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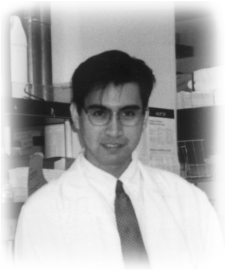

Scott Avecilla glories in bringing fields of thought and research together. After integrating data from both chemistry and biology labs here at UCI, Avecilla went on to the National Institute of Health-funded Medical Scientist Training Program where he found himself "working at the increasingly vital interface between science and medicine." The findings presented here detail research aimed at achieving the ability to regulate gene expression; if that is accomplished, Avecilla explains, "certain cancers and viral infections can be turned from life threatening acute conditions to chronic, manageable disorders."

Key Terms

- Cellular Uptake
- Confocal Microscopy
- DNA Recognition
- Gene Therapy
- Nuclear Localization
- Polyamide

Evaluation of Cellular Uptake of Synthetic DNA Binding Ligands

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Abstract

Since the historic discovery of genes, science has sought to achieve the unthinkable: to alter the genetic code to cure human disease. With the decoding of the blueprints for the human species, the genome, tools to utilize this fantastic information need to be developed. Such tools may come in the form of polyamides, chemicals that have shown the remarkable ability to bind specific genes. These amazing qualities suggest that polyamides could turn genes on or off at will, with the goal of shutting down disease causing genes. With the recent demonstration that polyamides are able to target and inhibit specific genes *in vivo*, it is vital to characterize their cellular uptake and localization. Using microscopy, polyamides were found to be cell permeable, but the bulk of the molecules gather around rather than in the nucleus. Continuous exposure to polyamides during several rounds of cell division appears to be insufficient to allow polyamides to accumulate in the nucleus *in vivo*. Additional evidence for *in vivo* nuclear exclusion is the striking contrast between intracellular localization of polyamide in live and dead cells, as the nuclear fluorescence is dramatically enhanced upon standard fixation. Thus, despite the small size of polyamides and high DNA affinity, their cellular permeability does not necessarily include nuclear localization/retention at concentrations sufficient to detect by fluorescence microscopy.

Faculty Mentor

When I was in graduate school, my adviser told me that when you obtained an expected result you carried out an exercise, but if you obtained an unexpected result, you performed an experiment. Scott's project began as control "experiments" to show that a novel small molecule (a polyamide) was concentrated where the genes reside, in the nuclei of cells. Similar polyamides had previously been shown to dramatically inhibit the AIDS virus, indicating they had enormous potential as therapeutic agents for many diseases. Scott's "control" exercise became an experiment and he proceeded to prove that the molecules are bound to cytoplasmic structures called mitochondria in living cells but that fixation of cells prior to microscopy resulted in massive relocation of the molecules to nuclei. These results could explain the spectacular gene-specific effects of polyamides despite their general affinity for nuclear genes. Furthermore, understanding the basis of mitochondrial binding can lead to design of polyamides that do enter the nucleus and, therefore can be used therapeutically at much lower concentrations precluding possibly deleterious side effects.

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School of Biological Sciences

Introduction

A major thrust of twenty-first century medicine will revolve around the utilization and manipulation of the human genome, the mapping of which is nearing its historic completion. Drugs that specifically target DNA sequences in order to control gene expression have the potential to actually cure disease rather than simply treat the symptoms. Because a wide variety of diseases like HIV and cancer have genetic origins, these and many others may be permanently treated with this chemical gene therapy. Various synthetic analogues of the naturally-occurring DNA binding ligand Distamycin A, have recently been developed with the capability to sequence-specifically recognize double stranded DNA by binding in the minor groove (Dervan and Burli, 1999; Reddy et al., 1999). Among these analogues, polyamides have received special attention due to their high affinity, sequence discrimination, and relatively small size.

Polyamides are a class of peptide-like oligomers consisting of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im), amide-linked monomeric units that interact with the minor groove of DNA via van der Waals forces and hydrogen bonding (Figure 1). Specific sequences of DNA can be targeted by synthesizing a polyamide with the monomer sequence following the predetermined pairing rules discovered by Dervan *et al.* (1997). Briefly, the Im-Py pair targets the GC base pair, the Py-Im pair binds CG targets while the Py-Py pair degenerately binds to either AT or TA base pairs. Strong DNA affinity, high sequence discrimination, and relatively small sizes make polyamides excellent candidates for site-specific DNA modification. Because the affinity of polyamides for DNA is on the same order of magnitude as endogenous transcription factors, they have the potential to modulate gene expression by blocking the activation of transcription or via transcription factor recruitment (Gottesfeld et al., 1997; Mapp et al., 2000). Recently, the *in vivo* HIV-1 viral replication inhibition was demonstrated (Dickinson et al., 1998).

Due to their highly specific effects on gene expression, it was assumed that polyamides were not only cell permeable but also localized in the nucleus. However, the characteristics of cellular uptake and subcellular distribution of polyamides have not been shown explicitly. In order to advance this exciting technology to the next level as a potential pharmaceutical or reagent in functional genomics, it is vital to characterize their cellular uptake.

Polyamides are aromatically conjugated in nature, thus they possess an intrinsic fluorescence when excited by ultraviolet radiation (~ 345 nm) that is slightly enhanced upon binding to DNA (Baliga and Crothers, 2000). By harnessing this phenomenon, the cellular uptake and localization of native polyamides could be monitored without the need to synthesize fluorescent or radioactive derivatives. Spectrofluorometry was used to characterize the natural fluorescent properties of polyamides while the cellular uptake and localization were visualized by laser-scanning confocal microscopy. Several polyamides with a range of affinities and DNA targets were tested to ascertain the broad *in vivo* characteristics of cellular uptake in mammalian cells (Figure 1).

Materials and Methods

Synthesis of Polyamides

Three representative polyamides were synthesized for study: bis-distamycin (Ac-Py-Py-Py-γ-Py-Py-Py-β-Dp), S32 (Im-Im-Im-Im-γ-Py-Py-Py-Py-β-Dp), and HA [High Affinity] (ImPyPyPy-γ-PyPyPyPy-β-Dp) [Py = *N*-methylpyrrole, Im = *N*-methylimidazole, γ = γ-aminobutyric acid, β = beta-alanine, Dp = dimethylaminopropylamide, and Ac = acetyl]. These polyamides were chosen due to the disparity in their sequence targets and affinities, 5'-$(A/T)_5$-3', 5'-(A/T)$(G)_4$(A/T)-3', and 5'-(A/T) G $(A/T)_4$-3' respectively (Van Dyke et al., 1982; Swalley et al., 1996; Trauger et al., 1996). Bis-distamycin, S32, and HA were synthesized as previously described (Baird and Dervan, 1996). Purification of the crude solid phase cleavage product was by reversed phase high performance liquid chromatography (RP-HPLC) using a Waters DeltaPak 25mm x 100mm C18 column, 0.1% (wt/v) trifluoroacetic acid (TFA) with acetonitrile as eluent at 5 ml/min flow rate. For S32, it was found that addition of 20% TFA (approx. 2 ml) to the crude product was necessary prior to injection to fully remove the aminolysis reagent, dimethylaminopropylamine. Polyamides were found to be greater than 95% pure by analytical RP-HPLC (Rainin Microsorb MV, 100A, 4.6 mm x 250 mm) and characterized by UV spectroscopy, ^{1}H-NMR, and matrix assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF-MS). Concentrations were determined spectrophotometrically for polyamides, using the approximate extinction coefficient ε = 8000 per ring. Stock solutions of approximately 100 μM in water were made and kept at –20 °C to avoid hydrolysis and decomposition. Distamycin A, Hoechst 33258, and Biliverdin were obtained from Sigma Chemical, Molecular Probes, and ICN Pharmaceuticals, respectively, and used without further purification. Stock solutions of 100 μM (Distamycin A) and 1 mM (Hoescht 33258) in water were prepared and stored as above. A 5 mM stock of Biliverdin in DMSO was diluted to 100 μM in water prior to use.

