Chapter 3 DNA-enzyme, Ribozyme and siRNA-Rz bispecific construct against CXCR4 and modulation of its expression by antisense ODNs

According to the 2008 global AIDS epidemic report of UNAIDS, there were 33 million people living with HIV world wide. Of these a major portion, 22 million people are those from the sub-Saharan region of Africa. The number of deaths in adults and children globally was estimated as a staggering 2 million in 2007, due to this deadly virus. Several gene therapy approaches have been employed to prevent HIV infection with varying successes. These include RNA decoys (Bahner et al., 1996), ribozymes (Rzs) (Bai et al., 2001, 2002, Feng et al., 2000, Rossi et al., 1999), siRNA (Martinez et al., 2002, Anderson et al., 2003a, Banerjea et al., 2008), targeting either viral regulatory or accessory protein encoding mRNAs or the host cell proteins such as the primary CD4 receptor (Anderson et al., 2003b) or the co-receptor CCR5. The patients with the Δ-32 mutation in the CCR5 co-receptor gene showed normal phenotype and relative resistance to HIV infection, this prompted many to target CCR5 co-receptor. This mutation was reported for the first time in India by Banerjea et al in 1998. This co-receptor has been targeted by many others as well as by Banerjea and co-workers at NII, ND (Goila and Banerjea, 1998; Anderson et al., 2003; Qin et al., 2003). While CCR5 is the major co-receptor present on macrophages, infected primarily by the M-tropic viral strains, CXCR4 co-receptor present on T-cell lines is utilized by the X4 viral isolates. Also, although the primary infection is established by the M-tropic strain, the X4 isolates predominate in the later stage of subtype B infection and may be important for progression to AIDS. There have been reports where the Δ-32 heterozygous population exhibit only partial resistance (Huang et al., 1996) and this protection is not absolute as the HIV is found in the individuals
homozygous for this allele (Michael et al., 1998). Therefore, there is a need to have a therapeutic approach in which CXCR4 coreceptor is also targeted.

The ability of nucleic acids as biological catalysts has become well established in the last few years. Several nucleic acid based approaches that include ribozymes (Rz), DNA-enzymes (Dzs), siRNAs and RNA decoys are currently being tried to knock down intracellular expression of target genes (Akkina et al., 2003; Banerjea et al., 2004). Rzs are catalytic RNA molecules that were discovered initially as self-cleaving entities as in ribonuclease P of E.coli and the intervening sequence of *Tetrahynema thermophila* (Cech et al., 1981). The two most extensively worked Rzs are-hairpin Rz and the hammer-head Rz. The hammerhead Rz is approximately 30 nucleotides long and capable of cleavage of target RNA in cis as well as in trans. The hammerhead Rz catalyses a general acid base reaction in which the divalent metal ion plays a pivotal role. It is a transesterification reaction which yields a 2'3' cyclic phosphate as the 5' end product and 5'OH as the 3' end product (Liu et al., 2007). Dzs are short catalytic DNA molecules that have been reported to perform a wide array of functions. The most exploited of all these functions has been its ability to cleave any target RNA in a sequence-specific manner (Basu et al., 2000; Berger et al., 1999). 10-23 Dzs (Santoro and Joyce, 1997) are the most extensively used Dzs that have been utilized as *in vivo* therapeutic agents to cleave specific mRNA targets (Baum and Silverman, 2008). The catalytic mechanism of RNA-cleaving is thought to be similar to that of hammer-head Rz (Santoro et al., 1997). Another class of small nucleic acid molecules is the small interfering RNA which includes 21-22 bp long short RNA molecules with a characteristic 2 nucleotide long overhang at their 3'end. In mammalian cells they are actually produced as a longer dsRNA precursor which is then cleaved by RNase III endonuclease, Dicer (Zhang et al., 2004). siRNA molecules associate with a protein complex known as the...
RISC assembly, where the antisense strand complementary to the target gene is recognized. The RISC complex contains the argonaute family of protein which cleaves the target mRNA between its 10th and 11th base relative to the 5' end of the antisense strand (Meister et al., 2004). RNAi technique has tremendous potential to study the function of various genes by knocking down its expression. Several Rzs, Dzs and shRNAs (short hairpin RNAs) have been designed against the viral co-receptor CXCR4 (Anderson and Akkina, 2005; Basu et al., 2000) and also other viral genes.

