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Controlling the Intracellular Localization of Fluorescent Polyamide Analogues in Cultured Cells

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Abstract—The intracellular distribution of fluorescent-labeled polyamides was examined in live cells. We showed that BODIPY-labeled polyamides accumulate in acidic vesicles, mainly lysosomes, in the cytoplasm of HCT116 colon cancer cells and human rheumatoid synovial fibroblasts (RSF). Verapamil blocked vesicular accumulation and led to nuclear accumulation of the BODIPY-labeled polyamide in RSFs. We infer that the basic amine group commonly found at the end of synthetic polyamide chains is responsible for their accumulation in cytoplasmic vesicles in mammalian cells. Modifying the charge on a polyamide by replacing the BODIPY moiety with a fluorescein moiety on the amine tail allowed the polyamide to localize in the nucleus of the cell and bypass the cytoplasmic vesicles in HCT116 cells.

Naturally occurring pyrrole-containing polyamides such as distamycin and netropsin bind with high affinity to the minor groove of DNA. Synthetic polyamide analogues of these natural products also bind to the minor groove of DNA and can be designed with sequence recognition capability. Since polyamides have been shown to interfere with protein–DNA interactions, they are currently being evaluated for use in regulating gene expression (reviewed elsewhere).[1] However, for polyamides to be effective in gene regulation, they must reach their target DNA inside the cell. In eukaryotic cells, polyamides must not only cross the plasma membrane, but they must also make their way through the cytoplasm and across the nuclear envelope to bind to the target DNA. To this point, there are few examples and only circumstantial evidence that suggest that polyamides bind to nuclear DNA targets in living eukaryotic cells and it should be noted that all studies of this kind are dependent on cell type.[2–5] Here, we examined the intracellular distribution of fluorescent-labeled polyamides to evaluate their potential and practical use as gene regulators in mammalian systems.

To examine uptake and intracellular distribution, polyamides were labeled with fluorescent probes (Fig. 1, compounds **1** and **2**, BODIPY and fluorescein, respectively), cultured cells were treated with **1** and **2**, and the intracellular distribution was determined by fluorescence microscopy.[6–8] Figure 2 shows the fluorescence-staining pattern of HCT116 human colon cancer cells and human rheumatoid synovial fibroblasts (RSF) treated with 10 μM **1** overnight and counterstained with DAPI just prior to examination. In treated cells, fluorescence from **1** showed a punctuated cytoplasmic pattern (Fig. 2C and E) that did not overlap the DAPI stained nuclear DNA (Fig. 2D and 2F). Figure 2A shows that autofluorescence was undetected when untreated cells were examined using the BODIPY/FITC filter set (Fig. 2A, synovial fibroblasts), demonstrating that the fluorescence was due to **1** and that **1** entered the cells and accumulated in cytoplasmic compartments, not the nucleus. Similar punctuated cytoplasmic fluorescence was observed in HepG2 hepatocytes and RAW macrophage cells (data not shown). In addition, a similar intracellular distribution has previously been reported for a BODIPY-labeled polyamide in SKOV-3 cells and a number of closely related BODIPY-labeled hairpin polyamides, including an analogue of **1** with the BODIPY moiety located at an internal rather than the terminal site, in a number of human and insect cell lines.[5,9]

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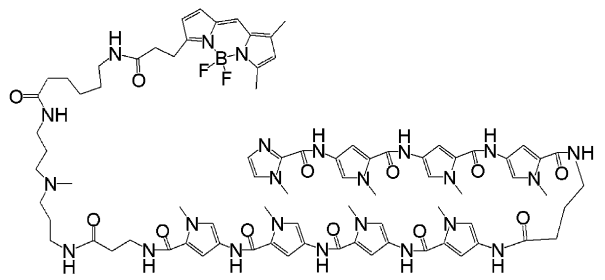
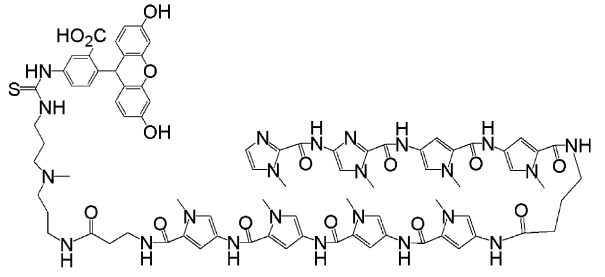


