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

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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.

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.
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 μM. 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 2A. Flow cytometric analysis of p210<sup>Bcr-Abl</sup> expression in cell line BV173 after 5 d of treatment with oligonucleotides. AS: αB2A2.21MET; SE: B2A2.21MET; NT: not treated. Upper panel: expression of p210<sup>Bcr-Abl</sup> relative to actin expression; lower panel: p210<sup>Bcr-Abl</sup> 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 $ab2A2.21MET$ 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 (Kirkland et al. 1994), 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 (Smetsers et al., 1995). Cell line BV173 expresses only $p210^{bcr-abl}$ and not $p145^{bcr-abl}$, 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-Ab1 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 $p210^{bcr-abl}$ 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-Ab1 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 Herbigyn 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 $p145^{bcr-abl}$ was reduced in the Herbigyn 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 $p145^{bcr-abl}$.

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.

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