Novel Cationic Transport Agents for Oligonucleotide Delivery into Primary Leukemic Cells

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Novel cationic compounds forming complexes with oligodeoxyribonucleotides (ODNs) were prepared, and their ability to transport ODNs into cultured primary leukemic cells was tested. Two cationic porphyrin derivatives (**2** and **3**) were found to be at least 1 order of magnitude more efficient in this respect than commercially available agents. The ODN transporting capacity of novel compounds was dependent on the magnitude and the nature of their positive charges as well as on the porphyrin/ODN molar ratio. Porphyrin−ODN complexes were internalized into cells, and their dissociation was demonstrated by accumulation of fluorescein isothiocyanate−ODN fluorescence in the nucleus. Importantly, porphyrin **3** significantly protected complexed ODN against degradation and efficiently mediated the specific antisense effect on targeted v-Myb expression, resulting in reproducible growth inhibition of treated cells. Low toxicity, serum compatibility, and water solubility of porphyrin **3** make this compound a promising novel tool for modulation of gene expression in primary leukemic cells.

Introduction

Synthetic antisense oligodeoxyribonucleotides (ODNs) can be used as sequence-specific inhibitors of viral and cellular gene expression. The functional efficacy of ODNs critically depends on target sequence selection and effective intracellular concentration. The latter depends on the degree of cellular uptake, intracellular distribution, and stability of ODN in the cell environment.[1,2] The transport of ODNs across the cell membrane into a desired cellular compartment represents one of the major difficulties of the antisense strategy. Polyanionic and hydrophilic ODNs cannot diffuse through cell membranes but are actively taken up into cells by a combined action of receptor-mediated endocytosis and fluid-phase pinocytosis. However, both processes are rather inefficient and result in trapping significant amounts of ODNs in intracellular vesicles. Many different attempts have been made to improve cytoplasmic and/or nuclear delivery of ODNs and are summarized in recent reviews.[3−7] Despite a recent improvement in delivering ODNs to cells in culture, nucleic acid based therapy is still often limited by the poor penetration of the nucleic acids into the cytoplasm and nuclei of the cells. To date, the most widely used and successful approach involves complexation of ODNs with cationic lipids, which enhances both the cellular uptake and activity of ODNs.[8,9] However, our experiments as well as reports of others show that leukemic cells are resistant to cationic lipid−oligonucleotide delivery methods (unpublished data[10]). Therefore, there is a need to find a potent vehicle that would deliver ODNs into these primary leukemic cells. Porphyrin derivatives have recently been shown to display promising properties in this respect.[6,11,12]

The formation of complexes of water-soluble positively charged porphyrins with DNA has been a subject of many studies (ref 13 and references therein). Also, some expanded porphyrins, mainly sapphyrins, were shown to exhibit a strong but reversible interaction with DNA.[14−16] These properties of oligopyrrolic macrocycles as well as their lipophilic character inspired us to examine porphyrins as potentially useful tools in delivering ODNs across cellular membranes.

In this work, we attempted to synthesize and select cationic porphyrin-based compounds that would efficiently deliver antisense oligonucleotides into primary leukemic cells and mediate their inhibitory effect on the target gene expression. As a model system, primary leukemic cells transformed by the v-Myb oncoprotein and isolated from the bone marrow of leukemic chicks were chosen. Two cationic porphyrins, out of 11 related compounds, efficiently delivered v-myb antisense ODNs into model leukemic cells and mediated repression of the v-Myb oncoprotein in these cells.

Results

Synthesis and Characterization. The syntheses of the novel compounds (Figure 1) were performed using two strategies. First, bromomethylporphyrins were reacted with tertiary amines, giving the corresponding quaternary ammonium salts (agents **1** and **2**). The second strategy was based on coupling the carboxylated porphyrins and sapphyrins with a corresponding amino component via an active ester or acid chloride (agents **3**, **4**, **9**−**11**). The newly prepared porphyrins were characterized by ^{1}H and MS, UV−vis, and fluorescence spectra, as well as elemental analysis as described in Experimental Section. The compounds were dissolved in water or water−DMSO mixture (9:1), and their interactions with ODNs were measured.

To find out whether the designed agents can form stable but dynamic complexes with ODN and thus be further utilized for transport into cells, the binding studies of antisense v-myb ODN were performed. The