1

Im-Py-Py-Py-γ-Py-Py-Py-Py-β-Ta-BODIPY-FL-X



2

Im-Im-Py-Py-γ-Py-Py-Py-Py-β-Ta-FITC

Figure 1. Fluorescent-labeled polyamides.

BODIPY-labeled Polyamides Localize in Acidic Cytoplasmic Vesicles

The distribution of **1** in HCT116 and RSF cells suggested that **1** is trafficked to a specific compartment within the cytoplasm. To determine which cytoplasmic compartment sequestered **1**, dual-staining co-localization studies were performed with **1** and organelle-specific, red fluorescent probes.[10] As shown in Figure 3A, D, and G, fluorescence from 1 (green) was detected in cytoplasmic granules as above, which (1) did not overlap with MitoTracker fluorescence (Fig. 3B, overlay 3C), (2) showed partial co-localization with the Golgi-specific probe fluorescence (Fig. 3H, overlay 3I), and (3) completely co-localized with LysoTracker fluorescence (Fig. 3E, overlay 3F) showing that **1** accumulates in lysosomes and a portion of the Golgi apparatus. The partial overlap of fluorescence from **1** with the Golgi-specific probe and complete overlap with LysoTracker is consistent with the fact that LysoTracker is specific for acidic organelles, and stains not only the lysosomes but also the *trans*-Golgi. That **1** has a weakly basic cationic nature is consistent with its accumulation in acidic organelles. It is well documented that weakly basic cations with pK_a's near neutral freely penetrate membranes, but once inside acidic organelles, become protonated and are thereby trapped in those compartments.[11] This accumulation in acidic organelles, in turn, can prevent or reduce nuclear accumulation of a nuclear-targeted basic drug such as daunorubicin and doxorubicin, and likely accounts for the absence of detectable **1** (using the methods described here) in the nucleus of RSF and HCT116 cells.[12]

Verapamil Induces Nuclear Localization and Accumulation of Polyamides in RASFs but not HCT116 Cells

Sequestration of drugs in acidic vesicles has been described as one of the major mechanism responsible for multidrug resistance.[11,12] While not an absolute, the ability of a cell to sequester drugs into vesicles is a phenotype frequently co-expressed with the cell's ability or increased capacity to efflux drugs or substrates via the plasma membrane transporter, *p*-glycoprotein (P-gp). Because of this, we attempted to induce nuclear accumulation of 1 using P-gp inhibitors including verapamil, bepridil, cyclosporin A, and ketoconazole. RSF and HCT116 cells were treated with 1 as before, but in the presence of the P-gp inhibitors.[7] None of these showed any observable ability to enhance nuclear accumulation of **1** or have any observable effect on the intracellular distribution of **1** in HCT116 (data not shown), indicating that **1** is not likely a substrate for P-gp-mediated efflux in these cells. Bepridil, cyclosporin A, and ketoconazole also had no effect on the intracellular distribution of **1** in RSF cells (data not shown). However, verapamil treatment induced a dramatic reduction in cytoplasmic fluorescence (Fig. 4A) accompanied by a redistribution of **1** to the nucleus in RSF (Fig. 4B). The dramatic reduction in cytoplasmic fluorescence indicates that verapamil blocked vesicular sequestration of **1** and suggests that vesicular sequestration of **1** in untreated cells is the main reason that **1** does not reach its intended intracellular target (nuclear DNA) in untreated cells. Importantly, we infer that the nuclear envelope does not act as a barrier to **1**. The mechanism by which verapamil blocks vesicular sequestration of **1** in RSF cells is unclear. However, verapamil not only inhibits P-gp-mediated drug efflux, but also affects intracellular Ca^{2+} concentration which, in turn, influences a number of intracellular events including vesicular trafficking.[13] In addition, verapamil itself is a weakly cationic base and has been shown to accumulate in acidic vesicles in response to the proton electrochemical gradient across the vesicular membrane.[14–16] It is therefore likely that verapamil-induced nuclear accumulation of **1** results from disruption in the trafficking or the general homeostasis of acidic vesicles. Such mechanisms have previously been shown to increase the sensitivity of drug resistant cells to nuclear-targeted weakly basic cationic drugs.[12]

