Cancer-selective antiproliferative activity is a general property of some G-rich oligodeoxynucleotides

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ABSTRACT

Oligodeoxynucleotide libraries containing randomly incorporated bases are used to generate DNA aptamers by systematic evolution of ligands by exponential enrichment (SELEX). We predicted that combinatorial libraries with alternative base compositions might have innate properties different from the standard library containing equimolar A + C + G + T bases. In particular, we hypothesized that G-rich libraries would contain a higher proportion of quadruplex-forming sequences, which may impart desirable qualities, such as increased nuclease resistance and enhanced cellular uptake. Here, we report on 11 synthetic oligodeoxynucleotide libraries of various base combinations and lengths, with regard to their circular dichroism, stability in serum-containing medium, cellular uptake, protein binding and antiproliferative activity. Unexpectedly, we found that some G-rich libraries (composed of G + T or G + C nucleotides) strongly inhibited cancer cell growth while sparing non-malignant cells. These libraries had spectral features consistent with G-quadruplex formation, were significantly more stable in serum than inactive libraries and showed enhanced cellular uptake. Active libraries generally had strong protein binding, while the pattern of protein binding suggested that G/T and G/C libraries have distinct mechanisms of action. In conclusion, cancer-selective antiproliferative activity may be a general feature of certain G-rich oligodeoxynucleotides and is associated with quadruplex formation, nuclease resistance, efficient cellular uptake and protein binding.

INTRODUCTION

Cancer is a leading cause of death throughout the world. Although many drugs have been developed to treat cancer, the majority of the oncology pharmacopeia causes serious, dose-limiting side effects due to damage to healthy cells. There exists a clear need for better, more specific cancer therapies, not only to increase efficacy against cancer cells but also to decrease the suffering of patients who are subjected to these drug regimens.

Nucleic acid aptamers are emerging as a new class of targeted therapeutic agents and are the focus of a rapidly growing field of medical research (1–4). These small synthetic oligomers of DNA or RNA form stable three-dimensional structures and bind to defined molecular targets via shape-specific recognition. Thus, in essence, their mechanism of action is similar to that of protein-based monoclonal antibodies. However, due to their smaller size and different chemical composition, aptamers have a number of potential advantages over antibodies. These include ease of manufacture and storage, facile conjugation, better tumor penetration, more rapid systemic clearance and non-immunogenicity. In recent years, aptamers have been identified as potential treatments for a variety of diseases, including macular degeneration (5,6), autoimmune disease (7), disseminated intravascular coagulation (8,9) and prion diseases (10). In the oncology field, the most clinically advanced aptamer, and the first anticancer aptamer to be tested in humans, is a 26-nt G-rich DNA known as AS1411 (formerly, AGRO100). This nucleolin-targeted aptamer, which was discovered by our group, has shown promising clinical activity without any major side effects in Phase I and ongoing Phase II clinical trials (11).

New aptamers against specific targets are most often engineered via systematic evolution of ligands by exponential enrichment (SELEX) (1,12,13), an iterative methodology designed to create target-specific aptamers from a

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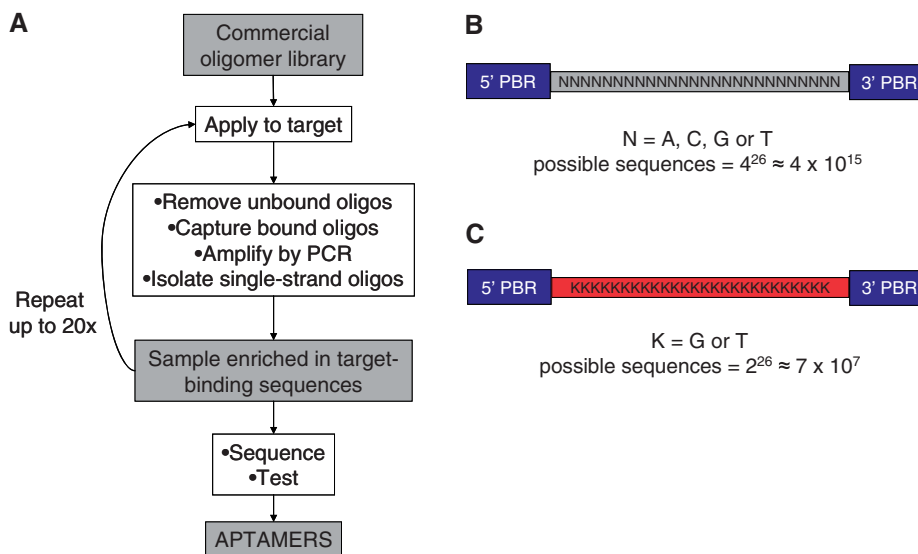
Figure 1. Overview of SELEX procedure and oligonucleotide libraries. (**A**) Schematic of SELEX procedure. (**B**) Diagram of N library. Primer-binding regions (PBR) flank a length of random sequence, for which each base has equal likelihood of being an A, C, G or T. In theory, the library contains 4^n different sequences, where n is the length of the random sequence (in this example $n = 26$). (**C**) Example of a guanine-rich GT library. The random sequence is restricted such that each base may be either a G or a T. This library should contain 2^n different sequences (in this example $n = 26$).

combinatorial library (Figure 1A). For DNA aptamers, a single-stranded oligodeoxynucleotide library consisting of two known primer-binding sequences flanking a random sequence of usually 20–40 nt in length (N library, where N = A, C, G, or T; Figure 1B) is applied to the target of interest. The molecules that do not bind are washed away, and the remainder are harvested and amplified by polymerase chain reaction (PCR). The aptamer-enriched pool of single-stranded molecules is then isolated from the PCR product, reapplied to the target and the process is repeated. In this manner, highly specific aptamer sequences are usually evolved after 10–20 cycles. For RNA aptamers, a similar procedure with additional steps for transcription and reverse transcription can be utilized. Typically, the target of interest for SELEX is a purified protein or small molecule, but the development of aptamers against intact cells or organisms can be also achieved (1,14).

One shortcoming of traditional SELEX is the high number of iterations required, which consumes time and resources, and increases the likelihood of artifacts (since every round of SELEX requires PCR and must be carefully protected from contamination). Moreover, for *in vivo* use, evolved sequences must typically be modified to enhance their pharmacokinetic properties and to increase resistance to serum nucleases. This can be achieved post-SELEX, or by incorporating modified bases or backbones during the SELEX procedure (1,15). However, these approaches have some drawbacks: post-SELEX modification can reduce aptamer activity or increase toxicity, and not all modified nucleotides are compatible with the polymerases used during SELEX. An additional shortcoming of aptamers (which is also shared by antibodies) is their inefficient cellular internalization, which generally limits their clinical targets to extracellular or cell surface molecules.

