Antagonism of Immunostimulatory CpG-Oligodeoxynucleotides by 4-Aminoquinolines and Other Weak Bases: Mechanistic Studies

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ABSTRACT

Oligodeoxynucleotides with unmethylated CpG motifs are immunostimulatory. Chloroquine and a number of structural analogs specifically and powerfully inhibit this effect at nanomolar concentrations. We explored the mechanism of this inhibition, with 4-aminoquinolines, quinacrine, 9-aminoacridines, and novel dibasic analogs, many of which are fluorescent. WEHI 231 murine B-lymphoma cells accumulated analogs up to a concentration several hundredfold higher than the medium. Uptake was rapid, nonsaturable, reversible, and partially inhibited by monensin, an agent that collapses pH gradients within cells. Uptake did not correlate highly with efficacy as inhibitors of CpG-oligodeoxynucleotide (ODN)-induced effects, suggesting that analogs act by a specific action. Confocal microscopy revealed analogs concentrating in large peripheral organelles. CpG-ODN is taken up by cells into acidified, small, perinuclear vesicles. This uptake is thought to be necessary for immunostimulatory activity. Cellular uptake of fluorescent CpG-ODN was not inhibited by the analogs. The pH of intracellular CpG-ODN (6.4) was not affected by analogs at the concentration required for inhibition, but pH was increased by higher concentrations. UV spectroscopy revealed no binding of analogs to CpG-ODN. Nuclear Overhauser effect spectroscopy revealed that an analog bound to phosphatidylcholine vesicles, with the ring structure of the analog buried within the lipid and the side chain facing the aqueous environment. We conclude that the analogs do not inhibit the action of CpG-ODN by preventing the uptake or acidification of CpG-ODN. It seems more likely that the analogs inhibit the efficacy of CpG-ODN by a specific action within acidified vesicles, possibly at the interface of a phospholipid membrane.

Bacterial DNA stimulates the polyclonal proliferation of B cells and the production of cytokines by monocytes and other cells (Messina et al., 1991; Krieg et al., 1995; Ballas et al., 1996; Klinman et al., 1996; Krieg, 1996; Pisetsky, 1996; Yi et al., 1996; Macfarlane et al., 1997). This immune stimulation is attributable to unmethylated CpG motifs that are more common in bacterial DNA than in vertebrate DNA. Selected single-stranded oligodeoxynucleotides having an unmethylated CpG motif (CpG-ODN) also are immunostimulatory and mimic bacterial DNA, especially when synthesized with a nuclease-resistant phosphorothioate backbone (Krieg et al., 1995).

How cells detect the presence of CpG-ODN and bacterial DNA is unknown. Like other DNA molecules, CpG-ODN are taken up into acidified organelles within cells (Tonkinson and Stein, 1994; Beltinger et al., 1995; Gray et al., 1997), and this internalization of CpG-ODN seems to be required for immunostimulatory activity (Krieg et al., 1995; Manzel and Macfarlane, 1999).

We have reported that the antimalarials chloroquine, quinacrine, and hydroxychloroquine (Plaquinil), and a series of their structural analogs specifically and powerfully inhibit immune stimulation by bacterial DNA and CpG-ODN (Macfarlane and Manzel, 1998; Strekowski et al., 1999). These results are of interest because some of these compounds (especially hydroxychloroquine) induce remissions of rheumatoid arthritis and systemic lupus erythematosus (Fox, 1993; Wallace, 1994), and may be useful in the prevention of graft-versus-host disease in bone marrow transplant recipients (Schultz and Gilman, 1997). The mechanism of this remittive action is not known.

The analogs that inhibit CpG-ODN-induced effects are all weak bases. Such bases partition into acidified vesicles within cells (Duve et al., 1974). Chloroquine has been well studied in this regard: it concentrates in lysosomes and col-

ABBREVIATIONS: CpG-ODN, oligodeoxynucleotide containing one or more immunostimulatory CpG sequences; NOESY, nuclear Overhauser effect spectroscopy; DMPC, 1,2-dimyrstoyl-sn-glycero-3-phosphocholine.
lapses the pH gradient (Ohkuma and Poole, 1981), leading to the hypothesis that organelle acidification is required for intracellular recognition of CpG-ODN (Yi et al., 1998).

Both chloroquine and quinacrine as well as analogs bind to duplex DNA by intercalation in a relatively sequence-non-specific fashion (Wilson, 1998). Compounds with only two fused aromatic rings (such as chloroquine) are poor intercalators and bind to DNA less avidly than quinacrine and other related tricyclic systems. Protonation of the terminal amino group and the ring nitrogen in chloroquine and quinacrine significantly enhances their binding to DNA, and both compounds bind to duplex DNA much more tightly at low salt concentrations that at higher salt.