Fluorescein-labeled Polyamides Accumulate in the Nucleus of HCT116 Cells

Based on the results above, the exclusion of **1** from the nucleus of HCT116 cells and RSF (in the absence of verapamil) appears to be due to vesicular sequestration and not inability to cross the nuclear envelope. In general, it is the neutralization of the charge on weakly basic cationic drugs that allow them to cross membranes, including that of the acid vesicles. In an attempt to block diffusion of a polyamide through the vesicular membranes and, therefore, to block vesicular accumulation of the polyamide, we modified a related polyamide to contain an acid anionic moiety, fluorescein

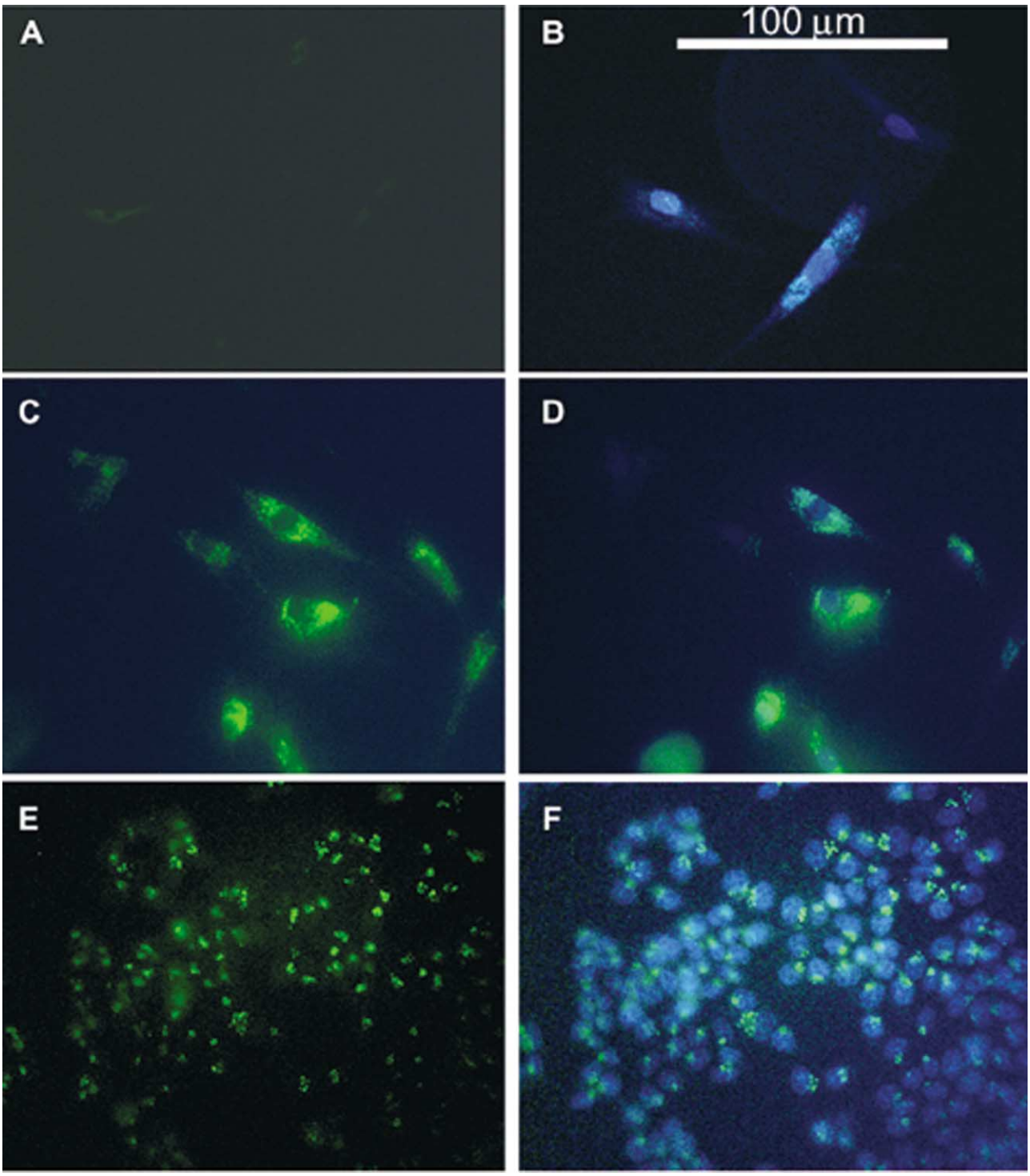


Figure 2. Fluorescence images of HCT116 and RSF cells treated with a BODIPY-labeled polyamide and DAPI. Cells were cultured overnight at 37 °C without (A & B) or with (C-F) 10 µM BODIPY-labeled polyamide (**1**) and counterstained with DAPI just prior to imaging. For cells treated with DAPI only, fluorescence is not detectable using the fluorescein filter set (A) but is detectable mainly in the nucleus using the DAPI filter set Using the fluorescein filter set green fluorescence from **1** is observable within RSF cells (C) and HCT116 cells (E), but not in untreated cells (A). Using the DAPI filter set the green fluorescence from **1** is shown to be excluded from the DAPI-stained (blue fluorescence) nuclei (D & F).

(compound **2**) which is not susceptible to neutralization at physiological or near physiological pH. Gel shift assays were used to show that the addition of the fluorescein moiety did not to block the polyamide from binding to DNA (data not shown), consistent with previous work that showed that fluorescein-labeled linear polyamides bind DNA.[17] HCT116 cells treated with **2** are shown in Figure 5. Fluorescent-filled vesicles are absent (Fig. 5B), while nuclei are brightly stained (Fig. 5C). We conclude that the additional anionic moiety did block 2 from crossing the membranes of acidic vesicles, thereby blocking vesicular accumulation. Presumably, with this pathway blocked and because of the permeable nature of the nuclear envelope, **2** entered the nucleus