In an attempt to address some of these limitations, we have considered the use of alternative starting libraries for SELEX. Rather than the random N library containing billions of sequences, the vast majority of which will not turn out to be clinically useful aptamers, we propose beginning with smaller libraries, especially those which may be enriched in molecules that contain secondary structure motifs, such as hairpins or G-quartets. In particular, we hypothesized that libraries enriched in guanine (G) residues (for example, see Figure 1C) would contain a higher proportion of quadruplex-forming sequences, which may impart increased functionality, nuclease resistance and cellular uptake compared to the N library. An additional driving force for this study was the realization that very G-rich sequences such as AS1411 (5′-d[GGTGG TGGTGGTTGTGGTGGTGGTGG]), which was discovered by chance, would rarely occur in the standard N library, making it unlikely to have been discovered by standard SELEX. Furthermore, our previous work involving the 'bench-to-bedside' development of AS1411 and the preclinical research of several other groups who are researching a variety of different G/T-containing oligonucleotides (11) led us to focus on G/T DNA libraries for our studies on the effect of oligonucleotide length.

As a prelude to SELEX studies using alternative oligodeoxynucleotide libraries, we have sought to establish the baseline characteristics of various libraries and to test our hypothesis that G-rich libraries will be enriched in quadruplex-forming sequences with improved nuclease resistance and cellular uptake. In this project, we have examined 11 oligodeoxynucleotide libraries of varying base compositions and lengths, as well as three monobasic oligomers (Figure 2). We have performed studies to evaluate various characteristics of the libraries, including a well-established colorimetric assay to determine their

	Name	Length	%A	%C	%G	%T
Non-G-rich	N 26mer	26	25	25	25	25
	A 26mer	26	100			
	C 26mer	26		100		
	T 26mer	26				100
	AT 26mer	26	50			50
	CT 26mer	26		50		50
G-rich	GT 10mer	10			50	50
	GT 18mer	18			50	50
	GT 26mer	26			50	50
	GT 34mer	34			50	50
	GT 0.33 26mer	26			33	67
	GT 0.67 26mer	26			67	33
	GA 26mer	26	50		50	
	GC 26mer	26		50	50	

Figure 2. Oligonucleotides and libraries used in this study. Single-stranded DNA oligonucleotide libraries and monobasic oligomers used in this study. Libraries with >25% guanines are considered 'G-rich'.

intrinsic antiproliferative activities in three different cancer cell lines compared to non-malignant cells. As far as we know, we are the first to perform such a comprehensive screen of oligonucleotide libraries. In addition, we report here the unexpected discovery that certain G-rich libraries have high levels of intrinsic, cancer-selective antiproliferative activity.

MATERIALS AND METHODS

Oligodeoxynucleotides and libraries

All oligonucleotides and oligonucleotide libraries were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and are shown in Figure 2. The relative proportion of bases was specified for libraries, which can be synthesized by mixing phosphoramidite precursors in the desired ratio. The specified bases are then randomly incorporated into the sequence. Lyophilized libraries were resuspended in filter-sterilized water, then heated at 65°C for 10 min and vortexed vigorously to dissolve. For uptake studies, three thymidine bases (TTT) were added to the 3′-end of the library sequence to avoid quenching the 3′-fluorophore, which can occur when it is proximal to a G-quartet (Choi,E., Nayak,L. and Bates,P. unpublished data). A G_{26} oligodeoxynucleotide was originally proposed for study, but is not commercially available due to difficulties in synthesis of oligodeoxyguanosines of this length.

Cell culture

Four cell lines were comprehensively studied: A549 lung adenocarcinoma, DU145 prostate adenocarcinoma, MCF7 mammary carcinoma and Hs27 non-malignant skin fibroblasts. All cell lines were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in complete cell culture medium of Dulbecco's Modified Eagle Medium

(DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-treated (15 min at 65°C) fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The MCF7 cell line alone was grown using charcoal-stripped serum (Invitrogen). All cells were cultured at 37°C in 5% CO_2. Methods for cell culture and antiproliferative assays using MCF10A cells can be found in the Supplementary Data.

Antiproliferative activity assay

In the four cells lines, each library/oligomer was tested in triplicate at eight concentrations of oligonucleotide (0, 2, 4, 6, 8, 10, 12 and 14 μM) using a modified previously published MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay protocol (16). Briefly, cells were seeded in 96-well tissue culture plates and incubated overnight. To account for intrinsic differences in growth rate, the A549 and DU145 lines were plated at 1000 cells per well, MCF7 at 2000 per well and Hs27 at 3000 per well. Oligomer library stock solutions were then added to each well to yield the desired final concentration. Plates were incubated with the oligomer libraries for 5 days, during which the cell culture medium was not changed. On Day 5, cell viability was determined and the background corresponding to medium alone (no cells) was subtracted. For each concentration, the mean and standard error of triplicate wells was plotted. Assays were repeated at least twice in triplicate to ensure reproducibility of the results.

Circular dichroism spectroscopy

Oligomer library stocks were diluted to a concentration of 5 μM (strand concentration) using either water (buffer was not included in order to be representative of the stock solutions used to treat cells), or aqueous solutions containing 100 mM sodium chloride or 100 mM potassium chloride. Samples were incubated at 95°C for 5 min, then cooled on ice until analysis. Circular dichroism (CD) spectra were collected at 20°C using a Jasco J-715 spectropolarimeter between 340 and 220 nm wavelengths, using 200 nm/min scanning speed, 2 s response time, 5 nm bandwidth and 1-cm pathlength. For each set of samples, the same solution used to prepare the oligomers was used as the reference. The resulting spectra in millidegrees (mdeg) were converted to molar CD ($\Delta\varepsilon$), referring to per mole of nucleotide. Methods for analysis of $(dC)_{26}$ at various pH can be found in the Supplementary Data.

Nuclease-resistance in serum-containing medium

Each oligomer library was 5′-end-labeled with ^{32}P using T4 kinase (Invitrogen) and unincorporated γ-^{32}P-ATP was removed from samples using TE-Midi SELECT-D G-25 columns (Shelton Scientific, Shelton, CT, USA), according to manufacturers' directions. Collection tubes containing end-labeled libraries were kept on ice, or stored at −20°C. The radioactivity of end-labeled libraries was measured in a scintillation counter on the day of the experiment. For each library to be tested, four reaction tubes containing complete cell culture medium without antibiotics were

prepared. One million counts per minute of end-labeled oligomer was added to each of four tubes. Samples were incubated at 37°C, and tubes were flash-frozen in a dry ice/ethanol bath at 0, 1.5, 24 and 72 h. Frozen samples were stored at −80°C until all samples had been harvested. Samples were then thawed on ice and run on 12% denaturing polyacrylamide gels. Band density was measured over each lane using the UN-SCAN-IT gel densitometry application (Silk Scientific, Orem, UT, USA). The percent sample remaining was calculated taking the signal of the zero time point lane to be 100%.