In this article, we explore how selected analogs are taken up into cells and whether they inhibit the uptake of CpG-ODN or influence the pH of the vesicles into which CpG-ODN concentrate. We also demonstrate that (at acid pH) analogs do not interact with single-stranded DNA (by UV spectrosopy), but do partition into the lipid layer of small unilamellar phospholipid vesicles [by an NMR technique based on nuclear Overhauser effect spectroscopy (NOESY)].

We conclude that the analogs do not block the action of CpG-ODN by influencing the cellular uptake or acidification of CpG-ODN, but rather by a specific mechanism, possibly involving an interaction at a phospholipid surface in an acidic environment.

**Experimental Procedures**

**Cell Culture and Analogs.** WEHI 231 murine B-lymphoma cells were maintained at log phase in medium as described previously (Macfarlane and Manzel, 1998). The structures of the analogs we used are shown in Fig. 1. The pyrimidine derivatives 215, 227, 228, and 231 (Strekowski et al., 1991) and 2-naphthylquinolines 91, 267, 350, and 352 (Strekowski et al., 1992, 1994) were prepared with general synthetic methodologies developed for similar compounds. Synthetic details will be reported elsewhere. All compounds were at least 98% pure as indicated by elemental analysis and analysis of their proton NMR spectra. The dimeric compounds 322 and 329 have

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**Fig. 1.** Structures of analogs used. The numbers are those in the first column of Table 1.
been described (Ismail et al., 1996). The other analogs have been previously reported (Macfarlane and Manzel, 1998) and were kindly supplied by Dr. Jill Johnson from the National Cancer Institute, or purchased (see Materials). The analogs were dissolved in dimethyl sulfoxide (10 mM) and thereafter diluted in culture medium to the desired concentration.

Efficacy of analogs as inhibitors of CpG-ODN-induced responses was determined with [3H]thymidine uptake by WEHI 231 cells treated for 24 h with or without antisurface IgM (kills cells by apoptosis), ODN1760 (protects against this cell killing), and a range of concentrations of analogs (reverses protection by CpG-ODN) as previously described in detail in Macfarlane and Manzel (1998). At the concentration used, compounds were nontoxic to cells and they did not directly inhibit thymidine uptake.

Cellular uptake of analogs was estimated by fluorescence. WEHI 231 cells (5 × 10⁶ cells/ml) were incubated in RPMI 1640 without phenol red (Life Technologies, Rockville, MD) with the indicated concentration of analog for 30 min. The cells were pelleted by centrifugation, and the fluorescence of the supernatant was measured immediately after the addition of analogs or monensin (10 μM). The pH of the ODN-containing compartment was determined from the increase in the fluorescence that occurred when monensin was added, assuming that monensin equilibrates this compartment with the medium (pH 7.4). We calibrated this assay with a standard curve of fluorescence of fluorescein-labeled ODN 1760 at a range of pH values, which yielded a pKₐ of 6.98 in 25 mM Tris acetate/150 mM NaCl buffer.

Confocal Microscopy of Live Cells. Preliminary experiments revealed that the Catrimox-14 extract contained essentially all of the cell-associated analog. The fluorescence intensity and spectra were determined with a Parrand manual spectrophotometer at three pH values (3.4, 7.4, 10.0). Subsequent measurements of fluorescence were performed at pH giving the highest yield (Table 1). Fluorescence intensity was converted to concentration with standard curves prepared by diluting each analog (0.1–5 μM) in Catrimox-14- and RPMI 1640-containing buffer.

**Uptake of CpG-ODN.** WEHI 231 cells were incubated with 5 μg/ml Texas Red-labeled ODN 1760. After incubation with the additions indicated, the cells were centrifuged and resuspended three times into Hanks’ balanced salt solution at 37°C to remove surface bound ODN, and then resuspended and fixed in 2% paraformaldehyde for analysis in the University of Iowa Flow Cytometry Facility. Ten thousand events were recorded with a Coulter EPICS 753 at 488/600 nm, gating out dead cells. The mean cellular fluorescence was recorded.

The pH of CpG-ODN within cells was estimated by taking advantage of the fact that acidification suppresses the fluorescence of fluorescein (Okhuma and Poole, 1978; Tonkinson and Stein, 1994). WEHI 231 cells (1 × 10⁶ cells/ml) were incubated with fluorescein- labeled ODN 1760 (5 μg/ml; 2 h), and washed as described above. The fluorescence of the live cells was determined by flow cytometry immediately after the addition of analogs or monensin (10 μM). The pH of the ODN-containing compartment was determined from the increase in the fluorescence that occurred when monensin was added, assuming that monensin equilibrates this compartment with the medium (pH 7.4). We calibrated this assay with a standard curve of fluorescence of fluorescein-labeled ODN 1760 at a range of pH values, which yielded a pKₐ of 6.98 in 25 mM Tris acetate/150 mM NaCl buffer.

