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Comparison of different ribozymes for efficient and specific cleavage of BCR/ABL related mRNAs

Winand Lange*, Michael Daskalakis, Jürgen Finke, Gottfried Dölken

Universitätsklinik Freiburg, Innere Medizin I, Abt. Hämatologie und Onkologie, Hugstetter Str. 55, D-79106 Freiburg, Germany

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Abstract

In chronic myelogenous leukemia (CML) the reciprocal translocation of the long arms of chromosomes results in the formation of the unique BCR/ABL fusion gene which is believed to play a crucial role in the pathogenesis of CML. Different short synthetic ribozyme constructs were compared with regard to their efficiency to cleave the BCR/ABL target RNA. In the CML cell line K562 we were able to inhibit the $p210^{BCR/ABL}$ synthesis by a ribozyme which was about twofold more effective than the corresponding antisense molecule.

Key words: Ribozyme; BCR/ABL; Chronic myelogenous leukemia

1. Introduction

The Philadelphia chromosome, a reciprocal translocation of chromosomes 9 and 22 is a consistant genetic finding in about 95% of patients with chronic myelogenous leukemia (CML) [1]. The result of this event is the BCR/ABL fusion gene which codes for two mRNA types, B3A2 and B2A2, both of which are translated into a protein of 210 kDa ($p210^{BCR/ABL}$) which is unique to the malignant cell phenotype [2]. The increased tyrosin kinase activity of this protein and its subsequently altered intracellular regulatory functions play a crucial role in the establishment of CML as could be shown in different transgenic mouse models [3–5] and studies with bone marrow transplantation in irradiated mice transplanted with a BCR/ABL transfected bone marrow [6].

Ribozymes are capable of catalyzing RNA cleavage reactions in a sequence-specific way. Therefore they seem to be suitable tools to inhibit the expression of specific RNA transcripts as could already be shown with several different constructs [7–11] targeting mainly viral RNAs. The so-called hammerhead ribozymes contain a conserved region which has to be flanked at both the 5′ and the 3′ ends by sequences that are complementary to the target sequence. Using this approach almost any target RNA can be cleaved after GUN [12] and with less efficiency after NUN (N = any nucleotide except G) [13] sequences. The cleavage reaction depends on the presence of divalent metal ions at neutral or higher pH and results in the production of two truncated RNA molecules with 5′ hydroxyl and 2′,3′ cyclic phosphate groups [14,15].

In order to be able to cleave the B3A2-type BCR/ABL mRNA and to decrease the synthesis of $p210^{BCR/ABL}$ in the CML cell line K562, which expresses only the B3A2 mRNA, four short ribozymes were synthesized which exclusively cleave this type of mRNA. The in vitro efficacy of the different ribozymes was evaluated. In addition a pure antisense oligonucleotide without ribozyme activity was compared to the true ribozymes in the K562 cell line experiments; lipofection was used as the transfection method.

2. Materials and methods

2.1. Ribozymes

The sequence information to construct the different ribozymes was taken from the published literature [16]. The different ribozymes and controls were synthesized from commercially available RNA phosphoramidites (Millipore, Eschborn, Germany) on a DNA/RNA synthesizer (Applied Biosystems, Model 394, Foster City, USA) according to the manufacturer's protocol. Then the oligonucleotides were incubated in NH_4 overnight at 55°C, lyophilized and resuspended in tetrabutylammoniumfluoride. Before use a final purification step by Biospin 6 columns (Bio-Rad, Richmond, USA) followed which resulted in oligonucleotides resuspended in dH_2O.

Four different ribozymes, R536, R736, R736S and R837, were tested in in vitro cleavage experiments (Fig. 1). A fifth construct, R736C, was identical to R736, except for a G to A base exchange at position 10 (numbering according to Williams et al. [17]); this leads to complete abolishment of cleavage capacity, leaving this construct as a pure antisense control. The cleavage capacity of the above mentioned ribozymes was compared to an in vitro transcribed NT7 ribozyme which was described previously [18].

*Corresponding author. Fax: (49) (761) 270 3206.

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2.2. Transcriptions

^{35}S-labeled B3A2-type BCR/ABL transcripts were synthesized from a pBluescript II derived vector as previously described [18].

2.3. In vitro cleavage reactions

Radioactive transcripts and unlabeled ribozymes were incubated at 37°C at a 1:1 molar ratio (concentration of 50 nM) in a 20 μl reaction volume containing 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA and 1 U RNase Block II (Stratagene, La Jolla, USA). Aliquots of the reaction mixes were removed after 1 h, 6 h and 16 h incubations and separated in 6% denaturing polyacrylamide gels; gels were dried and subjected to autoradiography for 6 to 48 h using Kodak XAR film. The different bands corresponding to RNA fragments were quantitated by laser densitometry scanning (Molecular Dynamics Image Quant 3.3).

