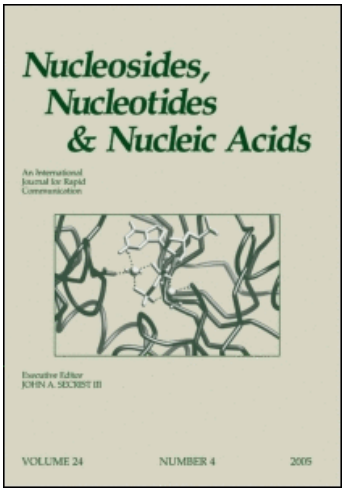

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Binding Features of BCL2-Targeted Oligodeoxynucleotides with K562 Cells

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ABSTRACT

Delivery of various oligodeoxynucleotides into cells is mediated by binding to certain surface proteins followed by receptor-mediated endocytosis. Moreover, oligonucleotides are able to provoke perturbation of cell surface proteins and growth factor receptors among them. Here we described binding sense BCL2 oligodeoxynucleotide targeted to translation start of BCL2 mRNA (ODN) with K562 cells. At low concentration ODN bound efficiently with K562 and penetrated into the cells via binding cell surface with rather high affinity and priming new binding sites. The loose binding constant at 4°C was 1.8×10^9 M^{-1} both for binding ODN in solution and ODN-associated liposome. The number of loose binding sites under both treatments was rather high: 4.6 to 6.6 pmoles per 10^6 cells. The extent of ODN penetration into the cells showed higher potential site numbers than initially seen and reached 8.6 pmoles per 10^6 cells for four hours incubation at 37°C.

Key Words: BCL2 sense oligodeoxynucleotide; K562 cells; Loose and tight binding; Penetration into the cells; Binding constant and binding sites.

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INTRODUCTION

Delivery of various oligodeoxynucleotides into cells is mediated by binding certain surface proteins followed by receptor-mediated endocytosis.[1,2] Moreover, ODNs are able to provoke perturbation of cell surface and growth factor receptors among them.[3] Some ODNs are known to bind with cell surface proteins, many factor receptors as well as cytoplasmic and nuclear transcription factors. There are TGFβ, EDFR, VETFR, heparin-binding growth factor, bFTF, tyrosine kinase receptors, Rel A subunits of NF—κB.[3,4] According to previous research,[3,5] ODN carrying GGGCGGGA interacts with Sp1 transcription factor and modulates its expression. Being a member of the ETS family of transcription factors, Sp1 participates in regulation of tyrosine kinase activity.[6] According to[7] SODN carrying GGGCGGGA interacts with Sp1 transcription factor and modulates its activity.

$P210^{BCR/ABL}$ oncoprotein as an active tyrosine kinase causes chronic myeloid leukemia (CML).[7] Modulation of tyrosine kinase activity is practical approach to renew normal cell functioning. STI 571, an inhibitor of tyrosine kinases, is used in CML treatment.[8] We studied the interaction of sense BCL2 ODN potentially targeted to Sp1 with BCR/ABL^{+}K562 cells and showed ODN efficient binding with the cell surface and penetration into the cells that was accompanied by priming new binding sites.

MATERIALS AND METHODS

Oligodeoxynucleotide (ODN) [^{3}H] labeled sense BCL2 as 18-mer was used: 5′—AAGGATGGCGCACG TGA—3′[^{3}H] that was labeled to 42 Ci/mmole.

K562 cells were a gift from the Institute for Cytology, Russian Academy of Sciences, St.Peterburg. K562 (10^6 cells/ml RPMI 1640 without any growth factors or fetal bovine serum) were incubated for 1, 2 or 4 hours at 37°C with $2{,}9 \times 10^{-8}$ M ODN itself or with ODN-associated liposome (TF—50 reagent, Promega). ODN and liposomes were mixed up to ratio$1^{-}/7^{+}$ 40 min at room temperature according Promega protocol. Cells that accepted ODN were isolated by sedimentation of the reaction mixture at 2100 rpm/min at 4°C for 20 min. Cells that accepted ODN and supernant as well as other probes of each step of treatments were tested by [^{3}H] counting and then normalized to 1×10^6 cells.

Total bound ODN with cells was used for testing binding types. Loose bound ODN was estimated by successive equilibrium dissociation of ODN totally bound with 10^6 cells in 1 ml of PBS (0.15 M NaCl + 3 mM KCl + 9 mM Na_2HPO_4 + 2 mM KH_2PO_4, pH 7.4) for 30 min at 4°C, followed by sedimentation at 4°C for 20 min and [^{3}H] counting both cells and supernants. Treatment was repeated 3–4 times for the each probe. Tightly bound ODN was estimated by elution of 10^6 cell probe that was obtained after extraction of loose bound ODN by 1 ml acid salt solution pH 2.5 (AS) for 5 min at 4°C.[9] The amount of ODN that penetrated into the cells (internalized ODN) was tested by [^{3}H] in cell lysate with 5 M KOH (0.5 ml, 5 min at 60°C) after prior elution of tight bound ODN with AS (0.2 M CH_3COOH + 0.5 M NaCl, pH 2.5).