**TABLE 1**

<table>
<thead>
<tr>
<th>Our No., Source</th>
<th>Fluorescence versus Quinacrine</th>
<th>Name</th>
<th>50% Efficacy</th>
<th>Cellular Uptake Ratio</th>
<th>Molecules/Cell × 10⁻⁶ at 50% Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>329, d</td>
<td>1.2, b</td>
<td>Ism 16</td>
<td>14.5</td>
<td>147</td>
<td>1.28</td>
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<tr>
<td>24, a</td>
<td>0.7, n</td>
<td>10591-T2</td>
<td>30.2</td>
<td>161</td>
<td>2.92</td>
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<tr>
<td>17, a</td>
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<td>Quinacrine</td>
<td>10.2</td>
<td>601</td>
<td>3.69</td>
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<tr>
<td>25, b</td>
<td>0.7, a</td>
<td>10593-V2</td>
<td>19.1</td>
<td>438</td>
<td>5.03</td>
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<tr>
<td>322, d</td>
<td>0.6, b</td>
<td>Ism 9</td>
<td>105.0</td>
<td>86</td>
<td>5.47</td>
</tr>
<tr>
<td>29, b</td>
<td>1.7, n</td>
<td>30020-N/2</td>
<td>57.5</td>
<td>164</td>
<td>5.69</td>
</tr>
<tr>
<td>30, b</td>
<td>1.7, n</td>
<td>74602-V4</td>
<td>52.4</td>
<td>299</td>
<td>9.44</td>
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<tr>
<td>27, b</td>
<td>3.3, a</td>
<td>50018-L/1</td>
<td>60.3</td>
<td>583</td>
<td>21.1</td>
</tr>
<tr>
<td>19, a</td>
<td>0.8, pH 2.0</td>
<td>Quinacrine</td>
<td>23,000</td>
<td>24</td>
<td>331.1</td>
</tr>
<tr>
<td>267, c</td>
<td>2.2, a</td>
<td>OZ-66</td>
<td>&gt;3000</td>
<td>362</td>
<td>&gt;654</td>
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<tr>
<td>227, c</td>
<td>1.4, n</td>
<td>MC-241</td>
<td>&gt;3000</td>
<td>63</td>
<td>&gt;115</td>
</tr>
<tr>
<td>228, c</td>
<td>1.3, n</td>
<td>MC-132</td>
<td>&gt;3000</td>
<td>27</td>
<td>&gt;49</td>
</tr>
<tr>
<td>215, c</td>
<td>0.7, a</td>
<td>RW-15</td>
<td>&gt;3000</td>
<td>70</td>
<td>&gt;126</td>
</tr>
<tr>
<td>231, c</td>
<td>0.3, n</td>
<td>DH-77</td>
<td>&gt;3000</td>
<td>29</td>
<td>&gt;52</td>
</tr>
<tr>
<td>91, c</td>
<td>N.D.</td>
<td>LS-8</td>
<td>9.5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>350, c</td>
<td>N.D.</td>
<td>OZ-123</td>
<td>5.9</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>352, c</td>
<td>N.D.</td>
<td>MHQ-6</td>
<td>16.3</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>15, a</td>
<td>Nonfluorescent</td>
<td>Chloroquine</td>
<td>110.0</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>16, a</td>
<td>Nonfluorescent</td>
<td>Hydroxychloroquine</td>
<td>407</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>

N.D., not determined.
tional preparations. The compound 91 content in the 1,2-dimyristoryl-
sn-glyero-3-phosphocholine (DMPC) vesicles was limited to 8 mol% to avoid phase separation in the bilayer.

Phase-sensitive two-dimensional NOESY spectra of sonicated DMPC vesicles to which compound 91 was bound were obtained with a Varian Unity Plus spectrometer operating at a proton frequency of 500 MHz. The pulse sequence developed by States et al. (1982) was used for data acquisition. The probe temperature was set at 35°C for all experiments. An optimum spectral width of 4773.3 Hz was determined for the initial NOESY experiment and maintained for all subsequent acquisitions. An optimum balance of sensitivity and resolution was obtained by using 256 t1 increments, each consisting of 2048 data points that were signal-averaged over 128 scans with a relaxation delay of 2 s and 215-ms acquisition time while mixing times of 50 and 150 ms were used. Very similar NOESY spectra were obtained with both of the aforementioned mixing times. To minimize the possible appearance of artificial cross-peaks due to spin diffusion, NOESY spectra obtained with the 50-ms mixing time only are considered and presented herein. Suppression of the residual HOD signal was accomplished by saturation with the receiver at a power setting of 24 Hz [1/4(νFW90)]. Data processing was performed with the program VNMR supplied by Varian Associates Inc. (Lexington, MA). The free induction decays were zero-filled to 2K data points in the t1 dimension. The data presented as NOESY spectra were analyzed with a sinewell window function shifted by 60°. Linear prediction (Gray, 1990) was used to obtain the first two points in the flame ionization detectors and to extend the number of increments from 256 to 512 in the F1 dimension. With the algorithm of Brown (1995), baseline correction was applied to both dimensions after fourier transformation of data in the t2 and t1 dimensions. A fourth-order polynomial was usually used for these corrections. The two-dimensional contour plots are shown in pure absorption phase.