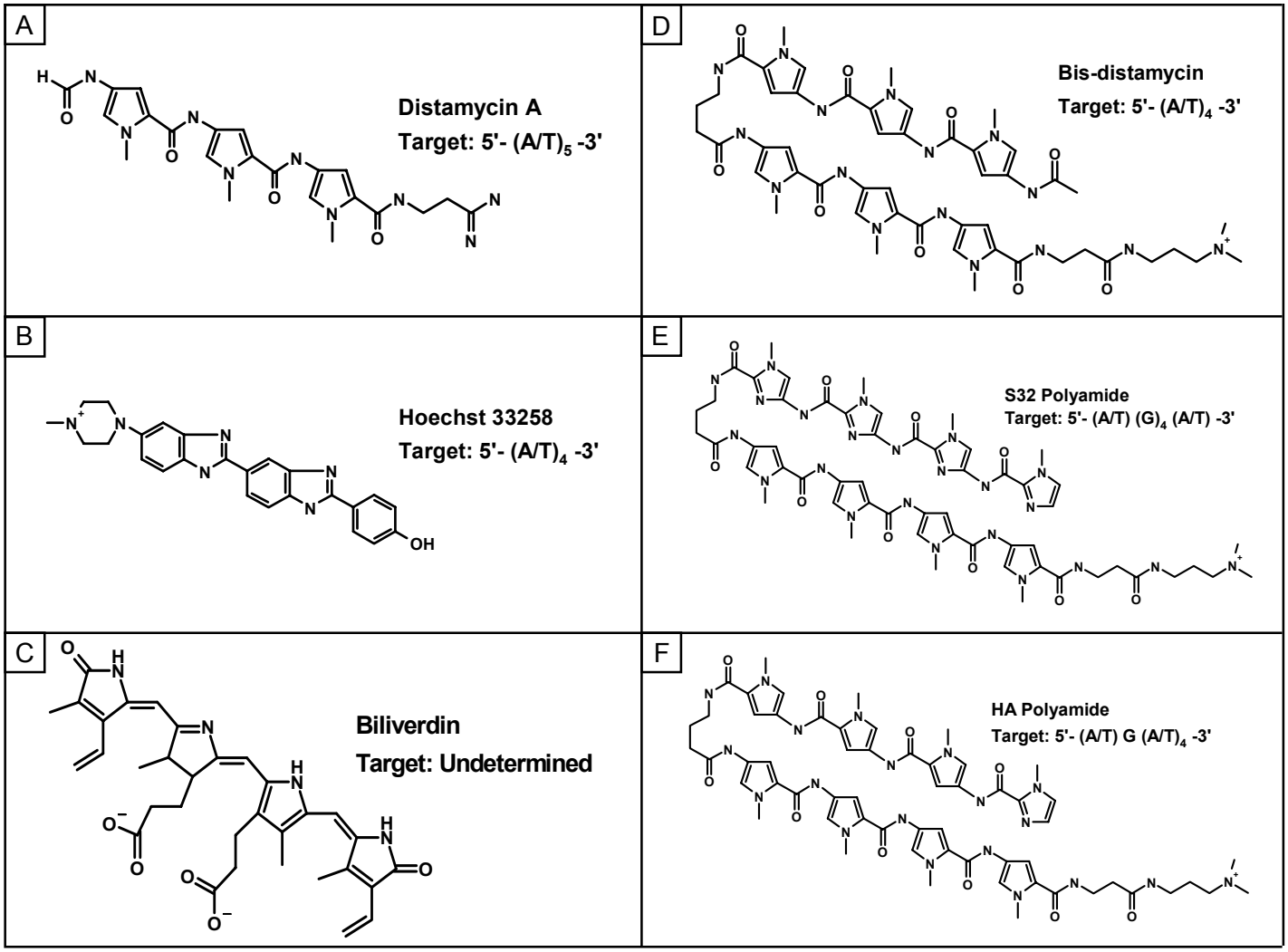


Figure 1

Chemical structures of DNA minor-groove binding drugs. (**A**) Distamycin A, (**B**) Hoechst 33258, (**C**) Biliverdin, (**D**) Bis-distamycin PyPyPy-γ-PyPyPy-β-Dp, (**E**) S32 Polyamide ImImImIm-γ-PyPyPyPy-β-Dp, and (**F**) HA Polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp. Imidazole(Im), pyrrole(Py), γ-aminobutyric acid(γ), β-alanine(β), and dimethylaminopropylamide(Dp).

Spectrofluorometric Characterization of Polyamide

The polyamides were dissolved in sterile phosphate buffered saline (PBS) to final concentrations of 0.1 μM, 1 μM, and 10 μM for the studies. Maximal absorbance was obtained with an SLM/Aminco 8100 steady-state fluorescence spectrometer. The maximally absorbed wavelength was used as the initial excitation wavelength (~330 nm). Subsequently, both the excitation and emission spectra for the polyamides were measured. Additionally, sonicated calf thymus DNA was added (from 1 μg to 37 μg or 1.6 μM - 44 μM bp as indicated) to a 1 μM polyamide solution to monitor the effect of DNA binding on the fluorescent characteristics of polyamides.