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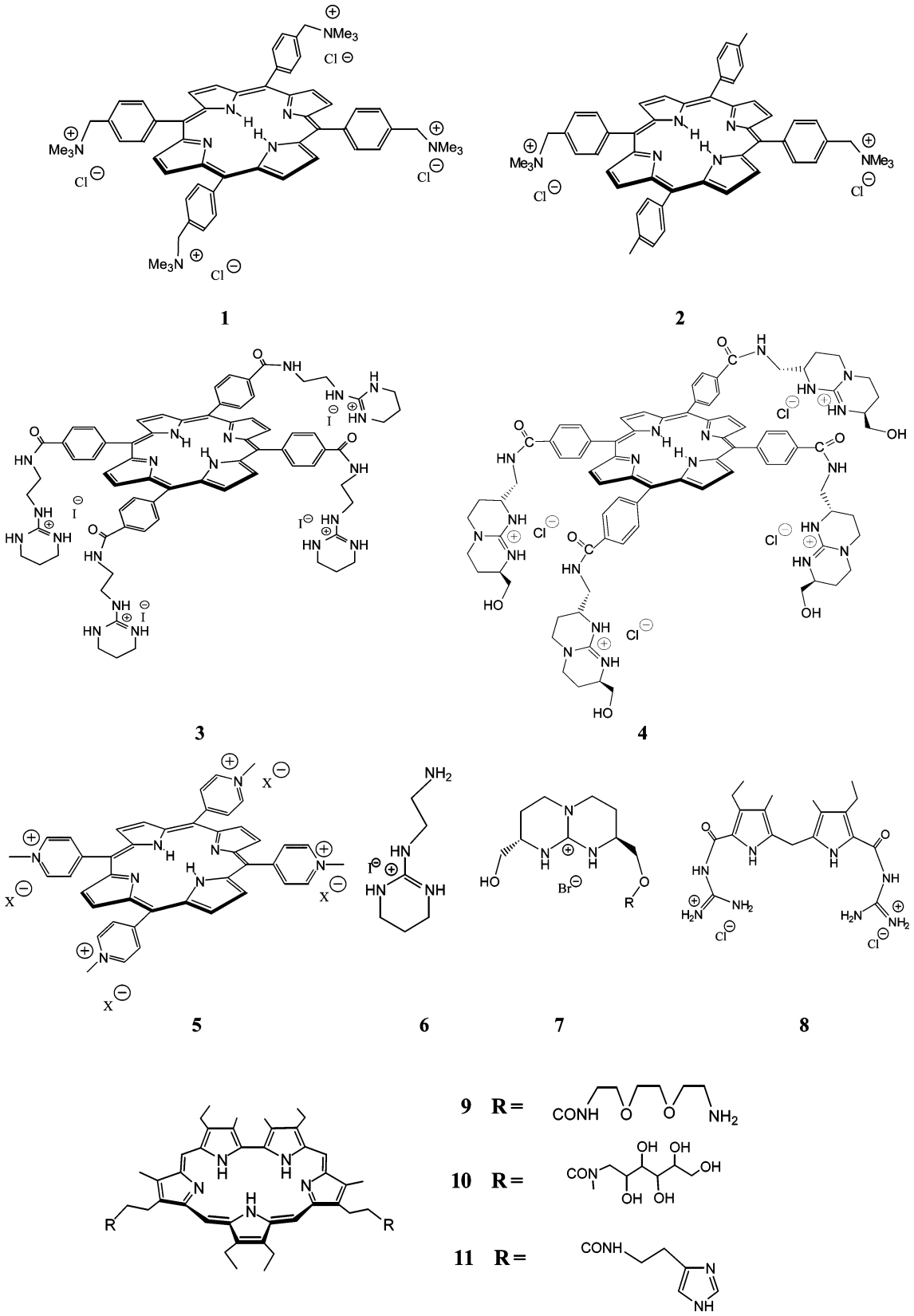

Figure 1. Structures of the tested oligonucleotide transport agents **1**−**11**.

interaction of cationic porphyrins with DNA results in the formation of organized porphyrin assemblies, which can be easily monitored by visible and circular dichroism spectroscopy. For comparison, the known delivery agent, tetrakis(pyridinium)porphyrin (agent **5**), was also included. All the tested compounds **1**−**11** changed their UV−vis spectra as a result of complex formation. The data obtained with porphyrins **1**−**5**, guanidine derivatives **6**−**8**, and water-soluble sapphyrins **9**−**11** confirmed earlier observations.[11,13−22] Because only compounds **2** and **3** were found to be active in ODN transport (see below), their absorption spectra are

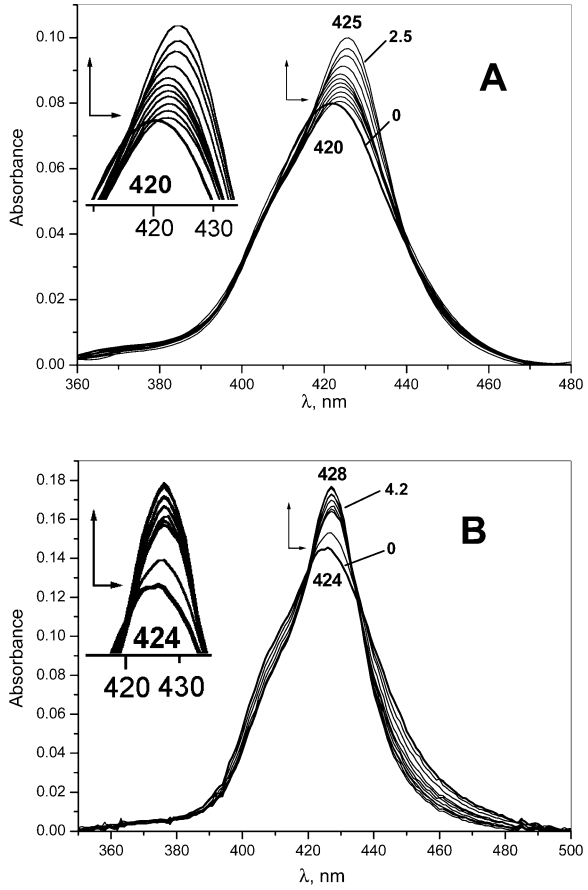

Figure 2. Absorption spectra of 2 μmol/L porphyrins **2** and **3** with increasing concentrations of v-myb antisense ODN. (A) Molar ratios of porphyrin **2**/ODN are 1:0, 1:0.05, 1:0.1, 1:0.2, 1:0.3, 1:0.35, 1:0.5, 1:1, 1:1.5, 1:2, 1:2.2, 1:2.5 (traces 0 and 2.5 indicate limiting values). (B) Molar ratios of porphyrin **3**/ODN are 1:0, 1:0.5, 1:1, 1:1.5, 1:2, 1:2.2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.2 (traces 0 and 4.2 indicate limiting values).

presented. The absorption of porphyrin **2** in the presence of increasing concentrations of ODN was measured in the range 380−460 nm (Soret band region) (Figure 2A). As a result of complex formation, the Soret band was red-shifted from the original value of 420 to 425 nm, with the clear isosbestic point at 416 nm, indicating a simple equilibrium between unbound and bound porphyrin **2**. The apparent association constant K_{ass} was determined to be 2.2×10^6 M^{-1} by the method described earlier.[13] Similar analysis yielded $K_{ass} = 2.68 \times 10^5$ M^{-1} for porphyrin **3** (Figure 2B). Porphyrins **2** and **3** also exhibited strong interaction with ds DNA (data not shown). The formation of ODN−porphyrin complexes was also demonstrated by HPLC chromatography (data not shown).

