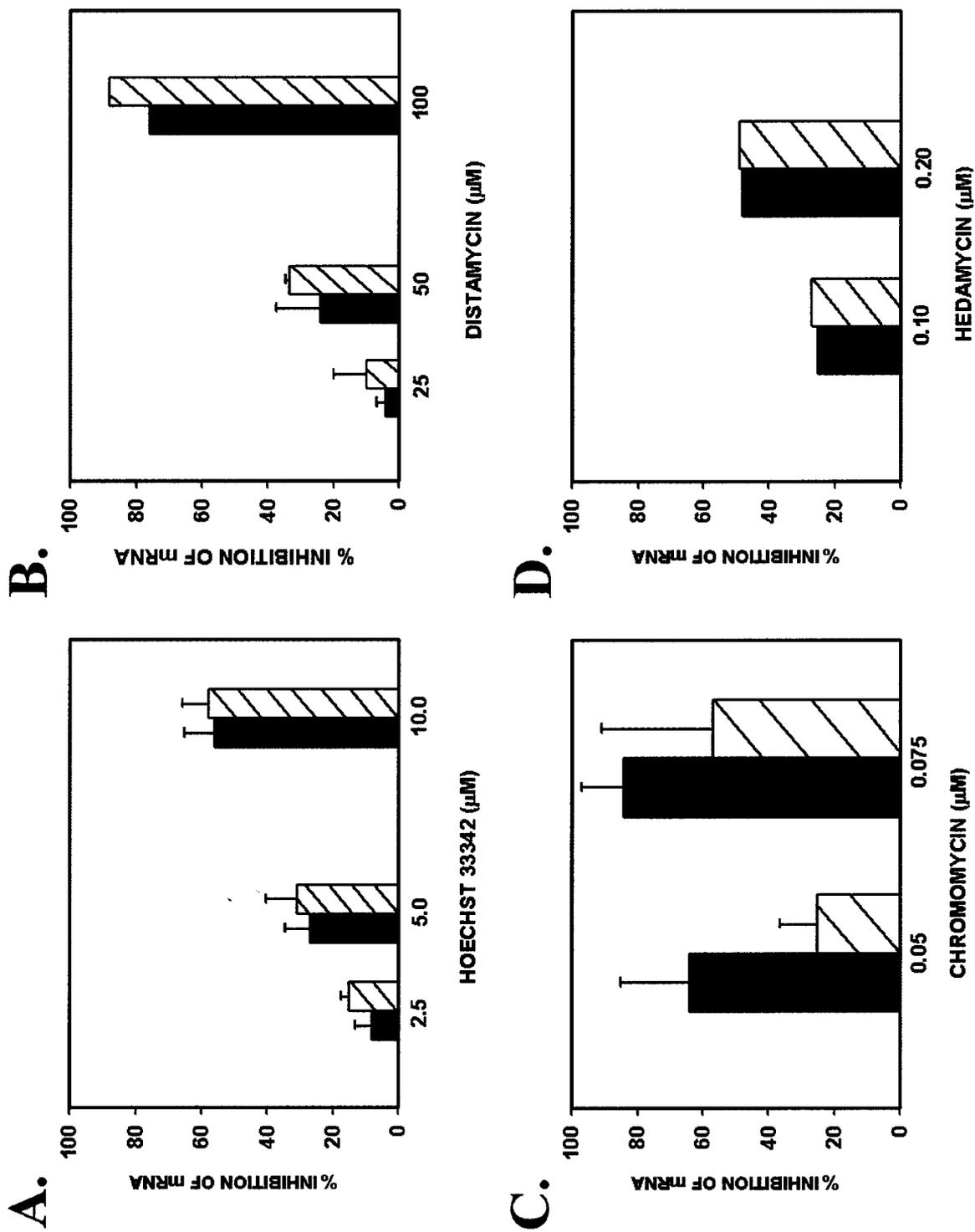
Figure 7

Figure 8
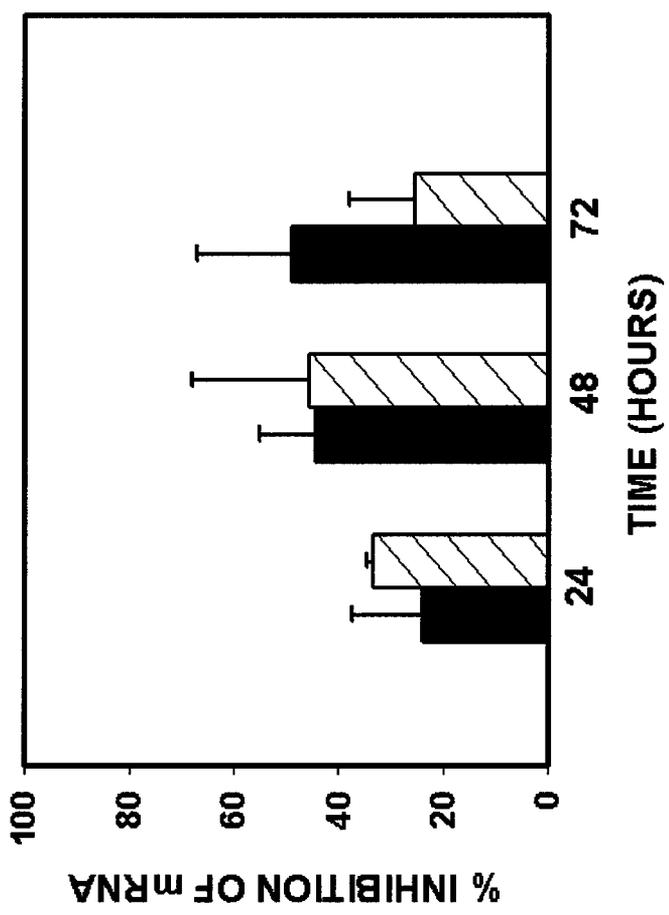
Appendix B:

Manuscript draft to be submitted at a later date to Cancer Research:

"Polyamide **22**, a Sequence Specific DNA Binding Ligand is Sequestered in the Cell Nucleus Following Uptake in SKB3 Human Breast Cancer Cells".

Title: Polyamide **22**, a Sequence Specific DNA Binding Ligand is Sequestered in the Cell

Nucleus Following Uptake in SKB3 Human Breast Cancer Cells.

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Running Title: Sequestering of polyamide 22 in the cell nucleus

Key Words: polyamides   fluorescence   permeability

localization   nuclei

Footnotes:

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Abstract:

Polyamides, a new class of sequence specific DNA minor groove binding compounds were evaluated for their permeability to intact cells. Polyamide **2**, a compound originally designed to target the ETS transcription factor DNA binding domain within the HER2/neu promoter, was modified by the addition of a bodipy fluorescent tag to create polyamide **22**. The fluorescent **22** displayed similar DNA binding characteristics as the parent compound. Following addition of **22** to a human breast cancer cell line SKBR3, quantitation by spectrofluorophotometric analysis revealed that up to 25 percent of the compound entered the cell. Fluorescence microscopy demonstrated that **22** was rapidly taken up by cells and by 15 minutes it was localized almost exclusively in the nucleus while avoiding the nucleoli. When cells were incubated with bodipy alone, fluorescence was only seen in the cytoplasm, suggesting that the nuclear signal observed with the **22** treatments was not caused by release of the tag prior to polyamide entry into the cell. Polyamide **22** could be competed by the parent compound and only minimally or not at all with other minor groove binding agents. Fluorescence intensity was also reduced when cells were treated first with **22** for four hours followed by a 6 hour chase using a high concentration of **2**. The pattern of **22** localization when compared to other DNA minor groove binding agents closely resembled Hoechst 33342 and DAPI which are widely used to stain the overall chromatin inside the cell nucleus. High resolution confocal microscopy confirmed the sequestering of polyamide **22** in the extranucleolar regions of the cell nucleus and revealed staining patterns that resembled higher order chromatin domains.

Introduction

Polyamides represent a novel class of DNA binding ligands that makes it possible to target unique DNA sequences (1-3). These agents bind the minor groove of DNA as anti-parallel side by side dimers with binding constants in the 10^{10} range for high binding affinity binding sites (1, 4). Sequence recognition of all four DNA bases is achieved by positioning an N-methylimidazole opposite guanine or N-methylpyrrole opposite cytosine as well as an N-methylpyrrole or hydroxymethyl pyrrole opposite adenosine or thymidine respectively (5). By linking DNA recognition elements with an appropriate hairpin turn to accommodate dimer binding, polyamides can be directed to a specified DNA sequence up to 16 base pairs in length (6). The exquisiteness of the DNA recognition, is illustrated by the finding that a one base mismatch between a polyamide and its DNA binding site results in over an order of magnitude decrease in binding affinity (7).

While the chemistry of polyamide recognition of DNA has been pursued in depth, utilization of these agents on biological targets is just beginning. Recent studies demonstrate that it is possible to inhibit transcription factor DNA complexes by directing polyamides to the binding site of the transcription factor DNA binding domain (8, 9). In the case of polyamides directed towards the TFIIIA DNA binding site within the promoter region of the 5S RNA gene, inhibition of complex formation was accompanied by a loss of gene expression (10). Other studies demonstrate that a rationally designed polyamide could interfere with binding of early protein 2 to its cognate binding site within the cis recognition sequence of the cytomegalovirus major immediate early promoter (8). Further versatility of polyamides as DNA targeting agents are revealed in studies that show inhibition of targeted genes in intact cells. For polyamides directed towards the 5S RNA promoter, patterns of inhibition of 5S RNA gene expression were similar whether treatment was under cell free conditions or carried out with intact cells (10). In another study, viral replication was strongly inhibited in cells treated with polyamides synthesized for binding to RNA polymerase II promoters of genes needed for HIV type 1 RNA synthesis (11).