2.4. Cells

K562 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated fetal calf serum (Seromed), 2 mM glutamine, 10 μg/ml streptomycin and 100 U/ml penicillin; cells were kept in a moist atmosphere with 5% CO_2 at 37°C and fed three times per week.

2.5. Ribozyme lipofection

1×10^7 K 562 cells were seeded into tissue culture flasks in a volume of 10 ml. The different ribozymes and controls were transfected with the lipofection agent DOTAP (Boehringer Mannheim, Germany) according to the manufacturer's protocol at a final concentration of 0.81 μM. After four hours the volume was adjusted to 20 ml with medium. Cells were harvested 72 h later.

2.6. Detection of p210$^{BCR/ABL}$

Cells were counted and washed once in phosphate-buffered saline (PBS). The different aliquots were adjusted to exactly the same cell number (1.2×10^7) and 12 ml of ice-cold PBSTDS (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) was added . After an incubation for 10 min the cells were further disrupted by repeated aspirations through a 21-gauge needle. After two centrifugation steps, one for 30 min at 1,500 rpm and one for one hour at 40,000 rpm the supernatant was used for immunoprecipitation with 20 μl of a polyclonal rabbit anti-BCR antibody [19,20] (kindly provided by J. van Denderen) for 2 h at 4°C. As a control antibody we used a pig anti-rabbit-IgG antibody for immunoprecipitation. After adding 40 μl of protein G plus/protein A agarose beads (Dianova, Hamburg, Germany) the solution was incubated for 30 min at 4°C and centrifuged for 15 min at 2500 rpm. The pellet was washed twice in PBSTDS (without SDS) and finally resuspended in 50 mM Tris-HCl pH 7.0. Autophosphorylation followed for 10 min at 30°C in a solution containing 20 mM PIPES, pH 7.0, 20 mM $MnCl_2$ and 20 μCi of [γ-^{32}P]ATP. The agarose beads were washed twice in PBSTDS (without SDS) and resuspended in 20 μl of gel loading buffer (65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% Bromphenolblue) [21]. After heating at 80°C for 5 min the reaction mixture was separated by SDS-PAGE in a 7% gel at a constant current of 80 mA. A prestained molecular weight marker (Bio-Rad) was included in each gel. After electrophoresis the gel was dried and quantification by laser densitometry scanning followed after autoradiography for 24 to 96 h.

3. Results and discussion

Four synthetic ribozymes with flanking regions of different length (Fig. 1), from 5 to 8 bases in stem III and 9 to 10 bases in stem I as well as one construct with a modified stem II/loop II were compared for their efficiency to cleave the BCR/ABL target molecule. A previously used ribozyme, NT7, which was transcribed from a short T7 promotor carrying gene served as comparison [18]. To differentiate between ribozyme and pure antisense effects in the cell line experiments we designed a

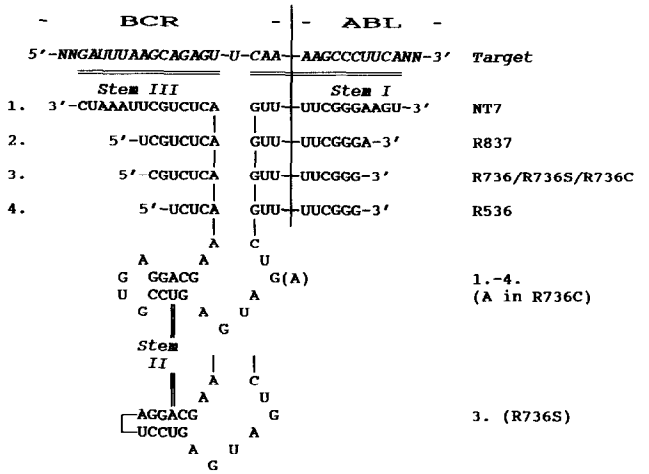

Fig. 1. The B3A2-type BCR/ABL RNA target, ribozymes and controls. The constructs differ in the lengths of the flanking regions in stems I and III (NT7, R837, R736, R536); R736S is identical to R736, but has a modified stem II; R736C has a G to A base exchange at position 10 compared to R736, hereby destroying the cleavage capacity of the molecule.

construct without cleavage capacity in which the ribonucleotide G was replaced by A at position 10, rendering this molecule, R736C, a pure antisense oligoribonucleotide (Fig. 1).