RESULTS

One of the approaches to inhibition of tumor BCR/ABL$^+$ cell proliferation is a treatment of the cells with ODNs. There are many observations that oligonucleotides with GC-rich sequences are active modulators of tyrosine kinase.[5,6] To study binding to K562 cells, bearing the BCR/ABL oncogene, we used (here applied) [^{3}H] labeled BCL2 ODN (18-mer) that was the sense analogue of the antisense oligonucleotide targeted to the translation start of BCL2 mRNA. The BCL2 ODN had sequence homology with the ODN that bound specifically to Sp1 transcriptional factors and modulated tyrosine kinase activity.[5,6]

Quantitative characterization of oligonucleotide binding to cells is considered usually as loose and tight binding and ODN penetrated into the cells (ODN-internalized).[9] Direct evaluation of ODN binding with cells is rather conditional. It is rather difficult to separate ODN-associated cells without some ODN dissociation or absorption of additional ODN. We measured [^{3}H] labeled free ODN and ODN bound with cells as described above. Using the same low ODN and cells concentration the ODN-associated K562 cell separated cells were considered as total bound ODN with cells. Then the latter underwent to equilibrium dissociation in 1 ml PBS several times (for 30 min at 4°C and 20 min centrifugation at 4°C). This condition we considered to establish equilibrium in the suspension. According to Ref. [10] equilibrium is reached after 20 min incubation at the same conditions.

Table 1 shows rather high binding at ODN in solution and ODN with liposomes: 40–60% of ODN bound with cells at 29 nM ODN (Table 1). The dynamics of total ODN binding with cell in ODN solution and with ODN-associated liposome demonstrated a more than 1.5-fold increase in binding of both ODN in solution and with liposome (Fig. 1). One and two hour incubation changed binding a little, showing a lag period for ODN acceptance. After four hours the incubation increased binding distinctly. To discriminate loose and tight surface binding we used two or four extractions by 1 ml PBS followed by AS extraction. The latter enables the elution of the surface-bound ligands, leaving the intracellular ligands.[9] The extractions indicated rather a high but scattered share of loose and tight binding (Fig. 2, Table 1). As to the amount of ODN that penetrated into the cell it was the same for one-two hour incubation and increased significantly with further incubation. The ODN internalized per 10^6 cells increased from 4 to 8 pmoles for 1–4 hour incubation, while from 1 to 2 hours it changed a little (Table 1 and Fig. 2), demonstrating a lag-period in internalization as reflected in changes in ODN binding. Total binding was enhanced to 7–12 pmoles, internalized one increased from 4 to 8 pmoles per 10^6 cells, but loose and tight binding stayed rather low. It should be mentioned that loose and tight binding were rather scattered. We found that estimating these binding separately at any point could not show real magnitudes because it was destroyed by fast ODN internalization and presumed absence of significant differences between loose and tight binding at 4°C. We did not exclude that tight binding underwent fast internalization. It seems that to consider the sum of loose and apparent tight was a better approach. A separate experiment with multiple treatments by PBS led to exhausting of any tight binding. Nevertheless the value of ODN binding increased with time both for binding ODN in solution and with ODN associated with liposome (Fig. 2 and Table 1).

Table 1. ODN binding with K562 cells in solution and in liposome formulation.

		ODN in solution				ODN-liposome associated			
		Binding type				Binding type			
Incubation time	Evaluation type	Total	Louse	Tight	Internalized	Total	Louse	Tight	Internalized
1 hour	[ODN] b pmole/10^6 cells	6,85	1,08	0,62	4,43 ± 0,9	9,3	2,16	0,74	6,05
	[ODN] b/[ODN] o, %	23				32			
	[ODN] b/[ODN] tb, %				74				65
2 hours	[ODN] b pmole/10^6 cells	8,03	1,24	2,03	4,54	9,0	2,14	2,75	4,03
	[ODN] b/[ODN] o, %	27				31			
	[ODN] b/[ODN] tb, %				56				45
4 hours	[ODN] b pmole/10^6 cells	11,53	1,63	1,84	8,07 ± 1,3	14,74	2,16	4,0	8,58
	[ODN] b/[ODN] o, %	40				51			
	[ODN] b/[ODN] tb, %				69				58

[ODN] b — bound ODN:
[ODN] tb — total bound ODN:
[ODN] o — initial concentration 2,9 $\times$ 10^{-8} M:
[K562] — 10^6 cells/ml:
1. Binding performed in RPMI-1640 medium at 37°C:
2. Values obtained for 10 ODN bound K562 cells after 20 min centrifugation at 4°C.