The cited examples provide indirect evidence that polyamides can work in a cellular environment, but the findings do not directly assess some fundamentally important features about polyamides, such as their ability to be taken into cells and where localization might occur. In general, minor groove binding agents show differing ability to be taken up by cells and also where they bind varies. The classic minor groove binding compound Hoechst 33258 binds to both DNA and RNA and is found in the cell nucleus as well as in the cytoplasm (12). In comparison a structurally similar molecule Hoechst 33342 enters cells even more readily and is found almost exclusively within the cell nucleus (12).

To address the question of how polyamides compare to classical minor groove binding agents in regard to cellular uptake and localization, a polyamide containing a fluorescent Bodipy tag was synthesized (22) so it could be tracked within cells. This compound was a modification of 2 a polyamide under study as an inhibitor of the binding of the ETS protein ESX to a regulatory sequence within the proximal promoter of the HER2/neu oncogene (13-15). The fluorescent 22 was compared to the parent compound to show that the addition of the Bodipy does not significantly alter the properties of the polyamide in regard to DNA binding and inhibitory activity on gene expression. Analysis of 22 uptake was carried out using the human breast cancer cell line SKBR3, which overexpress HER2/neu, the target of the parent polyamide 2 (16). A quantitative assessment of ligand uptake was determined from spectrofluorophotometric measurements on 22 treated cells, while a qualitative analysis was made by examining cells under a fluorescence microscope. Uptake was determined both as a function of 22 concentration and time, while assessment of localization was made using both standard epifluorescence and high resolution confocal microscopy. The study demonstrates that polyamide 22 readily enters mammalian cells and localizes predominantly in the extranucleolar regions of the cell nucleus.

Materials & Methods:

Cells:

SKBR-3 cells were obtained from ATCC (Rockville, MD). Cells were grown in McCoy's 5a medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum and cultured at 37°C with 5% CO_2. Normal human diploid fibroblasts (neonatal skin cells where a gift from Dr. Jane Clifford)

were grown in DMEM supplemented with 0.5 mM sodium pyruvate, 1% L-glutamine and 10%

fetal bovine serum and cultured at 37°C with 10% CO_2.

Ligands:

Drugs:

Stocks of Hoechst 33342 (Sigma), Hoechst 33258 (Sigma), and DAPI (Boehringer Mannheim)

were prepared in ddH_2O.

Synthesis:

Footprint analysis:

MSA:

Mobility shift assays, as described previously, were used to detect differences in the biological

activity between the ligands as determined by their effectiveness in preventing transcription

factor protein binding to DNA (14). Briefly, an oligonucleotide the ESX consensus binding site

(polyamide targeted sequence) from the HER2/neu promoter was incubated with purified ESX

(30 minutes at room temperature) and reactions were resolved on a native polyacrylamide gel. A

ligands's ability to prevent ESX/DNA complexes was assessed by a 30-minute incubation of the

oligonucleotide and the drug prior to the 30-minute incubation with ESX protein. Using

Molecular Dynamics ImageQuant software, rectangles were drawn around the ESX/DNA band

and the density determined for controls and ligand treated samples. Percent of the control

complex formed was determined by comparing the signal of the ESX/DNA band of ligand

treated samples by the average signal generated by 4 untreated control ESX/DNA bands.

Spectrofluorophotometer studies:

SKBR3 cells were plated in 60mm dishes at a sub-confluent density of 5×10^5 cells/dish. 72

hours after plating, the cells entered log phase and the media was changed followed by addition

of polyamide **22** at 0.5 μM for 15 minutes, 1 and 4 hours. Cells were washed in PBS, trypsinized

and pelleted. Cells pellets were then washed 3X in PBS, resuspended in lysis/extraction buffer

containing 0.3N HCl: 96% EtOH (1:1) and incubated overnight at 4°C. Following collection of

supernatant by centrifugation, fluorescent signal was read on a Shimadzu recording

spectrofluorophotometer model RF-540 at the following parameters: excitation wavelength of

504 nm and emission wavelength of 512 nm. Standard curves of free polyamide **22** were

prepared in the same lysis/extraction buffer and were utilized for determining amount of recovered polyamide **22** from the cells.

Epifluorescence Microscopy:

SKBR3 cells were plated at 1×10^4 cells (in 100μl) on a flame sterilized coverslip (No.1, 22x22) and allowed to adhere for 1 hour at 37°C with 5% CO_2, prior to the addition of 1ml of media. 72 hours after plating, ligand or drug was added at the indicated concentrations and times. Harvesting of the coverslips were carried out in the dark following treatment. Coverslips were PBS washed 3X, fixed in ice-cold acetone for 15 minutes, followed by rehydration in ice-cold PBS for an additional 15minutes. Coverslips were then rinsed in water and mounted (GelMount, Fisher) on a slide. Slides were visualized on an Olympus BX-40 epifluorescent microscope, NB filter (for polyamide **22**), WIY filter (for Hoechst 33342, Hoechst 33258 and DAPI) and images captured using an attached Kodak EOS-DCS5 digital camera.

Confocal Microscopy:

Slides were prepared as described above and examined under a Biorad MRC-1024 confocal microscope system equipped with a krypton-argon laser and Nikon (Optophot[TM]) microscope. The objective was a Zeiss 60X oil immersion planApo with a 1.4 numerical aperture and the 488 nm emission band from the krypton-argon laser was used for excitation of the bodipy fluorochrome. Optical sections of 512 x 512 pixels x 8 bits/pixel were collected through the samples at 0.5 um intervals.

RESULTS:

Effects on ligand binding to DNA following the addition of the fluorescent tag, Bodipy.

While the DNA footprint analysis revealed that polyamide **22** recognition and binding activity was not severely altered by the presence of the fluorescent tag, mobility shift assays (MSAs) were employed to compare each ligand's biological activity, in regard to preventing ESX/DNA complex formation. Briefly, an oligonucleotide containing the targeted ESX consensus-binding site from the HER2/neu promoter was incubated with purified ESX (30 minutes at room temperature) and reactions were resolved on a native polyacrylamide gel. Binding of ESX to the oligo resulted in a slower migrating ESX/DNA band compared to free oligo. Ligand activity in preventing ESX/DNA complex formation was determined by incubating the ligand with the oligo (30 minutes at room temperature) prior to the addition of ESX and an additional 30-minute incubation period. The percent of ESX/DNA complex formation compared to control was determined for each polyamide concentration and the results are shown in Figure 2B. Polyamide **2** at 10 nM resulted in a 40% reduction of complex formation compared to non-ligand treated controls and at 25nM further reduced complex formation. On the other hand, treatment with polyamide **22** at 30 nM resulted in no detectable reduction compared to control, however, at 50 nM, polyamide **22** reduced complex formation by ~50%. These MSA results indicated a 3-fold difference between polyamides **2** and **22**'s ability to bind the oligonucleotide and prevent ESX/DNA complex formation. Moreover, each ligand was also tested in a cell-free transcription assay and demonstrated that polyamide **22** was 3-fold less active than polyamide **2** at inhibiting HER2/neu transcript production (data not shown).

Polyamide 22 uptake in SKBR3 cells.

The cell-free results suggest that the fluorescently labeled polyamide had the same sequence preference and similar biological activity, albeit slightly decreased, to the parent compound, polyamide **2**. Hence, polyamide **22**, is a reasonable representative model for polyamides in whole cells studies, and with its fluorescent tag, readily available for determining cellular uptake of these types of agents, which is still unclear. For these studies we utilized the SKBR3 cell line, which over-express HER2/neu (the polyamide targeted sequence) and exposed them to polyamide **22** at 0.5 μM for various times. The fluorescent signal of the supernatant

from lysed cells was measured on a spectrofluorophotometer at an excitation wavelength of 504 nm and emission of 512 nm. The amount of recovered polyamide **22** from cells was determined by comparison to a standard curve of the fluorescent signal of free polyamide **22**. The results, shown in Figure 3, indicate that the cells took up the ligand in a time dependent manner; with ~15% of the polyamide treatment taken up within 15 minutes and a further increase up to ~30% by 4 hours.

Polyamide 22 localization in SKBR3 cells.

While the spectrofluorophotometer results indicate that the cells take up the ligand, it does not indicate location within the cells. To evaluate localization of **22**, epifluorescent microscopy studies were undertaken. Briefly, SKBR3 cells that were grown on glass coverslips were exposed to polyamide **22** at 0.5 μM for 4 hours then washed several times with PBS, followed by fixation in acetone, re-hydration in PBS, rinsed and mounted to slides prior to viewing on an epifluorescence microscope. The results are shown in Figure 4 using two images. Figure 4A is the phase/contrast image for visualization of cytoplasmic boundaries, nuclei and nucleoli in the nuclear interior, while Figure 4B is the fluorescent image of the same field. The fluorescently tagged polyamide **22** is localized primarily in the nucleus with avoidance of the nucleoli, the dark structures identified by the black arrows in Figure 4A and the corresponding white arrows in 4B. The finding that fluorescence was detected only in the cytoplasm of cells treated with bodipy alone (Figure 4C and D) suggest that the nuclear fluorescence was due to polyamide **22** localization and not a consequence of breakdown of the ligand and subsequent movement of bodipy into the nucleus.

Time study of polyamide 22 uptake and localization.

The spectrofluorophotometer studies detected a slight increase in cellular uptake over time (Figure 3) and the initial fluorescence microscope studies demonstrated nuclear localization, at least for the 4 hour time point examined. Time course studies were carried out to determine cellular distribution of polyamide **22** over time. SKBR3 cells were exposed to polyamide **22** at 0.5 μM from 5 minutes up to 24 hours and the results are shown in Figure 5. At the earliest time point of 5 minutes, fluorescence was detected, albeit weakly, in the cell nucleus with little or no detectable staining in the cytoplasm. The intensity of nuclear fluorescence increased rapidly for

up to one hour and then increased more gradually up to 24 hours (Figure 5). At all time points little or no cytoplasmic fluorescence was detected. These results are consistent with the spectrofluorophotometric measurements shown in Figure 3 and further demonstrate that the progressive increase in fluorescence with time is predominantly due to a corresponding accumulation of polyamide **22** in the nucleus. Similar results were obtained when polyamide 22 was incubated with a normal human diploid cell line (NHDF 4085) (results not shown) indicating that the results obtained with the SKB3 human breast cancer cells are generally characteristic of polyamide 22 localization in mammalian cells.