The major problem with mRNAs have been their complex secondary structures which makes the target sequence inaccessible to catalytic nucleic acids and shRNAs (Cairns et al., 1999; Schubert et al., 2004). It is now more evident that some sequences may be more prone to cleavage by Rz, Dz or siRNAs which needs to be experimentally determined (Kurreck et al., 2002). Target RNAs are usually long, consist of multiple stem-loop structures and up to 90% of all the putative sites are inaccessible to either Rz or Dz or siRNAs (Dash and Banerjea, 2004). The efficiency with which the hammer head Rz cleaves its target depends on the availability of free nucleotides in the substrate to pair with the Rz (Campbell et al., 1997). There have been different strategies that have been employed to enhance the cleavage efficiency of both the Dz and the Rz. These include the oligonucleotide effectors and antisenses. Oligonucleotide effectors (regulators) bind to both the Rzs and Dzs and substrate RNA and enhance the binding efficiency of enzyme and substrate via a three way enzyme-regulator-substrate complex (Wang and Sen, 2001). The antisense oligonucleotides (ODNs) bind adjacent to the Dz binding site in the target RNA. We have recently shown in vitro data as evidence of antisense molecules enhancing the cleavage efficiency of Dz against HIV-1 gag RNA (Sood et al., 2007) and also that of Dz and Rz designed against the X gene of Hepatitis B virus (Gupta et al., 2007).
Therefore, there is a need to have multi-target approaches either employing the same nucleic acid tool or different tools in combination. Earlier constructs with antisense RNA and multimeric Rzs were used against HIV-1 (Ramezani et al., 2006). Very recently siRNA against HIV-1 gene along with anti-gp120 aptamer was shown to have potent anti-HIV-1 activity (Zhou et al., 2008). Several mono (Martinez et al., 2002) and bi-specific siRNA constructs, as originally described by Anderson and Akkina, have been described earlier which targeted CD4, CXCR4 and CCR5 (Anderson et al., 2003). The unique feature of this bi-specific siRNA constructs is that the two small-hairpin siRNAs were joined together by an intracellular cleavable linker as originally described by Sioud and Leirdal in 2002 and impressive inhibition of HIV-1 replication was observed. Study involving the bispecific construct (Rz + shRNAs) against X gene of Hepatitis B has been recently published by Gupta et al in 2008.

In the present study, we designed Dz and Rz against the HIV-1 co-receptor CXCR4 and explored the possibility to use these two approaches synergistically as earlier studies had demonstrated only partial reduction in co-receptor expression. Also, we designed antisense ODNs to further modulate the cleavage efficiency of both the Dz and Rz. Also, efforts were made to design a construct which combined the earlier described hammerhead Rz against the CXCR4 coreceptor with a small hairpin siRNA against the same gene using the short cleavable linker, described by Sioud and Lierdal. It is shown in this objective that this construct interfered strongly with the expression of target gene expression both at RNA and protein levels. Furthermore, by disabling either Rz or siRNA, it was possible to modulate the intracellular gene expression in a controllable manner.
Experimental Procedure

Cell lines used
The human CD4+ T cells (Jurkat suspension culture) were propagated in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (HyClone, Logan, UT, USA), and U373 MAGI-CXCR4-CEM (obtained from NIH AIDS Research & Reagent Program, NIH, MD, USA) cell line and HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS.

Cloning of human CXCR4 gene
Full length CXCR4 gene was amplified from U373-MAGI-CXCR4-CEM (henceforth referred to as CXCR4-MAGI) cells by using two terminal primers and the PCR amplified product was ligated into a T-tailed vector pTarget under bacteriophage T7 & CMV promoter (Promega Biotech., Madison, WI) (Figure 3-1A).

The following primers were synthesized:
1) Forward primer 5'ATG GAG GGG ATC AGT ATA TAC ACT TCA G 3'
2) Reverse primer 5'CTA GCT GGA GTG AAA ACT TGA AGA CTC 3'

![Figure 3-1: Cloning of CXCR4 gene (full length and truncated) into expression vector](image-url)
Further a truncated variant of CXCR4 gene (502 bp) was subcloned into pCDNA 3 expression vector under mammalian CMV and T7 promoter (Figure 3-1B) using the internal BamHI restriction enzyme site present in the open reading frame. All the plasmids were purified on a Qiagen column (Qiagen, Gmbh, Hilden, Germany) before sequencing or for the purpose of transfection into mammalian cells. Figure 3-2A represents the amplification of full length CXCR4 gene (1059 bp) using the forward and reverse primers. The positive clones were confirmed by restriction digestion of the plasmids containing inserts with BamHI and XhoI releasing the 1059 bp CXCR4 insert from the pTarget backbone (lane 3 of Figure 3-2B). The positive clone was further confirmed by sequencing.