Cell Culture

For the uptake studies, all cells were grown at 37 °C in a humidified incubation chamber with 7.5% CO_2. The monkey epithelial cell line Vero, the mouse fibroblast L-929 cells and human breast cancer MCF-7 cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES buffer. The rat kangaroo epithelia line of PtK2 cells was cultured in modified Eagle medium (MEM) with 10% FCS and 10 mM HEPES. Typically, to prepare the cells for the uptake experiments, an 80-90% confluent culture was trypsinized and gently shaken for 5-10 min to detach the cells from the culture flask. The trypsin was neutralized by an equal addition of culture media (containing protease inhibitor). The resulting single cell suspension was plated at the

appropriate density on modified petri dishes with cover glass slip bottoms and grown for 24 hr. The plated cells were then washed twice with PBS and the appropriate media containing the polyamide, Distamycin A, Hoechst 33258, or Biliverdin was added at various concentrations (0.1 μM, 1 μM, or 10 μM). The cultures were incubated with the drug for various time periods ranging from 1 min to 48 hr. The drug exposure was limited by washing the cultures twice with PBS and replacing the polyamide with regular media. Note that the Biliverdin was introduced in acidified media (pH ~ 6.0) to increase its cell permeability. For the chromosomal and fixed nuclei binding studies, mitotic chromosome spreads of L-929 were prepared as described (Wray, 1973) and intact Vero nuclei were obtained as described (Jasinskas and Hamkalo, 1999). The chromosomes and/or nuclei were incubated in 1 μM solutions of drug for 30 min and then observed under confocal microscopy. Cell fixation was performed by exposing PBS washed cultures (3X with cold PBS) to ice cold methanol (2 ml) for 5 min. After methanol treatment, the methanol was aspirated and the samples were allowed to dry for 30 sec. The samples were further washed in PBS to prevent sample dehydration. Additionally, cell cultures were similarly fixed with paraformaldehyde. Labeling experiments for fixed cells were identical to procedures described above; note that a reduced incubation time of 10 min was sufficient for labeling.

Uptake Experiments

A BioRad MRC 1024UV Laser Scanning Confocal Microscope (UV-LSCM) equipped with an Argon Ion water-cooled UV laser with excitation wavelengths of 351 nm and 363 nm, using the UBHS filter cube in conjunction with an E2 filter, was used. Transmitted images were collected using a photo multiplier tube and collected in one color. Cells were focused with a Nikon DiaPhot inverted microscope, equipped with a Fluor NA 0.8-1.3 adjustable objective, at a magnification of 40X. Images were collected by averaging four scans using the Kalman filter. Immediately prior to microscopy, the culture media in the samples was replaced with PBS. In all experiments, Hoechst 33258 was used as a positive nuclear counterstain. In the fixed-cell experiments, Rhodamine 123 (10 μM) was used as an agent to define cellular borders. For time-lapse microscopy, cells were monitored at 1 min intervals to observe *in vivo* intracellular polyamide distribution. The cell cultures were maintained at 37 °C using a heated microscope stage.

Discussion and Results

Fluorescence Characteristics of Polyamides

The first parameter to establish in the uptake study was whether and to what extent polyamides fluoresce. The fluorescence of polyamides was determined by spectrofluorometric analysis of two representatives of the polyamide class. Using spectrofluorometry (Figure 2) it was found that polyamides emit light in the visible spectrum (413 nm and 440 nm) when excited by ultra violet light (341 nm and 347 nm respectively). This spectral profile closely matches that of the known minor-groove binding dye, Hoechst 33258. Thus, the fluorescence was harnessed to monitor cellular uptake, which is advantageous for two reasons. First, the drug uptake is monitored directly, avoiding fluorescently labeled conjugates that could have altered uptake profiles. Second, the cells can be imaged live without the need for fixing, which reduces the chances of introducing fixation artifacts. One major concern of using the fluorescent properties of polyamide to track cellular uptake is the phenomenon of fluorescence quenching upon binding to double-stranded DNA. In order to monitor the effect of DNA binding on polyamide's fluorescence, the emission spectrum of a polyamide-DNA solution was measured while gradually increasing the concentration of DNA. Instead of quenching, a modest increase in polyamide fluorescence upon binding to DNA was found, in agreement with the recent findings of Baliga and Crothers (2000).

Cellular Uptake Characterization of Polyamides

Dosage

While there have been several recent publications describing the gene modulation activity of polyamides (Gottesfeld et al., 1997; Dickinson et al., 1998), there have been no published accounts that directly assay the cellular uptake and localization of this promising class of compounds. The next parameter to be established was the concentration of polyamide necessary for visualization by confocal microscopy. Previous studies indicated that a dosage of 1 μM polyamide in the culture media was effective for achieving gene-specific modulation (Dickinson et al., 1998). Therefore, a range of concentrations from 0.1-10 μM was tested in various cell lines to determine the minimum amount of drug required for labeling, and 1 μM was found to be optimal (data not shown). Because 1 μM polyamide was used in the above gene modulation experiments, the cellular uptake and localization experiments at the therapeutic concentration should be relevant to biological results.