Oligonucleotide Uptake. The ODN uptake was assessed in primary leukemic cells, and the BM2 cell line was used for comparison. The radioactively labeled v-myb antisense ODN (0.25 μmol/L) was complexed with transport agents **1**−**11** (1−10 μmol/L) and incubated with cells. After incubation, the cell-associated radioactivity indicating cellular uptake was determined. The ODN uptake varied dramatically with the used transport agent (Table 1). The most efficient ODN uptake was detected with compounds **2** and **3** at the agent/ODN molar ratio of 10:1, while other agents displayed either weak or no effect. The agents **2** and **3** exhibited an order

Table 1. Transport Efficiency and Cytotoxicity of Novel Transport Agents

	relative ODN uptake[a]		cytotoxicity IC_{50} [b] (μmol/L)		
transport agents	PLC	BM2	PLC	CEF	BM2
1	4.2	2.5	12	10	10
2	67.5	16.7	>20	>20	>20
3	43.2	10.8	>20	>20	>20
4	13.4	NT[d]	>20	15	15
5	1.2	3.8	>20	15	NT[d]
6	3.2	5.1	>20	20	NT[d]
7	0.77	2.5	8.5	NT[d]	NT[d]
8	1.8	1.3	>20	15	>10
9	4.4	NT[d]	14	10	NT[d]
10	9.1	NT[d]	4.2	10	10
11	2.1	NT[d]	8.5	8.5	NT[d]
Cytofectin[c]	1	1.5	NT[d]	NT[d]	NT[d]
Fugene[c]	1.9	2.5	NT[d]	NT[d]	NT[d]
Oligofectamine[c]	3.4	3.7	NT[d]	NT[d]	NT[d]

[a] Uptake of ODN complexed to transport agents compared to free ODN. Primary leukemic cells (PLC) and BM2 cells were incubated for 2 h with 0.25 μmol/L of ^{32}P-labeled v-myb antisense ODN either free or complexed to cationic transport agents **1**−**11** at final concentrations of 2.5 μmol/L. After lysis, cell-associated radioactivity was determined as described in Experimental Section. Each value is the mean of at least three independent experiments. [b] The concentration of agents in which the relative cell viability reached 50% compared to that of the untreated control cells. The viability was determined by the MTT assay as described in Experimental Section. [c] Commercial transfection agents used for ODN delivery. [d] NT = not tested.

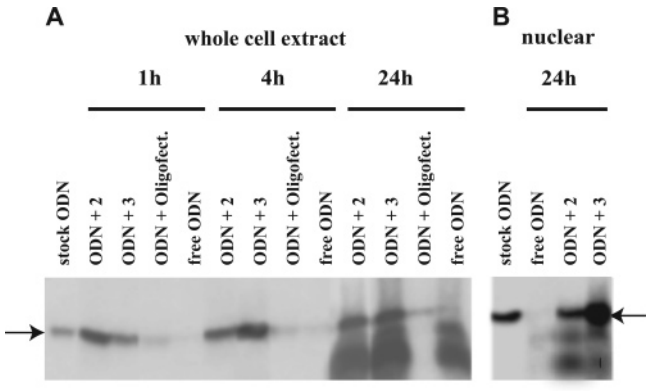

Figure 3. Intracellular stability of ODN. The whole-cell and nuclear fraction were prepared from primary leukemic cells treated for 1, 4, and 24 h with ^{32}P-end-labeled ODN in free or complexed form and then analyzed by denaturing 12% polyacrylamide gel electrophoresis followed by autoradiography: (A) whole-cell extract; (B) nuclear extract. The arrow indicates the mobility of full-length ODN. The control lane, stock ODN, contained 0.05 pmol of stock radiolabeled ODN used for application to the cells. Lanes ODN + 2 and ODN + 3 represent cells treated with complexes ODN with porphyrins **2** and **3** (molar ratios of 1:10). Lane free ODN represents cells treated with free ODN. Lane ODN + Oligofectamine represents cells treated with complexes of ODN with Oligofectamine.

of magnitude higher efficiency in ODN transport into primary cells than cationic lipids Cytofectin, FuGENE, and Oligofectamine (Table 1). The kinetics of ODN transport into cells was very fast, and the cell-associated radioactivity reached the maximum after 30 min of incubation. For more details see the Supporting Information. A systematic comparison of the cytotoxicity of transport agents was demonstrated by the IC_{50} value determined by a standard MTT assay (Table 1).

The integrity of intracellular ODN was studied in whole-cell and nuclear extracts by electrophoretic analysis (Figure 3). In whole-cell extracts, agents **2** and **3**