**Figure 3-2 : PCR amplification and cloning of CXCR4**

**Target site selection for ribozymes and DNA-enzymes**

CXCR4 mRNA sequence was subjected to secondary structure generating software programme, namely, m-FOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). The various secondary structures generated by this program were studied for most conserved loop regions containing target sites.
(AU or GU for being targeted by Dzs.) and selected as represented in Figure 3-3. The inset in the figure reveals AU as the target site for the Dz and GUC for the Rz. The target site chosen was a ’GUC’ at nucleotide position 255 of the 1059 bases long CXCR4 gene for the Rz-255. The nucleotide site with ’AU’ as target was chosen at position 139 of CXCR4 gene for the 10-23 Dz named as Dz-139.

**Construction of hammer-head ribozyme**

Hammerhead Rz, Rz-255 was designed using the following strategy. The oligonucleotide encoding the Rz containing the hammerhead catalytic motif flanked on either side by 8 nucleotides complementary to the target RNA was synthesized chemically. The Rz construct made is shown in Figure 3-4 in red colour. Rz encoding DNA was PCR amplified using the primers R1 and R2 (sequence given below). The forward primer R1 and reverse primer R2 had restriction enzyme sites, HindIII and BamHI engineered at their termini. The PCR amplified Rz encoding DNA was initially cloned into a T-tailed vector-pGEM-T-Easy and then they were subcloned into pcDNA3 using the engineered restriction sites, directly under T7 and CMV promoters.
Forward primer R1: 5’ CGGAAGCTTACAAAGAGCTGATGAGT 3’

Reverse primer R2: 5’ GCCGGATCCGCCGACCTTTTCGTCC3’

PCR conditions were same as described in materials and methods. The recombinant plasmids were checked for the insert by digestion with Hind III and BamHI. The positive clones were confirmed by sequencing.

The complete sequence of Rz-255 (ribozyme nucleotide) is shown below:

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5' CGGAAGCTTACAAAGAGCTGATGAGTCCGTGAGGACGAAAGGTCGGCGGATTCCGG 3',
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**Synthesis of 10-23 DNA-enzymes**

The target site chosen for cleavage was a purine followed by a pyrimidine, 'AU' at nucleotide position 139 of CXCR4 gene sequence. Dz-139 had the 15 nucleotide conserved 10-23 catalytic motif, flanked by seven nucleotide residues on both sides of 'AU', that act as hybridizing arms to the target sequence (Figure 3-4, blue in colour). These hybridizing arms provide specificity to the Dz (the A residue is left unpaired and the cleavage is expected to occur after the A residue-- shown by an arrow) (Figure 3-4). The complete sequence of Dz-139 is shown below:
Construction of chimeric-siRNA-Rz construct against CXCR4 gene

For making the bi-specific construct, an oligonucleotide was synthesized that consisted of Rz-255, a short spacer sequence identified by Sioud and Leirdal in 2002, followed by the small hairpin type of siRNA that ends with the complementary sequences for the T7 primer. To this oligonucleotide, the T7 primer was hybridized and the desired RNA was synthesized in vitro using T7 RNA Polymerase as described earlier (Donze and Picard, 2002) using the transcription kit (henceforth this is referred to as wt bispecific construct). A schematic representation of in vitro transcription of the wild type bispecific construct and its subsequent transfection into HEK-293 cells is depicted in Figure 3-5, panel A. Various mutants of the wt-bispecific construct was designed as shown in Figure 3-6. A four point mutant version of the siRNA was made which retained the normal sequences of the Rz (Figure 3-6, panel B shown as bold and underlined—henceforth referred to as mt-siRNA-wt-Rz or chimeric mutant-1). In the other construct, siRNA portion matched the wt construct but the Rz was disabled by changing a single nucleotide (G to U—henceforth referred to as wt-siRNA-mt-Rz or chimeric mutant-2, panel C) in the hammerhead catalytic motif. Another mutant version was designed in which both the siRNA portion and the Rz-255 were mutated by introducing the required changes of mutated siRNA and mutated Rz, panel D, referred as mt-siRNA-mt-Rz or chimeric mutant-3. In vitro synthesis of these RNAs indicated that they were of correct size.
Figure 3-5: Designing and in vitro synthesis of the wt-bispecific construct
Antisense Oligonucleotides

21 nucleotide long oligodeoxynucleotides (ODNs) were designed complementary to CXCR4 RNA upstream and downstream of the hybridizing arms of Dz-139 and Rz-255. They were named as ODN#1A, ODN#2A, ODN#3 and ODN#4. ODN#1 and 2 were also designed upstream and downstream to Dz-139, but they were designed with a nucleotide gap between them and the hybridizing arm of the Dz-139. Again, another antisense just downstream to the siRNA binding site (no gap) was designed, complementary to the CXCR4 RNA. The sequence and the location of these ODNs is depicted in Figure 3-7.
**Figure 3-7**: Site of action for Dz-139, Rz-255, siRNA-Rz and antisense oligonucleotides