DNA Thermal Melting. Thermal melting experiments were conducted at 260 nm with a Cary spectrophotometer interfaced to a microcomputer. A thermostir fixed into a reference cuvette was used to monitor the temperature. The DNA oligomer was added to 1 ml of buffer [2-(N-morpholino)ethanesulfonic acid with 150 mM KCl, pH 4.4] in 1-cm path length reduced-volume quartz cells. Experiments were generally conducted at a concentration of 5 × 10^{-5} M bases. Experiments with chloroquine 15 and quinacrine 17 were at a ratio of 0.3 mol of compound per base.

Materials. CpG-ODN 1760 has the sequence 5'-ATAATCGACGT-\text{GTAAGCAGAG}-3', synthesized with a phosphorothioate backbone. The fluorescent ODNs were ODN 1760 with either fluorescein or 9-amino-1,10-phenanthroline-5-carboxylic acid at a ratio of 0.3 mol of compound per base. The DMPC was obtained in powder form from Avanti Polar Lipids, Inc. (Canton, MA). Charged microscope slides were purchased from Fisher Scientific Co. (Canton, MA). Charged microscope slides were purchased from Fisher Scientific Co. (Canton, MA). Hydroxychloroquine was purchased from Copley Pharmaceutical, Inc. (Canton, MA). Hydroxychloroquine was purchased from Copley Pharmaceutical, Inc. (Canton, MA). Like quinacrine, many of these analogs are fluorescent (Table 1), enabling us to monitor their uptake by cells. To do this, we prepared standard curves of the fluorescence intensity of each analog in cell culture medium and in the cell extract buffer, measurements being taken at the pH listed in Table 1. We incubated WEHI 231 cells with a range of concentrations of the analogs, and measured the concentration of the analog remaining in the supernatant and the amount of analog in the extract of a cell pellet, with the standard curves.

Figure 2 shows some of the results. The cells incorporate substantial amounts of the analogs (often more than half of the added reagent). We found a linear relationship between uptake of each of the compounds and its free concentration, without evidence that the uptake process was saturable in the low micromolar range. We found that (nonfluorescent) chloroquine (up to 10 μM) did not inhibit the uptake of other analogs, which also suggests that the transport mechanism is not saturable. This result with WEHI 231 cells differs from the uptake of chloroquine by red blood cells infected with malaria parasites, which others have reported to be saturable and inhibitable by analogs, suggesting that infected red blood cells have a high-affinity transport system for chloroquine (Fitch et al., 1974). The uptake of the fluorescent analogs was rapid, reaching equilibrium in <2 min (data not shown). The efflux of analogs from cells when they are diluted into fresh medium was also rapid (data not shown).

We found that uptake of quinacrine 17 was partially inhibited by the addition of monensin, an ionophore that collapses pH gradients across biological membranes. Monensin also induced the loss of quinacrine from cells preloaded with the analog. Both effects were half-maximal at a monensin concentration of ~1 μM (Fig. 3). Similar results were obtained with other analogs (data not shown). This result is consistent with equilibration of analogs (as weak bases) into acidic vesicles.

By measuring the volume of a cell pellet on a known number of cells, we estimated that the volume of each cell is ~1000 fl. Using this value, we calculated that the cells accu-

Results

Cellular Uptake of Analogs. In the course of our work (Macfarlane and Manzel, 1998; Strekowski et al., 1999), we examined the ability of numerous analogs of chloroquine and quinacrine to block the activity of CpG-ODN. We selected from this library a number of such compounds with a range of structures and a range of potency as inhibitors of CpG-ODN. These analogs include novel compounds (Fig. 1).

Fig. 2. Uptake of analogs by cells. WEHI 231 cells were incubated with fluorescent analogs (0.1–20 μM) for 30 min. The concentration of the analogs in the supernatant medium (free) and cellular pellet (bound) was then determined as described in Experimental Procedures.
mulated analogs to a concentration several hundredfold higher than in the medium (Table 2). Similar results have been reported for chloroquine (Fitch et al., 1974; Ohkuma and Poole, 1981).