Cellular uptake

Uptake experiments were performed using oligomer libraries labeled at the 3′ end with FAM (6-carboxyfluorescein) for representative libraries that were inactive (N 26mer and GA 26mer) or active (GC 26mer and GT 26mer). For each sample, 3×10^5 cells were plated and incubated overnight. The following day, fresh medium was added to each plate. FAM-labeled libraries were added to make a final concentration of 5-μM oligonucleotide and plates were incubated for 1 h. The medium containing unbound oligo was removed. Cells were washed twice and treated with trypsin (Invitrogen) to remove surface-bound oligonucleotides and harvest cells. Cells were kept on ice and protected from light until analysis. Propidium iodide (PI, 2 μg per sample, BD Biosciences, San Jose, CA, USA) was added as a marker of viability. Ten thousand cells were counted, after gating to exclude PI-positive (non-viable) cells from analysis. Cells were analyzed using a FACSCalibur cytometer (BD Biosciences). The relative fluorescence of cells was determined using the FlowJo software program (Tree Star, Ashland, OR, USA).

Protein binding using electrophoretic mobility shift assay

Libraries were end-labeled with ^{32}P as described for the nuclease-resistance assay and then electrophoresed on a non-denaturing 20% polyacrylamide gel to check labeling. Whole-cell extracts from A549, DU145, MCF7 and Hs27 cells were prepared using a modification of a previously published method (17) as follows: cells were grown until confluent in 75 cm² flasks. The cells were washed, then scraped with 100 μl HEDG buffer [20 mM HEPES-NaOH, pH 7.8, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT)] containing 10% glycerol, 1 × protease inhibitor cocktail (Sigma, St Louis), and 0.42 M NaCl. The solution was harvested and subjected to three freeze–thaw cycles (liquid nitrogen versus room temperature). Samples were centrifuged at 16 000g at 4°C for 45 min to pellet cell debris. An equal volume of HEDG with glycerol and protease inhibitor but without NaCl was added to reach a final concentration of 0.21 M NaCl. An aliquot (100 000 c.p.m.) of radiolabeled oligomer was combined with 5.0 μg of cell extract from A549, DU145 and MCF7 cells, or with 3.2 μg from Hs27 cells, and 5X protein binding buffer [1X is 20 mM Tris–HCl, pH 7.4, 140 mM Kl, 2.5 mM MgCl$_2$, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 8% (v/v) glycerol]. Samples were incubated at 37°C for 15 min and run on a non-denaturing 5% polyacrylamide gel. Gels were fixed in a solution of 10% (v/v) methanol + 10% (v/v) glacial acetic acid in water for 10 min at room temperature, then exposed to a storage phosphor screen for 2 h at room temperature before scanning. Gels were also exposed to X-ray film overnight at −80°C.

RESULTS AND DISCUSSION

GT libraries of all types and the GC 26mer library have selective antiproliferative activity against cancer cells

The antiproliferative activity of 11 oligomer libraries and three monobasic oligomers at various concentrations against four cell lines was determined using the MTT assay. The results are summarized in Figure 3A, which shows the percentage reduction in cell number at 6-μM oligonucleotide strand concentration. This format was selected for clarity of presentation and 6 μM was chosen because it best illustrated the relative activities of the various libraries.

Some libraries containing G + T or G + C were found to strongly inhibit cancer cell proliferation at low micromolar concentrations, whereas non-G-rich libraries, including the standard N library, had little effect on the cancer cells (Figure 3A). All libraries tested had minimal antiproliferative activity against the non-cancerous Hs27 cells (Figure 3A and B). These novel data provide surprising evidence that some oligomer libraries possess intrinsic antiproliferative activity, even before isolation of individual aptamer sequences.

The GT 18-mer, 26-mer and 34-mer libraries had the highest level of antiproliferative activity against the three cancer cell lines (Figure 3A). The GT 10-mer, GT 0.33 26-mer and GT 0.67 26-mer libraries had similar specificity, but were less effective at inhibiting the cancer cells (Figure 3A). Figure 3B and C illustrate these results for A549 cells, and similar patterns of activity were observed in the other cancer cell lines (Figure 3A and data not shown). The GC 26-mer library had potent antiproliferative activity against A549 and DU145 cells, and had a weaker effect on MCF7 cells (Figure 3E). In contrast to the other G-rich libraries, the GA 26-mer library had very little antiproliferative activity against any cell line (Figure 3A and data not shown). Based on the shape of the dose-dependence curves (for example, see Figure 3C, which plateaus at a non-zero value), it appears that active libraries have a cytostatic rather than cytotoxic effect. This was also observed previously for individual antiproliferative G-rich oligonucleotides (16).

From these data, we designated all of the GT libraries and the GC 26-mer library as 'active' against cancer cells. In the remaining experiments, we compared these active libraries against the remaining 'inactive' libraries in order to identify characteristics that were associated with the unexpected antiproliferative activity. To confirm the cancer-selectivity of the active libraries, we also examined all libraries and oligomers in an additional non-tumorigenic cell line, MCF10A, which is derived from benign breast epithelial cells. The results indicated

A **Percent Inhibition in Cell Number at 6 μM Concentration**

	Library	A549 lung cancer	DU145 prostate cancer	MCF7 breast cancer	Hs27 non-malignant
Non-G-rich	N 26mer	0	6	27	11
	A 26mer	0	0	2	6
	C 26mer	0	0	1	13
	T 26mer	0	7	24	14
	AT 26mer	0	28	21	18
	CT 26mer	4	10	10	21
G-rich	GT 10mer	37	35	53	6
	GT 18mer	63	58	63	6
	GT 26mer	72	60	69	11
	GT 34mer	71	68	63	9
	GT 0.33 26mer	47	29	53	14
	GT 0.67 26mer	47	27	51	5
	GA 26mer	0	18	18	4
	GC 26mer	70	72	31	3
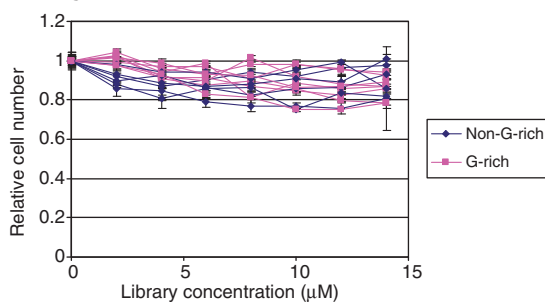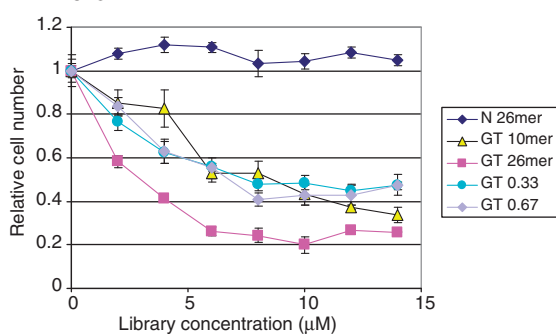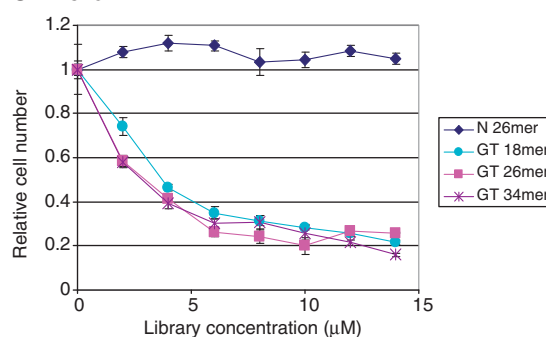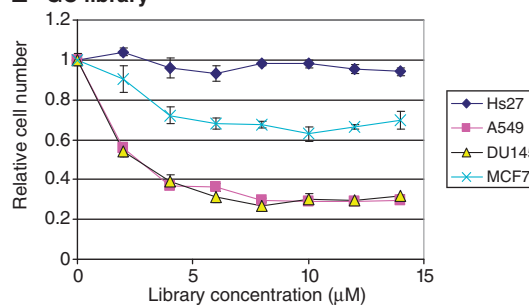
Figure 3. Antiproliferative activity of libraries and oligomers. (**A**) Tabulation of percent decrease in cell number at 6 μM (strand concentration), calculated taking untreated wells to be 100%. Dark-gray shading indicates >50% decrease; light-gray shading indicates between 30% and 50% decrease. (**B**) Growth curves showing the effects of all libraries, non-G-rich and G-rich, on non-cancerous fibroblasts (Hs27). Relative cell numbers at each concentration are compared to no treatment. (**C**) Growth curves showing the effects of GT libraries of different lengths on A549 lung cancer cells. The N library is shown for comparison. (**D**) Growth curves showing the effects of various GT libraries on A549 lung cancer cells. The N library is shown for comparison. (**E**) Growth curves showing the effects of the GC 26-mer library on four cell lines.