Polyamide 2 competition with polyamide 22 for cellular uptake and binding.

Competition and chase studies utilizing polyamide **2** were performed to investigate the possibility that the presence of the fluorescence tag influenced the cellular uptake and localization. If polyamide uptake and localization is independent of the fluorescent tag than the parent compound should be able to compete for cellular uptake, which would be detected by a decrease in fluorescence. Competition studies utilized SKBR3 cells grown on coverslips treated with either polyamide **22** alone at 0.5 μM (Figure 6A) or polyamide **22** with 10-fold excess of polyamide **2**, (0.5 μM and 5.0 μM, respectively; Figure 6B). In the presence of polyamide **2**, detectable fluorescence was significantly decreased compared to polyamide **22** alone. Moreover, competition studies with increasing amounts of polyamide **2** resulted in a further decrease in fluorescence signal to barely detectable levels (data not shown).

If binding in the nucleus is independent of the presence of the fluorescent tag than polyamide **2** should be able to bind to the same target, effectively chasing the polyamide **22** from the binding site. Chase studies employed a 4-hour pre-treatment of cells with polyamide **22** at 0.5 μM followed by a 6-hour chase by either water (Figure 6C) or polyamide **2** at 5.0 μM (Figure 6D). These results also showed a significant decrease in fluorescence when polyamide **2** was present.

Comparison of polyamide 22 with other DNA minor groove binding agents for localization and staining in SKBR3 cells.

Polyamides are designed to bind to DNA and the epifluorescence microscopy results suggest that polyamide **22** may be reaching its target in the nucleus, while avoiding, the nucleoli, a site concentrated in RNA. Comparison of polyamide **22** localization and binding patterns to the patterns of other known DNA minor groove binding agents may suggest the molecular binding site of the polyamides. DNA minor groove binding agents chosen for these studies include Hoechst 33342, Hoechst 33258 and DAPI, which have known fluorescent properties and have been well studied for cellular localization (17). SKBR3 cells were grown on coverslips, exposed to each agent for 1 hour, harvested and visualized as described in Material and Methods. The results are shown in Figure 7 with the phase/contrast images on the left-hand side and the fluorescent signal of the same field on the right-hand side. Comparison of the two images illustrates the localization patterns of each agent. Figure 7A and B is the result of a polyamide **22** treatment at 1 μM, and displays the ligand's nuclear localization accompanied by avoidance of the nucleoli. Hoechst 33342 (Figure 7C and D) and DAPI (Figures 7G and H) showed a similar extranucleolar pattern of fluorescence in the nucleus with little or no detectable cytoplasmic staining. In contrast, Hoechst 33258, which has almost identical structure and fluorescent properties to Hoechst 33342 but readily binds to RNA as well as DNA, resulted in visible cytoplasmic staining (red/orange) with a significantly decreased nuclear fluorescence compared to Hoechst 33342 (Figure 7E and F). We conclude that polyamide **22** localizes to the cell nucleus of mammalian cells much like the classic DNA specific minor groove binding agents Hoechst 33342 and DAPI which are widely used to stain the overall chromatin pattern inside the cell nucleus. Thus polyamide **22** also stains the overall chromatin in the nucleus.

Laser scanning confocal microscopy following treatment with polyamide 22.

To confirm and extend the epifluorescence microscopic studies we performed time studies of polyamide uptake using laser scanning confocal microscopy. Representative midplane optical sections following incubation of SKBR3 cells with polyamide **22** between 4 to 28 hours are shown in Figure 8. The results conclusively demonstrate that the polyamide **22** is virtually exclusively targeted to the extranucleolar regions of the cell nucleus with no detectable cytoplasmic staining over the time span examined. The multiple nucleolar regions, characteristic of cancer cells, are clearly seen as the very large black areas devoid of fluorescence. Interestingly, a fine granular pattern of staining is often detected (see, e.g, Figure 8B), that may

reflect higher order arrangement of the chromatin domains stained by polyamide **22**. Arrangement of the granular-like stained structures into higher arrays of structures is also suggested around the nucleolar regions, along the nuclear periphery and in various other extranucleolar regions inside the cell nucleus (see e.g., Figure 8B).

Discussion.

Polyamides are a conceptually new form of DNA minor groove binding agent, which through modular design can achieve a remarkable degree of sequence recognition (1). Numerous papers have been published on the chemistry describing how polyamides can be designed to recognize unique sequences of DNA based upon selective binding motifs to all four DNA bases, although relatively little is known about the ability of these agents to target biological molecules in cells (2, 5, 18). While there are several examples of cells treated with polyamides resulting in inhibition of gene expression there is no direct evidence that these agents are taken up by cells (9, 10). This study is the first to track the uptake of a polyamide into a cell and to determine its distribution.

To determine uptake, a well characterized polyamide, **2** originally designed to target a DNA sequence within the HER2/neu promoter was tagged with fluorescent Bodipy. Based upon spectrofluorophotometric analysis of SKBR3 cells treated with **22**, significant uptake occurs within 15 minutes and by four hours about 25 % of the compound is taken up (Fig. 3). Microscopic analysis of these cells revealed that by 15 minutes much of the drug was localized within the nucleus and by four hours compound in the cytoplasm was barely detectable (Fig. 5). The appearance of the fluorescent signal in the nucleus was not the result of degradation of **2** and the subsequent release of free tag, since bodipy alone only localizes to the cytoplasm (Compare Figs. 4A&B with C&D). It was also found that uptake was temperature dependent and that incubations at 4° resulted in no detectable uptake into the cell (data not shown). Finally, a similar profile of **22** uptake into normal human diploid fibroblasts cells was seen, demonstrating that polyamide permeability into mammalian cells is not limited to the neoplastic SKBR3 cell type (Leslie and Beerman, unpublished observation).

The observation that treatment with a 10:1 mixture of the nonflourescent **2** and **22** respectively markedly reduced the fluorescence signal intensity suggests that both agents share a common uptake pathway and that the presence of the Bodipy tag is likely not altering the

mechanism for cellular uptake and retention. Studies showing that **2** can chase the fluorescent **22** signal indicate that the parent and fluorescent compound have similar nuclear binding sites. The poor ability of a conventional DNA minor groove binding agent Hoechst 33342 to chase the **22** signal from the nucleus could indicate that the strong DNA binding properties of polyamides are a factor in where these molecules bind (data not shown) and are retained inside the cell nucleus.

Polyamide **22** shows a characteristic pattern of strong signal throughout the nucleus with the exception that almost no fluorescence is observed within the nucleolus region (Fig. 4A/B). This compares quite well to the uptake patterns for both Hoechst 33342 and another minor groove binding agent DAPI (Fig. 7) which are widely used for staining the overall chromatin inside the cell nucleus. In contrast, Hoechst 33258, which differs from Hoechst 33342 by the substitution of a ethoxy group for the hydroxyl group on the phenol ring, shows a very limited amount of uptake within the nucleus (12, 19). It has been reported that the 33342 Hoechst and DAPI bind almost exclusively to DNA within the nucleus while the 33258 compound can bind to RNA and DNA and is taken up both into the nucleus but even more strongly into the cytoplasm (12). Similarly, the levels of uptake between the two Hoechst compounds varies, with 33342 being much more efficiently taken up (three fold) by mammalian cells (20). At this point, it is not known whether other types of polyamides will possess the uptake characteristics and nuclear localization patterns of **22** which is like Hoechst 33342 or that variants will be seen such as those that whose binding is reminiscent of Hoechst 33258 or potentially other patterns of distribution.

Laser scanning confocal microscopic analysis enabled us to more precisely visualize the intranuclear pattern of polyamide **22** fluorescence. The results illustrated in Figure 8 confirm the extranucleolar localization in the nucleus and the absence of detectable signal in the cytoplasm. Moreover the overall nuclear staining pattern of the chromatin appears as granular-like structures that appear to be arranged in higher order arrays that often surround the nucleolus, nuclear periphery and in various other regions in the nuclear interior. Further studies of these putative granular-like structures decorated by polyamide **22** are being planned using computer imaging segmentation and three-dimensional computer imaging approaches (21). Preliminary measurements indicate that the repeating chromatin structures have x-y dimensions of about 0.5 microns (Berezney and Somanathan, unpublished findings). This is in the same size range as the replication/transcription sites detected in mammalian cells (22-25). Previous studies have further indicated that replication sites are characteristic features of higher order chromatin domains (23,

26). Thus the staining patterns of polyamide **22** may be revealing a fundamental aspect of higher order chromatin organization and function in the cell nucleus.

While the initial purpose of this study was to identify where a polyamide resides in mammalian cells, some of the features of the localization suggest that the unique sequence specific nature of the polyamides may be a useful approach for designing compounds that bind to specific regions of functional chromatin. Moreover these compounds offer the potential for direct visualization of such functional elements as origins of DNA replication or promoter sites of transcription within the nucleus of the intact cell.

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Figure Legends:

Figure 1. Creation of the fluorescently labeled polyamide, 22. Polyamide **22** was created by the addition of Bodipy to polyamide **2**.

Figure 2B,C. Comparison of 2 and 22 biological activity using mobility shift assays.
Polyamides **2** (*B*) and **22** (*C*) were incubated with an oligonucleotide containing the targeted ETS binding sequence from the HER2/neu promoter for 30 minutes at room temperature followed by an additional 30 minutes incubation with purified ESX protein. Reactions were resolved on a 4% native polyacrylamide gel and the percent of control ESX/DNA complex formation was determined.

Figure 3. Spectrofluorophotometric determination of polyamide 22 uptake into cells.
SKBR3 cells were treated with **22** at 0.5 μM for the indicated times. Cells were then washed, trypsinized and the cell pellets washed 3X in PBS. Pellets were then resuspend in lysis/extraction buffer and incubated overnight at 4°C. The supernatant was collected following centrifugation, fluorescent signal quantitated on a spectrofluorophotometer and the amount of polyamide **22** recovered from the cells determined by comparison to a standard curve of free polyamide **22**.

