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Cellular Uptake of *N*-Methylpyrrole/*N*-Methylimidazole Polyamide-Dye Conjugates

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Abstract—The cellular uptake and localization properties of DNA binding *N*-methylpyrrole/*N*-methylimidazole polyamide–dye conjugates in a variety of living cells have been examined by confocal laser scanning microscopy. With the exception of certain T-cell lines, polyamide–dye conjugates localize mainly in the cytoplasm and not in the nucleus. Reagents such as methanol typically used to fix cells for microscopy significantly alter the cellular localization of these DNA-binding ligands.

Introduction

Cell-permeable small molecules with the ability to target predetermined DNA sequences and interfere with gene expression would be useful tools in molecular biology and, potentially, human medicine. Polyamides containing aromatic amino acids *N*-methylpyrrole (Py), and *N*-methylimidazole (Im) are synthetic ligands that have an affinity and specificity for DNA comparable to naturally occurring DNA-binding proteins.[1,2] Based on pairing rules for recognition in the minor groove, polyamides can be designed to target a large number of predetermined DNA sequences. Polyamides have been shown to inhibit several classes of transcription factors, and thus regulate transcription in cell-free systems.[3] Modification of the DNA binding polyamide scaffold has produced agents capable of sequence-specific DNA-alkylation,[4,5] recruitment of Topoisomerase I,[6] and gene activation.[7,8] Significantly, polyamides have recently been shown to bind specific DNA sequences in isolated nucleosomes.[9]

Current impetus in our laboratories is directed at evaluating the potential of these molecules as potent regulators of specific gene transcription in cell culture assays. Polyamides targeted to the HIV-1 promoter inhibited viral replication in human peripheral blood lymphocytes.[10] Laemmli and coworkers have shown that polyamides targeting heterochromatic satellite DNA induced specific gain- and loss-of-function phenotypes in *Drosophila* presumably as a result of chromatin opening.[11,12] Together these studies indicated that polyamides are cell-permeable and, assuming that DNA is the cellular target of polyamides, localize to the nucleus at least in some cases.

In order to determine the potential of polyamides to inhibit the transcription of endogenous genes in human cells, our initial studies focused on the HER2/*neu* gene. Over-expression of the HER2/*neu* gene is implicated in human breast cancer and associated with poor prognosis.[13,14] Hairpin polyamides targeted to the Ets binding site of the HER2/*neu* promoter were shown to inhibit binding of the transcription factor ESX at nanomolar concentrations and to inhibit transcription of the HER2/*neu* gene in cell-free experiments.[15] However, despite the demonstrated in vitro activity, no transcription inhibition was observed in HER2-over-expressing SKBR-3 cells.[16] In order to test whether this result might be due to poor cellular uptake or nuclear localization, a series of polyamide conjugates incorporating the fluorophore Bodipy FL were synthesized. Confocal microscopy was used to study the cellular distribution of polyamide–Bodipy conjugates in

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SKBR-3 cells. In initial microscopy studies, cells were incubated with polyamide–Bodipy conjugates and visualized after treatment with cell-fixing reagents. Polyamide–dye conjugates were observed to localize in the nucleus of fixed cells. However, it was discovered that the common procedure of fixing cells prior to imaging had a drastic effect on conjugate localization, and that in living SKBR-3 cells without fixation, nuclear localization was not observed within detection limits. This suggested that a study of the cellular localization of polyamides in a variety of living cells was warranted. Here we report our initial findings on this key issue for gene regulation by polyamides.

Results

Polyamide–bodipy conjugates

Bodipy FL was chosen as a fluorophore because of its enhanced photostability relative to fluoroscein and its relatively small size.[17] The fluorescence of Bodipy is independent of pH in the physiological range, and the free dye is not known to localize to the nuclei of cells which should limit false positive results. Three polyamide–Bodipy FL conjugates, eight-ring hairpins **1** and **2** and a 2-β-2 hairpin (**3**) were synthesized (Fig. 1). Polyamides **1** and **2** are the same size but differ in sequence and the number of Im/Py rings. The polyamide precursors were synthesized by solid-phase methods[18] with one Py residue modified to yield a free amine upon cleavage from the resin.[19] The free amine of the precursor polyamides was allowed to react with the succinimidyl ester of Bodipy FL (Molecular Probes) affording polyamide conjugates **1**–**3**. The purity and identity of the dye conjugates were verified by analytical HPLC, UV–vis spectroscopy, and MALDI-TOF mass spectrometry. Additionally, DNase I footprinting experiments[20] were performed to assay the ability of the conjugates to bind DNA. Despite the short tether between the bulky dye and the polyamide backbone, the Bodipy conjugates still maintain high affinity and specificity for their match sites ($K_a = 10^7$–$10^9\ M^{-1}$) although with a loss of 10–100-fold in affinity relative to their parent polyamide. The fluorescence of conjugates was unaffected by the presence of cognate DNA or acidic pH. There was a 2-fold increase in fluorescence in methanol as compared to buffer.

