Aptameric inhibition of p210$^{bcr-abl}$ tyrosine kinase autophosphorylation by oligodeoxynucleotides of defined sequence and backbone structure

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ABSTRACT

Protein tyrosine kinases play key roles in cellular physiology. Specific inhibitors of these enzymes are important laboratory tools and may prove to be novel therapeutic agents. In this report we describe a new class of tyrosine kinase inhibitor, synthetic oligodeoxynucleotides (ODNs). An ODN is described which specifically inhibits p210$^{bcr-abl}$ tyrosine kinase autophosphorylation in vitro with a K_i of 0.5 μM. Inhibition is non-competitive with respect to ATP. The effects upon inhibitory activity of ODN structure modifications are described. The inhibition described is not mediated by classical antisense mechanisms and represents an example of the recently recognized aptameric properties of ODNs.

INTRODUCTION

Protein tyrosine kinases (PTKs) transfer the gamma phosphate of either ATP or GTP to specific tyrosine residues of acceptor proteins (1). These enzymes and their substrate proteins are important mediators of cellular signal transduction and their aberrant function is frequently associated with deregulated growth and neoplasia (2−8). In fact, the first oncogenic protein characterized, isolated from the transforming retrovirus Rous sarcoma virus, was the PTK src (9). Many of the subsequently discovered oncogenes are also PTKs. PTK inhibitors, therefore, may represent an important class of anti-neoplastic agents (10−12). The majority of PTK inhibitors consist of agents that either inhibit nucleotide or tyrosine containing substrate binding, while the remainder effect catalysis by other means (13−14). Since a high degree of homology exists among the nucleotide binding domains of many ATP-utilizing enzymes, existing inhibitors of nucleotide binding lack specificity for particular PTKs, and in some cases cannot distinguish between PTKs and other kinases.

Chronic myelogenous leukemia (CML) cells contain a unique chromosomal translocation, the Philadelphia chromosome, resulting in the expression of a novel PTK, the p210$^{bcr-abl}$ protein (15). This PTK has been directly implicated in the ontogeny and progression of CML (16). While studying the biological effects of antisense oligodeoxynucleotides (ODNs) targeting the p210$^{bcr-abl}$ fusion mRNA, we observed that certain ODNs will directly inhibit p210$^{bcr-abl}$ kinase activity in vitro. This study defines the parameters of this phenomenon and suggests that synthetic ODNs may represent a novel class of specific PTK inhibitors.

EXPERIMENTAL PROCEDURES

Cell cultures

K562, NIH 3T3, and A431 cells were purchased from the American Type Culture Collection (Rockville, MD). PC3M prostate cancer cells were a generous gift from J.Trepel (National Institutes of Health, Bethesda, MD). K562 and PC3M cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY) while NIH 3T3 and A431 cells were cultured in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) with 10% heat inactivated FBS. Cells were maintained at 37°C, 6% carbon dioxide with biweekly media changes.

Source of PTKs

The p210$^{bcr-abl}$ kinase, src kinase and cdc-2 kinase were immunoprecipitated from K562 cells. The p145^{abl} kinase was immunoprecipitated from PC3M cells, PDGF receptor kinase from 3T3 cells, and EGF receptor kinase from A431 cells. MAP kinase was utilized as a pure protein and lck kinase was immunoprecipitated from peripheral blood lymphocytes of a normal volunteer.

Oligodeoxynucleotide synthesis

ODNs were synthesized by cyanoethyl phosphoroamidite chemistry on an Applied Biosystems model 380B DNA synthesizer. Sulfurizing reagent (Glen Research, Sterling VA) was used according to the manufacturer's instructions. Samples were ethanol precipitated, washed in 70% ethanol, and resuspended in sterile water. Aliquots were resolved on a denaturing polyacrylamide gel to check for homogeneity. The

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sequence of **ODN1** is 5'-GTCCACCATGGCGCGGCCGGC-3' and unless otherwise specified is phosphorothioate capped in all experiments; it is complementary to nucleotides −12 to +9 of the p210$^{bcr-abl}$ mRNA (+1 is the translation start site). All phosphorothioate capped ODNs contain two interbase phosphorothioate linkages at both 5' and 3' ends, except the tetramer GGCG which only contains one phosphorothioate linkage at each end. ODNs modified with 2'-methoxy ribose were purchased from Integrated DNA Technologies Inc. (Coralville, IA).