that although the MCF10A cells were slightly more sensitive to antiproliferative effects than the Hs27 skin fibroblasts, they were markedly less sensitive than the cancer cell lines, including MCF7 breast cancer cells (Supplementary Figure S1).

Libraries with antiproliferative activity share features of CD spectra

CD of DNA arises from the helical arrangement of the bases and therefore conformational characteristics (e.g. A-, B- or Z-forms of duplexes, and C- or

G-quadruplexes) can often be inferred from CD spectra, as described in an excellent recent review article (18). In our experiments, we observed that the CD spectra of active libraries typically displayed a negative peak at ~240 nm, a strong positive peak at 260–265 nm and an additional maximum or shoulder (depending on the cation present) in the region 285–290 nm (Figure 4A). In addition, the spectra of the active libraries changed significantly between different salt solutions, with the largest peaks seen in the 100 mM potassium chloride solution. While CD data alone cannot be used to absolutely assign structure, it is widely accepted that positive peaks close to 260 nm or 290 nm are associated with G-quadruplex formation (18,19). Similarly, the conformational polymorphism suggested by the differences in CD spectra in the presence of various cations is typical of G-quadruplexes. Obviously, the shape of the spectrum for an oligonucleotide library is derived from the combined CD spectra from millions of sequences. Thus, the characteristic spectrum of the active GT libraries (Figure 4A) presumably represents contributions from molecules with various arrangements of G-quartets (maxima at 260 and 290 nm) and non-quadruplex single-stranded molecules (maximum at 275–280 nm). Somewhat surprisingly, the spectrum of the GC library (Figure 4A) was quite similar to the GT libraries and shared features suggestive of G-quadruplex formation, although the presence of duplexes or hairpins in the GC-library cannot be excluded.