Confocal microscopy

Confocal microscropy was used to study the distribution of polyamide–Bodipy conjugates in SKBR-3 cells. Using a procedure that included fixing the cells in methanol before imaging, rapid uptake and nuclear localization was observed.[21] However, in a control experiment without fixation, significantly reduced fluorescence located in a punctate pattern in the cytoplasm was observed (Fig. 2a). With the observation that methanol influences cellular localization of polyamides, an effort to screen a variety of *live* cells with the Bodipy–polyamide conjugates was initiated. Cells were incubated with 5 μM concentration of conjugate **1**–**3** or free Bodipy FL as control in a total volume of 100 μL for

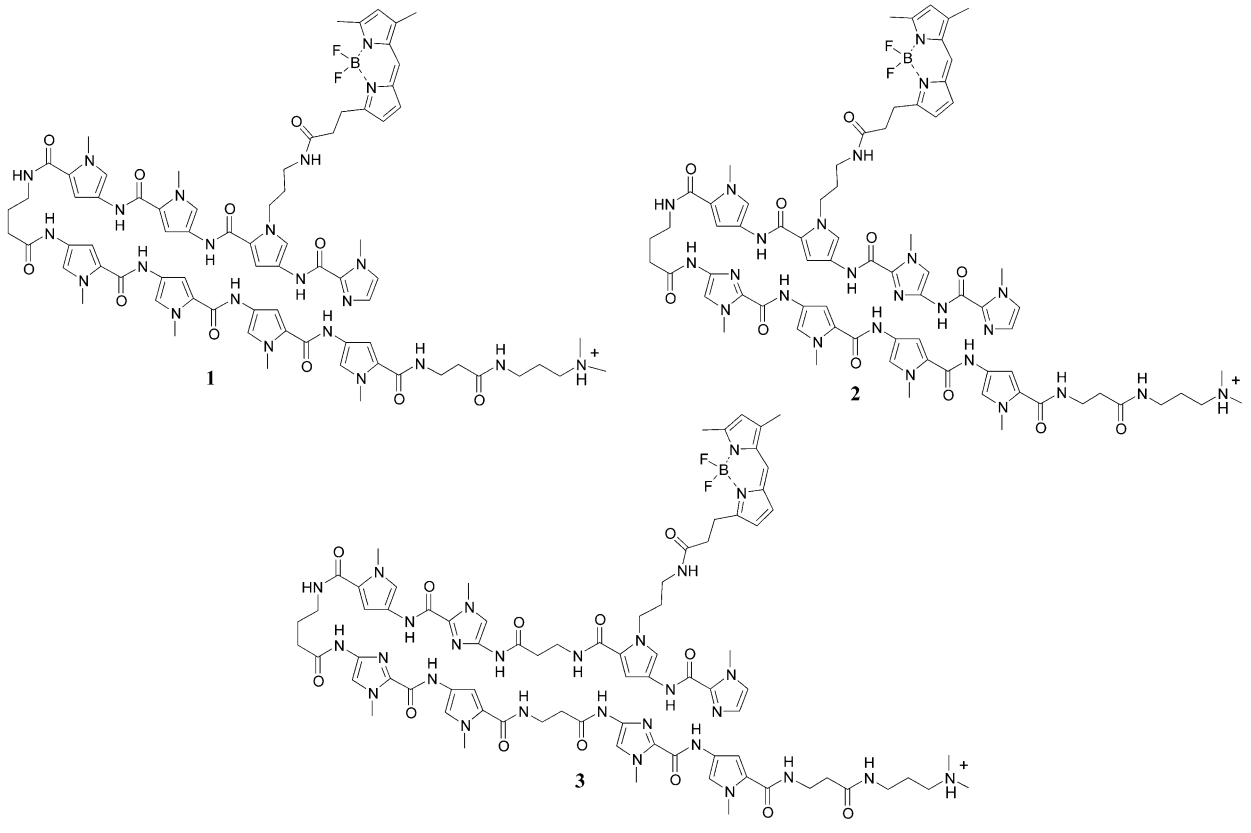
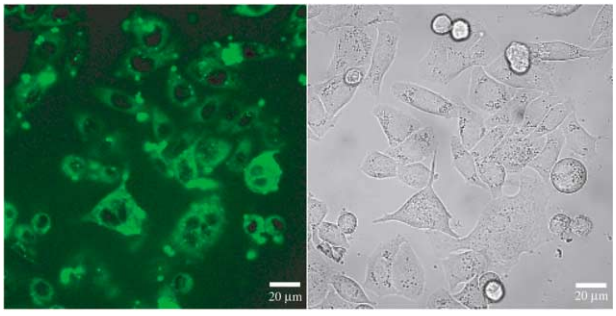
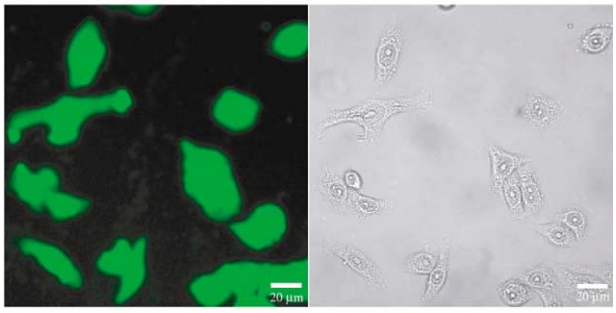