Preparation of cell lysates

Cells were washed in phosphate buffered saline (PBS) and $0.5-5 \times 10^6$ cells were solubilized in 1 ml of the specified cell lysis buffers. Unless otherwise stated, all cell lysis buffers contained the following protease inhibitors: 20 μg/ml leupeptin, 1 μg/ml aprotinin, and 1mM phenylmethylsulfonylfluoride. For p210$^{bcr-abl}$ and p145^{abl} immunoprecipitation, cells were lysed with ABL lysis buffer (25 mM imidazole, pH 7.2, 0.5% NP40, 50 mM NaCl, 1 mM Na$_3$VO$_4$) and lysates were clarified at 14,000 g, 4°C for 30 min. Cell lysis for all other proteins was performed according to the references describing the various immunoprecipitation kinase assays.

Immunoprecipitation kinase assays

Immunoprecipitation kinase assays of p210$^{bcr-abl}$ or p145^{abl} were performed as described, with modifications (15). Briefly, for p210$^{bcr-abl}$, 1 μg of anti-bcr antibody (clone 7C6, Oncogene Science, Uniondale, NY) was added to lysates prepared from $0.5-1 \times 10^6$ K562 cells. For p145^{abl}, 1 μg of anti-c-abl antibody (clone 24-21), which binds to the C-terminal domain of p145^{abl}, was added to lysates prepared from 5×10^6 PC3M cells. After incubating 1 h at 4°C, complexes were precipitated by incubating with protein A−Sepharose coated with rabbit anti-mouse immunoglobulin (Cappel Research Products, Durham, NC) for 30 min. The complexes were then washed as follows: ABL lysis buffer×2, PBS×1, and ABL assay buffer×1 (24 mM imidazole, pH 7.2, 1 mg/ml bovine serum albumin, and 10 mM MnCl); for p145^{abl} assays ABL assay buffer contained 20 mM MnCl. To assess the activiy of p210$^{bcr-abl}$ on substrate phosphorylation, acid inactivated enolase (Sigma Chemical Co., St Louis, MO) was added as described (15).

Epidermal growth factor receptor (EGFR) was immuno-precipitated with 10 μg anti-EGFR antibody (clone LA1) and human recombinant epidermal growth factor (both from Upstate Biotechnology Inc., Lake Placid, NY). The assay was performed as described (17) but without removing the EGFR from the protein A−antibody solid support. Instead, epidermal growth factor, at a final concentration of 100 nM, was added to the kinase reaction mixture. Platelet derived growth factor receptor (PDGFR) was immunoprecipitated with anti-human type A/type B PDGFR antibody (Upstate Biotechnology Inc.) and immune complex kinase assays performed as referenced (18) except that protein A−Sepharose coated with rabbit anti-mouse immuno-globulin was used instead of formalin-fixed *Staphylococcus aureus* to bind PDGFR−antibody conjugates. C-src was immunoprecipitated with anti-v-src antibody (Ab-1; Oncogene Science) and assayed as previously described (19) with the addition of enolase as exogenous substrate. Lck was immuno-precipitated using anti-lck antibody (Upstate Biotechnology Inc.) and assayed as described (19). Cdc-2 kinase (20) was isolated with affinity purified rabbit antibody (a generous gift of Jean

Wang, University of California, SanDiego, CA) and assayed with casein that was dephosphorylated as described by Mayer (21). MAP kinase was assayed as previously described (22) using purified enzyme (Upstate Biotechnology Inc.).