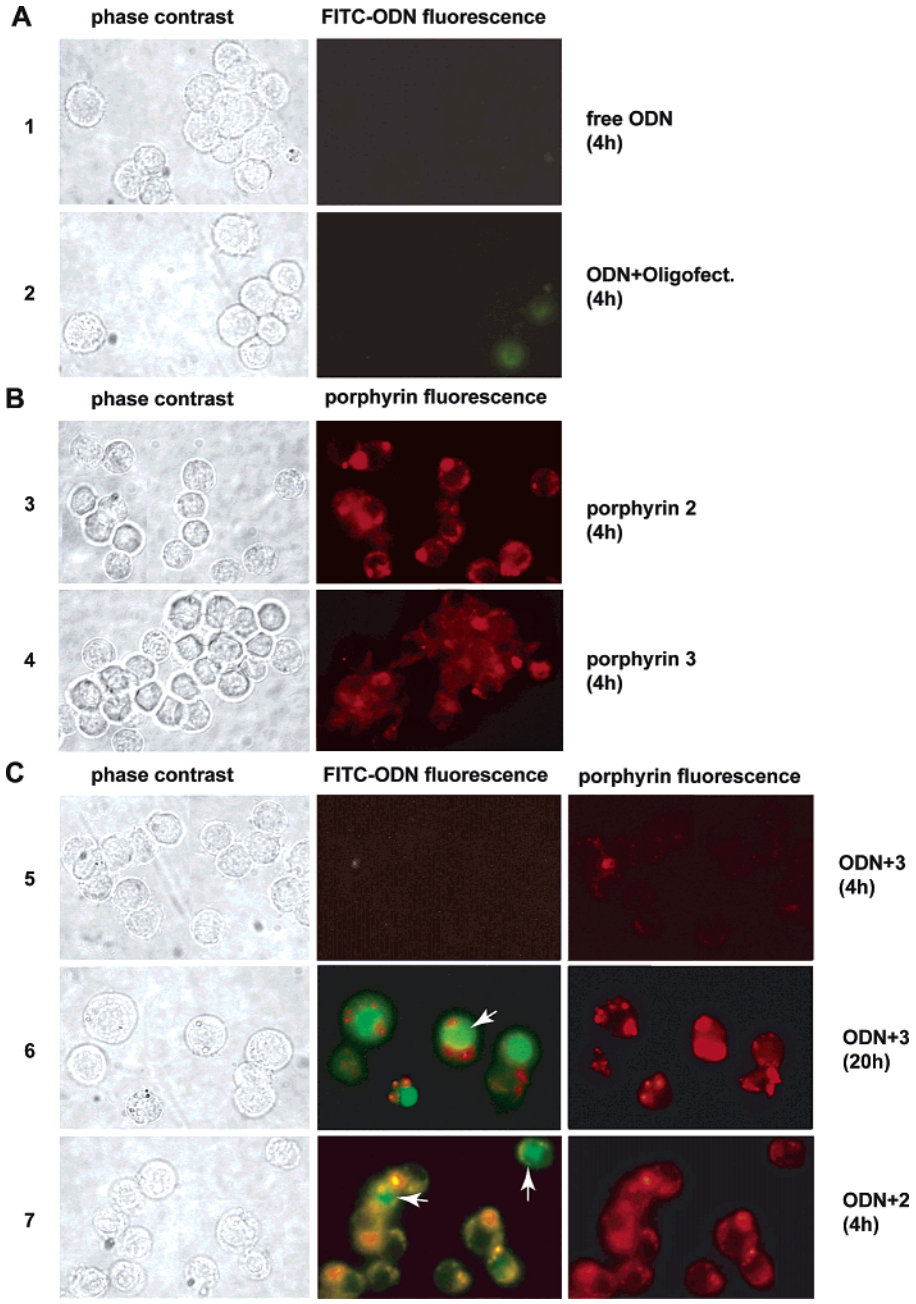

Figure 4. Intracellular uptake of FITC−ODN, porphyrins, and their complexes in live primary leukemic cells. After incubation for indicated time periods, the cells were examined live under a fluorescent microscope using either phase contrast or a filter for green FITC−ODN fluorescence or a filter for red porphyrin fluorescence. The oligonucleotide accumulation (green fluorescence) within the nuclei of the cells is indicated by white arrows. Large porphyrin assemblies are visible, even with an FITC−ODN filter, as orange spots.

protected radiolabeled ODN completely against nucleases within the first 4 h after the complex administration. A significant proportion of undegraded ODN was detectable even after 24 h (Figure 3A), while at that time only degradation products of free ODN were detected. In the nuclear fraction, the ODN was also notably stabilized (Figure 3B).

To monitor the subcellular localization and intracellular trafficking of ODN, primary leukemic cells were incubated with fluorescein isothiocyanate (FITC) labeled v-myb antisense ODN either free or complexed with agents **2**, **3**, or Oligofectamine. The cells were analyzed at different time points by fluorescence microscopy. Between 4 and 24 h after ODN addition, no fluorescence was observed on cells incubated with free ODN and only a faint signal was visible on cells exposed to Oligofectamine complexes in serum-free media (Figure 4A, panels 1 and 2; some data not shown). Because porphyrins upon excitation emit red fluorescence, their association with cells could be easily monitored (Figure 4B, panels 3 and 4). However, when porphyrins bind to the phosphate backbone of ODN, their molecules form extended aggregates on the ODN surface. The aggregation process, depending on the side chains of the porphyrin, is accompanied by considerable reduction of fluorescence.[17,30] Similarly, the fluorescence of FITC−ODN is effectively quenched by bound porphyrins.[11] Thus, the formation of porphyrin−ODN complexes results in an extinction of fluorescence, while dissociation of complexes is accompanied by the fluorescence reap-

pearance. Indeed, porphyrin **3**−FITC−ODN complexes were hardly detectable 4 h after their addition to cells (Figure 4C, panels 5), whereas 8−20 h later the complexes dissociated and ODN accumulated within the nuclei of almost 80% of the treated cells and the free porphyrin moiety in the cytoplasm in vesicular structures (Figure 4C, panels 6). At that stage, the majority of investigated cells were alive (92−95%) and able to exclude propidium iodide or trypan blue counterstain, thus demonstrating that nuclear accumulation of fluorescein-labeled ODN is not related to the cell death. Interestingly, porphyrins **2** and **3** differed in their ability to dissociate from fluorescent ODN over time (panels 5 and 7).