Figure 1.



(a)



(b)

Figure 2. (a) Conjugate **1** in live SKBR-3 cells. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm. (b) Conjugate **1** in SKBR-3 cells after the addition of MeOH (1:1 MeOH/media). The fluorescent image is on the left, bright field image on the right. Bar: 20 μm.

20 h under normal growth conditions for each cell-line (see Legend, Table 1). Most incubations were performed in culture dishes equipped with glass bottoms for direct imaging (MatTek Corporation). Adherent cells were allowed to grow in the glass bottom culture dishes for 24 h prior to the 20-h incubation with the dyes. To reduce evaporation due to the extremely small total volume in these experiments, cells which grow at room temperature without a humidified CO_2 incubator were incubated with **1**–**3** in 96-well plates and then transferred to glass bottom culture dishes for imaging. Imaging was performed with a Zeiss LSM 5 Pascal inverted confocal microscope, equipped with a 40× oil immersion objective lens. Images were captured at 0.88 μs/pixel scanning rate. The results for seven cell-lines are shown in Table 1. Cell-lines, ranging from cultured insect and human cancer cells to primary human CD4+ T-cells, were chosen based on availability, relevance to previous studies, and ongoing gene inhibition studies in cell culture.

Under non-fixing conditions, typically two distinct populations of fluorescent cells were observed in a given field. Live cells generally showed relatively dim fluorescence in the cytoplasm, while dead cells generally showed relatively bright fluorescence throughout the cell, including the nucleus. To determine the amount of dead cells in a given sample, solutions of cells on glass bottom culture dishes (with and without pre-incubated **1**–**3**) were treated with 1 μM Sytox Orange for 10 min and then imaged directly. As shown in Figure 3, the bright nuclear-stained Sf9 cells are dead or damaged as indicated by localization of the membrane impermient Sytox Orange dead cell stain (Molecular Probes). For each experiment shown in Table 1, a control sample of cells was incubated under the same conditions as the sample containing the conjugate. These cells were then imaged to gauge background fluorescence and then treated with Sytox Orange to determine percentage of dead cells in the control sample. Given that cells are imaged directly in growth media, background fluorescence can be significant, but in all cases fluorescence of the dyes and polyamide–dye conjugates far exceeded background. A comparison of the amount of dead cells in the control sample and the dye-containing sample allowed the conclusion that 5 μM **1**–**3** did not cause cell-death under the experimental conditions, but that polyamide–Bodipy conjugates readily localize in the nucleus of dead cells.