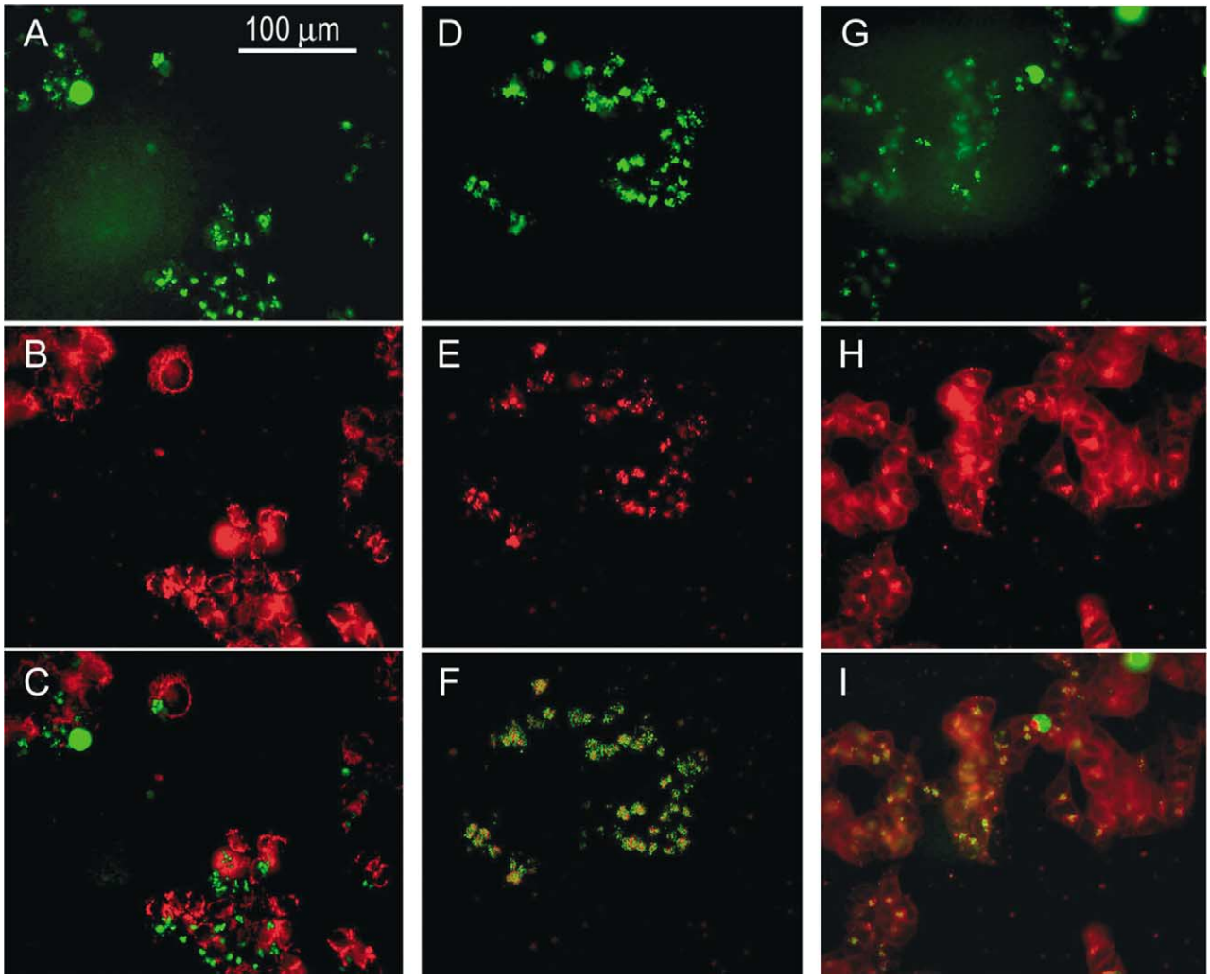


Figure 3. Co-localization of BODIPY-labeled polyamide with organelle-specific fluorescent probes in HCT116 cells. Cells were cultured overnight at 37 °C with 10 μM BODIPY-labeled polyamide (1) (A, D, G) and counterstained with MitoTracker® Red CM-H_2Xros (B), LysToracker® Red DND-99 (E) or BODIPY TR ceramide (H). Fluorescence images of the same field were captured using the fluorescein filter and the rhodamine filter set, and two images were overlaid (C, F, I).