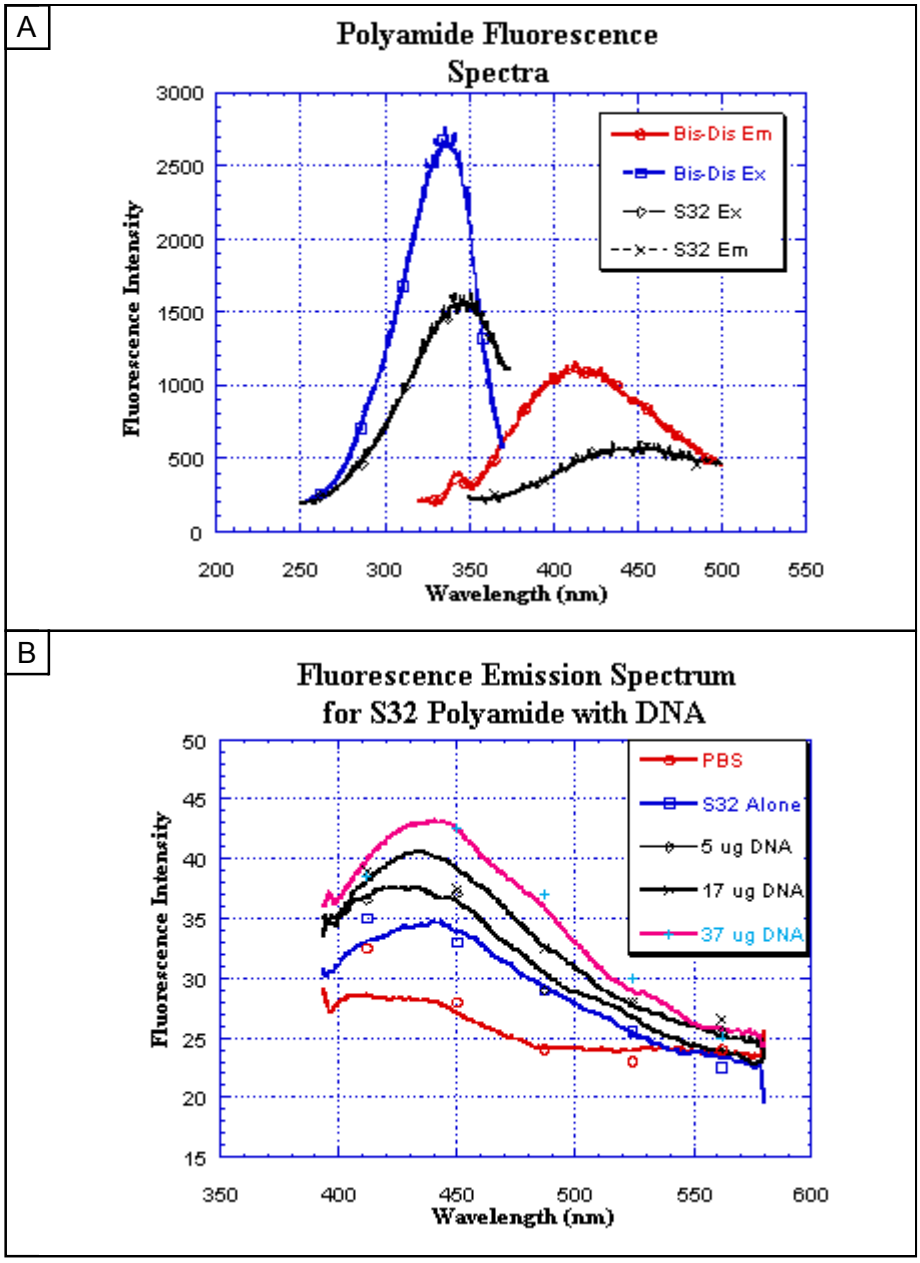


Figure 2

Fluorescence spectra of Polyamide. (**A**) The excitation and emission spectra of Bis-distamycin and S32 polyamides. (**B**) The emission spectrum of S32 with increasing amounts of ds DNA.

Kinetics

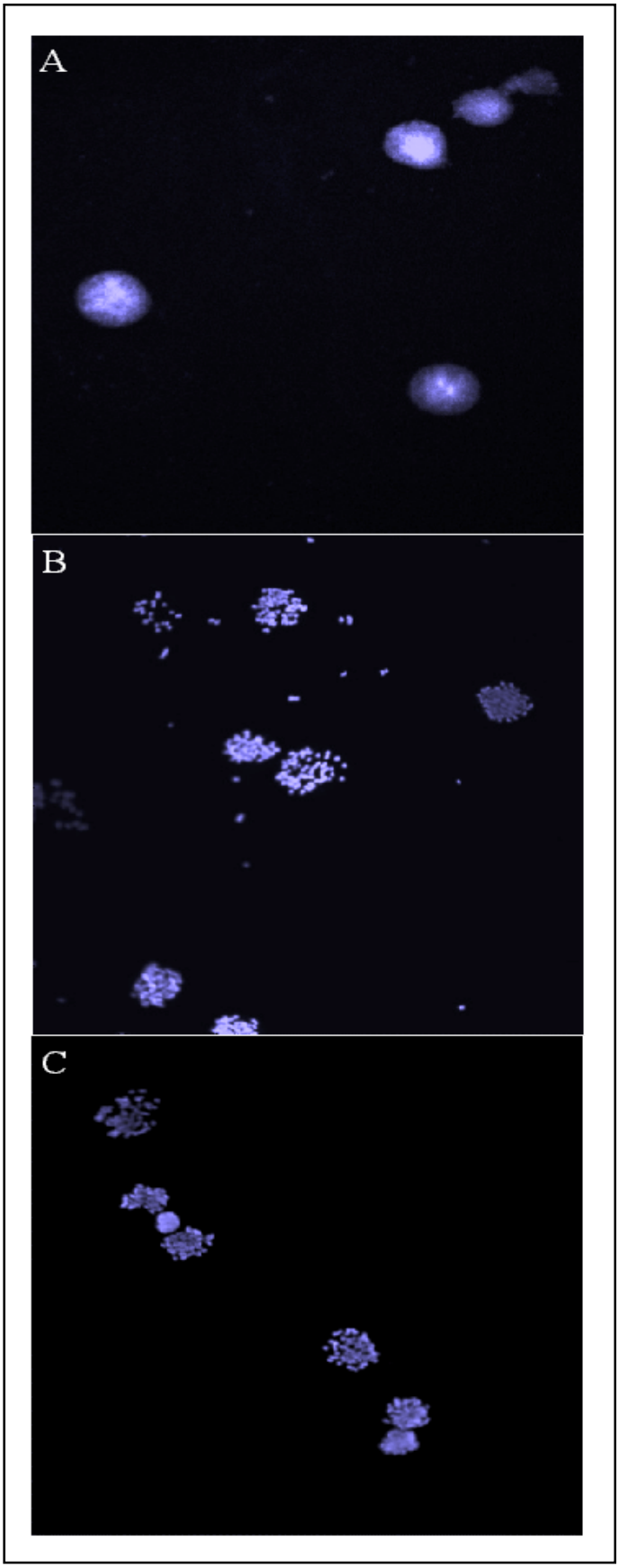
The rate at which polyamides enter cells was the next uptake variable to be determined. The relative signal intensity of the labeled cells was monitored for various time periods ranging from 1 min to 48 hr (data not shown). It was found that an incubation of 2 hr was sufficient for polyamides to localize intracellularly at the level observed for 24- and 48-hr incubations. In order to allow the compounds access to cells undergoing mitosis, most incubations were performed for 24 hr.

Chromosomal/Nuclei Staining

Polyamides have been shown to bind to DNA, but they have not been previously demonstrated to bind to DNA complexed with protein and chromatin, as is the case in living cells. Thus, to determine if polyamides could bind to condensed chromatin, L-929 mitotic chromosome spreads were incubated with 1 μM concentrations of S32 polyamide, Distamycin A, and Hoechst 33258 (positive control). Upon confocal observation (Figure 3), we found that polyamides bind to chromatin. In addition, intact nuclei (Figure 4) were stained to observe whether polyamides are 1) permeable to the nuclear envelope and 2) can bind to non-condensed chromatin. The nuclei were stained with 1 μM concentrations of the drug for 30 min prior to observation, and the confocal images (Figure 4) clearly show that polyamides are in fact permeable to the nuclear envelope and that they bind to interphase chromatin.

Cell Uptake Experiments

Finally, to determine the uptake profile of the molecules in live cells, several mammalian cell lines (PtK-2, Vero, and MCF-7) were exposed to polyamide (Figures 5, 6 and 7, respectively). The well-characterized vital DNA/nuclear stain, Hoechst 33258 (Arndt-Jovin and Jovin, 1989), was used as a positive control for a DNA minor-groove binding agent that localizes in the nucleus. From these representative images, it is evident that polyamides are cell permeable. However, the great majority of the drug appears to localize in the perinuclear space. Previous work describing both the *in vitro* and *in vivo* gene modulation properties of polyamide presumed DNA binding to be an essential part of its activity. Because the drug exposure allowed for a minimum of one cell division, it is presumed that the polyamide had direct access to the chromosomes during mitosis. The time-lapse microscopy experiments (Figure 8) further demonstrate that despite "free access" to the DNA during mitosis, polyamides do not localize in the nucleus. Thus, simple access to the genetic material does not appear to be sufficient for *in vivo*