Antisense Inhibition of the v-myb Expression. Prior to tissue culture experiments, the most efficient antisense v-myb ODN was selected in transcription/translation cell-free systems (unpublished). Primary leukemic cells, synthesizing high amounts of the v-Myb oncoprotein, were then treated for 6−24 h with free anti-myb ODN (1 μmol/L), free porphyrin **3** (10 μmol/L), or anti-myb ODN−porphyrin complexes at a molar ratio of 1:10. As controls, untreated leukemic cells were used. Following treatment, cells were harvested for RNA and protein and subjected to Northern and Western blot analyses. Northern blot analysis revealed a 2.5-fold reduction of v-myb mRNA synthesis after the treatment with complexes of anti-myb ODN−porphyrin **3** (AS+3) for 20 h. The porphyrin alone, free anti-myb ODN, and porphyrin **3** complexes with scrambled ODN or inverted anti-myb ODN (SCR+3, INV+3, respectively) displayed no inhibitory effects (Figure 5A). AS+3 treatment resulted in a moderate reduction of v-myb mRNA already 12 h after addition of complexes to the cells. The inhibition reached a maximum at 20 h (Figure 5A) and returned to the normal level 32 h after the treatment (data not shown). The levels of nontargeted C/EBPβ and GAPDH mRNAs were not affected. In parallel with v-myb mRNA, the v-Myb protein level was also significantly reduced (to 30%) by AS+3 but not by control ODNs (Figure 5B). The protein content of each sample on a blot was verified by staining with Ponceau S (not shown). Reprobing the Western blot with polyclonal anti-MAV antibody or anti-CEBP/β antibodies (Figure 5B) clearly demonstrated that the synthesis of other proteins has not been affected by the AS+3 treatment. In addition, the reduction of v-Myb expression was related to the growth inhibition of cells treated with antisense ODN but not with control ODN or porphyrin (Figure 5C).

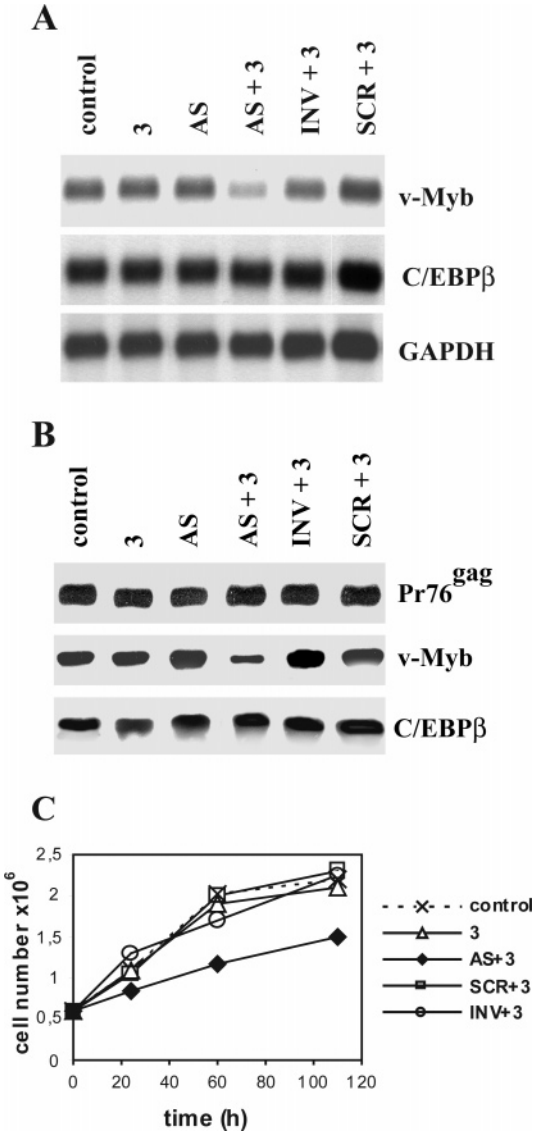

Figure 5. Specific inhibition of v-myb expression in primary leukemic cells. (A) v-myb, C/EBPβ, and GAPDH mRNA expression levels. Cells treated with ODN in free or complexed form with porphyrin **3** were harvested, and 5 μg of isolated total RNA was subjected to Northern blot analysis as described in Experimental Section. The membrane was sequentially hybridized with v-myb, C/EBPβ, and GAPDH (loading control) probes. (B) Western blot analysis of v-Myb and control proteins. The cell lysates from 2 × 10^5 cells were analyzed by SDS−PAGE electrophoresis and transferred to nitrocellulose membranes. Blots were cut into sections and incubated with antibodies specific for v-Myb (target) or antibody recognizing the gag polyprotein Pr^{76} (protein loading control). To demonstrate the specificity of the antisense effect, the same membrane was also probed with anti-C/EBPβ antibody. The lanes are defined as follows: (control) untreated cells; (**3**) cells treated with 5 μmol/L porphyrin **3**; (AS) cells treated with 0.5 μmol/L free v-myb antisense ODN; (AS+3) cells treated with antisense ODN complexed to porphyrin **3** (1:10); (INV+3 and SCR+3) cells treated with inverted antisense ODN and scrambled ODN complexed to **3**, respectively. (C) The cell growth inhibition with v-myb antisense ODN−porphyrin **3** complexes. The cells were treated every second day as described in Experimental Section, and the numbers of viable cells were determined on the day following the treatment. The cumulative cell numbers of one representative experiment are shown.