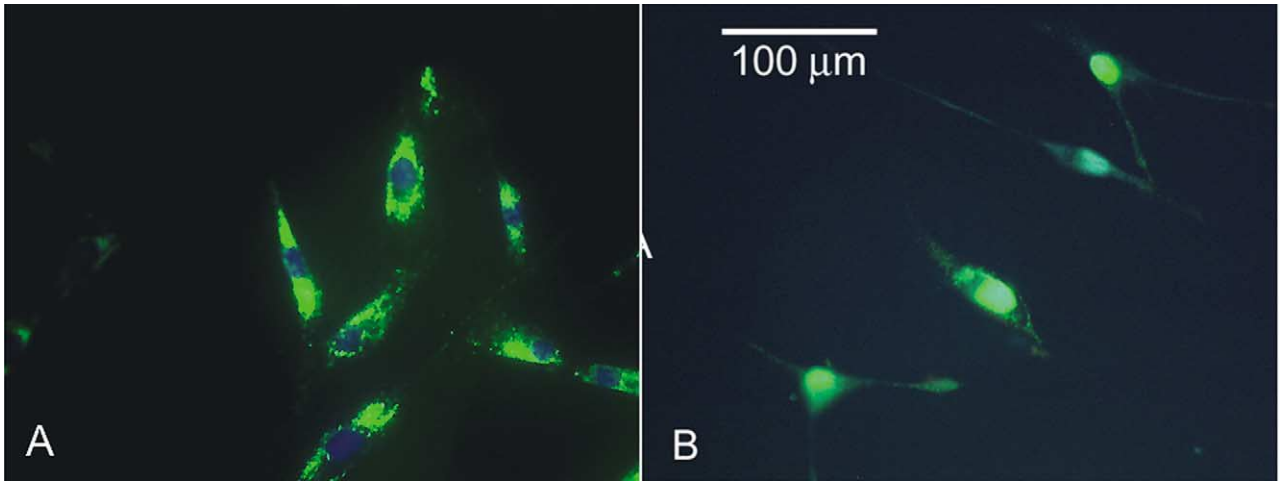


Figure 4. Verapamil induces localization of polyamides to nuclei of synovial fibroblasts. Cells were cultured overnight at 37 °C with 10 μM BODIPY-labeled polyamide (**1**) in the presence or absence of 100 μM verapamil and counterstained with DAPI just prior to imaging. Using the fluorescein filter set green fluorescence from 1 is observed in the cytoplasm in the absence of verapamil as before (A) and in the nuclei in the presence of verapamil (B) within RSF cells.

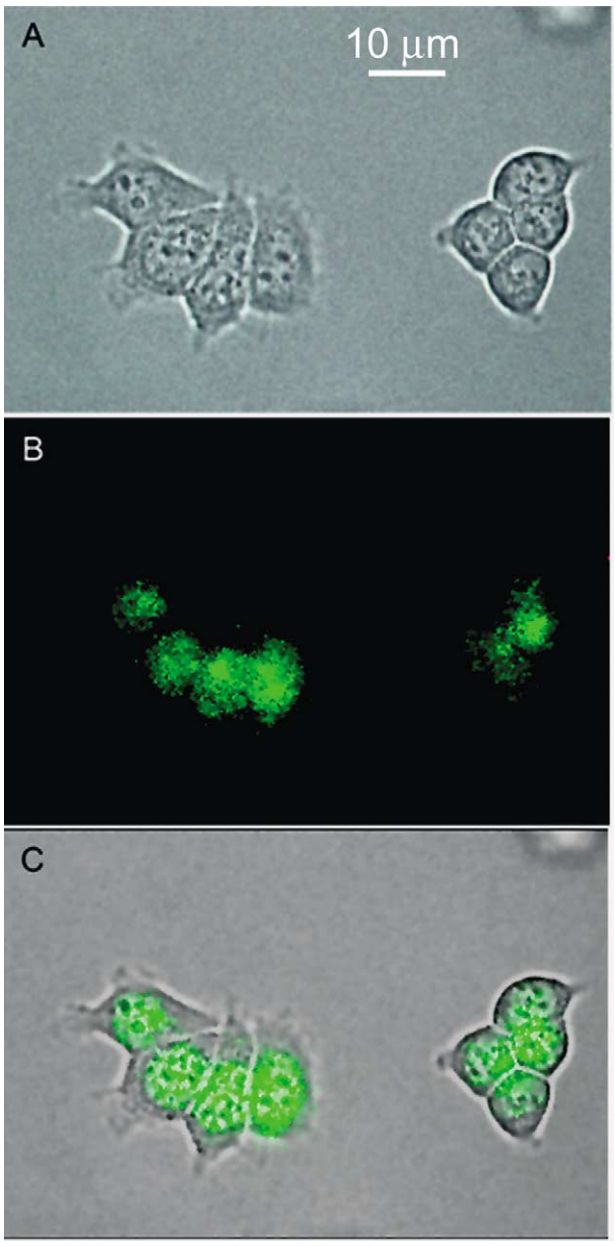


Figure 5. HCT116 cells treated with a fluorescein-labeled polyamide. Cells were cultured overnight at 37 °C with 10 μM fluorescein-labeled polyamide (**2**). The green fluorescence from **2** is observed in the nuclei of the cells (B; C overlay of A & B) when imaged using the fluorescein filter set.