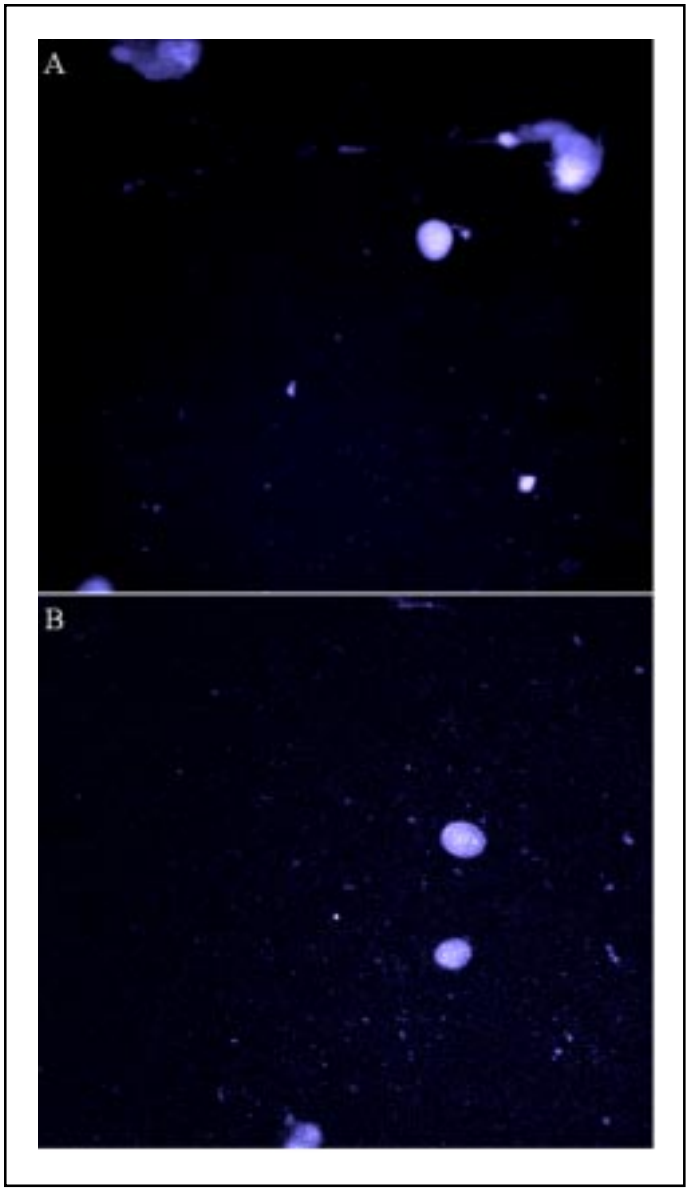


Figure 3 (Left)
Confocal imaging of L-929 chromosomes. (**A**) Hoechst 33258 staining (positive control). (**B**) Distamycin. (**C**) Staining with S32 polyamide (Bis-distamycin stains comparably to S32, not shown). All chromosomal stains performed at 1 μM concentrations of drug.

Figure 4 (Above)
Confocal images of isolated Vero nuclei stained with 1μM drug for 30 min. (**A**) Hoechst 33258 staining (positive control). (**B**) S32 Polyamide staining (Distamycin, Bis-distamycin, and HA gave similar results, not shown).

nuclear localization. In fixed-cell experiments (Figure 9), polyamides do, in fact, concentrate in the nucleus. It therefore appears that a yet to be determined cellular process or structure prevents polyamide nuclear localization. Note that in the fixed cell experiments and in the related controls, HA, a subnanomolar affinity polyamide, was used to illustrate that nuclear exclusion is not dependent upon target binding affinity.

We hypothesize that the apparent exclusion of bulk polyamide from the nuclei in the majority of viable cells may be a result of a sequestration/efflux mechanism that removes/prevents polyamide-like compounds from being retained in the nucleus. This hypothesis is further supported by the intact nuclear staining experiments, since we have shown that polyamides are permeable to the nuclear envelope and that they bind to chromatin. The published results (Gottesfeld et al., 1997; Dickinson et al., 1998) show that polyamides do target nuclear genes; however, we have determined that they do not achieve a concentration in nuclei sufficient for fluorescence detection. Because these experiments were designed solely to image the intracellular localization of bulk polyamide, no assertions can be made regarding the functional efficacy of polyamide as a gene modulator. Bulk sequestration/efflux of polyamide from the nucleus would not necessarily preclude the effectiveness of polyamides for gene targeting due to their unusually high specificity for DNA.

The presence of high concentrations of polyamide in the nucleus could conceivably interfere with basal transcription and lead to transcriptional inhibition *en masse* and/or an inability to properly condense chromatin, which would result in cellular damage or death. Thus, the reported lack of cytotoxicity (Dickinson et al., 1998) of polyamides further supports our hypothesis of a sequestration/efflux system that keeps high amounts of polyamide-like compounds out of the nucleus. Another aspect of polyamide uptake is the formation of polyamide vesicular bodies, which may be due to polyamide self-aggregation or binding to cellular proteins. The discrete pattern of polyamide staining in live cells may be indicative of mitochondrial and/or cytoskeletal localization. A notable observation from the time-lapse cell division experiments is that the discrete pattern of fluorescence is maintained, which may indicate that bulk polyamides are sequestered permanently such that once bound (to a protein/organelle), they are unavailable for future binding to DNA. It is conceivable that there exists a natural mechanism of sequestration or storage of endogenous general structural analogues, like Biliverden (Figure 1C), which have strikingly similar properties to polyamides.