ODNs were added to kinase reaction buffers not more than 5 min prior to the addition of ATP. Reactions were initiated by the addition of [γ-^{32}P]ATP (3000 Ci/mmole; Amersham Corp., Arlington Heights, IL) and quenched by the addition of 5× gel loading buffer and heating to 95°C. Kinase reactions were run for 1−10 min at room temperature, except for EGFR, p145^{abl}, and MAP kinase, which were run at 30°C. The products were then electrophoresed on SDS−polyacrylamide gels: 7% for p210$^{bcr-abl}$, EGFR, and PDGFR, 8% for p145^{abl}, 10% for *src*, *lck*, and cdc-2 kinase, and 12% for MAP kinase. Gels were dried and reaction products were visualized by direct autoradiography at −70°C with Kodak X-0MAT AR film. To determine IC$_{50}$ and K_i values, gel slices containing the band of interest were excised and analyzed by Cerenkov counting.

RESULTS

The *in vitro* inhibition of p210$^{bcr-abl}$ kinase activity by **ODN1** can be seen in Figure 1A. Kinase activity is markedly inhibited by pre-incubation of the immunoprecipitated enzyme with 1 μM **ODN1**. To determine the nature of the enzymatic inhibition and to quantitate the potency of inhibition, enzyme activity was determined at various [γ-^{32}P]ATP concentrations in the presence or absence of 1 μM **ODN1**. Figure 1B depicts the double-
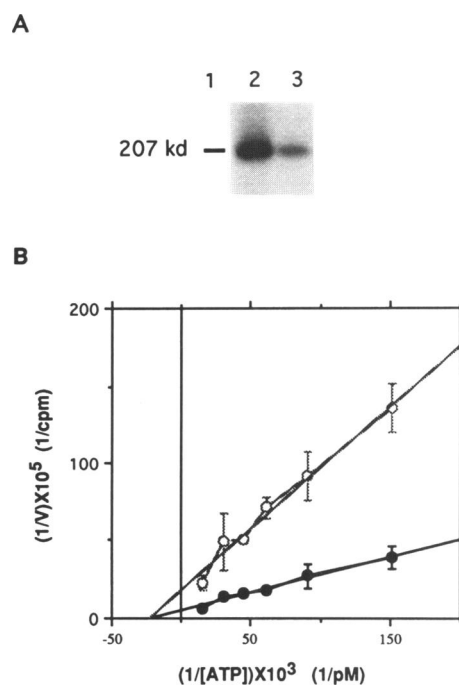

Figure 1. Inhibition of p210$^{bcr-abl}$ kinase activity by **ODN1**. (**A**) Autoradiograph of immunoprecipitation kinase reactions: lane 1, molecular weight standards; lane 2, no ODN; lane 3, 1 μM **ODN1**. (**B**) Double-reciprocal plot of p210$^{bcr-abl}$ kinase activity, as determined by Cerenkov counting of excised polyacrylamide gel slices, in the absence (closed circles) and presence (open circles) of 1 μM **ODN1**. The data represent the mean ± SD of a single experiment run in duplicate. Similar results were obtained in a separate experiment also run in duplicate.

reciprocal plot of enzyme activity as determined by Cerenkov counting of excised SDS gel slices. **ODN1** apparently inhibits the p210$^{bcr-abl}$ kinase in a purely noncompetitive manner with respect to ATP, with a K_i of 0.52 ± 0.12 μM.

The p210$^{bcr-abl}$ protein is the product of a chromosomal translocation leading to the formation of a fusion transcript (23, 24). In the fusion protein there is replacement of the normal abl N-terminal sequences with the N-terminal sequences of the bcr protein. This leads to a relative lack of regulation with a subsequent increase in PTK activity (25). There is evidence to suggest that both the loss of native abl sequences and interactions with N-terminal bcr sequences have effects upon kinase activity (26, 27). To determine if the bcr moiety is important in mediating the inhibitory effects of **ODN1**, its ability to inhibit p145^{abl} kinase activity was tested (see Table 1). The results demonstrate that both p145^{abl} and p210$^{bcr-abl}$ are equally sensitive to **ODN1**. To determine whether **ODN1** can also inhibit the ability of either p145^{abl} or p210$^{bcr-abl}$ to phosphorylate substrates, enolase was added to the kinase reactions; at **ODN1** concentrations of up to 50 μM there was no significant inhibition of enolase phosphorylation (data not shown).