Figure 4. Determination of cellular localization of polyamide 22 using epifluorescence microscopy. SKBR3 cells were treated with either **22** at 0.5 μ M (*A/B*) or Bodipy alone at 5.0 μ M (*C/D*) for 4 hours. Cells were then washed 3X with PBS, fixed in acetone followed by rehydration in PBS. Coverslips were then mounted to a slide and visualized using an epifluorescence microscope. *Scale bar*, 50 μm.

Figure 5. Cellular uptake of polyamide 22 over time. SKBR3 cells were treated with 0.5 μM of polyamide **22** at the indicated times points and harvested. *Scale bar*, 50 μm.

Figure 6. Comparison of polyamides 2 and 22 in cellular uptake and binding studies.
Competition between polyamides **2** and **22** for cellular uptake/transport utilized a 1 hour treatment of SKBR3 cells with: *A*, **22** alone (0.5 μM), or *B*, simultaneously with **22** and **2** at (0.5 μM and 5.0 μM, respectively). Chase studies for molecular binding site employed a 4-hour pre-treatment of SKBR3 cells with **22** (0.5 μM) followed by a 6-hour chase with: *C*, water or *D*, **2** (5.0 μM). *Scale bar*, 50 μm.

Figure 7. Comparison of cellular localization and binding pattern of polyamide 22 to other known DNA minor groove binding agents. SKBR3 cells were treated for 1 hour with: *A/B*, polyamide **22** at 1.0 μM; *C/D*, Hoechst 33342 at 2.0 μM; *E/F*, Hoechst 33258 at 2.0 μM; *G/H*, DAPI at 40 μM. *Scale bar*, 50 μm.

Figure 8. Localization of polyamide 22 in the cell nucleus using laser scanning confocal microscopy. SKBR3 cells were treated with 0.5 μM of polyamide **22** for: **A/B**, 10 hr; **C**, 10 hr;

and **D**, 28 hr. **B** is an enlargement of the boxed imaged in **A**. All images are 0.5 micron midplane sections.

Figure 1

B

% SHIFT COMPARED TO CONTROL
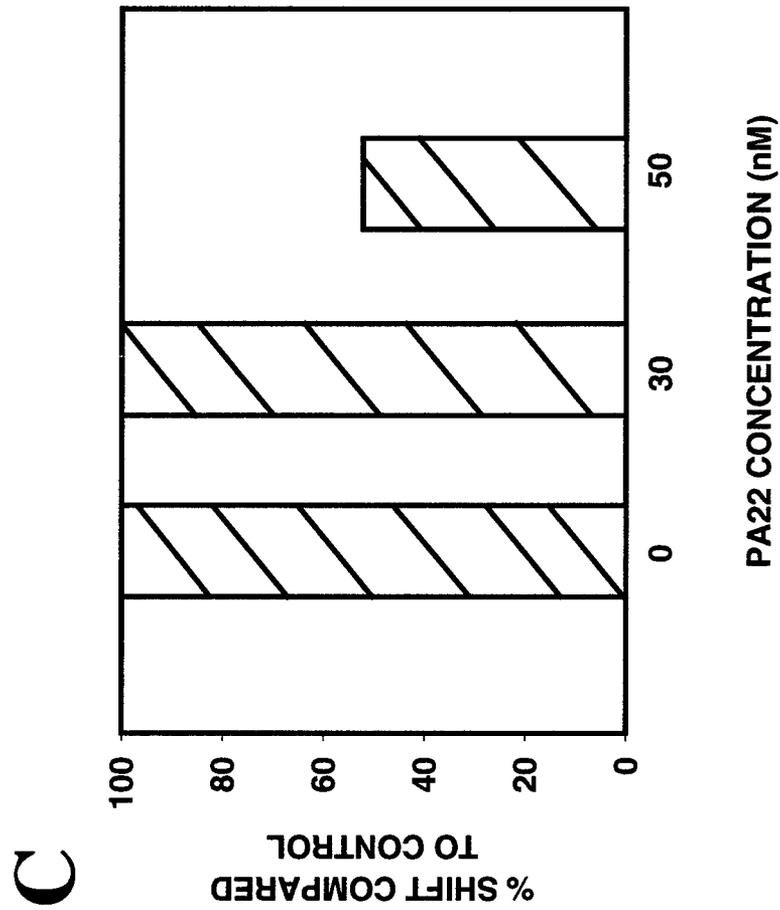
PA 2 CONCENTRATION (nM)

C

% SHIFT COMPARED TO CONTROL
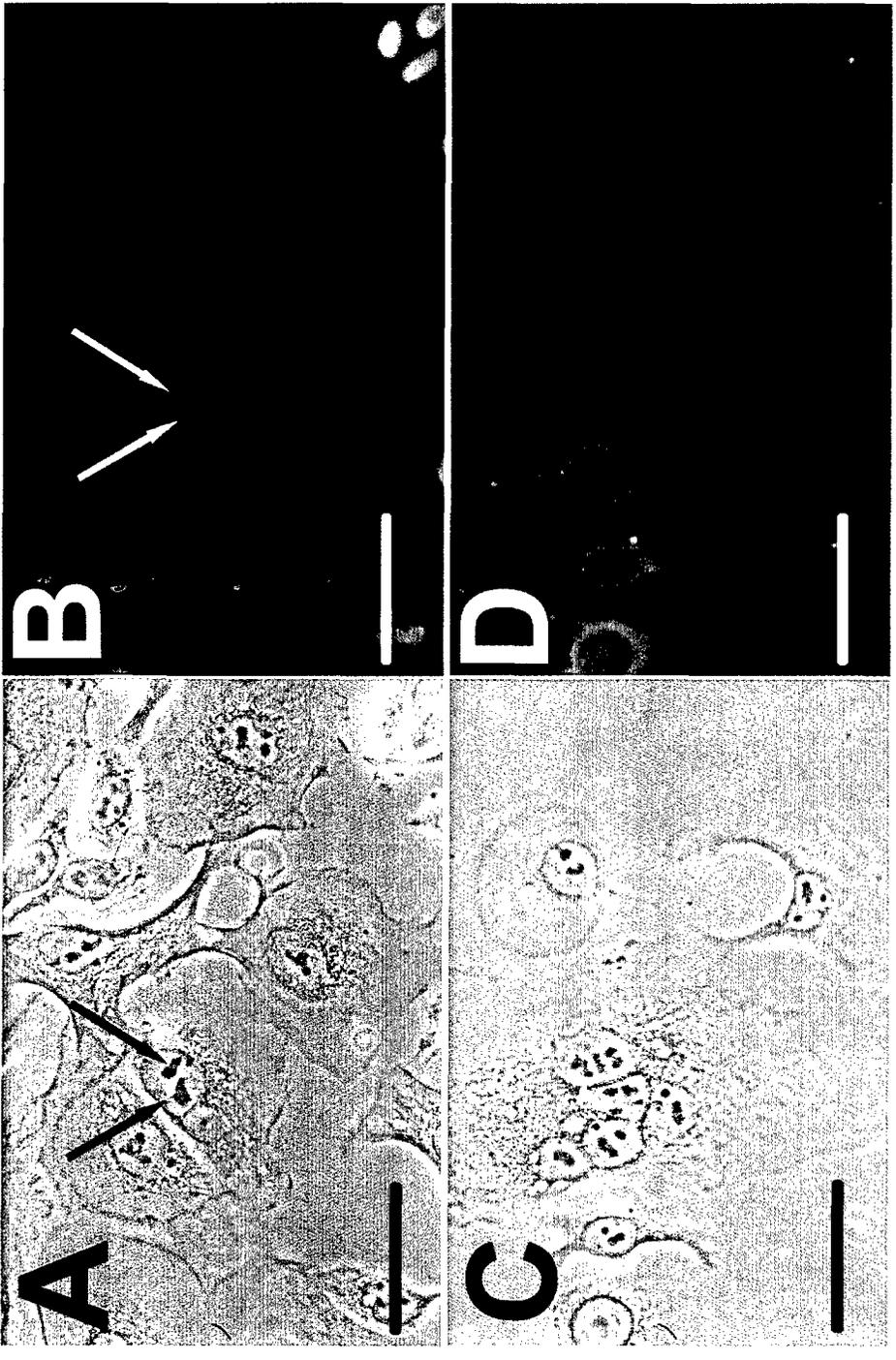
PA22 CONCENTRATION (nM)
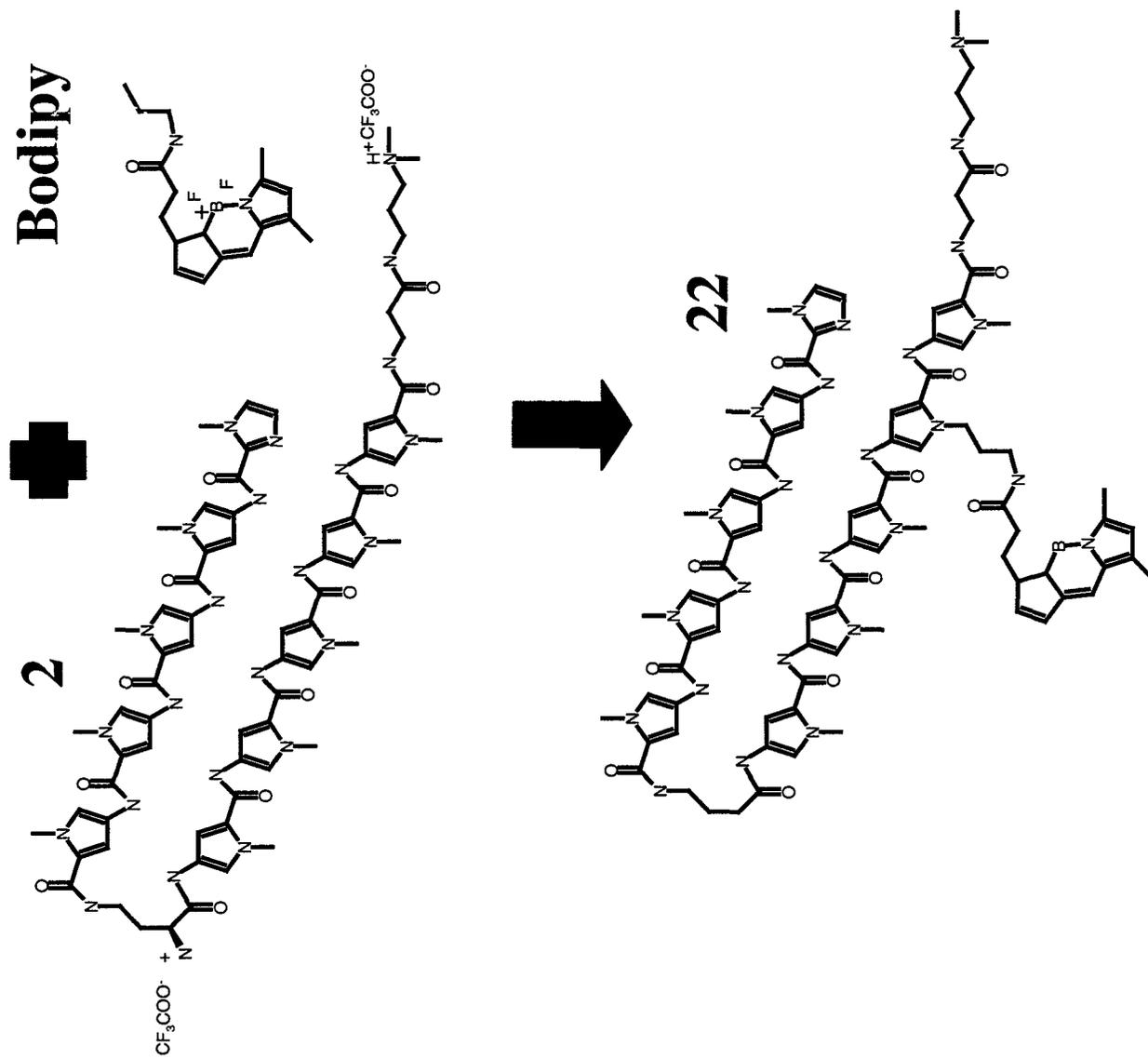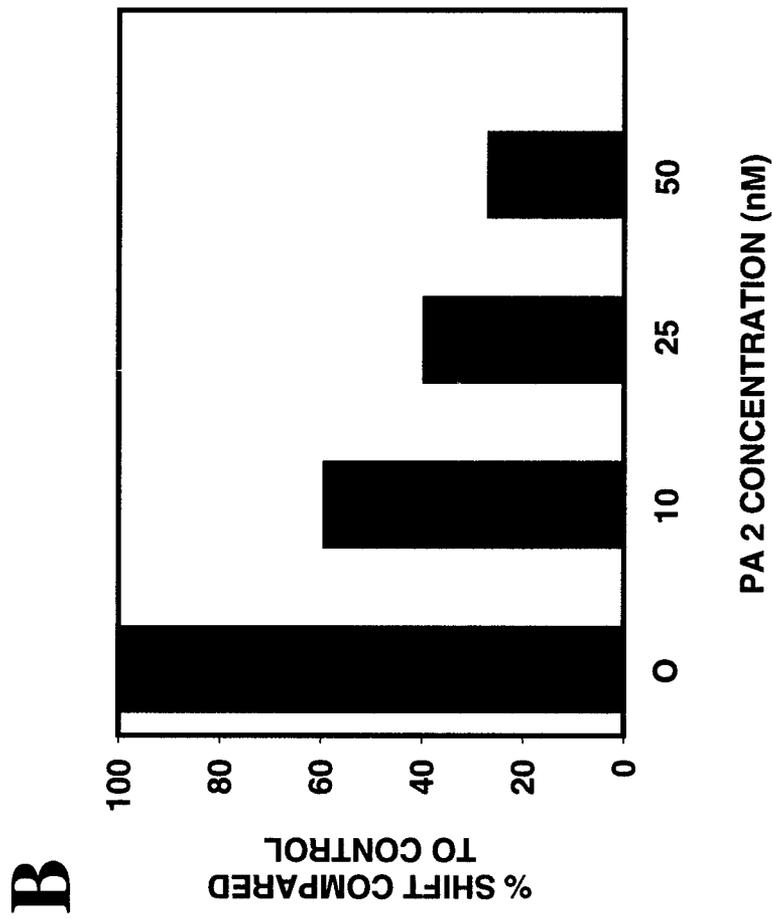
Figure 3.