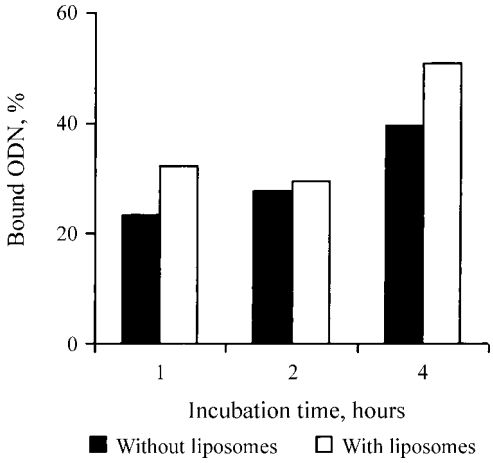

Figure 1. Dynamics of total binding ODN as 29 nM in solution or with ODN-associated liposome formulation with K562 cells as 10^6/ml.

Table 1 and Fig. 3 demonstrated that ODN internalization was always higher than the sum of loose and tight binding. It means that ODN internalization performed with higher rate than surface binding. As to loose binding it could be evaluated by equilibrium dissociation of the complex of ODN with K562 cells. Experiment sets of successive dissociation of total ODN bound to K562 cells under conditions that excluded internalization[11,12] showed that there was established an equilibrium dissociation process. It allowed us to obtain linear plots in Scatchard coordinates (ODN bound/ODN free versus ODN bound (Fig. 3).

From Scatchard plots the binding constant, K_b, and binding sites, b.s., per 10^6 cells were estimated for ODN loose binding with K562 cells in ODN solution (Fig. 3A) and with ODN-associated liposomes (Fig. 3B). K_b values were 1.80- and 1.88 $\times$ 10^9 M^{-1} at 4°C in PBS correspondingly; binding sites were 4.6 pmoles and 6.6 pmoles both per

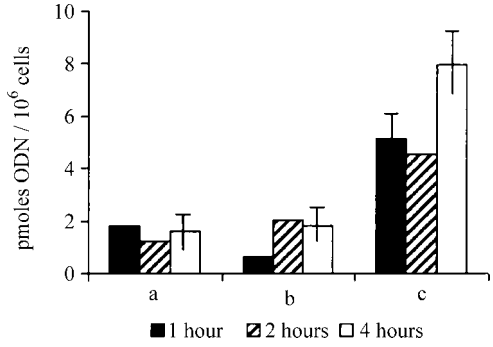

Figure 2. Characteristic of ODN binding with K562 cells in ODN solution. ODN as 29 nM. a—loose binding, b—tight binding, c—internalized ODN.

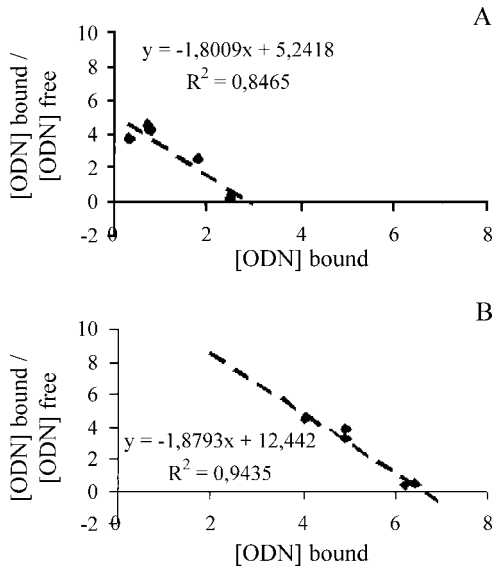

Figure 3. Scatchard plots for ODN loose binding with K562 cells as $0{,}625 \times 10^6$/ml (A) and 10^6/ml (B) in solution (A) and with ODN-associated liposomes (B), ODN as 29 nM. Evaluation of binding constant (K_b) and number of binding sites (b.s.) for loose ODN binding for 10^6 cells/ml. $K_b \times 10^9$ M^{-1}: A, 1.80, B, 1.88; b.s. pmoles/10^6 cells: A, 4.60, B, 6.62.