To determine if the inhibitory properties of **ODN1** are a function of sequence, four phosphorothioate capped scrambled sequences (based on **ODN1**) were synthesized and their effect upon the inhibition of p210$^{bcr-abl}$ kinase activity at 1 μM was compared to that of **ODN1** (Table 2). By comparing the percent inhibition seen with each ODN with its sequence, it appears that the presence of closely spaced GGC repeats contributes significantly to the ability of a particular ODN to inhibit p210$^{bcr-abl}$ activity. To test this hypothesis, we determined the ability of ODNs consisting only of GGC repeats to inhibit kinase activity (Figure 2A). With a 21-mer sequence (the same length

as **ODN1**) consisting of seven tandem GGC repeats, inhibitory activity equivalent to **ODN1** is seen. However, the tetramer GGCG had no inhibitory activity, again suggesting the requirement for GGC repeats.

To determine the significance of backbone structure to this phenomenon, **ODN1** was synthesized with four different backbone structures as follows: all phosphodiester, all phosphorothioate, phosphorothioate capped, and all phosphodiester with 2'-methoxy modified sugars. The ability of these sequences to inhibit p210$^{bcr-abl}$ kinase activity at 1 μM was then determined (Figure 2B). It is evident that backbone modification can significantly affect the ability of **ODN1** to inhibit the kinase. **ODN1** with an all phosphorothioate backbone was the most potent inhibitor, while the version of **ODN1** containing 2'-methoxy modified sugars was the least effective. The phosphorothioate capped and the all phosphodiester species have intermediate potencies. Prior nuclease digestion of the phosphodiester congener of **ODN1** completely abrogated its inhibitory potential (data not shown).

The specificity **ODN1**'s inhibitory activity was evaluated by testing its ability to inhibit several other PTKs and serine/threonine kinases. Table 1 lists the IC$_{50}$ values. The data demonstrate that all kinases are not equally sensitive to this ODN. Only one other PTK examined, the PDGF receptor, demonstrated sensitivity, although this kinase was almost a log less sensitive than either p145^{abl} or p210$^{bcr-abl}$. Other PTKs, including *src, lck* and the EGF receptor were not significantly inhibited at **ODN1** concentrations greater than 2 logs above the K_i for p210$^{bcr-abl}$. Interestingly, while PDGF and EGF receptors are PTKs with extensive amino acid sequence homology (28), they display a marked difference in sensitivity to **ODN1** (Figure 3).

As kinase inhibitory activity *in vitro* may not correlate with *in vivo* inhibitory activity, the effects of **ODN1** *in vivo* were assessed. K562 cells in liquid culture were incubated in the presence of either 50 μM **ODN1**, or with a scrambled version of **ODN1** that does not inhibit kinase activity (ODN 1D from Table 2). After 3 days, cells were washed and p210$^{bcr-abl}$ kinase activity was measured. It can be seen in Figure 4 that in **ODN1** treated cells the activity of p210$^{bcr-abl}$ is significantly decreased relative to the scrambled and untreated control cells. Neither cellular p210$^{bcr-abl}$ protein content nor cell growth was affected by ODN treatment. (data not shown).

DISCUSSION

This study demonstrates that synthetic ODNs can act as inhibitors of specific PTKs with K_i values in the sub-micromolar range. Other nucleoside-based PTK inhibitors are generally analogs of adenosine which interact with kinases at the active site (13). As

Table 1. IC$_{50}$ values of **ODN1** against various protein kinases

Protein kinase	IC$_{50}$ (μM)
Tyrosine kinases	
BCR-ABL	0.52 ± 0.12
ABL	0.62 ± 0.24
SRC	>100
LCK	>50
EGFR	>100
PDGFR	4.4 ± 2.3
Serine/threonine kinases	
cdc -2 kinase	>50
MAP kinase	>100

Enzyme activity was determined by Cerenkov counting of excised gel slices. Values are the mean ± SD of two separate experiments, each composed of dual determinations.