Most of the inactive libraries showed a negative peak at ~250 nm as well as a single positive peak at ~275 nm (Figure 4B). This is typical of both ordered single-stranded and double-stranded DNA (18). The spectra of the inactive libraries changed only minimally with
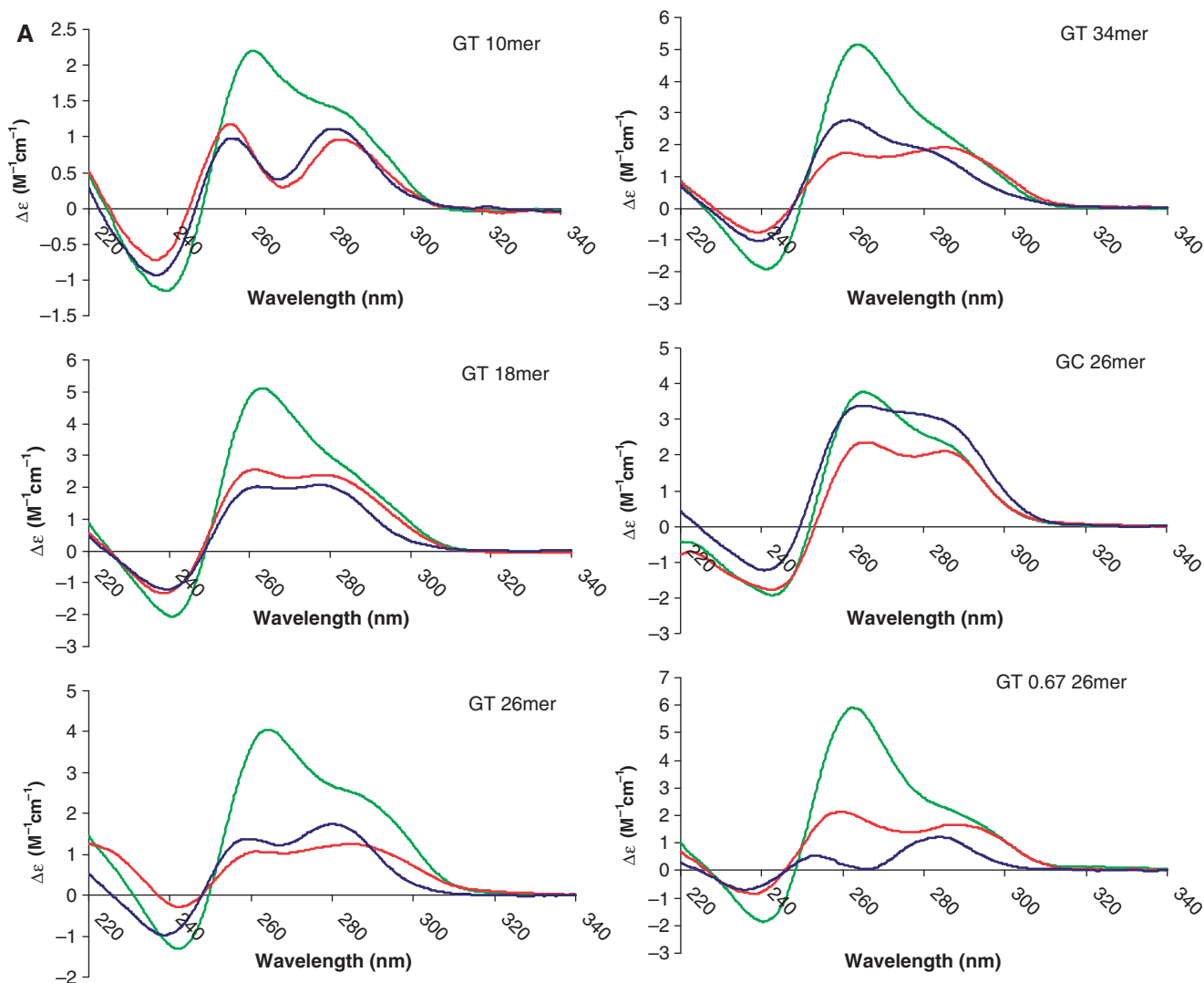
Figure 4. CD studies. Samples were prepared at a concentration of 5 µM in three different solutions: water (blue), 100 mM NaCl (red) and 100 mM KCl (green). (**A**) CD spectra of various active libraries showing a similar profile (a positive peak at 260 nm and a second positive peak or shoulder at 290 nm), which is characteristic of G-quadruplex formation. (**B**) CD spectra of inactive libraries showing a similar profile (positive peak in 270–280-nm region), characteristic of non-G-quadruplex oligonucleotides. (**C**) Libraries with anomalous CD. The inactive GA 26-mer library CD is different from both active and inactive profiles. The GT 0.33 26-mer library has some antiproliferative activity, but its CD spectrum is similar to that of the inactive libraries. Results are shown as molar CD (Δε), referring to per mole of nucleotide.
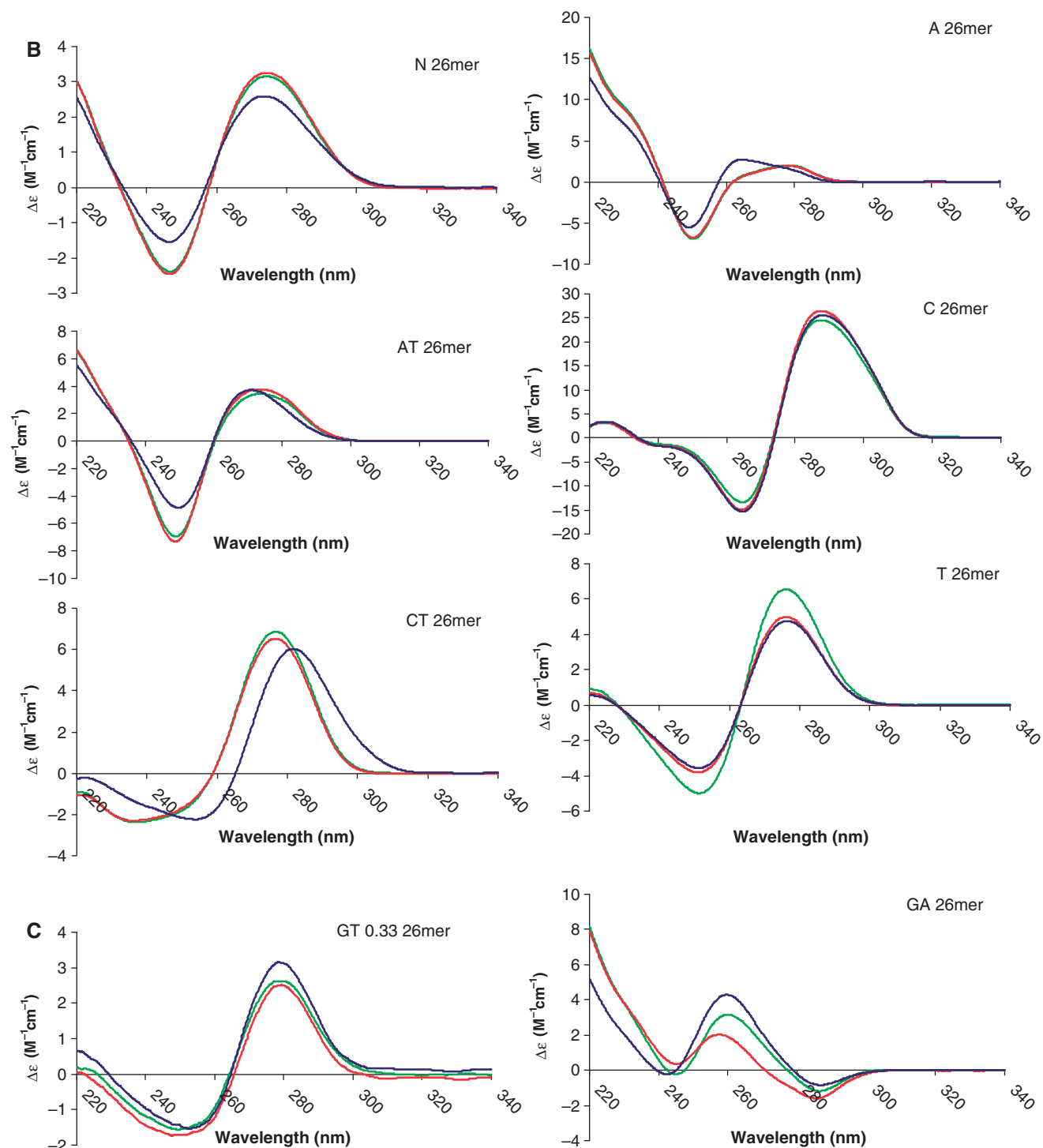
Figure 4. Continued.

different salt solutions, except for the CT library and $(dA)_{26}$, which showed slightly different spectral patterns in water compared to the salt-containing solutions. Interestingly, the $(dC)_{26}$ oligomer displayed a very large ellipticity and the peaks were slightly phase-shifted from the remaining inactive libraries, with a negative peak at 265 nm and positive at 285 nm. This subtle shift in CD

spectra may be due to the formation of secondary structures called i-motifs that have been reported in cytosine-rich molecules (20,21), albeit usually only under slightly acidic conditions. Our theory was supported by additional experiments in buffered solutions, which showed that the amplitude and shape of the CD spectrum for $(dC)_{26}$ was strongly pH-dependent and was

typical of the i-motif (18,21) at pH 5, 6 or 7, but there was a transition to the standard single-stranded DNA spectrum at pH 8 (Supplementary Figure S2).

The two exceptions to the general trends were the GT 0.33 26-mer library and the GA 26-mer library (Figure 4C); although the GT 0.33 26-mer library is included in the active libraries, its spectrum resembled the inactive libraries, consistent with the expected much smaller proportion of quadruplex-forming sequences and its lower level of antiproliferative activity. The spectrum of the inactive GA 26-mer library varied considerably between water, NaCl and KCl, but was distinct from any of the other libraries examined, being close to zero at 240 nm, with a positive peak at 260 nm and a negative peak at 285 nm. This has been reported to be typical of G + A containing oligonucleotides and associated with stacked guanosines, although it is not clear if this reflects the presence of quadruplexes or other ordered structures, such as alternatively base-paired duplexes (18).