**In vitro cleavage reaction**

Equimolar concentrations (100 pmol each) of UTP labeled substrate RNA and unlabeled Dz alone or together with ODNs (100pmol each) were mixed in 10μl of 50 mM Tris-HCl, pH 7.5, containing MgCl₂ (final concentration, 10 mM) and incubated at 37°C for 1 h. The cleavage products were resolved by electrophoresis on a 7% polyacrylamide-7 M urea gel. The pictorial representation of the *in vitro* cleavage reaction by Dz-139 and the expected product sizes are shown in Figure 3-8.
Figure 3-8: In vitro transcription of CXCR4 constructs and cleavage by Dz-139

Similarly, the in vitro cleavage reaction with Rz-255 involved the in vitro transcription of linearised Rz-255 pcDNA3 to produce in vitro synthesized Rz-255 RNA (substrate). Rz-255 was then incubated with labeled CXCR4 transcript in the presence of Tris-HCl (pH 7.5) and 10mM MgCl₂. The expected size of the cleavage products is depicted in Figure 3-9.

Figure 3-9: In vitro transcription of CXCR4 constructs and cleavage by Rz-255
**Intracellular reduction of the target RNA**

The intracellular decrease in CXCR4-specific RNA in CXCR4-MAGI cells that were transfected with the Dz-139 alone and in combination with different ODNs was determined by reverse transcriptase (RT) PCR techniques. 48 hours after transfection, total RNA was isolated using Trizol reagent (Gibco BRL) following the manufacturer's directions and divided into two equal parts. One set was used for estimating CXCR4 RNA and the other for the control RNA. The RNA was treated with DNase 1 (RQ1, Promega Biotech.) for 45 min at 37°C. The primers used for amplifying CXCR4 transcripts were

Forward primer 5' ATG GAG GGG ATC AGT ATT A TAC ACT TCA G 3'

Reverse primer 5' CTA GCT GGA GTG AAA ACT TGA AGA CTC 3'

The control RNA (509 bases), human glyceraldehyde-3-phosphate-dehydrogenase (huGAPDH), was amplified using the following set of primers:

Forward, 5'-ACCACCATGGAGAAGGCTGG-3', and

Reverse, 5'-CTCAGTGTAGCCCAGGATGC-3'.

The reduction in CXCR4 transcript with the Rz-encoding plasmid and various CXCR4 siRNA-Rz bispecific constructs was monitored by RT-PCR.

**Cell surface staining to determine reduction in CXCR4 protein expression**

0.5 x 10⁶ Jurkat cells were transfected with 100 pmole each of Dz and antisense ODNs using 3μl lipofectamine 2000 (Invitrogen) per well in a 6 well plate. 36 hours after transfection cells were treated with PMA (phorbol myristyl acetate) (5μg/ml). Sixteen hours after PMA stimulation, cells were harvested by centrifugation for 5 min at 1800 rpm. For cell surface staining, cells were resuspended in PBS and incubated with the PE-conjugated mouse anti-human
CXCR4 antibody (PE-CXCR4) (BD Pharmingen) for 1 hour at 4°C. After 1 hour, cells were washed twice with PBS, resuspended and analyzed by FACS. Similarly, 1 μg of Rz-255-pcDNA3 and 1μg of various mutant and wt bispecific construct (in vitro synthesized CXCR4 siRNA-Rz RNAs) were transfected into Jurkat cells either alone or along with their respective antisense ODNs. The reduction in cell surface expression of CXCR4 co-receptor was determined by staining with PE-CXCR4 and analyzed by Flow cytometry.

**Viral Challenge Assay**

The efficiency with which the Dz-139, Rz-255 and the CXCR4 siRNA-Rz constructs were able to reduce viral infection was determined by performing viral challenge assay. To perform this assay, U373-MAGI-CXCR4 cells were utilized which constitutively express CXCR4 co-receptor and contain an LTR driven β-galactosidase cassette, which is induced when Tat is produced upon HIV infection. To determine the efficiency of the catalytic nucleic acids (Dz-139 and Rz-255) and the bispecific constructs in inhibiting HIV infection, MAGI cells were co-transfected with either 50 picomole of Dz-139, 200ng of Rz-255 encoding plasmid or 200ng of various CXCR4 siRNA-Rz bispecific constructs and the virus encoding plasmid p89.6 (500ng) and the reduced virus infection was measured by determining the decrease in β-gal activity with respect to the activity measured when the p89.6 plasmid was transfected alone in a 24-well plate set up. The β-gal assay was performed as described in materials and methods.