Table 2. Inhibition of p210$^{bcr-abl}$ kinase activity *in vitro* by phosphorothioate capped ODNs of various sequence

	Sequence	Percent remaining activity
ODN1	GTCCACCATGGCGCGGCCGGC	36 ± 1.2
ODN1A	GGCGGCCGCGCTGCAACCCGT	42 ± 6.9
ODN1B	TGCCCAACGTCGCGCCGGCGG	59 ± 6.4
ODN1C	GGCCGTGCAGCTCGCACCGGC	74 ± 15
ODN1D	TCCGCGAACCCGGTGCGCCGG	79 ± 19

Percent activity remaining refers to percent of activity relative to control as determined by Cerenkov counting of excised gel slices as described in Experimental procedures. All ODNs were added to a final concentration of 1 μM. ODNs 1A–1D are scrambled versions of ODN1. Values are expressed as the mean ± SE of three separate experiments.
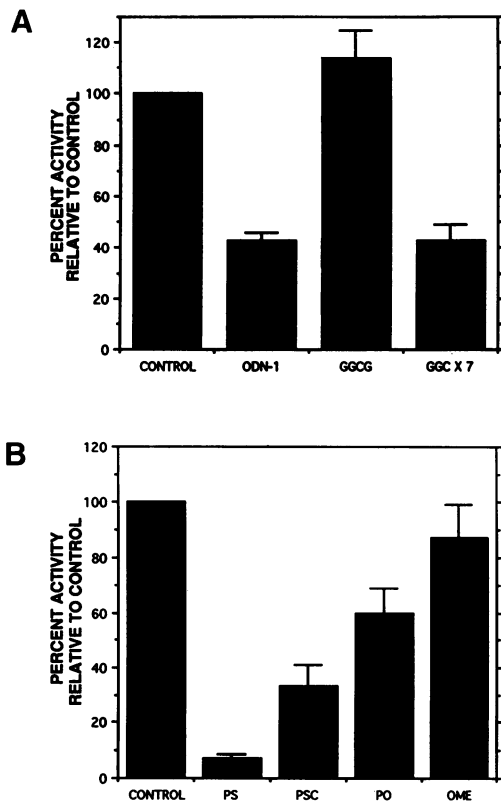
Figure 2. Inhibition of p210$^{bcr-abl}$ kinase activity *in vitro* by ODNs of altered sequence and backbone structure. (A) Effect of sequence. Activity was measured in the absence (control) or presence of the following phosphorothioate capped ODN species: **ODN1**, GGCG tetramer, and a 21-mer consisting of seven tandem GGC repeats (GGC X7). (B) Effect of ODN backbone modification. Activity was measured in the absence (control) or presence of **ODN1** with various backbone modifications: PO, all phosphodiester; PS, all phosphorothioate; PSC, phosphorothioate capped; OME, all phosphodiester 2'-methoxy ribose modified. The activity of p210$^{bcr-abl}$ kinase was determined by excision of gel slices as explained in Experimental procedures; all ODNs were present at 1 μM. Values are the mean ± SE of three separate experiments.

Figure 3. Inhibition of epidermal growth factor receptor (EGFR) (A) and platelet derived growth factor receptor (PDGFR) (B) autophosphorylation *in vitro* by **ODN1**. Immunoprecipitation kinase assays were performed as described in Experimental procedures, in the absence of (lane 2), or with increasing amounts of **ODN1** present as follows: lane 3, 0.1 μM; lane 4, 1 μM; lane 5, 10 μM; lane 6, 100 μM; lane 1, molecular weight standards.

Figure 4. Immunoprecipitation kinase assay of p210$^{bcr-abl}$ from K562 cells treated with ODNs for 3 days. K562 cells were seeded at 0.5×10^6/ml and allowed to grow for 3 days in the presence of either 50 μM **ODN1** (lane 2) or ODN 1D (lane 3); lane 1, no ODN. The kinase activity of p210$^{bcr-abl}$ isolated from an equal number of cells was then assessed as described in Experimental procedures.

such, they are competitive inhibitors that lack specificity. If they were acting solely as adenosine analogs, oligodeoxynucleotides would be expected to interact with the active site in a manner similar to such inhibitors.