Libraries with antiproliferative activity are significantly more stable in serum than inactive libraries

To assess nuclease resistance, oligonucleotides and libraries were radiolabeled at the 5'-end and incubated in complete medium (which contains serum-derived exonucleases) for various times. The primary purpose of this experiment was to determine nuclease stability under the exact conditions used in the MTT assays. This was considered essential, because if an oligonucleotide is degraded in the cell culture medium, it is unlikely to be active whatever other properties it has (unless the active component is derived from degradation of the oligonucleotide into nucleotides). The results (Figure 5A) showed that libraries that did not contain any G bases, as well as all of the monobasic oligomers tested, were highly susceptible to degradation in serum-containing medium, with most being fully degraded within 90 min. Libraries containing guanines in any percentage had at least some molecules still intact at 72 h, regardless of antiproliferative activity.

Although all active libraries displayed stability in the presence of serum nucleases, the degree of stability did not correlate with antiproliferative activity. For example, the moderately active GT 10-mer library was markedly less stable than the inactive N 26-mer library. However, although band density did not correspond to antiproliferative activity for the individual libraries, the mean band density of the active libraries at 24 and 72 h was significantly greater ($P = 0.026$ using Students' two-tailed t-test) than the inactive libraries (Figure 5B).

Active libraries had increased cellular uptake compared to inactive libraries as shown by flow cytometry

For this assay, four representative 26-mer libraries (the active GT and GC libraries, and the inactive N and GA libraries) were synthesized with a fluorophore at the 3'-end and used to assess cellular uptake by flow cytometry. Cells were washed and harvested with trypsin prior to analysis to remove any surface-bound oligonucleotides. The relative level of uptake in a given
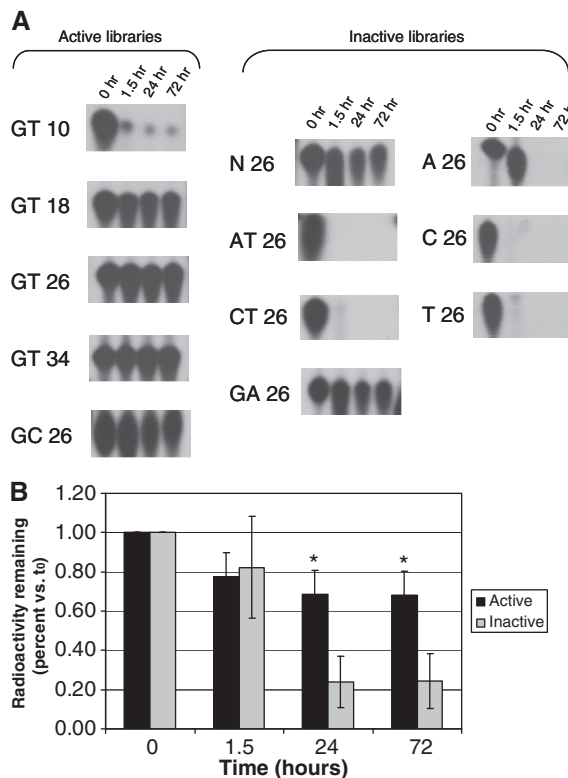
Figure 5. Nuclease-resistance in serum-containing medium. (**A**) Auto-radiographs showing electophoretic migration of radiolabeled oligomers or libraries following incubation in complete cell culture medium, which contains serum exonucleases. (**B**) Mean stability of active versus inactive libraries. The difference in band density between active and inactive libraries was statistically significant at 24 and 72 h (*$P < 0.05$ using Student's two-tailed t-test).

cell line can be estimated by determining the percent of cells that are 'positive' for the fluorophore (i.e. have a greater fluorescent intensity than the background signal, which arises from autofluorescence of the cells) after excluding non-viable cells. This type of analysis is especially useful for comparing the uptake of various libraries and oligonucleotides in a particular cell line (22), although its use for comparing uptake between different cell lines is somewhat complicated by differing levels of baseline autofluorescence between cell lines.

We observed that the active libraries, GT and GC, consistently yielded a higher proportion of fluorescent cells compared to inactive libraries in all cell lines (Figure 6). In general, the inactive N library had the lowest uptake of any library examined, and the inactive GA library had a similar or slightly higher uptake than the N library, but substantially less than the active libraries. For the GC 26-mer library, whereas its uptake in Hs27, DU145 and A549 cells was close to that of the GT library (albeit a different distribution), its uptake in MCF7 cells was considerably less than the GT library (Figure 6D). This result is noteworthy because the GC library also has reduced antiproliferative activity in this particular cell line (Figure 3E). Thus, these data suggest a correlation between relative uptake and antiproliferative activity, although with the caveat that, as mentioned above, this
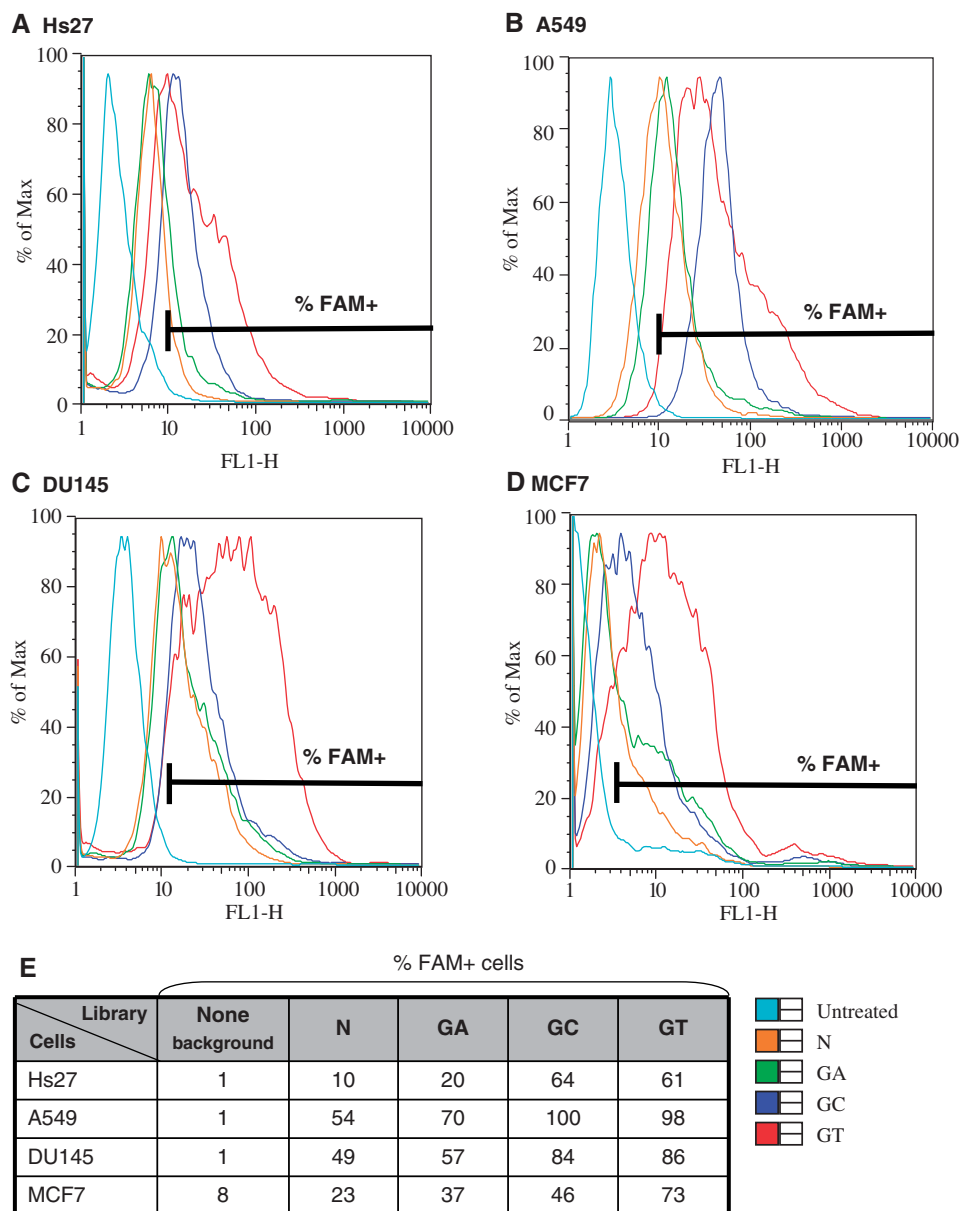
Figure 6. Cellular uptake of libraries and oligomers. Fluorescently labeled oligonucleotides (5 μM strand concentration) were incubated with cells for 1 h. Cells were harvested and stripped of surface-bound DNA using trypsin, then analyzed by flow cytometry to determine uptake. Uptake was quantified by determining the percentage of cells included in the black gate (positive for FAM after controlling for autofluorescence). (**A**) Uptake in Hs27 non-malignant fibroblasts. (**B**) Uptake in A549 cancer cells. (**C**) Uptake in DU145 cancer cells. (**D**) Uptake in MCF7 cancer cells. (**E**) Table of percent of FAM-positive cells for each sample.