In controls, free Bodipy FL was either non-cellular or localized in the cytoplasm, within the detection limits, in the seven cell-lines examined. All live cell-lines evaluated were permeable to conjugates **1**–**3** under the tested conditions (5 μM dye, 20-h incubation, 25 or 37 °C). The designation 'cytoplasm' in Table 1 is broadly defined as any cellular location other than the interior of the nucleus. The actual location and extent of non-nuclear uptake of **1**–**3** varied from the punctate pattern observed with **1** in SKBR-3 cells in Figure 2a to the

Table 1. Cellular localization of polyamide–Bodipy conjugates[a]

Conjugate	SKBR-3 human breast cancer	CEM human cultured T-cells	T-cells human primary T-cells	NB4 human leukemia	293 human kidney fibroblast	Sf9 insect	Kc Drosophila
1	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
2	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
3	Cytoplasm	Nuclear	Nuclear	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm

[a]Localization of polyamide–Bodipy conjugates in live cells as determined by confocal microscopy. The designation 'nucleus' indicates observation of fluorescence in the interior of the nucleus. The designation 'cytoplasm' indicates cellular, non-nuclear fluorescence (see text). Cells were imaged directly following 20-h incubation with 5 μM **1**–**3** under normal growth conditions for each cell-line. SKBR-3 is an adherent, human breast cancer cell-line grown in McCoy's 5A Medium, supplemented with 10% Fetal Bovine Serum and 1% penicillin–streptomycin, at 37 °C in 5% CO_2. CEM is a non-adherent human T cell-line (ATCC, # CCL-119) grown in RPMI 1640 Medium, supplemented with 10% Fetal Bovine Serum and 1% penicillin–streptomycin, at 37 °C in 5% CO_2. Primary human CD4+ T-cells[23] were incubated at 37 °C in 5% CO_2. NB4 is a non-adherent human acute promyelocytic leukemia cell-line grown in RPMI 1640 Medium, supplemented with 10% Fetal Bovine Serum and 1% penicillin–streptomycin, at 37 °C in 5% CO_2. 293 is an adherent human kidney fibroblast cell-line grown in Dulbecco's Modified Eagle Medium, supplemented with 10% Fetal Bovine Serum and 1% penicillin–streptomycin, at 37 °C in 5% CO_2. Sf9 is a non-adherent insect (*Spodoptera frugiperda*) cell-line grown in TNM-FH insect media, supplemented with 10% Fetal Bovine Serum and 1% penicillin–streptomycin, at 25 °C. Kc is a non-adherent *Drosophila* cell-line grown in HyQ-CCM3 supplemented with 10% Fetal Bovine Serum, at 25 °C.

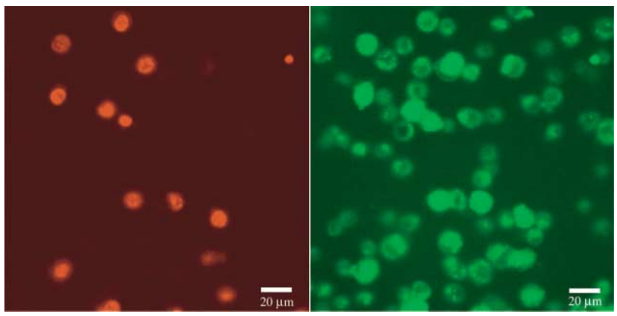


Figure 3. Conjugate **1** and dead cell stain Sytox Orange in Sf9 cells. The fluorescent emission of Sytox Orange (Rhodamine filter) is shown on the left. The fluorescent emission of conjugate **1** (FITC filter) is shown on the right. Note the different appearance of conjugate **1** in live and dead cells, as distinguished by uptake of Sytox Orange. Bar: 20 μm.

more general cytoplasmic staining seen with **2** in Sf9 cells in Figure 4a. Rapid nuclear localization was induced in both cases by the addition of methanol (1:1 methanol/media) directly to the solutions of cells and **1** or **2** (Figs 2b and 4b). The same behavior was observed for other polyamides and cell-lines as well as for other common fixing agents, including organic solvents such as ethanol and cross-linking agents formaldehyde and paraformaldehyde.[22]