Our data would suggest that **ODN1** is not binding at the ATP binding site. Kinetic analysis indicates that **ODN1** is inhibiting p210$^{bcr-abl}$ in a noncompetitive manner with respect to ATP. Furthermore, within the limitations of this study, selective inhibition was also demonstrated: PDGFR was almost a log less sensitive to inhibition than either p145^{abl} or p210$^{bcr-abl}$, and all other kinases tested were not significantly inhibited at concentrations 2 logs above the K_i for p210$^{bcr-abl}$. Since both p210$^{bcr-abl}$ and p145^{abl} are equally sensitive to **ODN1**, it would appear that **ODN1** interacts with the abl portion of the p210 chimeric protein.

Though **ODN1** appears to be a weak inhibitor of enolase phosphorylation *in vitro*, it is a potent inhibitor of p210$^{bcr-abl}$ autophosphorylation *in vitro* and *in vivo*. While the ability to inhibit substrate phosphorylation is relevant, enolase is not a physiologic substrate for the p210 protein, nor are the physiologic substrates kn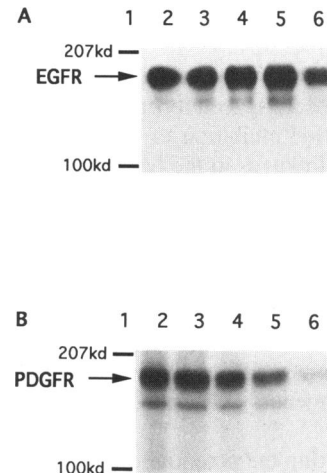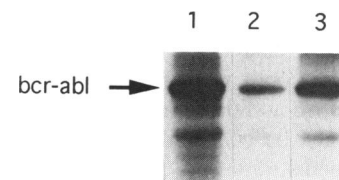own. On the other hand, PTKs participate in signal transduction pathways by associating with various members of signal transduction cascades, usually via interaction of target protein SH2 regions with an autophosphorylated site on the PTK. Thus, the phosphorylation state of a particular PTK is also an important regulator of overall enzyme activity (11, 29–31). Pendergast *et al.* have shown that autophosphorylation of tyrosine at position 1294 of p210$^{bcr-abl}$ is critical for cellular transformation (32). However, while replacement of this tyrosine residue with phenylalanine abrogates the transforming ability of p210$^{bcr-abl}$, this mutation has no effect on the specific activity of the enzyme. In support of this observation, we have observed that the protein phosphotyrosine content of CML cells incubated with **ODN1** is markedly reduced, and that this correlates with inhibition of clonogenicity in soft agar (Bergan and Neckers, manuscript in preparation).

The direct interaction of an ODN with a protein, accompanied by modulation of protein activity, has been described (33). Although several such 'aptamer' effects have been reported in the literature, the rules governing these interactions are not at all clearly understood (33–37). Nonetheless, a requirement for ODN sequence specificity can be demonstrated. Presumably, base

sequence determines final form and function, analogous to amino acid sequence in proteins.

Through non-exhaustive selection procedures, we have determined that closely spaced repeats of the consensus sequence GGC are critical for inhibition of p210$^{bcr-abl}$. The presence of phosphorothioate linkages in the backbone apparently enhances the inhibitory properties of the oligonucleotide. Enhanced affinity of phosphorothioate ODNs for proteins has been described and is probably contributing to this effect (34). The 2-methoxy modified species did not significantly inhibit the p210$^{bcr-abl}$ kinase, suggesting that negative charge is not the only determinant of aptameric inhibition. Previous reports have suggested that 2-methoxy species associate with proteins much less avidly than do other ODN congeners (34), and our findings seem to support this observation.

While complicating the potential use of ODNs as molecularly targeted 'magic bullets', the aptameric properties of this class of molecule, if properly understood and exploited, could in fact expand their ultimate utility. Techniques have now been developed to optimize the process of aptameric ODN selection (35). Such an approach has led to the identification of a specific ODN that inhibits thrombin function (33). A similar approach could potentially identify other ODNs that would be specific for one and only one PTK.

In summary, the ability of ODNs to potently and specifically inhibit PTK activity in an aptameric fashion makes these molecules appealing candidates for development as specific enzymatic inhibitors both for laboratory use and as potential therapeutic agents. In addition, the potential for the occurrence of such effects must be considered when conducting classical antisense experiments.

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