An antisense Bcr-Abl phosphodiester-tailed methylphosphonate oligonucleotide reduces the growth of chronic myeloid leukaemia patient cells by a non-antisense mechanism

TOON F. C. M. SMETSERS, ELLEN H. P. LINDELS, LOUIS T. F. VAN DE LOCHT, THEO M. DE WITTE AND EWALD J. B. M. MENSINK Department of Haematology, University Hospital St Radboud, Nijmegen, The Netherlands

Received 20 September 1996; accepted for publication 21 October 1996

Summary. The specificity of antisense oligonucleotides targeted to the mRNA breakpoint region of the Bcr-Abl oncogene, found in leukaemic cells from patients with chronic myeloid leukaemia, remains controversial due to non-specific effects. To prevent protein binding of oligonucleotides we designed and tested a methylphosphonate oligonucleotide with an attached 3' soluble phosphodiester tail. Growth of chronic myeloid leukaemia (CML) cell lines BV173, KCL-22 and cells of CML patients tested was inhibited by the b2a2 type antisense Bcr-Abl oligonucleotide and not with controls. Also the growth of control CD34+ cells of two healthy donors, control cell lines and cells from AML patients was only moderately affected or not affected. Bcr-Abl protein studies in combination with growth-determination experiments indicated that the antisense methylphosphonate Bcr-Abl oligonucleotide tested is a potent inhibitor of the growth of CML cells but works in a non-antisense manner.

Keywords: antisense, oligonucleotides, Bcr-Abl, methylphosphonate, apoptosis.

A variety of antisense oligonucleotides targeted to the bcr-abl mRNA breakpoint have been used. Modified oligonucleotides are more stable in serum, but non-sequence specific effects occur (Kirkland et al, 1993; Obrieb et al, 1994; Smetsers et al, 1995). This is probably caused by binding to proteins as a result of the negative charge of the oligonucleotides. Antisense phosphorothioates can bind to protein (for review see Stein, 1995). The uncharged methylphosphonates have been claimed to reduce cell growth by specific antisense effects (Tari et al, 1994). The big disadvantage of these oligos is their insolubility and they need to be delivered to cells in liposomes. In this study we used methylphosphonate oligonucleotides targeted to the Bcr-Abl mRNA breakpoint junctions. We tried to overcome liposomal delivery by adding a charged 3' phosphodiester tail to the oligonucleotides to make them soluble in culture medium.

They were dissolved in sterile IMDM and sterilized by filtration. The sequence of the oligonucleotides is as follows:

\[ \text{aB2A2.21MET : 5'-CTGAAGGGCTTCTTCCTTATT-3'} \]

\[ \text{B2A2.21MET : 5'-ATAAGGAAGAAGCCCTTCAG-3'} \]

\[ \text{d33A2.24MET : 5'-GGGCTTTTGAACTCTGCTTAAATC-3'} \]

Underlined characters represent methylphosphonate linkages; plain characters represent normal phosphodiester linkages.

CML cell lines culture, flow cytometric analysis and determination of the Bcr-Abl breakpoint type were carried out as described (Smetsers et al, 1994, 1995). Cryopreservation and separation of CD34+ cells were performed as described (Willems et al, 1996). SDS PAGE and immunoblotting were performed as described (Meijerink et al, 1995).

RESULTS

Antisense methylphosphonate oligonucleotides of 15 nucleotides and longer are not soluble in aqueous solutions. We modified the oligonucleotides described by Tari et al (1994). The oligonucleotides used here contain an additional five nucleotide long phosphodiester tail at the 3' end of the oligonucleotide and could be dissolved in culture medium.

MATERIAL AND METHODS

Antisense oligonucleotides were synthesized and purified by the Eurogentec corporation (Eurogentec, Seraing, Belgium).

Correspondence: Dr Ewald J. B. M. Mensink, Department of Haematology, University Hospital St Radboud, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.
The effect of the partial methylphosphonate oligonucleotides on the cell growth of several CML and non-CML cell lines was determined at an oligonucleotide concentration of $10 \mu M$. Fig 1A shows the cell numbers of these cell lines after 6 d in culture. Sense control oligonucleotide B2A2.21MET showed no reduction of cell growth of any of the tested cell lines. Treatment with antisense oligonucleotide αB2A2.21MET resulted in a decrease in cell number of

