Specificity of ribozymes against the bcr-abl mRNAs in vitro.

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Chronic myelogenous leukaemia (CML) is a disease characterized by the presence of the Philadelphia chromosome and the bcr-abl fusion gene, which result from a reciprocal translocation between chromosomes 9 and 22. Two forms of bcr-abl mRNA exist, b3a2 and b2a2, depending upon whether the translocation includes bcr exon 3 or not. The p210* protein, a deregulated tyrosine kinase, is implicated in progression of CML. We are investigating the use of ribozymes to down-regulate the bcr-abl gene expression.

Ribozymes are catalytic RNA molecules that can be designed as specific endoribonucleases [1]. We have made three hammerhead ribozymes, two designed to cleave the b3a2 mRNA at two distinct sites 12 nucleotides (nt) apart and the third ribozyme designed to cleave the b2a2 mRNA. The cleavage sites were chosen close to the bcr-abl fusion point to encourage the specificity of the ribozymes for the fusion mRNA relative to the wild-type abl and bcr mRNAs.

cDNAs for each ribozyme were cloned into the pGEM-3Z plasmid so that in vitro transcription with SP6 RNA polymerase produced the active ribozymes. In vitro ribozyme cleavage reactions were performed in conditions previously described [1] using in vitro generated transcripts of cDNA fragments of abl, bcr, and bcr-abl mRNAs as substrates.

We found that ribozymes designed to cleave either b3a2 or b2a2 mRNA 9nts 3' of the fusion point are non-specific; they cleave both bcr-abl and abl RNAs. However the third ribozyme, designed to cleave 3nts 5' of the fusion point of b3a2 is specific for its substrate [2]. Under none of the experimental conditions tried was complete cleavage of the substrate RNA observed, including when the three ribozymes were used together.

It was recently noted by Tabler et al., [3] that the shortening of one flanking arm of the hammerhead ribozyme can increase the ribozyme's specificity. Shortening the arm of the non-specific b3a2 ribozyme, from 10nts to 4nts capable of base-pairing with the substrate, resulted in an increase in specificity; the ribozyme no longer cleaved the wild-type abl RNA [2].

Using this asymmetric ribozyme design we had a DNA-RNA hybrid ribozyme made for studies with cell cultures (Figure 1). Flanking arms made of DNA, whilst the catalytic core of the ribozyme remaining RNA, have been shown by us (unpublished results) and others [4] to confer increased resistance to nuclease degradation. Using this hybrid ribozyme in vitro cleavage experiments have been carried out. Preliminary results suggest that the DRD-1 ribozyme is more specific for its substrate than the equivalent all RNA ribozyme. At 50°C the all RNA ribozyme is the more active, however at 37°C the hybrid and RNA ribozymes appear to have similar activities.

Using the cationic liposome LipofectAMINE™ the hybrid ribozyme has been transiently transfected into a bcr-abl expressing murine cell line 32DAb3a2. Preliminary experiments show that, although the ribozyme is taken up by the cells and is relatively stable for up to 24 hours, no change in bcr-abl mRNA can be detected by RT-PCR and no ribozyme cleavage products can be observed by an RNase protection assay.

The effect of the ribozyme on cell viability is also being investigated using the MTT assay [5]. Neither non-specific toxic effects on the parental 32D cells (no bcr-abl gene) nor a reduction in cell viability of the 32Db3a2 cells have been observed after the treatment of the cells with DRD-1 (Figure 2).

This work was supported by the Leukaemia Research Fund, UK and The Bryan Gunn Leukaemia Appeal.

References:
2) James HA, Mills KI and Gibson I, paper submitted.

Figure 1. DNA-RNA hybrid ribozyme DRD-1.

Underlined nucleotides are DNA, the catalytic core remains RNA.

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3' TCG TCT CAA GCT TCC GGG AA TGG C 5'

A       C         A
G       U         G
C       G         A
A       U         U
G       C         C
G       C         G
A       G         U
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Figure 2. Effect of DRD-1 on cell viability as measured by an MTT assay.

MTT was added to the cells (25μl of 5μg/ml in PBS). After 5 hours the unreacted MTT and cell culture was aspirated off and the resulting formazan crystals dissolved in 200μl DMSO. After 10 minutes the samples were read on a multwell plate reader, test filter of 550nm and reference filter of 690nm.