Discussion

Because the standard transfection agents fail to mediate the transport of ODNs into primary leukemic cells, our intention was to develop a delivery system for these cells. Novel cationic compounds were designed to combine a number of properties important for delivery of DNA into cells, i.e., relatively stable but reversible interaction with DNA, neutralization of DNA charge, solubility in water, lipophilicity, and protection of bound DNA against enzymatic degradation. With respect to these criteria, 11 compounds, **1**−**11**, were selected (Figure 1) and examined as delivery agents for antisense ODN. These compounds were based on porphyrin, sapphyrin, or guanidine derivatives and displayed different degrees of lipophilicity, endowing them with a

potential to cross the cytoplasmic membrane. The attention was focused on the number, location, and distribution of positive charges on molecules. Porphyrins **1** and **2** contain four and two positively charged quaternary ammonium groups, respectively, whose binding strength to ODNs is determined by the Coulombic attraction. The macrocycles substituted with monocyclic guanidine (**3**) and bicyclic guanidine (**4**) were compared for transport efficacy with the parental guanidine derivatives (**6**−**8**) carrying positive charges on monocyclic guanidine (**6**), bicyclic guanidine (**7**), or acylguanidine (**8**). They are known to selectively bind phosphates, and from a binding point of view, they also exhibit hydrogen bonding in addition to Coulombic interactions. Sapphyrin derivatives **9**−**11** are monoprotonated on the oligopyrrolic ring at pH 5−8 and form reversible complexes with ODNs. Moreover, sapphyrins have recently been shown to selectively accumulate within the cancer tissue.[23] It was revealed that the number and the nature of positive charges as well as their location were quite critical for the ODN transport efficacy of the tested compounds. Porphyrins with positive charges localized on the periphery (side chains of a macrocycle) were quite efficient in ODN transport (**2** and **3**), whereas sapphyrin derivatives with positive charges on the macrocycle ring were inefficient (**9**−**11**). Even the presence of additional positive charges on the periphery of compound **9** did not improve ODN transport. Moreover, porphyrin **2** with two positive charges on the periphery seems to have a slightly better transporting ability than substituents with four positive charges (**1**, **3**−**5**). It indicates that a very delicate balance between the number and position of positive charges and the lipophilicity of the molecules determines the efficiency of the transport into a specific cell type.

In complexes with porphyrin **2** or **3**, the phosphodiester bonds of ODNs are partly protected against nucleases because significant amounts of full length ODNs were also demonstrated after 24 h of incubation not only in the cytoplasm but also in the nuclear fraction (Figure 3). Thus, it appears that porphyrins enable ODN to traverse the cellular and endosomal membranes and to accumulate within the nuclei. Nuclear localization of ODN was independently demonstrated by uptake studies employing ODN conjugated to FITC (Figure 4). A substantial fraction of cells exposed to complexes FITC−ODN−porphyrin showed nuclear accumulation of FITC−ODN. To bind to the target sequence, ODN should be capable of also forming complexes with nucleic acids. Although the formation of ternary complexes (porphyrin−ODN−mRNA) cannot be completely ruled out, we find it more likely that ODN−porphyrin dissociation precedes the ODN binding to target sequences because there is a correlation between the appearance of free FITC−ODN fluorescence and the antisense effect (12−24 h after the treatment) (Figures 4 and 5). The specific antisense effect of anti-v-myb ODN was observed at the reduced mRNA and protein levels and at the reduced growth rate of transformed monoblasts, whose proliferation rate depends on the level of the v-Myb oncoprotein (Figure 5 and unpublished results). These suspension cells are normally very resistant to various gene transfection and ODN delivery methods. It is possible, therefore, that the specific quality of their cellular membranes might account for this resistance. Accordingly, the commercially available porphyrin **5**, which has been proved as a potent delivery vehicle for bladder carcinoma cells,[11] was totally ineffective in our experiments with primary leukemic cells. Importantly, the transporting abilities of porphyrins **2** and **3** were confirmed not only for avian leukemic cells but also for the human leukemic cell line HL-60 (for details see Supporting Information), thus indicating the more general applicability of these compounds. Interestingly, these porphyrins were more efficient in leukemic cells growing in suspension than in adherent fibroblasts.

Conclusion

Two cationic porphyrins (**2** and **3**) were found to be highly efficient in mediating ODN intracytoplasmic uptake. Porphyrin−ODN complexes were internalized into cells, and their dissociation was demonstrated by accumulation of FITC−ODN fluorescence in the nuclei and red porphyrin fluorescence in the cytoplasm or cytoplasmic vesicles. The biological activity of the v-myb antisense ODN delivered into v-myb oncogene-transformed leukemic cells via complexation with porphyrin **3** was demonstrated by specific suppression of v-myb expression and growth inhibition of treated cells whose cycling is dependent on the level of oncogene expression. Owing to water solubility, low toxicity, serum compatibility, and nuclease protective ability, cationic porphyrins **2** and **3** can be regarded as excellent starting substances with a potential for further development of chemical vehicles applicable to primary leukemic cells and/or primary hematopoietic cells.

Experimental Section

Instruments. UV spectra of tested compounds were obtained and their complexation properties were measured with a Varian CARY 400 SCAN UV−vis spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were obtained with a Varian Gemini 300 HC (300.077 MHz for ^{1}H NMR and 75.460 MHz for ^{13}C NMR spectra) at 23 °C in D_2O or hexadeuteriodimethyl sulfoxide. Chemical shifts of signals are presented in ppm. Mass spectra were recorded with a VG Analytical ZAB-EQ spectrometer. HPLC separations were performed in a Perkin-Elmer-200 instrument with UV detection at 256 nm. The injection volume was 50 μL, and the column was C8 and C18 reverse-phase (Merck), 250 mm × 4 mm, 5 μm, with methanol−water and and acetonitrile−water eluents.