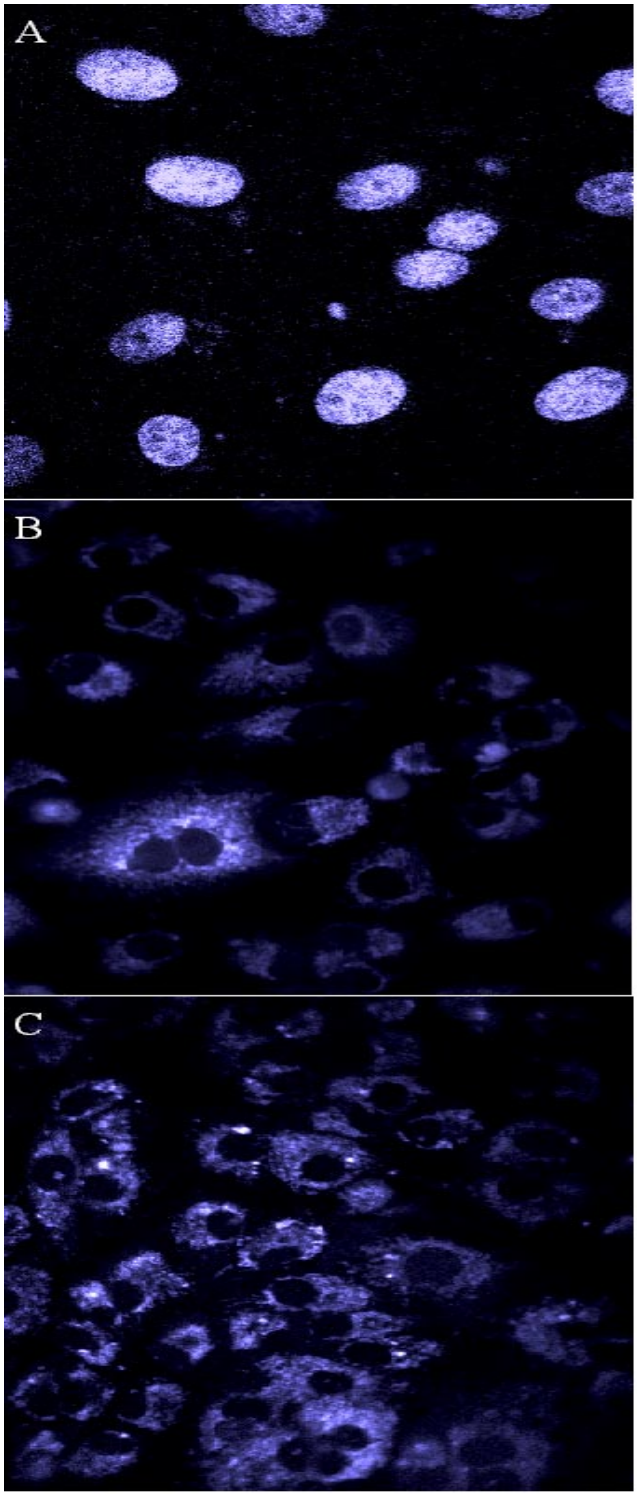


Figure 5
Confocal images of Vero cells exposed to 1 μM drug for 24 hr. (**A**) Hoechst 33258. (**B**) Bis-distamycin. (**C**) S32 Polyamide (Distamycin staining appears identical to Bis-distamycin, S32, and HA staining, not shown).

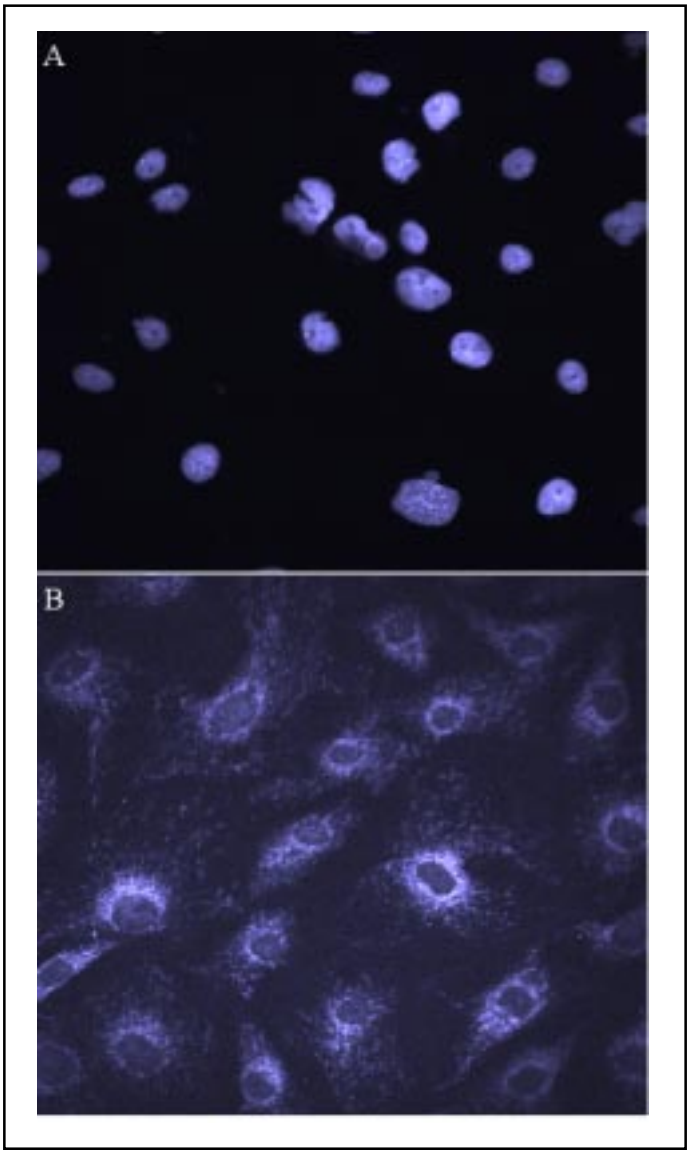


Figure 6

Confocal images of PtK-2 cell line uptake of drugs after a 24-hr exposure at 1 μM. (**A**) Hoechst 33258 positive control staining. (**B**) S32 polyamide (note that both Distamycin, Bis-distamycin, and HA cellular uptake closely mirrored S32, not shown).

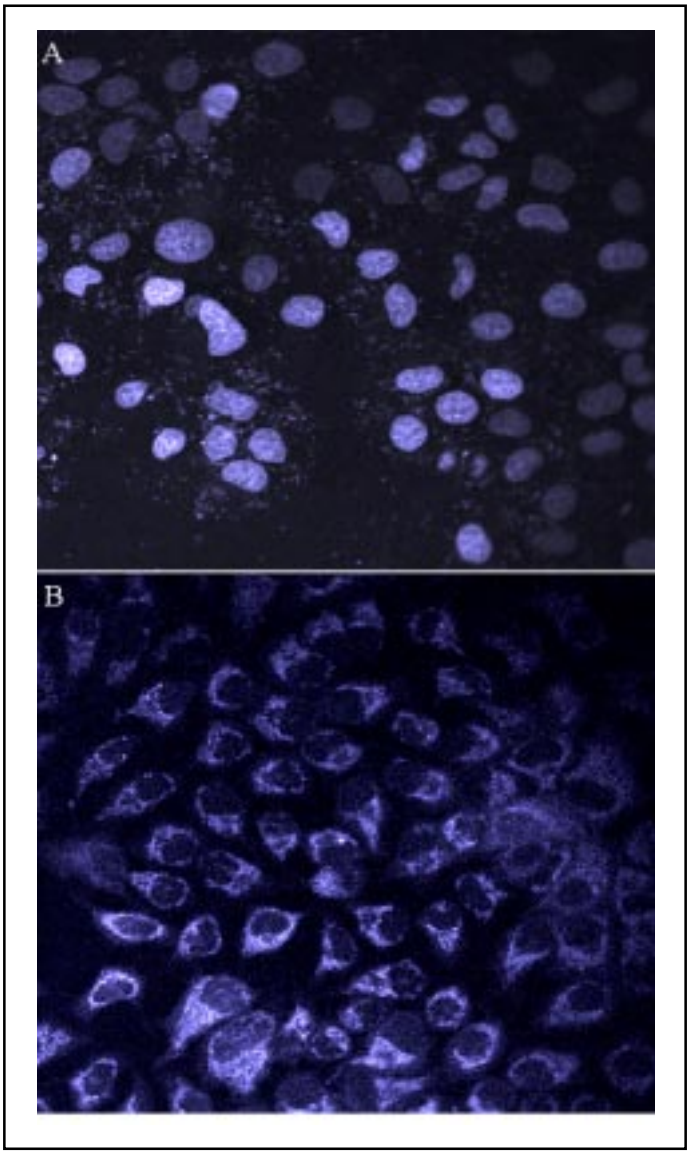


Figure 7

Confocal images of MCF-7 cells exposed to 1 μM drug for 24 hr. (**A**) Hoechst 33258. (**B**) S32 polyamide (again both Distamycin and Bis-distamycin staining appear identical to S32, not shown).