technique may not be optimal for comparing uptake between different cell lines. We noted the non-uniform shape of the curve for the GT library compared to other libraries, which may indicate a non-uniform uptake of the GT library (some cells take up much more oligomer than others), but the reason for this is unclear at present.

Active libraries and inactive libraries had quantitative and qualitative differences in protein binding

The protein binding patterns of 5′-radiolabeled libraries or oligonucleotides following incubation with cell extracts were examined by electrophoretic mobility shift assay

(EMSA) and are shown in Figure 7. To start, we first analyzed the libraries and oligomers in the absence of proteins. The results (Figure 7A) confirmed the labeling and integrity of each sample and showed a quite marked dependence of electrophoretic migration on base composition, as expected. A very general observation from the EMSA experiments was that inactive libraries and oligomers displayed fewer and less intense shifted bands, when compared to the active libraries across all samples. Several shifted bands of interest were noted and are marked in Figure 7B–F. Appearance of the band marked 'B1' was associated for the most part with nuclease-resistance libraries, but not nuclease-susceptible
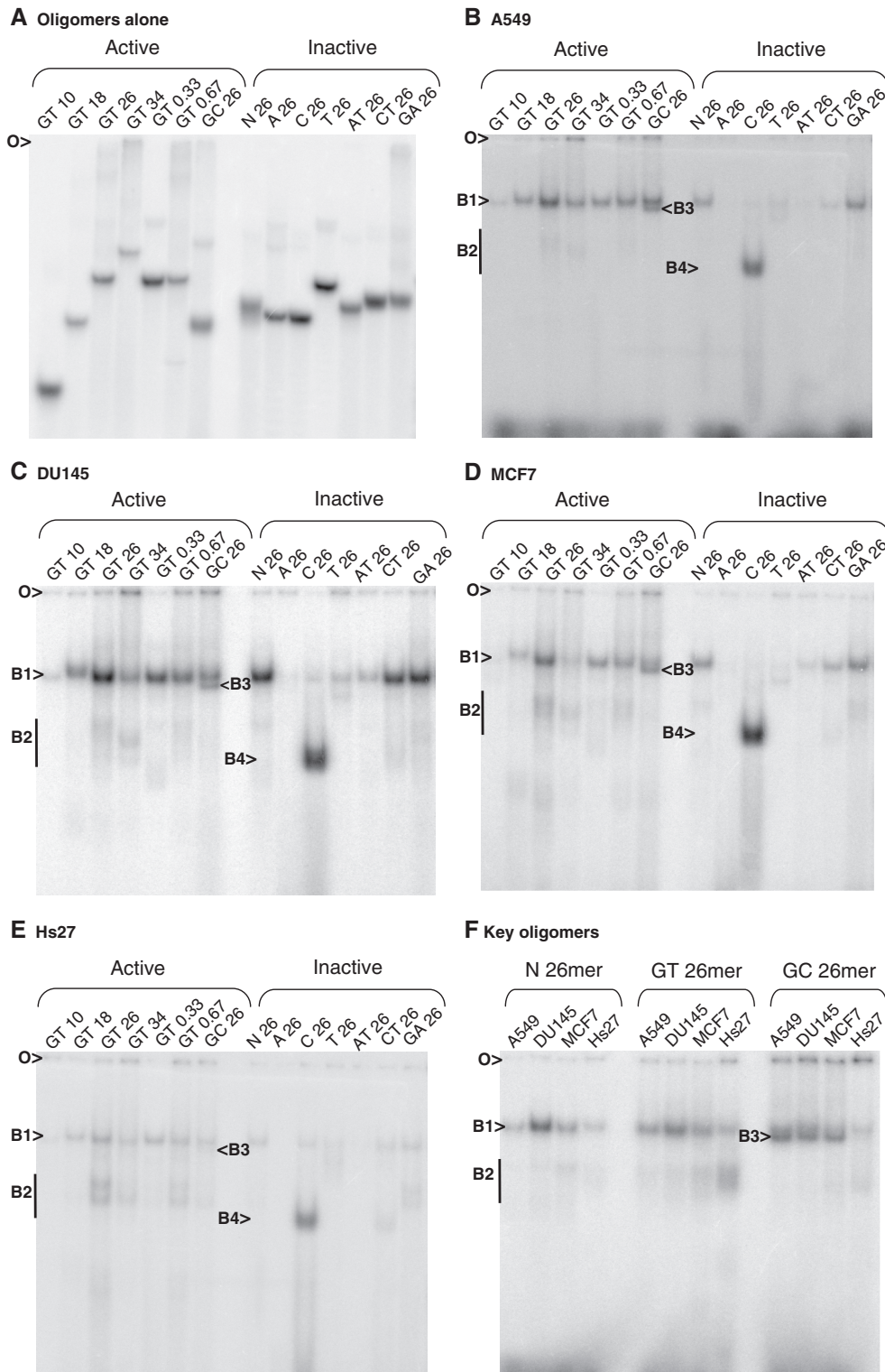
Figure 7. Protein binding of libraries and oligomers. Radiolabeled oligonucleotides were incubated with whole-cell extracts and analyzed by native gel EMSAs. Oligonucleotides were with: (**A**) no protein extracts (oligomers alone); (**B**) A549 extracts; (**C**) DU145 extracts; (**D**) MCF7 extracts; (**E**) Hs27 extracts; or (**F**) representative libraries were directly compared for binding to extracts (5 μg) from the four cell types. The arrows indicate DNA–protein complexes of particular interest (see text for discussion), and 'O' indicates the origin of the gel.