Preparation of Synthetic Carriers. For preparation of transport agents, we applied two general strategies. The first was based on quarternization of amines with bromomethyl-substituted porphyrins. The second applies to carboxylated macrocycles for formation of an amide bond in order to couple positively charged groups on the periphery of a macrocycle. We summarize the preparation of transport agents **1**−**11**. Porphyrin **1** was prepared as reported earlier.[13]

5,15-Bis(α-trimethylammonio-*p*-tolyl)-10,20-(*p*-tolyl)-porphyrin Dibromide Salt (2). A 0.1 mmol sample of 5,15-bis(4-bromomethylphenyl)-10,20-(*p*-tolyl)porphyrin (for preparation, see ref 17) was suspended in 10 mL of ethanol solution of trimethylamine (31−35% solution, Fluka) in a sealed tube. The resulting reaction mixture was heated to 85 °C for 8 h with vigorous stirring. After the mixture was cooled to room temperature, the precipitated product was filtered off, washed with a small amount of ethanol/THF (1:1) and dried. Product **2** was obtained in 96% yield. Porphyrin **2** was characterized by ^{1}H NMR, MS spectrometry, and elemental analysis. ^{1}H NMR (DMSO-d_6): δ 8.94 (s, 4H, β-pyrrole), 8.86 (s, 4H, β-pyrrole), 8.39 (m, 4H, *p*-tolyl), 8.11 (m, 4H, phenyl-CH_2), 7.98 (m, 4H, *p*-tolyl), 7.65 (m, 4H, phenyl-CH_2), 4.97 (d, 4H, CH_2N^+),

3.32 (s, 18H, CH_3N^+), 2.71 (s, 6H, CH_3), 2.98 (bs, 2H, NH-pyrrole). Anal. ($C_{54}H_{54}N_6Br_2$) C, H, N. MS (FAB, MALDI), *m*/*z*: 947 [MH^+], 393 [M − 2Br]/2. UV−vis (water/methanol 4:1): 414 (Soret, $\epsilon = 1.6 \times 10^5$ M^{-1} cm^{-1}), 522, 566, 600, 650 nm.

5,10,15,20-Tetrakis{4-[(2′-amino-1′,4′,5′,6′-tetrahydropyrimidine)-2-(ethylamino)carbonyl]phenyl}-21*H*-23*H*-porphine Tetrahydroiodide (3). The synthesis of compound **3** is based on the coupling of activated porphyrin tetraacid with the amino component under standard peptide bond forming conditions. The synthetic protocol describes the reaction of the aminoethyl derivative of cyclic guanidine, 2-(2′-aminoethyl)-amino-3′,4′,5′,6′-tetrahydropyrimidine,[18,19] with an active ester or acyl chloride of tetrakis(4-carboxyphenyl)porphyrin, yielding the desired porphyrin **3**. Thus, a solution of the active ester in dry dichloromethane (generated with diisopropylcarbodiimide and 1-hydroxybenzotriazole in DMF, 10 mL) was generated from 5,10,15,20-tetrakis[4-carboxyphenyl]-21*H*-23*H*-porphine (79 mg, 0.1 mmol). The product was obtained by coupling porphyrin tetrakis(active ester) with the aminoethyl derivative of cyclic guanidine [2-(2′-aminoethyl)amino-3′,4′,5′,6′-tetrahydropyrimidine (216 mg, 0.8 mmol)] in a dry 1,2-dimethoxyethane/chloroform mixture (1:1, 50 mL) with the addition of 0.5 mL of triethylamine as a base and 10 mg of 4-(dimethylamino)pyridine. The reaction mixture was stirred for 2 days at room temperature under argon. Alternatively, the porphyrin tetra(acid chloride) (generated with oxalyl chloride in dry dichloromethane with one drop of DMF at room temperature, under argon for 1 h) was used for reaction with the above-mentioned amino component. The reaction mixture was evaporated and the product was precipitated from THF/$CHCl_3$ (1:1, 10 mL). The product was filtered off and washed with dichloromethane. The product **3** (124 mg, 69%) was obtained as a brown-violet powder. The analytical sample was prepared by semipreparative reverse-phase HPLC with methanol/water 1:1 as a mobile phase. ^{1}H NMR (300 MHz, DMSO-d_6): δ −2.98 (s, 2H, NH pyrrole), 1.82 (q, 8H), 2.89 (t, 8H), 3.03 (m, 8H), 3.20 (m, 8 H), 3.54 (m, 8H), 7.15 (m, 4H), 7.27 (d, 4H), 7.63 (d, 4H), 8.09 (m, 8H), 8.32 (m, 8H), 8.82 (bs, 8H), 9.13 (bs, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 19.36, 20.69, 38.31, 45.17, 48.76, 124.97, 125.69, 127.86, 128.56, 133.84, 152.59, 171.51. MS (MALDI TOF), *m*/*z*: 1799 [MH^+]. Anal. ($C_{72}H_{82}I_4N_{20}O_4$) C, H, N.

Porphyrin **4** was prepared by reaction of the chloride of porphyrintetracarboxylic acid with aminomethyl-substituted bicyclic guanidine.[20] Porphyrin **5** was purchased from Fluka. Compound **6** was prepared as described earlier.[19] Compound **7** was reported previously.[21] Compound **8** was prepared by reaction of the corresponding dipyrrylmethane dimethyl ester with guanidine hydrochloride under basic conditions, leading to acylguanidine derivatives according to the procedure reported by Schmuck.[22] Water-soluble sapphyrins **9**−**11** were prepared by coupling the sapphyrin bis-acid with the corresponding amino component according to a procedure reported earlier.[23]

Cells. Ex vivo cultures of primary leukemic cells, v-myb-transformed monoblasts, were prepared from the bone marrow of 20-day-old chicks infected with a v-myb retrovirus as described previously.[24] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (Sebak, Switzerland), 2% chicken serum (Sigma), 20 mM HEPES, pH 7.3, 100 units/mL penicillin/streptomycin (Gibco-BRL), and further additives.[25] Cultures were maintained at 41 °C in a humidified 5% CO_2 incubator. Chicken embryo fibroblasts (CEF) and the v-Myb-transformed cell line BM2[26] were grown at 37 °C in the same medium.