The polyamide–dye conjugates were mainly in the cytoplasm, not the nucleus in five of the cells studied. For cultured human T-cell (CEM), broad cellular staining including the nucleus was observed with **1** (Fig. 5a). Fluorescence from conjugate **3** was also observed in the nucleus of live CEM cells, although to a lesser extent (Fig. 5b). Since conjugates **1** and **3** both contain the same Bodipy dye, the differential nuclear localization may be related to the polyamide structure. Polyamide **3** is an imidazole-rich 2-β-2 hairpin, while polyamide **1** is an eight-ring hairpin containing only an N-terminal imidazole residue. The imidazole rich eight ring hairpin **2** exhibited a cellular distribution more similar to **1** than to **3**. Thus, the addition of internal beta-alanines (eight ring to 2-β-2) may diminish nuclear localization.

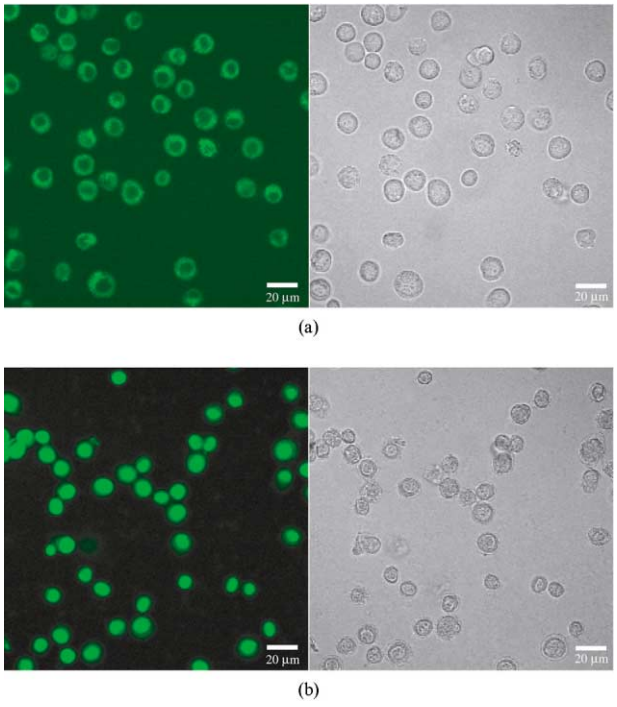


Figure 4. (a) Conjugate **2** in live Sf9 cells. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm. (b) Conjugate **2** in Sf9 cells after the addition of MeOH (1:1 MeOH/media). The fluorescent image is on the left, bright field image on the right. Bar: 20 μm.

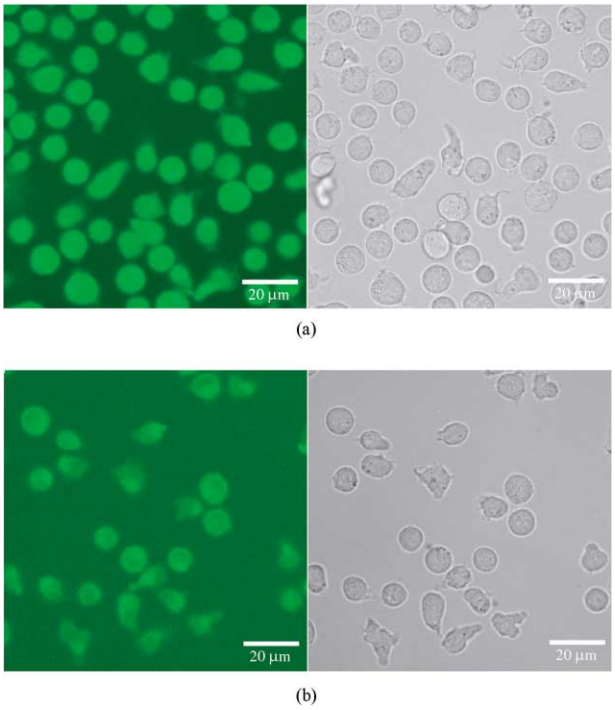


Figure 5. (a) Conjugate **1** in live CEM cells. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm. (b) Conjugate **3** in live CEM cells. The fluorescent image is on the left, bright field image on the right. Bar: 20 μm.