**Cleavage of wild type (wt) chimeric siRNA-Rz in the presence of mammalian cell extracts**

Cytoplasmic protein extract was prepared from 293 cells. Briefly the cells were washed twice with PBS and lysed at 4°C with lysis buffer (0.2% NP40 in PBS and protease inhibitors). After 20 min of incubation, cell lysate was
centrifuged for 5 min at 10,000 rpm and supernatant was collected and used as cytoplasmic protein extract for the cleavage of chimeric RNAs. All the bi-specific constructs (siRNA-Rz-255) templates with T7 promoter sequence was synthesized from Sigma. In vitro transcribed siRNA-Rz-255 RNA were synthesized from these DNA template oligonucleotides using T7 RNA polymerase and α-P³² rUTP as described in methods and methodology. After transcription, RNA was purified using chloroform and phenol and stored at -70°C. Labeled siRNA-Rz-255 RNA transcript was subjected to processing by cellular protein extract for its specific cleavage. Internally labeled siRNA-Rz-255 RNA was incubated with or without cellular protein extracts for half an hour at 37°C and then quenched with phenol/chloroform mixture. Samples were phenol extracted and analyzed on 15% polyacrylamide gel-7M urea by autoradiography.

**RNAse H assay to determine the binding efficiency of the Antisense oligonucleotides**

Antisense oligonucleotides were incubated with the α-P³² labeled CXCR4 transcript in a final mix of 10μl reaction containing 50 mM Tris-HCl, pH 7.5, MgCl₂ (final concentration, 10 mM) and 1 unit of RNaseH enzyme (Promega) and incubated at 37°C for 1 hour. The cleavage products were resolved by electrophoresis on a 7% polyacrylamide-7 M urea gel.

**Results**

**Cleavage of full-length CXCR4 RNA by DNA-enzyme-139 in the presence of upstream and downstream ODNs**

Recombinant clone of pTarget that harbored the 1059 bases (full-length) long substrate (CXCR4) gene was linearized with NotI, which is present in the multiple cloning sites (MCS) downstream of the cloned gene. When subjected to transcription in the presence of T7 polymerase, a 1103 nucleotide long
transcript was expected. This length included 26 nucleotides at the 5’ end and 18 nucleotides at the 3’ end of the cloned gene derived from the vector (see Figure 3-8 A). The DNA-enzyme was tested for its ability to cleave the in vitro generated substrate RNA. Lane 1, Figure 3-10A, shows the synthesis of a full length labeled CXCR4 RNA. When equivalent amounts of cold Dz-139 and labeled substrate were added in equal amounts (100 picomoles each), specific 5’P cleavage (938 bases) and 3’P cleavage products (165 bases) were observed (Figure 3-10 A, lane 2). Cleavage reaction was continued for 2 h at 37°C in presence of 10 mM MgCh. When ODN #1 (lane 3) and ODN#1A (lane 4) was included in the cleavage reaction (100pmoles each), significant enhancement of cleavage was observed with the latter ODN (1A – no gap) (about four fold increase in the 165nt long cleaved fragment). In the similar manner, ODN #2 (lane 5) and ODN # 2A (lane 6) resulted in 3 and 5 fold more production of the same cleaved fragment (compare the intensity of the 165 nt long RNA fragment in lane 2 with lanes 5 and 6). A combination of ODN #1 and #2 (lane 7) (50 pmoles each) (both possess a nt gap), no significant increase in the 165nt long cleaved RNA fragment but the same cleavage products increased significantly when a combination of ODN #1A and 2A (lane 8) (they possess no gap) were used. We can therefore conclude from the experiment that efficient cleavage was obtained by the Dz which was further augmented by ODNs and without gap ODNs performed significantly better over the ones with a single nucleotide gap. Since the 983nt long cleaved fragment is very close to the input substrate RNA, only intensity of the 165nt long fragment was compared to determine the cleavage efficiency in each lane.
Figure 3-10: Effect of antisense oligonucleotides with Dz-139 on CXCR4 full transcript and its truncated variant