Biliverdin, like some polyamides, also demonstrates striking anti-HIV-1 activity (Mori et al., 1991). The mechanism of this anti-HIV-1 effect is thought to be via inhibition of viral particle binding to cells. One can then ask whether high doses of Biliverdin (>1 μM) have anti-gene effects via binding to DNA, or whether polyamide can also exhibit the property of interfering with viral particle binding to target cells. The cellular uptake and chromosome staining patterns of Biliverdin (Figures 10A, B) closely matches those observed with the polyamides tested, which supports the argument of Biliverdin binding to DNA (Figure 10C). It is conceivable that evolution would select for a mechanism to exclude/sequester cellular metabolites capable of binding to DNA from the nucleus, to prevent interference of transcription. One such mechanism may lie in the structure of the Bilin Binding Protein (BBP) (Huber et al., 1987). BBP is a naturally occurring protein from the butterfly, *Pieris brassicae,* which has been shown to bind to Biliverdin. Most notably, BBP has recently been reengineered via combinatorial protein design (Beste et al., 1999) to bind to a non-natural ligand with nanomolar affinities, which suggests that the ligand binding domain may be promiscuous in its ligand selectivity. Protein sequence ho-

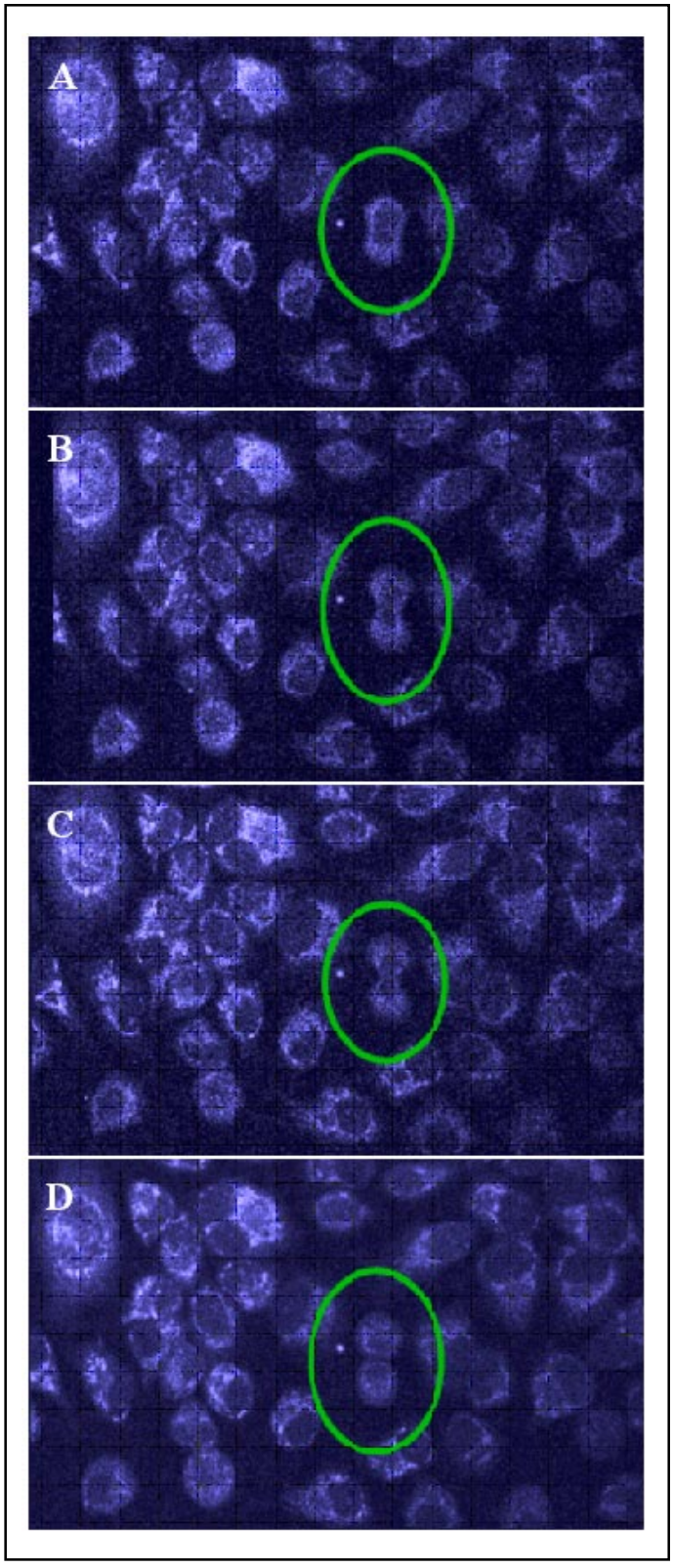


Figure 8

Time-lapse confocal images of MCF-7 cells undergoing mitosis (selected images showing progression of cell division, left to right). (**A**) 5 min (**B**) 10 min (**C**) 20 min (**D**) 30 min after beginning of mitosis.