Figure 4

Figure 5

5 min

15 min

1 hour

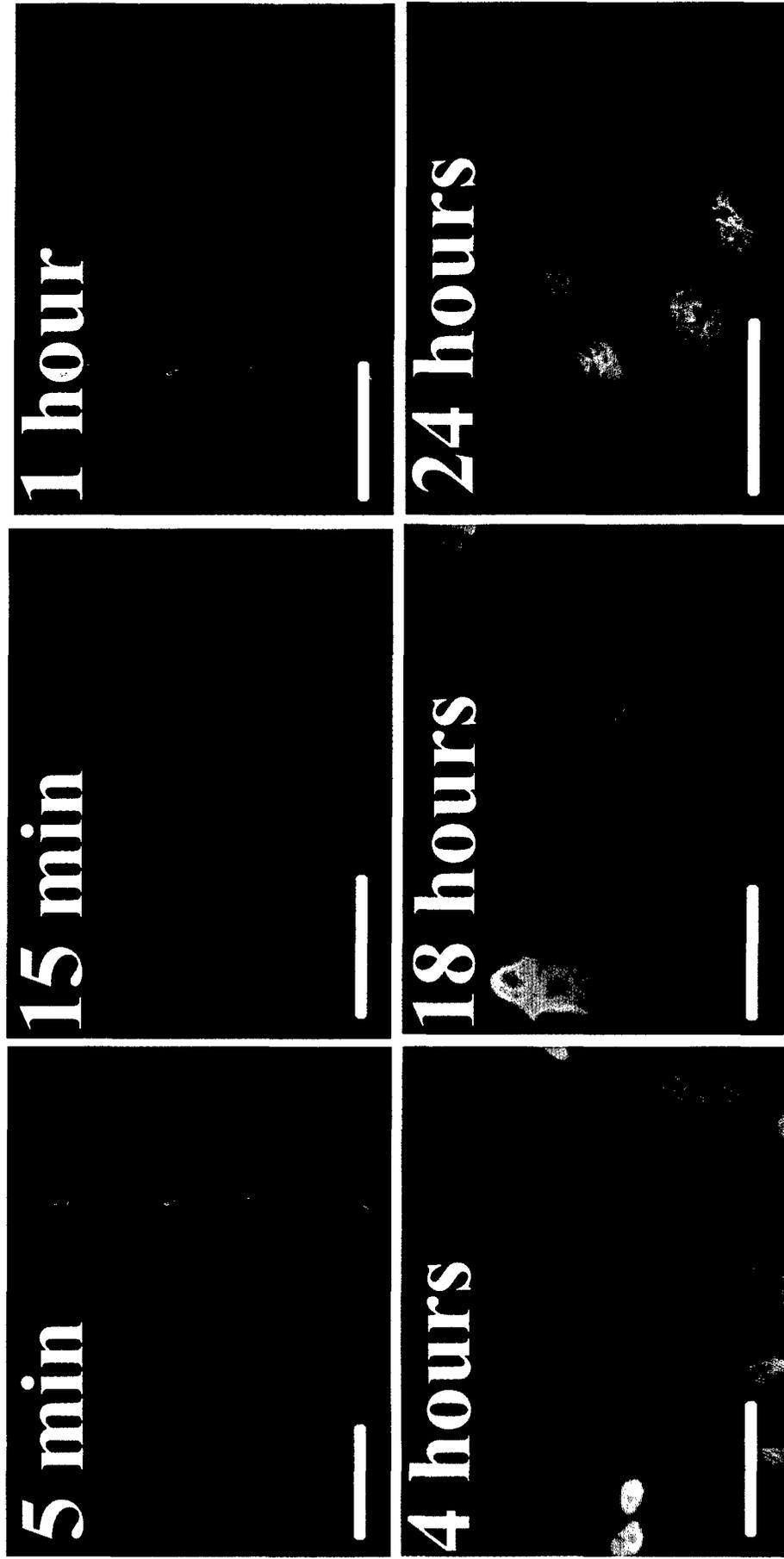4 hours

18 hours

24 hours
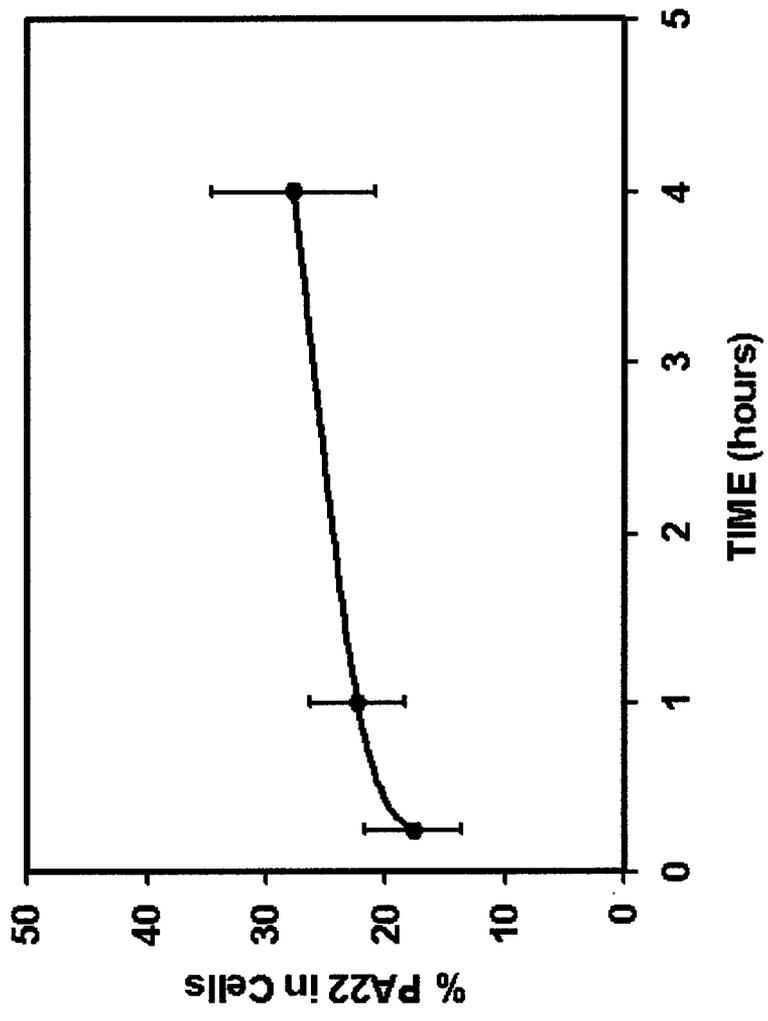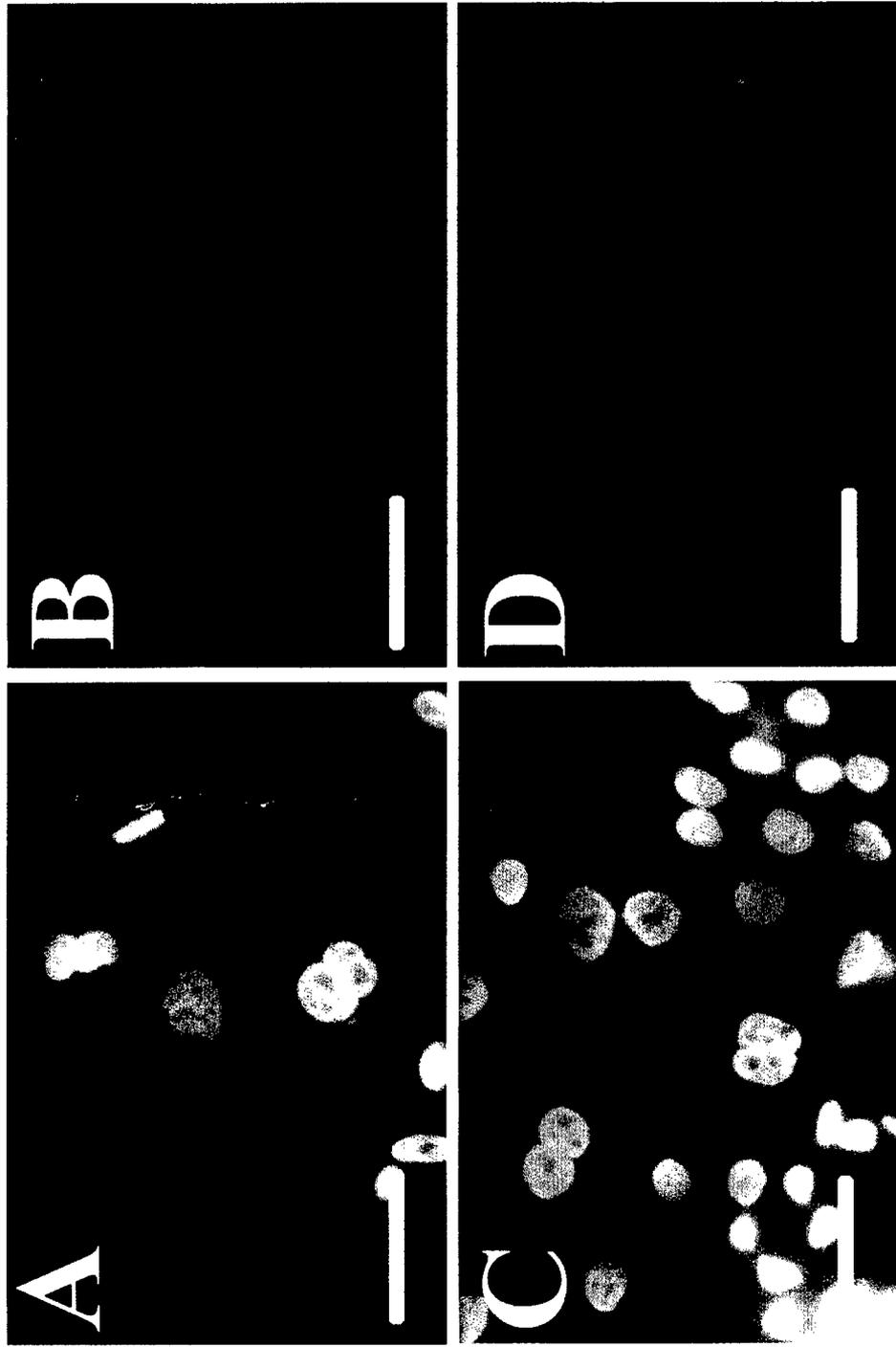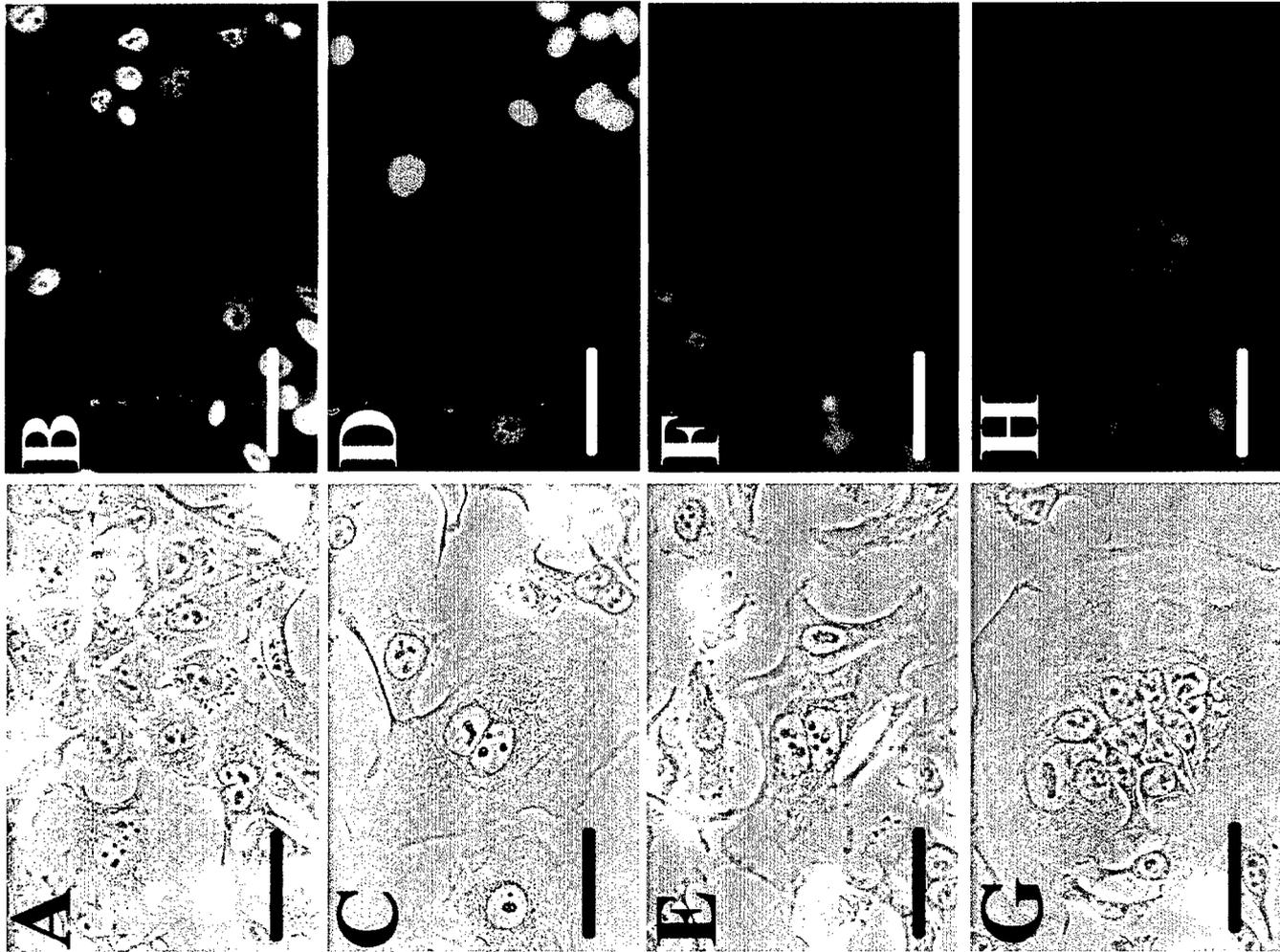
Figure 6