Comparison of Uptake and Activity. We measured the ability of analogs to inhibit an immunostimulatory effect of CpG-ODN with an assay we have described previously (Macfarlane and Manzel, 1998). This assay measures the reversal of the protection by CpG-ODN against surface IgM-induced killing of WEHI 231 cells. Several of the analogs were highly potent in this regard, inhibiting the CpG-ODN effect in the low nanomolar range. With the above-mentioned fluorescence uptake data, we compared this activity of analogs with their uptake, finding a poor correlation between these two parameters (Table 1). At half-maximal efficacy, cells accumulated a few million molecules of the most active analogs (e.g., 329, 24, and 17), but the accumulation of much larger amounts of inactive analogs did not inhibit CpG-ODN effects (e.g., 215 and 267).

Effect on CpG-ODN Uptake. We examined the effect of chloroquine and nonfluorescent analogs on the uptake of Texas Red-labeled CpG-ODN 1760. This derivatized ODN is active as an immune stimulator, although it is a little less active than its nonfluorescent parent (data not shown). We used flow cytometry to measure cell-associated fluorescence because signals derived from dead cells, which take up ODN avidly (Tonkinson and Stein, 1994), are easily gated out of the analysis. We found that analogs did not significantly alter the uptake of the fluorescent ODN (Fig. 4).

With confocal microscopy, we found that the fluorescence of Texas Red-labeled CpG-ODN 1760 is confined to numerous small organelles distributed throughout the cytoplasm with a slight predominance in the perinuclear region and Golgi apparatus. In our experiments (with cells fixed from live state), we did not see a relocation of fluorescence to the nucleus, nor labeling of microfilaments, as has been observed by others with digitonin-permeabilized cells (Shoeman et al., 1997) or subcellular fractionation (Beltinger et al., 1995). We attribute this discordant result to the tendency of dead cells to absorb polyanions, including ODN (Tonkinson and Stein, 1994), if the cells die before fixation. In any event, we found that chloroquine 15 did not influence the subcellular distribution of internalized CpG-ODN (Fig. 5).

Effect on CpG-ODN pH. The fluorescence of fluorescein is suppressed at acid pH. We used cells preloaded with fluorescein-labeled CpG-ODN to determine the pH of cell-associated ODN with flow cytometry. The addition of monensin immediately before flow cytometry results in increased fluorescence, attributable to the equilibration of the pH of the

### Table 2

Proton resonance chemical shift assignments for compound 91 and DMPC in unilamellar vesicles

<table>
<thead>
<tr>
<th>Proton Group</th>
<th>Chemical Shift, in D2O ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 -CH3</td>
<td>0.87</td>
</tr>
<tr>
<td>13 -CH2-</td>
<td>1.17</td>
</tr>
<tr>
<td>12 -CH2-</td>
<td>1.25</td>
</tr>
<tr>
<td>4-11 -(CH2)4–11-</td>
<td>1.29</td>
</tr>
<tr>
<td>3 -CH3</td>
<td>1.56</td>
</tr>
<tr>
<td>2 -CH2-</td>
<td>2.33</td>
</tr>
<tr>
<td>15 CHOCO-</td>
<td>5.26–5.31</td>
</tr>
<tr>
<td>16 H2COCO-</td>
<td>4.43</td>
</tr>
<tr>
<td>16 H2COPO-  (glyceride)</td>
<td>3.99</td>
</tr>
<tr>
<td>17 -OPOCH3- (choline)</td>
<td>4.27</td>
</tr>
<tr>
<td>18 -CHN-</td>
<td>3.56–3.63</td>
</tr>
<tr>
<td>19 N(CH3)3</td>
<td>3.12–3.19</td>
</tr>
</tbody>
</table>

**Compound 91** Naphthalene, quinoline 7–9

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**Fig. 3.** Effect of monensin. Cells were incubated with 1 μM quinacrine 17 or compound 227 for 30 min. Monensin at the indicated concentration was added either at the same time as the analog or at the end of the 30-min incubation, and the cellular concentration of the analog was determined by fluorescence.

**Fig. 4.** Influence of chloroquine on CpG-ODN uptake. Cells were incubated with 5 μg/ml Texas Red CpG-ODN 1760 and indicated concentration of chloroquine 15 for the time indicated. The cells were then washed three times and fixed. The cellular uptake of fluorescent CpG-ODN was estimated by flow cytometry. Note that chloroquine 15 has no significant influence on CpG-ODN uptake.
ODN with the pH of the medium and the diluent used in flow cytometry.

