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Design, Synthesis, and Intracellular Localization of a Fluorescently Labeled DNA Binding Polyamide Related to the Antibiotic Distamycin

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Abstract—The design and synthesis of the lipophilic (**9**) and fluorescent (**10**) conjugates of a structural analogue of distamycin and their in vitro cellular localization studies are reported. Confocal laser scanning microscopy (CLSM) indicates that **10** rapidly enters human ovarian adenocarcinoma (SKOV-3) cells with principal uptake in mitochondria and uniform cytoplasmic distribution.

The development of small, synthetic molecules that specifically bind to any sequence in the human genome in a predictable manner and that can regulate gene expression is an area of current research interest in molecular biology and potentially in human medicine. Despite the fact that there are many minor-groove binders, the natural product distamycin **I** (Fig. 1) remains the principal prototype candidate for further elaboration of the central theme of designing synthetic gene regulators. A simple code has been developed to rationally alter the sequence specificity of the minor-groove binding polyamide molecules.[1–4]

A considerable amount of synthetic and biological work has been done on polyamides related to distamycin in our group and in Dervan's group (viz. hairpin, cross-linked and extended bis-polyamides), which have shown promising and encouraging results on various biological targets.[5–9] Difficulties with the nondestructive delivery of these exogenous molecules through the plasma membrane into living cells have so far limited their potential application. Intracellular distribution of pharmaceutical agents provides inferential evidence of potential mechanism of activities.[10] Preliminary studies in this regard were performed in our group[11] by using spin labeling on netropsin by EPR spectroscopy. Recently confocal microscopy has emerged as an effective tool for studying the intracellular distribution of drugs by using the fluorescent properties of conjugated chromophores. In order to study the cellular uptake and subcellular localization of these minor-groove binding polyamides, we synthesized the lipophilic (**9**) and fluorescent (**10**) conjugate. The DNA binding properties of the conjugates **9** and **10** are expected to remain unaltered from distamycin since the conjugate maintains the basic assembly of distamycin: three pyrrole units, a leading *N*-formyl group and a dimethylaminopropylamine terminus (Scheme 1).

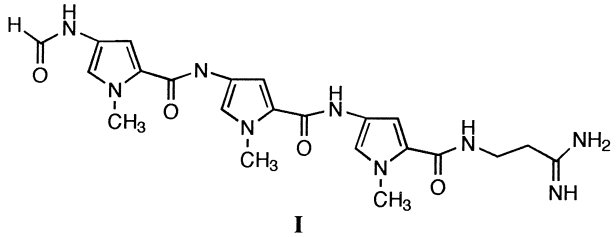

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Figure 1. Distamycin.

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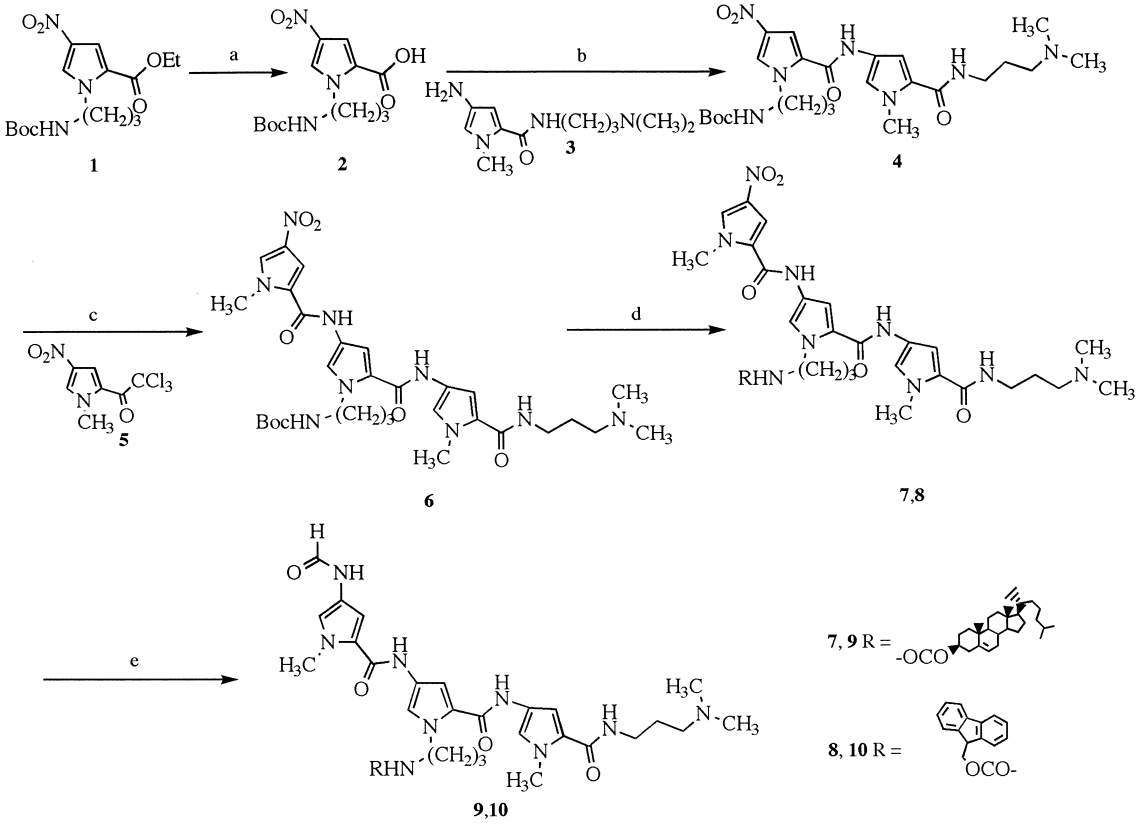