The BCR/ABL target RNA transcript was cleaved under standard conditions for up to 16 h. A corresponding autoradiograph is shown in Fig. 2. The cleavage rate varied as shown in Fig. 3 and Table 1. R736 with flanking sequences of 7 bases at the 5′ end (BCR) and 9 bases at the 3′ end (3 BCR, 6 ABL) had the maximum cleavage rate (68% after 16 h) of all ribozymes tested. Ribozymes R536 with a shorter stem III and R736S with a shortened stem II were less efficient. In the case of R536 this was probably due to an inability to stabilize stem III and subsequent early dissociation from its target. Ribozymes with longer stem I and/or stem III sequences were also less efficient than R736.

Substrate discrimination is a critical issue concerning ribozyme efficiency as was discussed in detail by Herschlag [22]. Some of the theoretical aspects of this could be confirmed by our experiments; a ribozyme with 7 and 9 bases in stem III and stem I had the highest cleavage rate.

Table 1
Cleavage rates (in percent) of the BCR/ABL target corresponding to the different ribozyme constructs after different time intervals; numbers were calculated from Fig. 2 after quantification by laser densitometry scanning

	1 hour (%)	6 hours (%)	16 hours (%)
R536	7	14	30
R736	15	36	68
R837	14	37	51
R736S	21	37	57
NT7	14	35	62

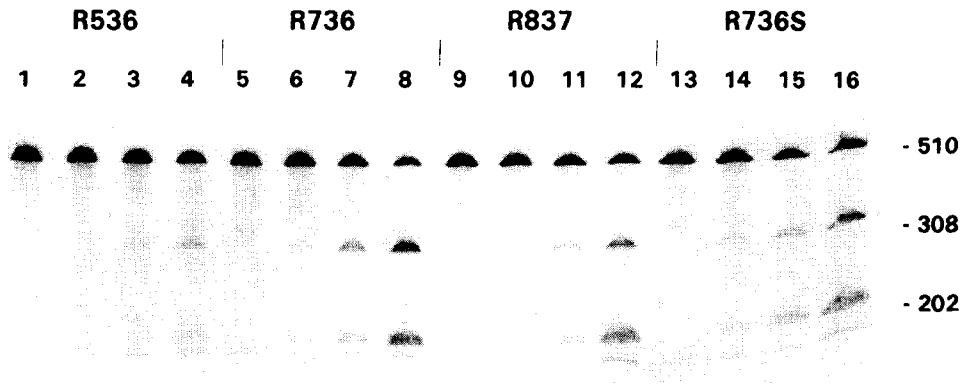

Fig. 2. Autoradiography of a ribozyme cleavage reaction with the different constructs. The target and the ribozyme are incubated at a concentration of 50 nM each. The B3A2-type BCR/ABL target has a length of 510 bases; products are 308 and 202 bases long. Reactions were performed in the absence of magnesium (lanes 1, 5, 9, 13) or with magnesium and stopped after 1 h (lanes 2, 6, 10, 14), 6 h (lanes 3, 7, 11, 15) and 16 h (lanes 4, 8, 12, 16).

In the case of the BCR/ABL target it was not possible to shorten stem I the same way as stem III due to the fact that the cleavage site was within BCR sequences just 4 bases 5′ to the BCR/ABL junction. Further shortening of stem I might allow cleavage of normal instead of rearranged BCR related RNAs.

Based on the above experiments we tested ribozyme R736 in the cell line K562 in order to inhibit the $p210^{BCR/ABL}$ synthesis. Compared to the lipofection agent DOTAP (100%) and the pure antisense control R736C (43%) the greatest reduction in the autokinase activity of $p210^{BCR/ABL}$ was observed with R736 (23%) (Fig. 4). This showed an almost 2-fold higher efficiency than the corresponding antisense molecule. This is in contrast to the results reported by Snyder et al. which showed complete abolishment of $p210^{BCR/ABL}$ [23]. The difference might be due to the greater stability of their ribozyme, as this was composed of a rather RNase resistant DNA/RNA hybrid molecule; in addition flanking sequences of 10 bases in stem III (8 deoxynucleotides and 2 ribonucleotides)