We used a standard curve to relate fluorescence with pH, enabling us to determine that the pH of the cell compartment occupied by ODN is ~6.4, a value very close to that published by Tonkinson and Stein (1994) with a similar method. With this method, we examined the effects of (nonfluorescent) analogs on the pH of the compartment. The results are shown in Fig. 6. It can be seen that the effect of 5 μM chloroquine (a concentration 50-fold higher than needed to suppress CpG-ODN responses) increases the pH of the CpG-ODN-containing compartment by only 0.3 pH units. No analog increased the pH substantially at the concentration required for activity, although they did collapse the pH at higher concentrations (Fig. 6).

Subcellular Localization of Analogs. Next, we examined the subcellular localization of fluorescent analogs with confocal microscopy. We were unable to find a method of fixation that immobilized the analogs within the cells. Instead, we incubated the cells with the analogs and examined them live. This procedure yields pictures of lesser quality than fixed cells. Despite this limitation, we found a consistent difference between the distribution of fluorescent CpG-ODN and of fluorescent analogs: the analogs occupy organelles that are larger and more peripherally located within the cell, and that appear not to incorporate CpG-ODN (Fig. 7). It is not clear from the images whether quinacrine also occupies the smaller vesicles that do take up CpG-ODN.

Interaction between Analogs and ODN. Structural nucleic acids with duplex or single-stranded folded conformations generally give sigmoid thermal melting curves characteristic of thermal unfolding of the structures. This unfolding results in a change in the UV spectrum. In studies kindly performed by Dr. W. David Wilson (Georgia State University, Atlanta, GA), CpG ODN 1760 gave a very small continuous increase in absorbance at 260 nm as a function of temperature, characteristic of some base unstacking in single-stranded DNA, and did not show any transition curve characteristic of a base-paired structure. Addition of chloroquine 15 or quinacrine 17 to the CpG ODN did not cause any significant change in the thermal melting curve of the free CpG-ODN (data not shown). It can, thus, be concluded that under these conditions, these compounds bind poorly, if at all, to the CpG ODN.
Interaction between Analogs and Lipids. In the absence of an interaction with DNA, we wondered whether our compounds could interfere with the activity of a membrane-bound protein, particularly by inserting themselves into the membrane. The structures of compound 91 and DMPC are provided in Fig. 8; the lipid NMR resonance assignments that are listed in Table 2 are keyed to the numbered groups on the DMPC structure in this figure. The assignments in Table 2 are derived in part from the work of Ellena et al. (1987) with additional assignments by us.

A typical spectrum, obtained at a pH of 7.4, is shown in Fig. 9 and is referenced to the terminal methyl resonance of the DMPC acyl chain (0.87 ppm); this assignment is relative to an external trimethylsilyl standard. NOESY cross-peaks between the compound 91 quinoline and naphthalene ring protons and those of the DMPC fatty acid unresolved methyl enes (1.3 ppm on the F1 axis, Nos. 4–11; Fig. 9A) are present. Additional cross-peaks are observed between the compound 91 aromatic ring protons and those of the lipid glycerol backbone methylenes (3.99 ppm, 16) and to those of the choline N-CH2 moiety (3.5 ppm, 18). Very weak, marginal cross-peaks are observed between the compound 91 aromatic system protons and those of the choline N(CH2)3 moiety (3.2 ppm, 19) and to those of the fatty acid terminal methyl group at 0.87 ppm on the F1 axis (14, Fig. 9A). The NOESY spectrum obtained at a pH of 4.7 is qualitatively similar to that obtained at a pH of 7.4 (data not shown). In the aliphatic region, the cross-peak at 4.25 ppm on the F2 axis is assigned to the interaction of the compound 350 aliphatic chain methylene groups with those of the OPO-CH2 methylene (17) (Fig. 9B).

A location model that is consistent with the NOESY data suggests that the charged N(CH3)2 moiety be located no deeper in the membrane bilayer than the onset of the hydrocarbon region, near the ester linkages in the DMPC lipids. The NOESY results indicate that the aliphatic chain of compound 91 is located near the interface of the choline head group and the glycerol backbone of the lipid. Such a location would allow for favorable electrostatic interactions between the protonated N(CH3)2 group and the negatively charged DMPC phosphate moiety.