Cleavage of truncated CXCR4 RNA (566 bases) by Dz-139

The experimental conditions for cleavage were essentially the same except that instead of using a full length \textit{in vitro} synthesized CXCR4 RNA, 566 bases long (truncated) labeled CXCR4 transcript was used as shown in Figure 3-8 B. Lane 1 of Figure 3-10 B shows the synthesis of 566 bases long CXCR4 transcript. Cleavage reactions were carried out for 1 h at 37°C in presence of 10 mM MgCl\textsubscript{2} concentration. Specific cleavage products (5'P, 389 bases and 3'P, 177 bases) could be easily seen in lane 2. In this experiment we co-related the efficiency of various treatments by estimating the remaining input substrate RNA in each lane. Cleavage reactions were carried out in presence of 100pmoles each of labeled CXCR4 RNA and Dz-139 in the absence and
presence of both the upstream and downstream ODNs without gap (100 pmoles each) as indicated at the top of each lane. A significant reduction in the input RNA substrate (>80% reduction in lane 3 when compared with the intensity in lane 1) could be observed. We conclude that ODNs were extremely effective in augmenting the Dz-139 mediated cleavage when the truncated substrate RNA was used. Here ODNs with no gap were utilized because they showed greater effect on the full length CXCR4 transcript.

**Rz-255 mediated cleavage of full length CXCR4 RNA in the presence of upstream and downstream antisense oligodeoxynucleotides**

Rz-255 is expected to cleave full-length CXCR4 RNA (1103nt) into specific RNA fragments (822 and 281 bases long) as schematically represented in Figure 3-9 A. When equimolar amounts (100 pmoles each) of *in vitro* synthesized Rz RNA and labeled substrate RNA were incubated for cleavage under standard conditions, two specifically cleaved fragments (822 and 281nt long RNA fragments) were obtained as expected (Figure 3-11, lane 2). When the same cleavage reaction was carried out in the presence of 100 pmoles (lane 3) ODN#3, approximately 4-fold increase was observed in the intensity of cleavage products (Compare lane 3 with lane 2). In the similar manner, the ODN#4 also caused an increase in the cleavage product when similar amounts was used (compare lane 4 with 2). However the increase in the cleavage efficiency was comparatively lower than what was observed with ODN#3 (compare lanes 3 and 4). There was an enhanced cleavage when both the ODNs were used together (lane 5). This effect was similar to the effect observed with ODN#3 alone. Thus it could be concluded that ODNs that hybridized immediately upstream and downstream of Rz-255, were able to significantly augment the Rz-255 mediated cleavage and that ODN# 3 was more effective than ODN# 4.
Rz-255 mediated cleavage of truncated CXCR4 RNA in the presence of upstream and downstream antisense oligodeoxynucleotides

When an equimolar amount (100 pmoles each) of Rz-255 is used for cleaving a truncated CXCR4 transcript (566 nt), specific RNA fragments (293 and 273 bases long) are expected (Figure 3-9 B). The cleavage efficiency is further enhanced using antisense ODNs which hybridize immediately adjacent to the two hybridizing arms of the hammerhead Rz-255. For performing the cleavage reaction, 566 nt labeled truncated CXCR4 transcript was in vitro synthesized (lane 6, Figure 3-11). When equimolar amounts (100 pmoles each) of Rz-255 and labeled substrate RNA were used for cleavage under standard conditions two specifically cleaved fragments (293 and 273 nt long RNA fragments) were obtained (lane 7). When the same cleavage reaction was carried out in the presence of 100 pmoles (lane 8) ODN#3, approximately 5-fold increase was observed in the intensity of cleavage products. (Compare lane 8 with lane 7). In the similar manner, the ODN#4 also caused an increase in the cleavage product when similar amounts was used (compare lane 9 with 7). There was an enhanced cleavage when both the ODNs were used together (lane 10). As similar increase in cleavage efficiency was observed in lanes 8, 9 and 10, it could be concluded that the effect of both the ODNs together was similar to that observed with them individually.
Figure 3-11: Effect of antisense oligonucleotides with Rz-255 on CXCR4 full transcript and its truncated variant

**Intracellular reduction of the CXCR4 RNA**

Whether or not Dz-139 was able to reduce intracellular levels of CXCR4 RNA upon transfection into a mammalian cell (CXCR4-MAGI) alone or in combination with various ODNs as indicated at the top of each lane (Figure 3-12A), was examined by quantifying the levels of CXCR4 RNA by RT-PCR from equal amounts of cell lysates. As expected, CXCR4-MAGI cells showed a very prominent CXCR4 specific RNA fragment and after Dz-139 treatment, the levels of CXCR4 RNA dropped very significantly. When ODN #1A was included, significantly reduced CXCR4 signal was observed. In presence of ODN #2A, a faint CXCR4 specific band was observed. When these two ODNs were introduced, no CXCR4 signal was detected. These are specific effects caused by various treatments as the levels of house keeping gene (huGAPDH) remained essentially unchanged in all the lanes. We conclude that almost
complete (~80%) intracellular knock down of CXCR4 gene expression was achieved by the combined treatment. These doses were not toxic to the cells as determined by trypan blue dye exclusion test.