Scheme 1. Reagent and conditions: (a) 0.1 N NaOH, EtOH, reflux, 0.1 N HCl; (b) DCC, HOBt, DMF, rt, 87.4%; (c) DMF, TEA, rt, 81%; (d) 80% TFA, CH_2Cl_2, cholesteryl chloroformate, DMF, rt, 90% or Fmoc-Cl, Na_2CO_3, DMF, rt, 84%; (e) 10% Pd/C, DMF, *N*-formylimidazole, −40 °C, 75%.

Our strategy for the synthesis of **9** and **10** conjugates requires an intermediate **6**, which possesses an *N*-protected linker arm attached to the central pyrrole.[12] We adopted reported methodology for the synthesis of **6**, with slight modification in order to improve the reported yields.

The acid **2** was obtained by refluxing the ester[12] **1** with 0.1 N NaOH followed by the acidification of the corresponding salt with 0.1 N HCl to pH 2. The resulting acid was then coupled with the reduced amine of **3** catalyzed by DCC/HOBt in DMF to afford **4** in 87.4% yield. The reduced amine corresponding to **4** was then directly coupled with **5** instead of the corresponding acid without any coupling agents to afford the intermediate **6** in 81% yield. The Boc protecting group on **6** was deblocked by 80% TFA,[12] and then coupled with cholesteryl chloroformate to afford **7** in 90% yield. An equivalent amount of Na_2CO_3 was used prior to the addition of Fmoc-Cl to the free amine of **6** to afford **8** in 84% yield (the yields of all the intermediates are after flash chromatography on silica gel). It was observed that use of an excess of the base in the reaction decreased the yield of **8**. The nitro groups of **7** and **8** were reduced by hydrogen over 10% Pd/C and the resulting amines were formylated by using *N*-formylimidazole at −40 °C to afford **9** and **10** in 75% yield, respectively.[13] The intrinsic fluorescent properties of **10** facilitated confocal laser scanning microscopy (CLSM) assessment of cellular uptake and intracellular distribution.

For uptake and intracellular distribution, a 100-fold concentrated stock solution of **10** was freshly prepared in tissue culture grade dimethylsulfoxide (DMSO), and added to SKOV-3 cells growing in 36 mm Petri plates treated for monolayer mammalian cell culture. The compound **10** was introduced at a final concentration 0.1 μM, a dose approximate with the IC_{50} value, as determined using a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric screening assay for cytotoxicity (unpublished results). Incubation under conditions outlined above ensued for 1 h, at which time the medium containing **10** was removed. The cell culture was gently washed three times with phosphate buffered saline (PBS), and a coverslip was placed directly upon the cell monolayer, using PBS/glycerol (1:1 by volume) as mounting medium. To conduct a control for cellular autofluorescence, parallel cell cultures were incubated as described, with 1% DMSO in the absence of **10**. Cellular uptake and intracellular distribution was monitored using a confocal microscope.[14] SKOV-3 cells incubated in the absence of **10** showed virtually no autofluorescence (data not shown). Minor autofluorescence was observed focally within the cytoplasm. The fluorescence of **10** in SKOV-3 cells is observed as shown in Figure 2.

The greatest fluorescence intensity is associated with mitochondria as demonstrated by bright fluorescent foci. Mitochondria appear round or sometimes rod-shaped, depending upon how they are situated within