The unambiguous cross-peaks between the aromatic ring protons of compound 91 and the unresolved DMPC fatty acid methylene protons (1.3 ppm on the F1 axis) indicates that a portion of the compound 91 molecules penetrate the bilayer...
Fig. 9. Expanded sections from a NOESY spectrum of DMPC unilamellar vesicles (10 mM) in the medium described in Experimental Procedures at a pD of 7.4. The NOESY spectrum obtained at pD of 4.7 is qualitatively similar to that shown. The sample contained 8 mol% compound 91 added after the vesicles were prepared. In A and B, the 1-mm threshold for cross-peak display was set at 0.02 and 0.03%, respectively, of the vertical scale multiplication factor. Spectrophotometer parameters are given in Experimental Procedures.
to at least the level of the fourth methylene group on the fatty acid chain and perhaps below this level (see below). Molecular modeling studies using SYBYL (Tripos Associates, St. Louis, MO) of a DMPC bilayer with the lipids in the all trans-conformation and with compound 91 in an energy-minimized extended conformation with the protonated N(CH$_3$)$_2$ nitrogen located near the phosphate group will allow an NOE cross-peak between protons on the naphthalene ring and the eight methylene protons with a 5-Å NOE cutoff distance criterion. These results will be presented in more detail elsewhere.

The lack of cross-peaks between the terminal methyl group and the aromatic protons does not preclude the eventual translocation of compound 91 through the bilayer and the accumulation of the molecules in the vesicle internal volume as has been observed in whole-cell studies based on similar quinolines tagged with fluorescent labels as described in a separate section of this article. The NMR results suggest in this instance that the steady-state population of compound 91 molecules near the middle portion of the bilayer, which would include the roughly 0.5 mol% of compound 91 in neutral form, is below the detection threshold for the NOESY experiment in the DMPC vesicle system.

**Discussion**

The 4-aminoquinolines chloroquine, hydroxychloroquine, and several of the other analogs we used were originally synthesized as antimalarials. This class of drug preferentially concentrates in red cells infected with malaria (Fitch et al., 1974), where the drugs inhibit heme polymerase, an enzyme required by the parasite to detoxify ferriprotoporphyrin IX (Wellems, 1993). Like many other weak bases, chloroquine is lysosomotropic, partitioning into the lysosomes of live cells to achieve concentrations that are thousands-fold higher than the medium (Duve et al., 1974). It is therefore reasonable to conjecture that the site of action of the anti-CpG-ODN effects of these agents also might be in an acidified compartment within the cells where they concentrate. In the course of our work, we have examined several hundred analogs of chloroquine, including compounds with novel structures, finding that many have potent anti-CpG-ODN activity. Some of these compounds, including quinacrine, are brilliantly fluorescent. For the work described herein, we selected from this library a number of fluorescent analogs with a range of potencies as inhibitor of CpG-ODN-induced effect.

We examined the uptake of the fluorescent analogs by WEHI 231 cells. As expected, they were incorporated into cells to a substantial extent, reaching cellular concentrations several hundredfold higher than the medium. Examination of the distribution of the fluorescence within the cell shows that the analogs concentrate into organelles (presumably lysosomes and other acidified vesicles) near the periphery of the cell. Because these organelles occupy only a fraction of the volume of the cell, the concentration of the analogs within these organelles must be higher than we report for the whole-cell volume.

The uptake of chloroquine by red blood cells infected with malaria appears to be mediated by a high-affinity, saturable process (Fitch et al., 1974). Our results with WEHI 231 cells are different. We found that the uptake of the analogs was rapid, reversible, and nonsaturable. Uptake was reduced by blocking the acidification of vesicles with monensin. These data are compatible with the movement of analogs by diffusion into acidified vesicles, but they do not rule out the involvement of a high-capacity, low-affinity nonspecific cation transport system (Koepsell, 1998).

We find that a broad range of cationic chemical structures are highly active as inhibitors of CpG-ODN-induced effects (Macfarlane and Manzel, 1998; Strekowski et al., 1999). The lack of a rigorous structural specification of the active analogs raises the hypothesis that the analogs act more as physical agents than as specific inhibitors of a receptor or an enzyme-active site. Thus, we examined the relationship between the uptake of analogs and their efficacy as inhibitors of CpG-ODN. We found a poor correlation between these two parameters. Several compounds (e.g., 267 and 215) had little or no efficacy even when >100 million molecules were incorporated per cell, whereas others (e.g., 322 or 24) were powerful inhibitors of CpG-ODN when only 3 million molecules/cell were incorporated. These results suggest that the efficacy of an individual analog is determined not only by the physical mass of the analog present within the cell but also by some specific structural features.

Like other nucleic acids, CpG-ODN are taken up into cells. Immobilized CpG-ODN is reported to be ineffective as a stimulator of immunity, leading to the hypothesis that CpG-ODN need to be internalized to be detected (Krieg et al., 1995). This has been disputed (Liang et al., 1996), but our own results (Manzel and Macfarlane, 1999) confirm the finding that immobilized CpG-ODN is ineffective. If uptake is required, it is of interest to determine whether the analogs inhibit the uptake of CpG-ODN, or cause it to be excluded from certain compartments within cells. We explored this with Texas Red-labeled CpG-ODN. This derivative is active as an immune stimulator, albeit with reduced potency to the parent ODN. As reported by others (Tonkinson and Stein, 1994; Beltinger et al., 1995; Gray et al., 1997; Hacker et al., 1998), the fluorescent CpG-ODN is rapidly taken up by cells, where it concentrates into numerous small vesicles with a slight perinuclear predominance. We found no evidence to suggest that the analogs influence either the uptake of fluorescent CpG-ODN or its subcellular distribution.