Given the in vivo results on HIV-1 inhibition and the observed nuclear localization in the cultured human T-cell line CEM, it was of interest to test the Bodipy conjugates with primary human CD4+ T-cells. A similar cellular distribution of conjugates **1**–**3** was observed in primary human CD4+ T-cells as in cultured CEM cells (Table 1).[23] However, it should be noted that over 20 different cell-lines have now been examined by the method described here, and nuclear localization of **1**–**3** was only observed in CEM and primary human T-cells.

Discussion

While polyamides are larger molecules ($M_r \sim 1200$) than typical pharmaceuticals, they are considerably smaller than some proteins which can cross through the nuclear pores.[24] Thus, it was believed that if polyamides could gain entry to a cell, they should be able to localize to their DNA targets in the nucleus, given their high affinity for DNA and fast association kinetics.[25] However, while the Bodipy conjugates examined in this study were found to be permeable to all cell-lines tested, nuclear localization was only observed in certain T-cells. In this

preliminary study the screening conditions chosen (5 μM conjugate, 20 h incubation) were arbitrary, and the detection limits in this study were lower than under fixed cell conditions. Given that the amount of polyamide in the nucleus necessary to generate a biological effect is unknown, a small amount of conjugate in the nucleus missed by our screen could be significant. As expected, free Bodipy FL was observed to be non-cellular or cytoplasmic in all cell-lines tested, which indicates that this dye is unlikely to yield false positive results. The observation in CEM and primary human T-cells that the extent of nuclear localization varies with polyamide structure suggests that polyamide redesign might influence cellular distribution. We cannot rule out the possibility that the choice of fluorescent dye may have biased the conjugates against nuclear localization. Further research is in progress to construct other fluorescent polyamides for the purpose of studying cell distribution. The positive results for HIV-1 inhibition in primary T-cells and negative results for inhibition of HER2/*neu* transcription in SKBR-3 cells, suggest that the Bodipy conjugates reflect the behavior of unmodified polyamides.

Lown and co-workers have recently investigated a fluorescently labeled derivative of distamycin in human ovarian adenocarcinoma (SKOV-3) cells.[26] Distamycin contains three pyrrole carboxamides and is considered the parent DNA binding polyamide molecule.[27] Nuclear localization was not observed within detection limits, and the primary cellular location of the fluorescent derivative was found to be the mitochondria. In conjunction with their studies targeting heterochromatic satellite DNA in *Drosophila,* Laemmli and coworkers have shown specific staining of chromosomes in isolated nuclei with polyamides conjugated with the fluorescent dye Texas Red.[11,12] More recently, these researchers have shown that Texas Red–tandem hairpin conjugates can specifically target both insect and vertebrate telomere repeat sequences, and can be used for rapid estimations of relative telomere length in vitro.[28] Extending these studies to intact cells, rapid uptake, nuclear localization, and staining of telomeres was observed in Sf9 cells. However, these experiments were performed under fixing conditions with methanol.[28]

There is no chemical reason for the dramatic effect of fixing agents to be specific for polyamide–Bodipy conjugates. It is possible that any molecule that has a high affinity receptor (i.e., DNA) from which it is excluded by cellular processes would exhibit the same behavior. However, in a very recent study with fluorescein-labeled oligonucleotides, greater nuclear localization was in fact observed in live cells as compared to fixed cells.[29] In surveying the literature, many published reports on cellular uptake and localization of designed ligands show data from fixed cells, without evidence of having performed a live cell control. These results suggest that such studies should demonstrate localization in live cells.

The finding that polyamide–Bodipy conjugates localize to the nucleus of CEM and primary human T-cells is significant. This result is consistent with the previous HIV-1 inhibition study,[10] and provides impetus for further screening to find other cell-lines amenable to nuclear localization. The mechanisms by which polyamide-Bodipy conjugates enter live cells and are excluded from the nucleus are unknown at this point. These mechanisms could be distinct and also could be variable between different cell-lines. Understanding the mechanisms of cellular entry and localization is an important issue for reengineering second-generation polyamides with specified nuclear uptake properties.

Conclusions

In the majority of live cells tested, polyamide–Bodipy conjugates were observed to localize mainly in the cytoplasm, not the nucleus. These results suggest that nuclear localization is likely a hurdle for the development of polyamides as in vivo modulators of gene transcription. We are currently in the process of developing modified polyamides with enhanced nuclear localization properties which will be reported in due course.

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