Fig 1. (A) Relative cell numbers of cell lines after 6 d of treatment with $10 \mu M$ of oligonucleotides. (B) Relative cell number of cells of CML patients and purified normal CD34+ cells after 6 d of treatment with $10 \mu M$ of oligonucleotides. (C) Relative cell number of cells of AML patients. Horizontal hatching: untreated; oblique hatching: αB3A2.24MET; open bars: B2A2.21MET; solid bars: αB2A2.21MET.
Fig 2A. Flow cytometric analysis of p210\textsuperscript{Bcr-Abl} expression in cell line BV173 after 5 d of treatment with oligonucleotides. AS: oB2A2.21MET; SE: B2A2.21MET; NT: not treated. Upper panel: expression of p210\textsuperscript{Bcr-Abl} relative to actin expression; lower panel: p210\textsuperscript{Bcr-Abl} expression relative to DNA.
CML cell lines BV173 and KCL-22 to 12% and 56% respectively, relative to the untreated control. Other cell lines were not affected. Oligonucleotide aB3A2.24MET failed to inhibit the growth of any of the cell lines tested. BV173 and KCL-22 are both cell lines with a B2A2 type breakpoint. The growth of these cell lines is inhibited by an antisense oligonucleotide targeted to this breakpoint. Cell line KYO-1 also expresses a bcr-abl mRNA containing this breakpoint, but its growth is not affected by the oligonucleotide. Because the behaviour of CML cell lines in culture can be different from primary CML cells, we also tested cells from CML patients, for which selection was based on expression of the bcr-abl mRNA, presence of >5% CD34+ cells and in vitro growth of cells. The four CML patient cells tested all showed a 20–30% reduced growth, relative to the untreated control, when treated with aB2A2.21MET (Fig 1B). Also one sample (from patient CML4) containing CML cells expressing the other B3A2 type bcr-abl mRNA was affected, indicating that the aB2A2.21MET oligonucleotide inhibition is not breakpoint specific. Purified CD34+ cells from healthy donors were not inhibited at all by aB2A2.21MET. This oligonucleotide only showed a moderate decrease of growth to about 70% in three out of the four randomly chosen AML patient cells (Fig 1C). Oligonucleotide aB3A2.24MET showed similar effects although they were less drastic than aB2A2.21MET. Sense control oligonucleotide B2A2.21MET did not induce an effect on the growth of any of the tested cells.

We used a flow cytometric assay to investigate whether the cell growth reduction by the tailed methylphosphonate oligonucleotide is caused by an antisense mediated reduction of the expression of the p210 Bcr-abl protein (Nemes et al, 1995). Cell line BV173 expresses only p210 Bcr-abl and not p145 Abl, which enables determination of only p210 Bcr-abl protein expression. Fig 2A shows flow cytometric analysis of BV173 cell samples after 5d of treatment with aB2A2.21MET and complementary sense oligonucleotide B2A2.21MET in medium containing 10% FCS. Expression of Bcr-abl together with actin, and with DNA was investigated. Only in the antisense aB2A2.21MET treated cells could a negative cell population with low expression of p210 Bcr-abl be detected. These cells, however, also had a low expression of actin and a population showed a low DNA staining (apoptotic cells). No cells were detected expressing low amounts of only actin or only p210 Bcr-abl. This indicates that cells simultaneously express lower amounts of actin and p210 Bcr-abl and some of these cells are apoptotic. A similar, but less profound, effect was observed in cell line KCL-22, about 10% p210 Bcr-abl negative cells were present (data not shown). Cell lines KYO-1 and K562 showed no Bcr-abl negative cells after treatment (data not shown). Cell line KYO-1 also expresses a b2a2 type Bcr-Abl mRNA but p210 Bcr-abl expression is not reduced.

Protein expression of BV173 and KCL-22 cells after 5d of incubation with oligonucleotides was also analysed using immunoblotting (Fig 2B). K562 cells after 2d of treatment with Herbimycin A was used as a control for decrease in p210 Bcr-abl expression. After immunoblotting and screening with the anti-abl antibody 8E9 the relative expression of p210 Bcr-abl with respect to p145 Abl was reduced in the Herbimycin A treated K562 cells, but not in antisense treated KCL-22 cells. In BV173 the relative expression could not be determined because this cell line does not express the normal p145 Abl.

Cell line BV173 is the most sensitive cell line to treatment with the aB2A2.21MET methylphosphonate oligonucleotide. It is also the only CML cell line tested that expresses a wild-type p53 (Bi et al, 1992). KCL-22 expresses a truncated mutated p53 protein (p53 mut, Fig 2B). The p53 protein plays a crucial role in the induction of cell death, especially when generated by DNA damage (Yonish-Rouach et al, 1991). No evidence for p53 and bax/bcl-2 mediated cell death however could be found, since the expression of the Bax and p53 protein was not increased in treated cells (Fig 2).

DISCUSSION

Oligonucleotide aB2A2.21MET is a growth inhibitor of CML cells. An antisense mechanism, however, is not responsible for this growth inhibition. Many differences exist between the tailed oligonucleotide and liposomal packed full methylphosphonates (Tari et al, 1994). Full methylphosphonates are breakpoint specific and reduce the growth of cell line K562. The use of liposomes, however, is likely to target the oligonucleotides directly to the cytoplasm. This can increase the specificity of the oligonucleotides in an antisense mediated effect. The use of the tailed oligonucleotide aB2A2.21MET has an advantage over the liposome encapsulated oligonucleotide in that it is more easy to

prepare and it reduces the growth of both breakpoint types of CML cells.

The mechanism of action of this oligonucleotide is not known. The oligonucleotide probably hybridizes to another RNA or some sequences may induce physiological effects. If CML cells are more sensitive to these effects than normal cells the oligonucleotide can induce the specific CML growth reduction that was observed in this study.

ACKNOWLEDGMENTS

This work was supported by a grant from the Dutch Cancer Society. We also thank the Maurits and Anna de Kock Foundation for financial support, Marc Lemaître and Michel Dechamps from the Eurogentec Corporation for their synthesis of the methylphosphonates and Dr J. Y. J. Wang (University of California at San Diego, La Jolla, Calif., U.S.A.) for providing the 8E9 antibody.

REFERENCES