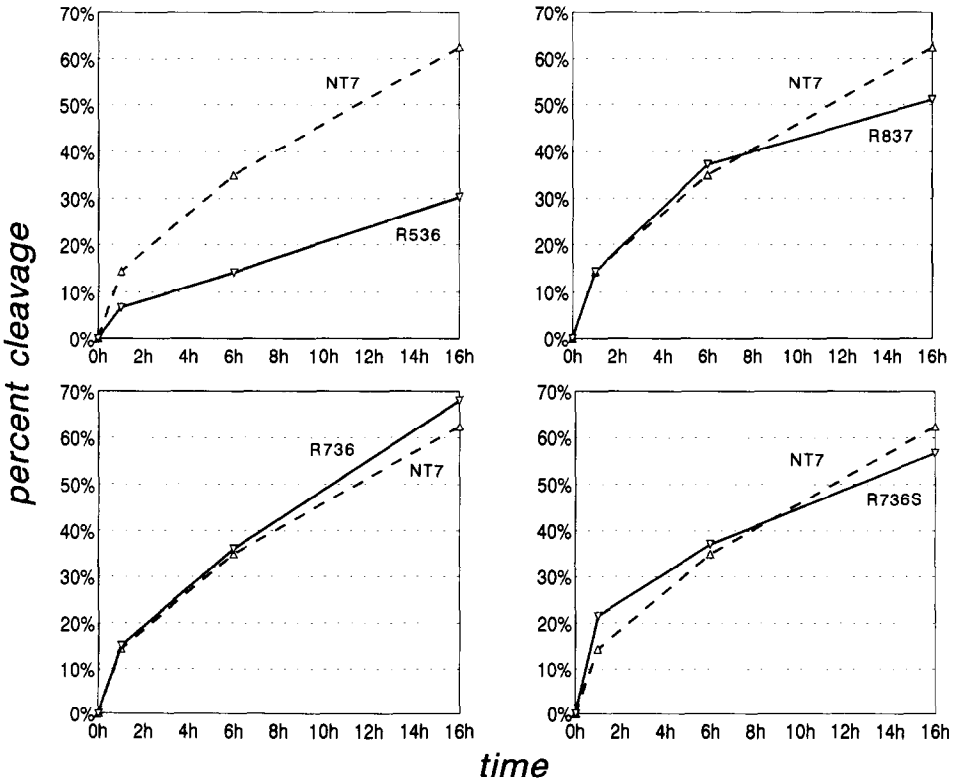

Fig. 3. Cleavage rates of the different ribozymes (R536, R736, R837, R736S) in comparison to the in vitro transcribed NT7 ribozyme. The cleavage rate (in percent) is given as the ratio of the sum of the cleavage products to the sum of the cleavage products plus the remaining target after certain time intervals. Results were calculated from Fig. 2 after quantification by laser densitometry scanning.

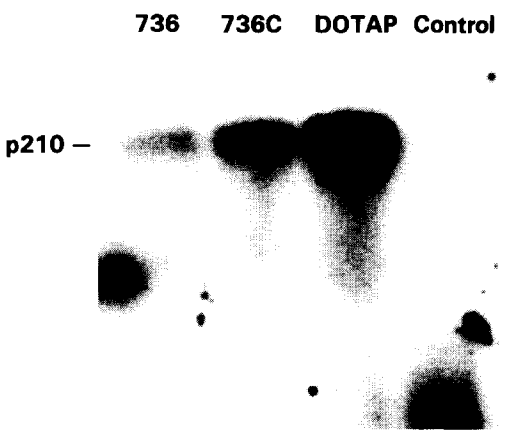

Fig. 4. Autokinase assay of $p210^{BCR/ABL}$ isolated from identical numbers (1.2×10^7) of K562 cells after incubation with ribozyme R736, the antisense oligonucleotide R736C without ribozyme activity and the lipofection agent DOTAP alone. 'Control' is the same as 'DOTAP', but immunoprecipitation was done with a pig anti-rabbit-IgG antibody. The least amount of $p210^{BCR/ABL}$ is shown with the R736 ribozyme (23%), about 2-fold less than with the antisense oligonucleotide R736C.

and 8 bases in stem I (7 deoxynucleotides and 1 ribonucleotide) might favour faster dissociation of the ribozyme from the cleavage products as DNA/RNA helices are less stable than RNA/RNA helices.

We have previously shown, that ribozymes are able to selectively cleave BCR/ABL rearranged RNAs [18]. In this report we describe the optimization of such a ribozyme; the possible large scale synthesis of such a short, but still very effective ribozyme, will soon allow therapeutic applications as ex vivo purging of autologous blood stem cells. Its selectivity to destroy only the malignancy associated transcript and the fact that short functioning ribozymes, which are easily lipofected into a target cell, do not need to be integrated as ribozyme genes into the genome are main advantages.

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