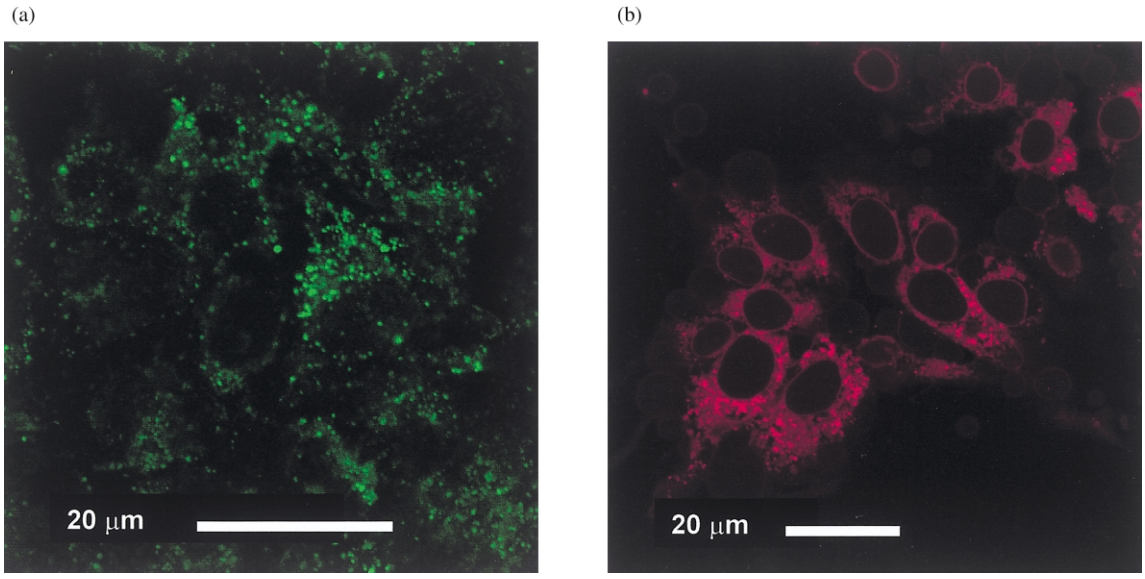

Figure 2. (a) Fluorescence of **10** in SKOV-3 cells in vitro; (b) mitochondrial fluorescence of SKOV-3 cells by rhodamine 123.

the optical section. Mitochondrial uptake of **10** is consistent with its cationic properties because the relatively high electronegativity of the mitochondrial membrane apprently restricts uptake to cationic compounds.[15] Mitochondrial uptake is confirmed by performing a control experiment with a mitochondrial dye rhodamine 123 as shown in Figure 2b. The remainder of the cytoplasm is uniformly labeled, indicating moderately weak uptake of the stain by the endoplasmic reticulum and the Golgi apparatus. There is no evidence of nuclear uptake, within the limits of fluorescence detection.

In conclusion we have synthesized a model prototype fluorescent polyamide and also studied its cellular uptake and intracellular localization by CLSM. The fluorescence results showed that these DNA minor-groove agents localized in organelles, mainly in the mitrochondria. These results provide useful information for efficient rational drug design. For example nuclear targeting of agents, which otherwise partition exclusively to the cytoplasm, can be affected and verified. As an extension of this work, this protocol will be applied to different forms of polyamides which have, for example, shown high binding affinity in 2:1 drug/DNA ratio.

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13. A solution of 200 mg (0.26 mmol) of **8** in 30 mL of methanol was hydrogenated over 100 mg of 10% Pd on charcoal for 2 h. The catalyst was removed by filtration, the filtrate concentrated to 15 mL and cooled to −40 °C and a solution of *N*-formylimidazole (prepared by 50 μL (1.3 mmol) of 98% formic acid in 1 mL of THF and 211 mg (1.3 mmol) of carbonyldiimidazole in 2 mL of THF stirred for 15 min) was

added. After 30 min at −40 °C the solution was concentrated in vacuo. Flash chromatography (3:7:0.3, MeOH/CH_2Cl_2/NH_4OH) afforded **10** (138 mg, 75% yield) as yellow crystals. Mp 110–112 °C. ^1H NMR (DMSO-d_6) δ 10.19 (s, 1H, exchanged with D_2O), 9.93 (s, 1H, exchanged with D_2O), 9.91 (s, 1H, exchanged with D_2O), 8.12 (s, 1H), 8.09 (t, $J=6.5$ Hz, 1H), 7.87 (d, 2H), 7.68 (d, 2H), 7.42–7.35 (m, 6H, one proton exchanged with D_2O), 7.20 (d, $J=6.0$ Hz, 1H), 7.18 (d, $J=6.0$ Hz, 1H), 7.01 (d, $J=6.0$ Hz, 1H), 6.91 (d, $J=6.0$ Hz, 1H), 6.80 (d, $J=6.0$ Hz, 1H), 4.30 (m, 4H), 4.20 (t, $J=6.5$ Hz, 1H), 3.84 (s, 3H), 3.78 (s, 3H), 3.15 (q, $J=6.5$ Hz, 2H), 2.45 (t, $J=6.5$ Hz, 2H), 2.22 (t, $J=6.5$ Hz, 2H), 2.12 (s, 6H), 1.86 (q, $J=6.5$ Hz, 2H), 1.60 (q, $J=6.5$ Hz, 2H). HRMS calcd for $C_{41}H_{48}N_9O_6$ 762.372 found 762.372 ($M^+ + H$, 100%).

14. Zeiss 510 NLO confocal/multiphoton microscope using the 40×1.3 NA standard oil objective, excitation wavelength 488 nm, 4.5 A output; beamsplitter HFT 488 and on LP 505 detection filter.

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