10^6 cells. The number of binding sites obtained for one hour incubation in ODN solution with 0.625×10^6 cells/ml was determined as 2.5 pmoles: Normalized to 10^6 cells it was 4.6 pmoles (Fig. 3). This figure was consistent with the amount of ODN internalized for the same time (Table 1). The K_b values for both cases were close in magnitude as well in order. The binding sites in pmoles/10^6 cells obtained for

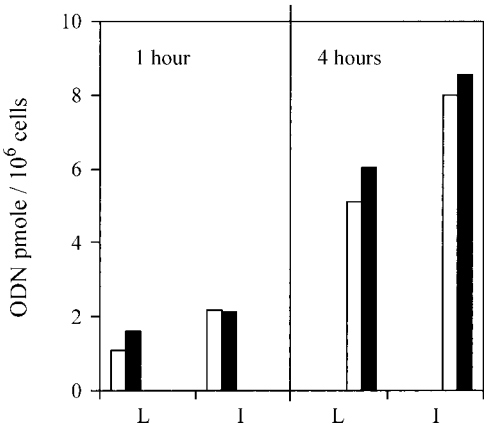

Figure 4. K562 cell binding with ODN in solution or with liposome-associated ODN as 29 nM. Light bars—ODN in solution. Black bars—ODN in liposome formulation. L—loose binding, I—internalized ODN.

the liposome formulation was 1.5-fold higher than in solution during the first hour of incubation.

These results in Fig. 3 and Table 1 show that all determined binding sites were realized for loose binding and for internalization for one hour incubation at 37°C. The small difference between one and two hours incubation binding made it evident that there was the same cell binding mechanisms. Four hours incubation demonstrated enhanced intensity of binding and an increased number of binding sites. Equilibrium dissociation of four hours ODN bound cells as well showed increased binding sites and changed binding mechanism that need further investigation.

Figure 4 and Table 1 show a little difference for loose ODN binding and internalized in solution and ODN-associated liposome. The extent of binding increased with incubation time, but practically stayed similar for solution and liposome ODN binding experiments. The binding sites were the same as treating ODN associated and ODN in solution, 8 pmoles/10^6 cells. Fig. 3B shows only 6.6 pmoles/10^6 cells for loose binding at 4°C, which was consistent with the amount of internalized ODN for the same time at 37°C. It should be emphasized that the amount of ODN bound in solution and with liposome was increased only for total binding (Fig. 1). All binding occurs in medium without growth factors and therefore binding in solution was carried out with free binding sites, but liposomes could interfere with binding. Nevertheless, the close magnitude of K_b values showed that affinity of ODN to the cells with liposomes was not higher than in solution.

Thus, we could summarized that:

- K562 cells 10^6/ml accepted totally up to 40% sense BCL2 oligonucleotide (ODN) at [ODN]o 10^{-8} M and up to 70% of total bound ODN penetrated into the cells. Three types of ODN binding were observed for incubation with the cells: loose, tight and receptor-mediated tight endocytosis (ODN internalized). The extent of total, loose and internalized ODN binding with K562 is enhanced with incubation time.
- Loose binding occurred with a binding constant, Kb $1{,}8 \times 10^9$ M^{-1} at 4°C in PBS for a large number of binding sites (4,6 pmoles/106 cells). These data assumed that loose binding with the cell surface occurred with rather high affinity and site specificity. However binding sites were several different specific targets probably.
- ODN binding in liposomes was increased only for total and loose ODN binding but not for internalized one. The level of ODN internalization into the K562 cells remained similar both with ODN solution and ODN associated liposomes.

The extent of ODN that penetrated into the cells (8 pmoles/10^6 cells) is higher than the number of sites for loose binding (4,6 pmoles/10^6 cells). This result and increasing extent of ODN internalized with incubation time showed that there was priming of new binding sites that performed rather slowly.

DISCUSSION

The cell receptor affinity for in appropriate ligand is usually characterized by a binding constant of the 10–12 orders of magnitude. The determination of K_b for BCL2

with K562 cells in our experiment gave the value 1.80×10^9 M^{-1}. This binding constant shows rather high affinity of the oligonucleotide (ODN) to the binding sites on the K562 surface.

Qualitative determination of three types of binding, including loose, tight and internalization revealed some controversies. The near linearity of Scatchard plots corroborates the similarity of binding sites for the studied ODN (BCL2).

The method for Scatchard plot, in our modification, with successive equilibrium dissociation is based on two checked facts;[10–12] that is 1) the 30 min incubation of cells, bearing some kinds of receptors, with ligands to them at low temperature (4–12°C) established an equilibrium between the ligand in solution and ligand bound with receptor; 2) treatment of isolated cells by an acid-salt solution that liberates all occupied binding sites.[9]

Thus we had a set of probes of the successive equilibrium-dissociation procedure, for which the concentrations of free and bound/free ODN were determined. The plots were linear and fit to a one-site binding model.

The number of ODN binding sites on the K562 cells for 4 hours incubation period is merely twice as more as for 1 hour. This fact may reflect another phenomenon, well known cell- ligand interaction. This consists in long-term (after > 15 min) up-regulation in receptor number and is called ''priming,'' that enhances cellular responses to a subsequent stimulus.[12] Additionally these data showed that there is some potential for ODN modulation of the tyrosine kinase pathway.

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