![Figure 3-12: Effect of Dz-139 and Rz-255 on expression of CXCR4 RNA by RT-PCR](image)

The efficiency of Rz-255 and its ODNs in degrading CXCR4 RNA was checked by performing RT-PCR. RNA was isolated from CXCR4-MAGI cells transfected with Rz-255 encoding plasmid alone and in combination with the antisense ODN#3 and ODN#4 and subjected to RT-PCR (Figure 3-12 B). Lane 1 depicts the expression of CXCR4 RNA in control CXCR4-MAGI cells while lane 2 shows the effect of Rz-255 on CXCR4 RNA expression. Though, Rz-255 alone is not efficient as observed in *in vitro* cleavage reaction, on addition of ODN#3 (lane 4) there was a 40% reduction in expression levels, similar to those obtained with ODN#4 (lane 5). Also, the cumulative effect of both the ODNs#3 and #4 (lane 6) dramatically reduces the expression level by greater than 90%. It should be noted that the antisense ODN#3 alone had no effect on the CXCR4 RNA expression levels (lane 3). The extent of house-keeping gene
(huGAPDH) in all the corresponding lanes remained essentially unchanged. From this experiment it could be concluded that Rz-255 and upstream ODN#3 combination was more effective in causing decrease in the levels of intracellular target RNA.

**Effect of Dz-139 and antisense ODNs on the surface expression of CXCR4 co-receptor on Jurkat T cells**

Following transfection of Jurkat T-cells with Dz-139 and antisense ODN # 1A and ODN # 2A, cells were analyzed by flow cytometry using flowjo program to determine the downregulation of CXCR4 co-receptor.

![Figure 3-13: Downregulation of CXCR4 expression on jurkat cells transfected with Dz-139 and ODN # 1A and ODN # 2A (non-shaded area represents control staining and shaded area represents the reduction in staining after various treatments)](image)

FACS analyses of various treatments are shown in Figure 3-13. There was a 49.7% reduction in CXCR4 expression with Dz-139 treatment alone (panel A); 81.3% reduction in presence of Dz + ODN #1 (panel B); 67.9% with Dz-139 + ODN 2A (panel C) and 85.2% with Dz-139 and both ODNs (ODN # 1A + ODN # 2A (panel D).
Unshaded area exhibits the control CXCR4 staining in untransfected cells. The shaded areas represent the extent of reduction in staining for CXCR4 by FACS analysis. We conclude that the combination treatment reduced the surface expression by additional 30 to 35% when compared with the reduction with Dz-139 treatment alone.

**Effect of Rz-255 and antisense ODNs on the Jurkat T cell surface expression of CXCR4 co-receptor**

Jurkat T-cells were transfected with Ribozyme 255 encoding plasmid and antisense ODN#3 and ODN#4, followed by flow cytometry analyses using flowjo program to determine the downregulation of CXCR4 co-receptor.

![Graphs showing downregulation of CXCR4 expression](image)

**Figure 3-14: Downregulation of CXCR4 expression on jurkat cells transfected with Rz-255 and its ODNs**

FACS analyses of various treatments are shown in Figure 3-14. Rz-255 alone was able to reduce the CXCR4 expression by only 9.87% (panel A), which was enhanced to 46.5% with the addition of ODN#3 (panel B). Also, ODN#4 was able to increase the effect of Rz-255 in causing 33.6% reduction in CXCR4 expression (panel C). When both ODN#3 and ODN#4 were added together...
with the Rz-255, a reduction of 47.2% was observed (panel D). Also, ODN# 3 alone did not significantly reduce the cell surface expression of CXCR4 (panel E).

Modulation of intracellular target gene expression by wild-type and mutant bispecific constructs