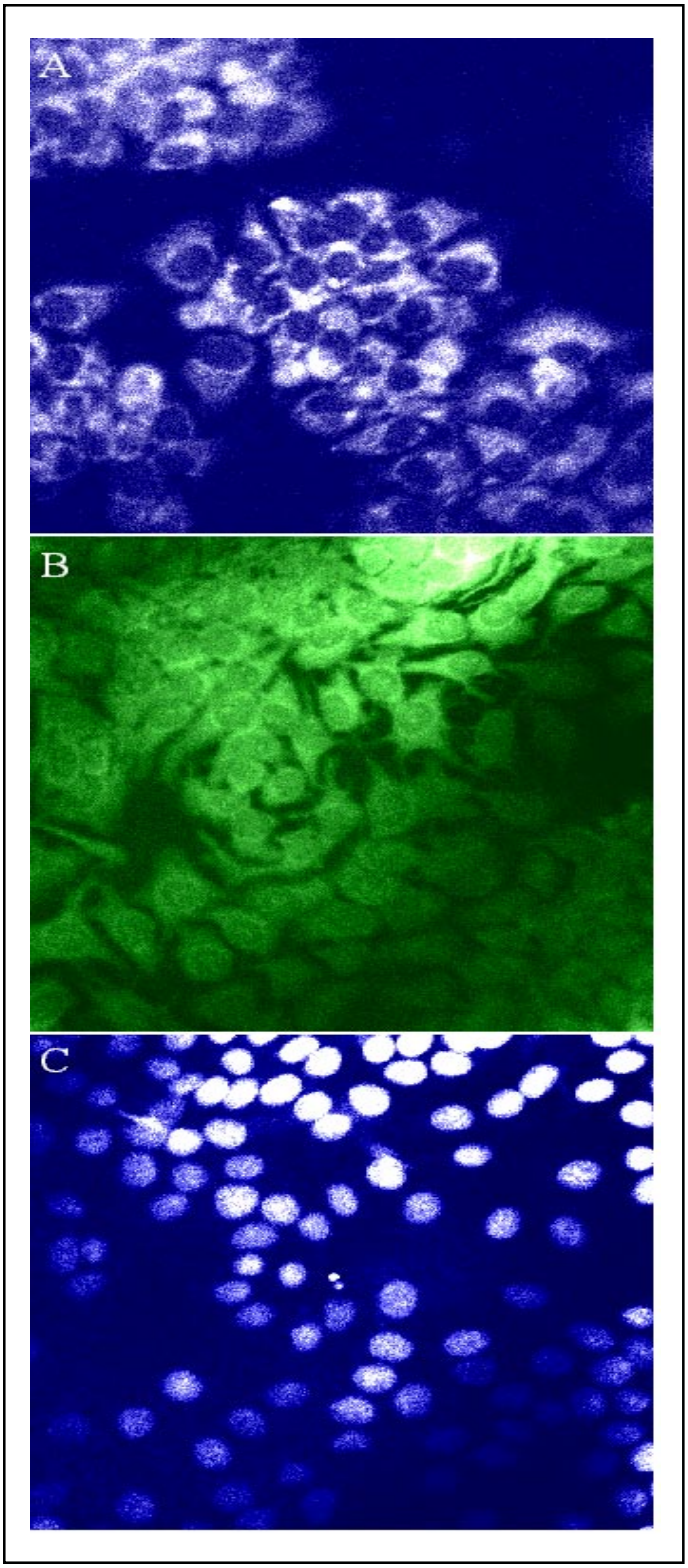


Figure 9

Confocal images of live and fixed MCF-7 cells exposed to 1 μM drug for 2 hr. (**A**) Live cells with HA polyamide (high affinity). (**B**) Fixed cells with 10 μM Rhodamine 123 (contrast agent to define cellular borders/structures). (**C**) Fixed cells with HA polyamide.

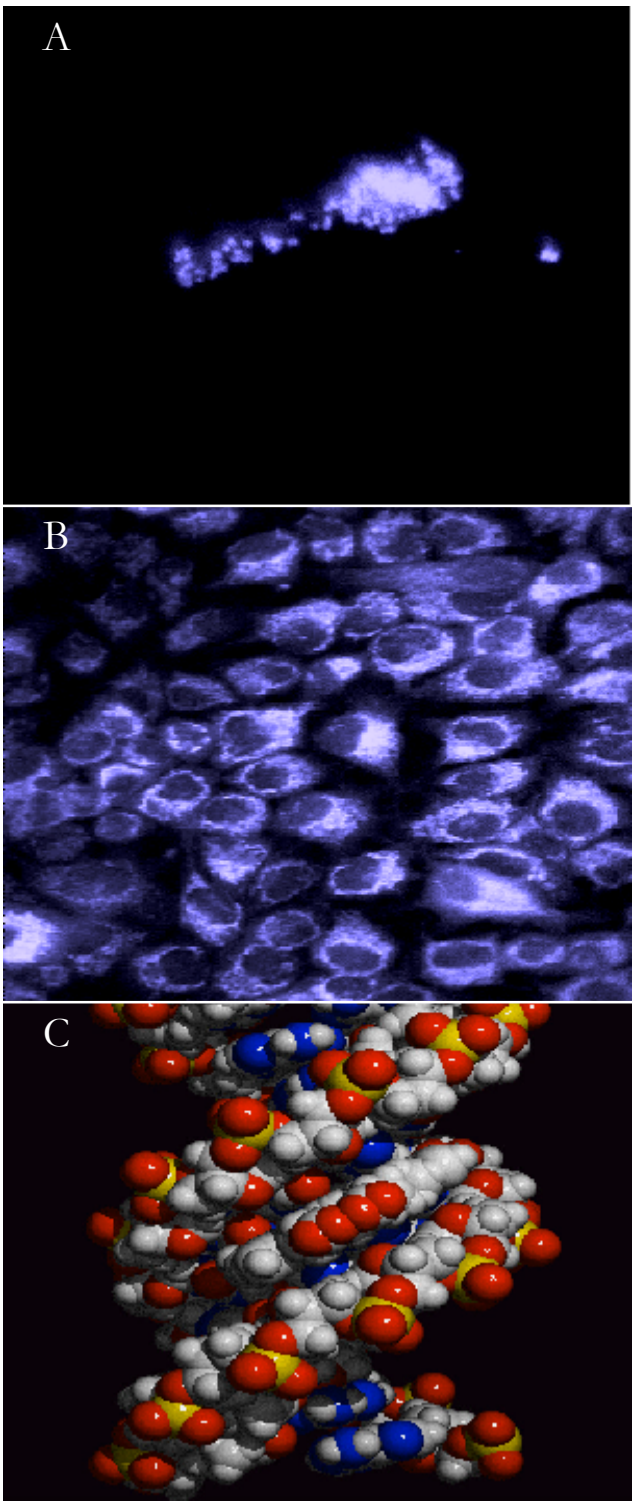


Figure 10

Biliverdin imaging. (**A**) Confocal images of chromosome spreads stained with 1 μM Biliverdin. (**B**) Confocal images of MCF-7 cells exposed to 1 μM Biliverdin. (**C**) Molecular model of Biliverdin bound to DNA.

mology to BBP was found with human apolipoprotein D (Peitsch and Boguski, 1990) which could then exhibit similar bilin-binding properties. In addition, there has been an independent report of Biliverdin binding to prostaglandin D synthase with high affinity (Beuckmann et al., 1999). Inherent in the combination of these findings is the suggestion that mammals have a protein sequestration system for Biliverdin, a potential DNA binding ligand. The observed polyamide nuclear exclusion could then be a result of being co-sequestered by the proposed mechanism. As previously alluded, there would be a high selective pressure to evolve a system to prevent DNA binding metabolites from having access to the genome and wreaking havoc upon transcription. Thus, in our attempt to document the cellular uptake of synthetic DNA binding ligands, potentially new insights into the evolution of nuclear exclusion/toxic-metabolite sequestration have surfaced.

Future Prospects

The distinctive pattern of cellular component staining will be pursued by co-labeling experiments with dyes that bind specifically to mitochondria, tubulin and/or actin. On a more fundamental level, attempts will be made to deliver the polyamides to the nucleus via conjugation to a nuclear localization signal (NLS). Bacterial gene modulation experiments are being performed to evaluate the potential of polyamide as a novel class of Designer Antibiotics.

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