and accumulated therein due to its high affinity for DNA. Again, this demonstrates that the nuclear envelope does not act as a barrier to polyamides.[18] Supporting the idea that polyamides can enter the nucleus directly in some living cells, in contrast to the finding that polyamides accumulate in cytoplasmic vesicles in a number of cell lines, Belitsky et al. have reported that cationic BODIPY-labeled hairpin polyamides accumulate in the nucleus of human T-cells and CEM human cultured T-cells.[5]

The results reported here show that in cases where polyamides accumulate in cytoplasmic vesicles, polyamides can be redirected to the nucleus by agents that disrupt the acidic vesicle homeostasis or by modifying the charge of the polyamide. When vesicular sequestration is inhibited, polyamides are free to accumulate in the nucleus. Since the nuclear DNA is the polyamide target, polyamides may indeed be useful molecules for regulating gene expression in mammalian cells by using one of these two strategies.

References and Notes

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6. Baird, E.; Dervan, P. *J. Am. Chem. Soc.* **1996**, *118*, 6141. The polyamides were labeled with BODIPY-FL-X, SE and FITC respectively using standard labeling conditions.
7. Human colon cancer HCT116 cells were cultured as monolayers and maintained at 37 °C in a humidified incubator with 5% CO_2 in buffered medium consisting of RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Gibco), 100 Units/mL penicillin–streptomycin and 25 μg/mL gentamicin. Human rheumatoid synovial fibroblasts (RSFs) were cultured in DMEM (Gibco 11995-040) supplemented with 15% FBS, 1% glutamine, and 50 μg/mL gentamycin. Cells (8×10^5) were plated on 25-mm round glass coverslips in 30-mm wells and incubated for 24 h to allow cells to adhere. Fluorescent polyamides were freshly prepared in DMSO to 10 mM, diluted to 1 mM with distilled water, added pre-warmed cell culture media to a final concentration 10 μM polyamide, 0.1% DMSO, and cells were incubated in this media for an additional 16 h as before. Where applicable, cells were also pretreated with one of the MDR inhibitors, verapamil, bepridil, cyclosporin A, or ketoconazole, at a concentration of 5–100 μM for 30 min before the addition of the fluorescent polyamides.
8. Live wet mounted cells were examined and photographed using an Olympus AX70 microscope equipped with fluorescence optics and a Sony 3CCD color video camera. DAPI was detected using a bandpass 405 ± 20 nm excitation filter, a 420 nm dichroic beam splitter, and a $\geq$450 emission filter (DAPI filter set). BODIPY and Fluorescein conjugated polyamides were selectively detected using a bandpass 485 ± 11 nm excitation filter, a 505 nm dichroic beam splitter, and a 530 ± 15 nm emission filter (fluorescein filter set). Organelle-specific probes were detected using a 546 ± 5 nm excitation filter, a 570 nm dichroic beam splitter and a 590 nm longpass emission filter (rhodamine filter set).
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10. Where applicable, after the 16 h incubation in the presence of polyamide and/or MDR inhibitor, either MitoTracker® Red CM-H_2XRos, LysToracker® Red DND-99, or BODIPY® TR ceramide was added directly to the cell culture for 15 min to 1 h as recommended by the supplier (Molecular Probes). Just prior to examination 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was added to cell cultures to 300 nM and samples were incubated for at least 5 min at room

temperature. The coverslips were rinsed several times in PBS and wet mounted.
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