The mechanism of action of CpG-ODN is under intensive study. B-cells exposed to CpG-ODN (but not control ODN) increase the rate of production of oxygen radicals, and up-regulate the activity or expression of a number of early response proteins, including c-Jun NH$_2$-terminal kinase, nuclear factor-κB, and c-myc (Yi and Krieg, 1998; Yi et al., 1993). This up-regulation (like all other responses to CpG-ODN) is blocked by agents, including chloroquine, that inhibit acidification of vesicles, leading to the concept that this acidification is necessary for the action of CpG-ODN (Yi et al., 1998). We explored the effect of chloroquine and other analogs on the pH of the CpG-ODN-containing compartment.

The fluorescein-labeled CpG-ODN we used is an active immunostimulator. Because its fluorescence is quenched by acid pH (with a $K_a$ we observed to be ~7.0), it can be used to determine the pH of the compartment(s) in which it resides. As reported by Tonkinson and Stein (1994), the addition of monensin to fluorescein-labeled phosphorothioate oligonucleotide to cells results in an immediate increase in fluorescence, attributable to the equilibration of the pH of the ODN-
containing vesicles with the medium. Our calculation suggests that the average pH of intracellular CpG-ODN is ~6.4, close to other estimates (Tonkinson and Stein, 1994). Our data also show that this pH is increased by chloroquine and its (nonfluorescent) analogs, but only when they are added at concentrations much higher than are required to inhibit the actions of CpG-ODN. At the concentration of analogs required to inhibit the immunostimulatory effect of CpG-ODN, the influence on the pH of the CpG-ODN-containing vesicle is negligible. For instance, analogs 352 and 350 inhibit CpG-ODN at 16 and 6 nM, respectively, concentrations that are 300- and 800-fold lower than those required to increase the pH of intracellular ODN by 0.4 U.

We have not yet determined how much of the analogs enter the CpG-ODN-containing compartment within the cell. There is little doubt from the confocal micrographs that the analogs enter a peripherally located large compartment that contains little or no CpG-ODN. However, our confocal micrographs do not indicate that the smaller, perinuclear vesicles containing CpG-ODN do not also contain analogs.

Planar weak bases may bind to double-stranded DNA by intercalation. We wondered whether our compounds could interfere with the detection of single-stranded CpG-ODN by binding to the CpG-ODN. Little information has been published on the binding of molecules such as chloroquine to phosphorothioate ODN at acid pH. With UV spectroscopy, we found little or no evidence that the presence of chloroquine or quinacrine altered the thermal unstacking of the ODN, providing objective evidence that these compounds have little affinity for CpG-ODN under the acidic conditions likely to prevail within the cell.

NOEY is a powerful method for determining the position of a ligand within a binding site. We used it to seek evidence that our compounds bind to a phospholipid bilayer because the surface area-to-volume ratio is high in the organelles into which CpG-ODN concentrates. This technique clearly demonstrates that the ring structure of the test analog penetrates into the bilayer to a depth of at least the fourth carbon of the phospholipid acyl chains, leaving the side chains of the compound free to interact with the phosphate group of the phospholipid head group at the aqueous interface. The same result was obtained at neutral and acid pH.

We conclude from these experiments that the analogs do not inhibit the action of CpG-ODN by interfering with the acidification of vesicles, nor with the uptake or subcellular distribution of CpG-ODN. Given that the CpG-ODN-containing vesicles are acidified, it is likely that the analogs are concentrated into the same vesicle as the CpG-ODN. It therefore seems likely that the analogs inhibit the detector system for CpG-ODN within vesicle by a specific mechanism, rather than by a bulk effect. Our finding that the analogs penetrate lipid bilayers in an orientation that leaves the side chain free to interact in the aqueous phase raises the possibility that the inhibition of the effect of CpG-ODN occurs near the phospholipid surface facing the acidified vesicle. Further studies are planned to elucidate the mechanism in detail.

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References


The above text includes a reference to the structure-activity relationship analysis of cation-substituted polyaromatic compounds as potential potentiators (amplifiers) of bleomycin-mediated degradation of DNA. This suggests that there is potential for the development of new drugs that could enhance the effectiveness of bleomycin in treating cancer.


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