Figure 7

Figure 33
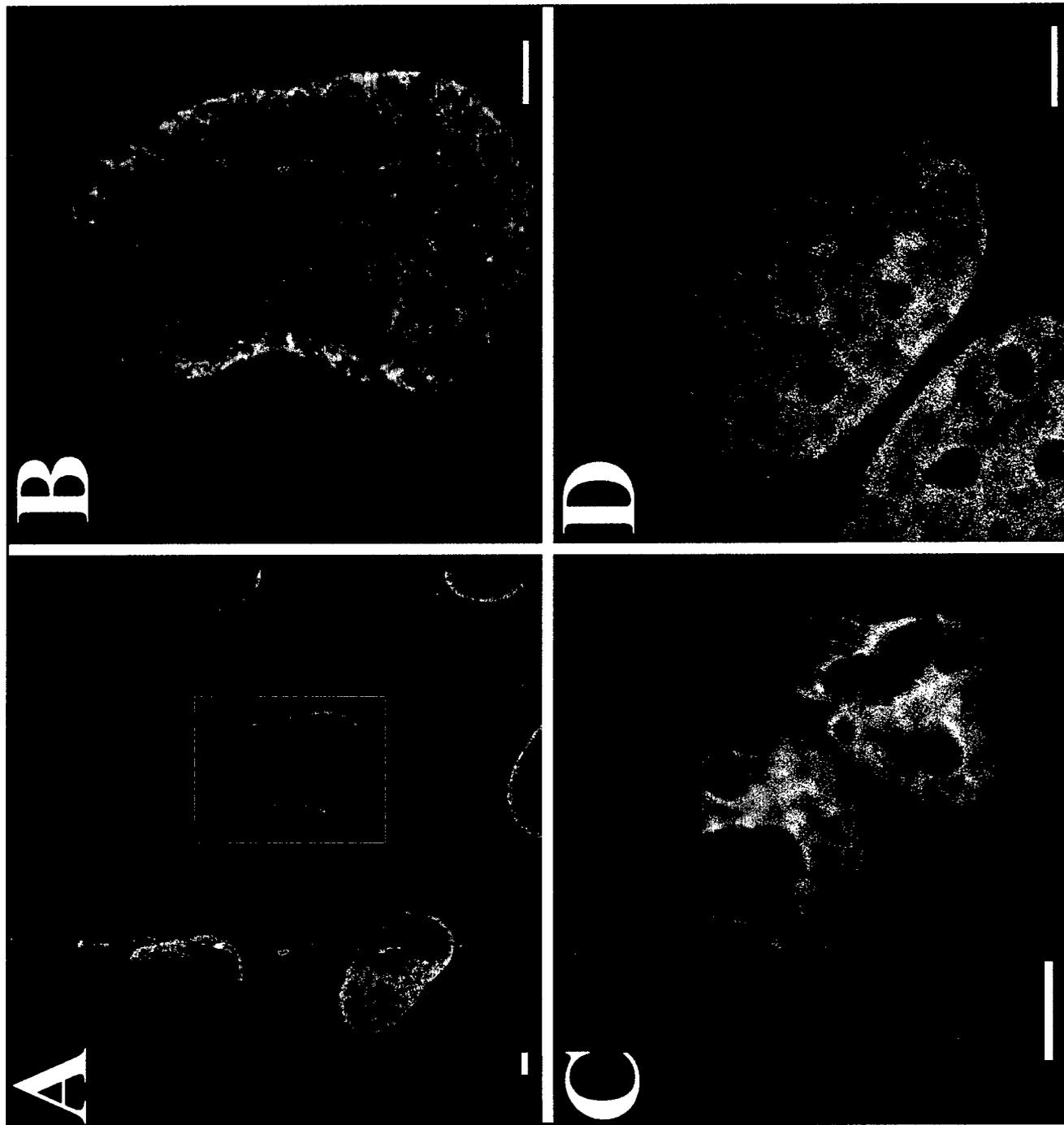
Appendix C:

Copy of award letter to Dr. Terry Beerman from NIH.

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

TERRY A BEERMAN   Date : 15-AUG-00
ROSWELL PARK CANCER INSTITUTE
DEPT OF PHARMACOLOGY & THERAPEUT
ELM & CARLTON STS
BUFFALO, NY 14263-000'

RE: iКiCA^80939-ui A1

DEAR DR. BEERMAN:

Congratulations! On behalf of the National Cancer Institute (NCI), I am pleased to
inform you that your application was rated in the peer review process as having
significant and substantial scientific merit. The purpose of this letter is to provide you
with early notification that the NCI has selected your grant application, as referenced
above, for funding in FY 2001. Under separate cover, NCI program staff will send
you a copy of the summary statement prepared by the Scientific Review Administrator
of the Initial Review Group that evaluated your application.

It is our goal to expedite the processing of your application. At this time we cannot
commit to either an exact dollar amount or an actual start date of the award. Both will
depend upon further NCI staff review of the application and summary statement concerns
as well as other issues such as Other Support and applicable program reductions directed
by NCI funding plan decisions.

If you have any general questions regarding this expedited award process, please call
Ms. Barbara Lamb, Manager, Awards, Records and Control Center, on 301-496-7756.

We wish you every success with your research.

Sincerely .

Leo F Buscher Jr

Leo F. Buscher Jr.
Chief Grants Management Officer
National Cancer Institute

cc : Business Official

DIRECTOR OF OPERSTIONS
ROSWELL PARK CANCER INSTITUTE
ROSWELL PARK DIVISION
ELM & CARLTON STS
BUFFALO, NY 14263

Appendix D:

Copy of platform presentation abstract given at Pharmaceutical Sciences Day at the State University of New York at Buffalo, March 2000.

UB

March 6, 2000

Stephanie Leslie
Department of Molecular Pharmacology and Cancer Therapeutics
Roswell Park Cancer Institute

Dear Registrant:

This will confirm that we have received your registration fee to attend Pharmaceutical Sciences
Day on Friday, March 17, 2000 in the Center for Tomorrow on U.B.'s North Campus.

We have received your abstract and have scheduled you to present as follows:

       TYPE: Oral Presentation

       TITLE: *Evaluation of the Polyamide PA2, a Novel DNA Binding Ligand in Cell-free
and Whole-cell Studies*

       TIME: 11:00 a.m.

The oral presentations have been scheduled at fifteen minute intervals. **This time schedule
allows for a ten-minute talk**, and up to five minutes of questions for each presenter. **We ask
your cooperation in strictly adhering to these guidelines.** There will be a slide projector, an
in-focus projection system with an IBM compatible PC (used for Powerpoint presentations),
and an overhead projector available for your use.

Coffee and registration will occur from 8:30-9:00 a.m. Opening remarks will begin at 9:00
a.m. We look forward to your participation. If you have any questions, please call 829-2800.

Sincerely,

Richard Rabin

Richard A. Rabin, Ph.D.
Director of Graduate Studies

PHARMACEUTICAL SCIENCES DAY
Graduate Student Symposium

University at Buffalo
School of Medicine and Biomedical Sciences
Center for Tomorrow
Friday, March 17, 2000

Abstract Reproduction Form

EVALUATION OF THE POLYAMIDE PA2, A NOVEL DNA BINDING LIGAND IN CELL-FREE AND WHOLE-CELL STUDIES.

Stephanie Leslie*, Dr. Roland Bulri, Dr. Peter Dervan and Dr. Terry Beerman
Laboratory of Dr. Beerman, Roswell Park Cancer Institute, University at Buffalo

Polyamides (PA) are a novel group of DNA binding agents, which can be targeted, to specific DNA sequences. These agents are synthesized in a linear array of pyrrole and imidazole amino acids, which form a hairpin structure that binds in the DNA minor groove. PA2 and a conventional minor groove binding drug, Hoechst 33342, were evaluated as TF inhibitors in cell-free and whole cell studies. PA2 was designed to bind to the HER2/neu promoter region, which is regulated by ESX, a new member of the ETS transcription factor family. By preventing ESX binding to the promoter, the polyamide should decrease ESX regulated HER2/neu expression. PA2 was first evaluated in mobility shift assays for its ability to prevent ESX binding to the HER2/neu promoter. PA2 prevented complex formation by 50% at concentrations as low as 10nM while the conventional minor groove binding drug, Hoechst 33342 required 1.4µM. PA2 was then evaluated in cell-free transcription assays to determine if it blocks HER2/neu transcript formation. To inhibit HER2/neu transcript formation by 50%, 4.5µM PA2 was required while Hoechst 33342 inhibited transcript formation at 3µM. These agents were next evaluated for their ability to inhibit HER2/neu expression in SKBR3 cells, which over expresses HER2/neu. Northern blot analysis was performed on RNA harvested from cells treated for 24 hours with either agent. Hoechst 33342 inhibited both HER2/neu and a housekeeping gene, GAPDH, message by 50% at a concentration of 9µM. However, PA2 at concentrations up to 10µM did not inhibit HER2/neu or GAPDH gene expression and even after 72 hours of exposure, PA2 was still incapable of inhibiting mRNA production. Subsequently, PA2 was fluorescently labeled to assess its ability to enter cells and to determine where it may localize. SKBR3 cells treated with the fluorescently labeled PA22 for 24 hours were visualized using fluorescent microscopy. The study showed PA22 entered the cells and localized to the nucleus while avoiding the nucleoli. Control studies with the fluorscent label (BODIPY) alone indicated that the fluorescence seen in the nucleus is from PA22 while BODIPY alone localized to the cytoplasm. A time course treatment with PA22 indicated that uptake was rapid (within five minutes) accompanied by diffuse staining in the cytoplasm while by four hours PA22 is only in the nucleus. Competition studies with PA2, the parent compound of PA22, were performed to evaluate whether PA2 could reduce PA22 uptake. PA2 at 10-20 times the concentration of PA22 strongly decreased fluorescence suggesting that PA2 and PA22 are taken up similarly. Further assessment of the pharmacological properties of polyamides is continuing to better understand their activity in cells.

Instructions:
1. TITLE IN CAPITAL LETTTERS
2. Full names of Authors underlined. Denote presenter with asterisk
3. Laboratory and University at which the research was performed
4. Abstract of the work: not to extend outside of the template

Please return this form by February 22, 2000 to:

Richard A. Rabin, Ph.D. (rarabin@buffalo.edu)
Department of Pharmacology and Toxicology
102 Farber Hall, University at Buffalo
Buffalo, NY 14214-3000

Please indicate your preference for presentation: ☒ Talk ☐ Poster ☐ Either
Indicate Status: ☒ Student ☐ Postdoc

Appendix E:

Copy of poster abstract presented at Sigma Xi Poster Competition at the State
University of New York at Buffalo, April 2000.