libraries or oligomers (Figure 5), suggesting it may be a general DNA-binding protein. However, this was usually most intense for the most active libraries and was present at much higher levels in the cancer cell lines compared to

the non-cancer cells (Figure 7F), suggesting it may contribute to the antiproliferative effects. The series of faster migrating bands indicated by 'B2' were much less intense than B1, but are of interest because they were present

predominantly for the most active libraries (GT26 and GT34). However, these bands were observed for Hs27 cell extracts, as well as for cancer cells (Figure 7F). The band marked 'B3' is particularly interesting because it is specific for the GC-library, which has strong antiproliferative activity, and it is present at high levels in all of the cancer cells, but is undetectable in the non-malignant cells (Figure 7F). The fastest migrating band we have highlighted, 'B4', is present only in the C26 sample. Although this sequence has no antiproliferative activity, this band is interesting because oligo–dC sequences may form unusual structures, involving the i-motif, and very few proteins that bind specifically to such structures have been previously reported (20). We plan to follow up these studies in the future by attempting to identify proteins of interest using mass spectrometry-based techniques.

Interpretation of the EMSA data in the presence of protein (Figure 7B–F) has the potential to be somewhat complicated because the presence of shifted bands may depend not only on protein binding activity, but on nuclease-resistance as well. The relative nuclease-susceptibility under these conditions was expected to reflect the results from our serum stability assay (Figure 5), but may not be exactly the same because cell extracts contain endonucleases, as well as exonucleases. To further investigate these issues, we performed nuclease-resistance assays in the presence of the DU145 cellular extracts that were used in the EMSA experiments. We examined stability after incubation for a short time (15 min), which mimics the EMSA conditions, and found no significant nuclease degradation (Supplementary Figure S3A). These results verify that the absence of a shifted band in the EMSA is due to the complex not being formed, rather than the oligonucleotides being degraded by nucleases. After longer incubation (18 h), which we anticipate will reflect the persistence of oligonucleotides inside cells, we observed (Supplementary Figure S3B) relative stabilities similar to those reported above for long-term stability in cell culture medium (Figure 5). This confirms our conclusions regarding the relationship between nuclease resistance, protein binding and antiproliferative activity (i.e. that, while active libraries are invariably stable and display strong protein binding, not all libraries that are stable and display strong protein binding are active). Furthermore, the data also suggest that protein binding may be responsible for nuclease resistance and not vice versa.

CONCLUSIONS

In this study, we have examined the biological properties and CD spectra of oligonucleotide libraries. The purpose of this exercise was primarily to test our hypothesis that G-rich libraries would be enriched in quadruplexes, which might make them useful starting points for the development of new therapeutic aptamers using SELEX or similar technologies.

The results of our analyses seem to confirm that G-rich oligodeoxynucleotide libraries do indeed contain large numbers of quadruplex-forming sequences, and they are more nuclease-resistant and efficiently internalized by cells compared to the standard 'N library' currently used in SELEX. Experiments are now underway to determine if G-rich libraries can be used for SELEX and whether they will allow faster evolution of aptamers for *in vivo* use.

While many of the observed properties of G-rich libraries were anticipated, the strong and cancer-selective growth inhibitory effects of some GT and GC libraries were unforeseen. When analyzing a library of molecules, it is important to remember that the results are the averages of the effects of all the different molecules within the library. Given our previous work, showing that several different quadruplex-forming oligonucleotides have cancer-selective antiproliferative activity (19,23), we expected that G-rich libraries might contain a small proportion of active molecules. However, while we anticipated that a G-rich library might contain some very potent molecules, we presumed that the vast majority of the estimated 67 000 000 sequences in such a library would be ineffective, such that the library as a whole would display at best a modest growth inhibitory effect against cancer cells. Thus, the finding that certain GT and GC libraries have very potent and tumor-selective antiproliferative effects against a variety of cancer cell types was somewhat surprising. Remarkably, the antiproliferative activity of these libraries was comparable to some individual G-rich oligodeoxynucleotides that are currently in pre-clinical development or clinical trials (11).

Although a full understanding of the antiproliferative properties of active libraries will require further efforts, the work that we have described provides some clues to the mechanism of action for these effects. The restriction of the activity to only certain GT and GC libraries indicates that it cannot be a non-specific effect of phosphodiester oligonucleotides or simply due to the presence of deoxyguanosine nucleotides (either in the context of an oligonucleotide or as nucleobases resulting from oligomer degradation), because some dG-containing libraries, including the GA and N libraries, had no activity. In addition, although active libraries are generally more stable than inactive libraries, nuclease-resistance alone cannot explain the antiproliferative effects because some of the inactive libraries displayed comparable stability.

In view of our observations, we now propose that antiproliferative activity requires all of the following: nuclease-resistance, efficient cellular uptake and binding to a specific protein (or proteins). It is not yet clear if quadruplex formation, *per se*, is necessary for activity, or whether it is simply that quadruplex-forming oligonucleotides generally have the aforementioned properties. One possible candidate for the complex in the EMSAs that appears to be most relevant for activity (band 'B1' in Figure 7) is nucleolin. This protein is highly expressed in cancer cells and is a general DNA binding protein, but binds particularly strongly to a variety of quadruplex-forming sequences (24–27). It is also the molecular target of an existing G/T oligonucleotide aptamer (11). However, further studies will be required to verify this possibility and to reconcile why some

libraries bind to this protein (if it is indeed band B1), yet do not have enhanced cellular uptake or antiproliferative activity. Interestingly, the GC library appears to have a different cancer cell selectivity compared to the GT libraries and forms a specific protein complex (band 'B3' in Figure 7), suggesting that its mechanism of action and molecular target may be distinct from the GT oligonucleotides.

In summary, the potent intrinsic and selective antiproliferative activity of the GT and GC libraries is an unexpected and wholly novel finding. Our results indicate that cancer-selective antiproliferative activity is a common characteristic of G-rich DNA oligonucleotides, which may explain why numerous different G-rich oligonucleotides have been reported as potential anticancer agents (11). Although many different mechanisms of action have been proposed for these, our results suggest there may be common mechanisms (related to nuclease resistance, protein binding and cellular uptake) that contribute to the activity of G-rich oligonucleotides. Further research is warranted to elucidate the mechanism of action for these cancer-selective effects, as this may lead to insights into basic cancer cell biology, as well as to the identification of novel molecular targets and new oligomer-based therapies for cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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