ODNs and Labeling. The sequences of the phosphodiester oligonucleotides used in this study were the following: antisense v-myb (AS), 5′-AAT AAT TCT ATC TTC CTC-3′, complementary to the v-myb oncogene sequence at positions 422−439 nt (NCBI accession number J02012); control scrambled ODN (SCR), 5′-CCA CAC TTG CAC CTA CT-3′; and inverted antisense ODN (INV), 5′-CTC CTT CTA TCT TAA TAA-3′. Radioactive labeling of 5′-ends was performed by phosphorylation as described.[27,28]

Quantitation of ODN Uptake. Primary leukemic cells or BM2 cells were seeded in 24-well plates at 1×10^6 cells/well in 0.4 mL of complete growth medium containing 10% serum 1 day prior to transport experiments. Complexes of 0.25 μM ^{32}P-end-labeled v-myb antisense ODN with novel transport agents (in various ODN/transport agent molar ratio) were formed in 100 μL of serum-free medium at room temperature for 30 min. Then, the complexes or free control ODNs were added to the cells and incubated with the ODN 0.5−24 h at 37 °C or 41 °C depending on the cell type. Simultaneously, complexes of ^{32}P-end-labeled ODN with optimized volumes of FuGENE 6 (Roche Diagnostics), Cytofectin GSV (Glen Research), and Oligofectamine (Invitrogen) were formed in accordance with the manufacturers' instructions. After incubation, the cells were harvested and ODN bound to the cell membrane was removed by the acid-salt elution method.[28] To determine cell-associated radioactivity, cell pellets were lysed in proteinase K lysis buffer[28] and analyzed by counting in a liquid scintillation cocktail Ecolume (ICN Biomedical) by a liquid scintillation counter (Beckman Instruments).

Detection of Intracellular ODN. For the ODN stability studies, cellular lysates were prepared from the cells treated for 1, 4, and 24 h with ^{32}P-end-labeled ODN in free or complex form as described above in proteinase K lysis buffer, then extracted with phenol, precipitated, and analyzed by 12% denaturing gel electrophoresis. Gels were dried and submitted to autoradiography using X-ray film (Eastman Kodak). The nuclear fraction from cells treated with radioactively labeled ODN was prepared using a protocol based on an NP-40 lysis procedure.[29]

Fluorescence Microscopy. The 1×10^6 v-myb-transformed monoblasts were plated in a 24-well culture vessel in complete DMEM media without phenol red. Next day, the cells were treated with 1 μM 5′-fluorescein-labeled v-myb antisense oligo (AS) in either free or complexed form with porphyrin **2** or **3** (5−10 μM). In parallel, ODN was transfected with Oligofectamine. After the indicated times, cells were harvested, washed twice in PBS with 1% BSA, covered with coverslips, and photographed live under a fluorescence microscope (Leitz DM IRB, Leica) with a digital camera (Olympus) through a 100× oil immersion objective. Two band-pass filters were used: BP 450−490 nm for FITC (fluorescein isothiocyanate) and BP 515−560 nm for porphyrins.

Western Blot Analysis. Lysates derived from 2×10^5 cells treated with 0.5 μM ODN and 5 μM porphyrin **3** were resolved by 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) followed by electrophoretic transfer to Hybond ECL nitrocellulose membrane (Amersham). Blots were cut into sections and, after blocking for 1 h in 2% BSA-TBST, were incubated for 1 h either with the polyclonal anti-Myb antibody or with the polyclonal antibody against MAV reacting preferentially with the gag polyprotein Pr^{76} (Korb, unpublished results) to verify equal protein loading. Subsequently, the blot was washed 3 times with washing buffer and incubated with the secondary antibody (goat anti-rabbit conjugated with horseradish peroxidase, Amersham) for 1 h. The membrane was then washed another 5 times with washing buffer, developed in ECL reagents (Amersham), and exposed to X-ray film (Eastman Kodak). To demonstrate the specificity of the antisense effect, the same membrane was also probed with anti C/EBPβ antibody to detect the level of the endogenous control C/EBPβ protein.

Northern Blot Analysis. The total RNA was isolated with the RNAwiz reagent according to the manufacturers' direction (Ambion) from 2×10^6 cells treated with free or complexed ODN for 20 h. RNA (5 μg) was fractionated on a 1% agarose gel containing formaldehyde, transferred by capillary action to nylon membrane GeneScreen (NEN), fixed to the membrane by UV cross-link, and sequentially hybridized with specific probes. The following probes were used: 0.8 kb Xba-EcoRI fragment of chicken v-myb, 609 bp Pst fragment of chicken C/EBPβ, and full-length cDNA of chicken D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The probes were labeled with [^{32}P]-dCTP (Amersham) by nick-translation and

hybridized overnight in ULTRAhyb (Ambion) at 43 °C. Filters were washed and exposed to BioMax MS film (Eastman Kodak) with an intensifying screen. The levels of mRNAs were quantified in an instant imager (Packard) and normalized to the GAPDH mRNA level.

Cytotoxicity Studies. The cytotoxicity was assessed by measurements of the IC_{50} value in a standard MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue indicator dye) assay (Promega). The cytotoxicity was determined by comparing the amount of MTT reduced by the cells treated with transport agents to that reduced by control untreated cells (100%).

Cell Proliferation Study. Primary leukemic cells seeded in a 24-well plate at 0.6×10^6 cells/well in 0.4 mL of complete growth media (supplemented with 5% serum) were incubated every second day for 7 h with complexes of 0.5 μM ODN and 3−5 μM porphyrin **3**. After incubation, the cells were washed and seeded in fresh media. On the day following the treatment, viable cells (excluding trypan blue dye) were counted and replated for continuation at the same cell density.

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Supporting Information Available: Binding properties of transport agents from UV−vis titration, comparison of transport abilities of novel cationic porhyrins **2** and **3** and cationic lipids (Figure 1), effect of novel cationic porphyrins on the cell viability (Figure 2), fluorescence microscopic images demonstrating the intracellular uptake of FITC−ODN−porphyrin complexes in the human leukemic cell line HL-60 (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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