SIGMA XI
THE SCIENTIFIC RESEARCH SOCIETY

Sigma Xi Student Research Competition

April 25, 2000

(Abstract Deadline: April 1, 2000)

Name Stephanie Leslie

Address: 19 Melberry Trail, Upper Orchard Park, NY 14127

Department: Molecular Pharmacology & Cancer Therapeutics

Affiliation:
Roswell Park Cancer Institute

Advisor:
Dr. Terry Beerman

Phone:(work) 845-3233 **(home)** 827-9780 **(email)**
sleslie@sc3101.med.buffalo.edu

Title of Poster: CELL-FREE AND WHOLE-CELL EVALUATION OF POLYAMIDE PA2, A NOVEL DNA BINDING LIGAND

Student Status: Circle One – Graduate or Undergraduate

Abstract:

Polyamides (PA) are a novel group of DNA binding agents that recognize and bind to specific DNA sequences. Base recognition and binding occurs through a linear array of pyrrole and imidazole amino acids, which form a hairpin structure that binds in the DNA minor groove. The PA (PA2) was designed to bind to the HER2/neu promoter region at an ETS transcription factor (ESX) binding site. In mobility shift assays, PA2 was a significantly more potent inhibitor of ESX/DNA complex formation than was the conventional minor groove-binding agent, Hoechst 33342. In contrast, as inhibitors of cell-free HER2/neu transcription, both agents were nearly equally inhibitory. In whole-cell assays employing the breast cancer cell line SKBR-3, Hoechst 33342 inhibited HER2/neu mRNA production by 50% at 9µM (by 24 hours) and inhibited cell growth by 50% at 7µM (by 72 hours). In contrast, PA2 showed neither inhibition of mRNA production nor cytotoxicity at concentrations up to 10µM. To address whether a lack of PA2 uptake explained the inactivity in whole cells, drug entry was monitored using a PA2 fluorescently labeled with BODIPY. Cells were treated for 24 hours with the fluorescent agent PA22 (PA2 + BODIPY = PA22) and visualized by fluorescent microscopy. These experiments revealed that PA22 was not only taken-up but localized to the nucleus while avoiding the nucleoli. Control studies with the fluorescent label (BODIPY) alone verified that the fluorescence seen in the nucleus was due to the PA, since BODIPY alone localized to the cytoplasm. Nuclear accumulation of PA22 appeared within 15 minutes after treatment and increased for up to 24 hours. Uptake studies performed at 37°C vs. 4°C indicated that PA22 uptake is an active cellular process. The presence of PA2 at a 10:1 ratio to PA22 effectively competed with PA22 for cellular uptake, suggesting that both agents utilize the same transport mechanism. A 4 hour pre-treatment of cells with PA22 followed by a 6 hour chase with 10 fold excess of PA2 showed a detectable decrease in fluorescence, suggesting that both PAs 2 and 22 bind to the same site. Additionally, chase studies using 10 fold excess of Hoechst 33342 also effectively competed PA22 binding in the nucleus suggesting PA22, like Hoechst 33342, binds to DNA. Further assessment of the pharmacological properties of PAs will continue in an effort to enhance their activity in whole cells.

Appendix F:

Copy of poster abstract presented at Department of Defense Era of Hope Meeting in Atlanta, June 2000.

EVALUATION OF DNA BINDING AGENTS FOR THEIR ABILITY TO INHIBIT ESX BINDING AND FUNCTION ON THE HER2/neu PROMOTER.

Stephanie Leslie[1], Dr. Roland Burli[2], Dr. Peter Dervan[2], Dr. Gary Scott[3], Dr. Chris Benz[3], and Dr. Terry Beerman[1]

[1]Roswell Park Cancer Institute, Buffalo, NY 14263; [2]California Institute of Technology, Pasadena, CA 91125; [3]University of California at San Francisco, San Francisco, CA 94143

sleslie@sc3101.med.buffalo.edu

DNA binding agents were evaluated for their ability to disrupt transcription factor (TF) binding to promoter DNA and down regulate cancer associated gene expression. The target gene is HER2/neu, which is overexpressed in ~30% of breast cancers. Recently, a new member of the ETS family of TFs, ESX, has been identified as a possible regulatory TF of the HER2/neu gene. Drugs with different sequence binding (sequence preference vs. sequence specific) were evaluated. Results in mobility shift assays indicate that sequence specific binding agents (polyamides) inhibit TF/DNA complexes better than sequence preference agents by an order of magnitude. In cell-free transcription assays there is less difference between the two types of binding agents.

In whole cell experiments, some sequence preference agents are very effective at inhibiting gene expression while first generation polyamides show limited ability to diminish gene expression. Subsequently, sequence specific polyamides were further evaluated to assess if their diminished whole cell activity was due to the agent's inability to enter the cell. SKBR3 cells were treated with a fluorescently labeled polyamide and visualized by fluorescent microscopy. Studies showed that the polyamides appear to localize to the nucleus (Figure 1). Control experiments indicate the polyamide is in the nucleus and not the fluorescent tag alone. Additionally, competition experiments indicate the un-labeled compound diminished accumulation of fluorescently labeled polyamide.

Figure 1: SKBR3 cells treated with fluorescently labeled polyamide. A: phase. B: fluorescent.

The goal of this research is to find properties of DNA binding agents which could be applied to the development of new, more effective and potent drugs to decrease cancer associated gene expression.
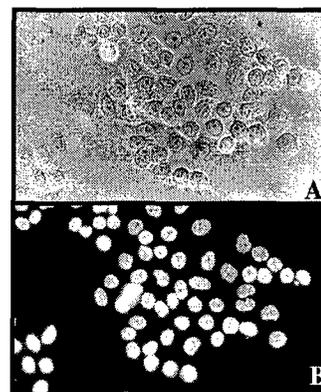
Appendix G:

Copy of Dr. Ronald Berezney's letter offering post-doctoral position in his laboratory at the State University of New York at Buffalo, Department of Biological Sciences.

June 14, 2,000

Ms. Stephanie Leslie
Department of Pharmacology and Therapeutics, GCDC233
Roswell Park Cancer Institute
Buffalo, NY 14263

Dear Stephanie,

I am delighted to offer you a position in my laboratory as a postdoctoral research associate beginning on **June 1, 2,001**. The initial appointment will be for one year at a salary of **$28,000 per annum** and is renewable for additional years as deemed appropriate with incremental yearly raises of $ 2,000. In addition to the salary you will receive a comprehensive package of fringe benefits such as medical, dental and prescription coverage.

We have discussed generally, potential projects for you to begin your research here. This would likely involve combining your current research experience on drug interactions with nuclear target sites and studies of nuclear architecture and genomic function. If you decide to accept this offer, we will initiate a series of conversations in the near future to begin to discuss research projects and plan specific directions for you to pursue. As a first step in that direction I will send you a copy of my recently funded NIH grant application. I would expect you to pursue at least two major projects with one based on fundamental aspects of nuclear organization and function and the second directly related to drug binding/targeting in relation to nuclear architecture/function. You are also requested to submit applications for post-doctoral fellowships in the area of drug interactions with nuclear architecture and function. Support on this post-doctoral position, however, is not dependent on successful funding.

Stephanie, with your previous experience, knowledge and success in research in the area of DNA binding drugs and nuclear targeting, I am confident that you will make important contributions to our laboratory efforts. At the same time I believe that you would have the opportunity to develop your scientific career in a new and exciting area of research that will have profound implications for our understanding of the dynamics of nuclear organization/function in relationship to the mechanisms of action of DNA binding drugs in intact human cells.

As an additional feature, our laboratory is in the forefront of developing computer imaging algorithms and programs for the multi-dimensional analysis of genomic organization and functions of DNA replication and transcription. These tools as well as further developments in this area will be made completely available to you in your research. Indeed your research is likely to contribute to their future development.

Please let me know as soon as possible whether or not you wish to accept this offer and do not hesitate to contact me directly if I could provide additional information that would be helpful for you in coming to a decision. I look forward to the possibility of you joining our laboratory.

Sincerely,

Ronald Berezney, Ph.D.
Professor of Biological Sciences

Appendix H:

Copy of Admissions letter from the Marine Biological Laboratory at Woods Hole regarding a position in their "Optical Microscopy and Imaging in Biomedical Sciences" course.

August 18, 2000

Stephanie J. Leslie
Roswell Park Cancer Institute
Department of Pharmacology & Therapeutics
GCDC Room 233
Buffalo, NY 14263

Dear Ms. Leslie,

The Committee on Admissions for the 2000 MBL course **Optical Microscopy and Imaging in the Biomedical Sciences** has made its decisions and I write to inform you of them. You have been selected as an alternate and your name is on the course waiting list. Your application was one of a very large number of outstanding ones received this year, making the selection process extremely difficult.

Alternates selected for MBL courses are not ranked, per se, so it is difficult to predict the likelihood of any individual being offered a position in the course. If an admitted student withdraws from the course, the admissions committee designates an appropriate replacement from the waiting list for that particular student. We strive to maintain a diverse enrollment in each course.

Although it is uncommon, *should an opening occur for you, we will notify you immediately.* This may occur at anytime (admitted students who cancel usually have some unexpected circumstance arise.) We ask waiting-list students to inform us should they decide to make plans which would prevent them from attending the MBL course if offered a position. At the same time, we want to recommend that you do not preclude yourself from any other opportunities in hopes of being offered a place in this year's **Optical Microscopy** course.

I hope this will help answer your questions concerning your status on the course waiting list. I would also like to congratulate you on having been selected as such; this year we received a very large number of applications from extremely well-qualified students around the world—you are among the small group who simply could not be turned down. It is really quite a distinction.

Please let me know if I may be of any further assistance.

Sincerely,

E.A. Dawidowicz
Director of Education

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   26 Nov 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA   22060-6218

SUBJECT: Request Change in Distribution Statement

1.   The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for this Command.   Request the limited
distribution statement for the enclosed accession numbers be
changed to "Approved for public release; distribution unlimited."
These reports should be released to the National Technical
Information Service.

2.   Point of contact for this request is Ms. Kristin Morrow at
DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl   PHYLIS M. RINEHART
                                        Deputy Chief of Staff for
                                        Information Management

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