HEK 293 cells were co-transfected with either 1µg of CXCR4 encoding plasmid alone (Figure 3-15 lane 1) or in combination with the various in vitro synthesized bispecific constructs, namely 1µg of wt-siRNA-wt-Rz (lane 2), 1µg of wt-siRNA-mt-Rz (lane 3), 1µg of mt-siRNA-wt-Rz (lane 4) and 1µg of mt-siRNA-mt-Rz (lane 5). RT-PCR based analysis was carried out using CXCR4 specific primers to check the efficiency of various bi-specific constructs. A prominent CXCR4 specific gene expression was observed in lane 1 as expected. Wt chimeric construct transfected cells showed about 60% reduction (compare lanes 1 & 2). Also, wt-siRNA-mt-Rz (chimeric mutant-2) transfected cells showed about 55% reduction (lane 3) similar to the mt-siRNA-wt-Rz (chimeric construct-1) (lane 4). On the contrary, mt-siRNA-mt-Rz (chimeric mutant-3) transfected cells showed only 20% reduction (lane 5). These are representatives of three independent experiments. It could thus be concluded from this experiment that the intracellular reduction in the target RNA was due to both siRNA and Rz component of the chimeric construct with the siRNA component contributing a little more than the Rz part. The levels of the control RNA (huGAPDH) remained same in all the corresponding lanes which suggest that chimeric constructs down regulated the target gene expression in a specific manner.

Effect of antisense ODN on efficiency of wt-chimeric construct to downregulate the expression of CXCR4 RNA

21 nt long antisense ODN#5 was designed immediately downstream the siRNA target site in CXCR4 sequence. The ODN#5 was then co-transfected
along with wt-chimeric construct (1μg) and CXCR4 encoding plasmid DNA (1μg) in HEK 293 cells and were assayed for CXCR4 RNA expression by RT-PCR, using CXCR4 specific primer (Figure 3-15 B). The wt chimeric construct was alone able to reduce the intracellular expression of CXCR4 by 50% (lane 2). This reduction was increased to 80% upon addition of the antisense ODN#5 (lane3). However, in presence of unrelated ODN (lane 4) the level of reduction in CXCR4 expression remained similar to the wt construct RNA alone at nearly 40%. Also, both ODN#5 (lane5) and unrelated ODN (lane 6) showed a reduction of 30% in the expression of CXCR4 RNA. Here the unrelated ODN used was specific for CXCR4 but designed far away from the target site of siRNA.

Figure 3-15: Effect of various bispecific constructs on expression of CXCR4 RNA by RT-PCR
Effect of CXCR4 siRNA-Rz bispecific construct on the cell surface expression of CXCR4 co-receptor

1μg of various bispecific constructs were transfected into the Jurkat T cells and the cell surface expression of the co-receptor was analysed by flow cytometry.

Figure 3-16 represents the data obtained with the wild type and mutant chimeric constructs. The shaded population in each of the panels of Figure 3-16 represents the staining obtained with anti-CXCR4-PE in the control, untransfected cells. The reduction in the staining of cells due to reduced cell surface expression of CXCR4 on jurkat cells, in response to various treatments was calculated by population comparison program of the flowjo software.

Figure 3-16: Downregulation of CXCR4 co-receptor by various bispecific constructs on jurkat cells

It was observed that the wildtype chimeric construct reduced CXCR4 cell surface expression by 51.5% (Panel A). Also, the wt-siRNA-mt-Rz construct...
(Panel C) gave a similar effect with a 54.2% decrease in the levels of CXCR4 surface protein in comparison to the control population. However, the mt-siRNA-wt-Rz construct (Panel B) gave a modest reduction of 32.5% in the expression of the co-receptor, while the mt-siRNA-mt-Rz construct (Panel D) proved almost ineffective in decreasing the cell surface expression of CXCR4. The contribution by these constructs proved to be sequence specific, where point mutations rendered the constructs less effective as compared to the wild type chimeric constructs.

**Antisense ODN#5 designed downstream to the wt-chimeric construct augmented reduction of cell surface expression of CXCR4**

21 nt ODN#5 was co-transfected with the wild type chimeric construct and the cell surface expression of CXCR4 was monitored by staining the transfected cells with CXCR4-PE and analyzing them by flow cytometry. The results are shown in Figure 3-17. The shaded histogram in each of the panels of the figure represents the control population of untransfected jurkat cells expressing CXCR4. The subsequent difference in the CXCR4 expression as a result of different treatments is seen as a shift in the histogram from the control (shaded) population. Panel A represents a reduction of 50.9% in the expression of CXCR4 with the wt-siRNA-wt-Rz. This reduction is further enhanced to 78.8% when ODN#5 is added along with the wt-chimeric construct (Panel B). The unrelated ODN had no such effect in enhancing the effect of the wt chimeric construct (53.4%) (Panel C). Also, the ODN#5 alone could not reduce the expression of CXCR4 on the surface of jurkat cells significantly (10.5% reduction observed) (Panel D).
**Figure 3-17:** Downregulation of CXCR4 expression on jurkat cells by WtsiRNA-wtRz bispecific construct and its ODN#5

**In vitro cleavage of bispecific wild type construct in the presence of cellular extracts**

The transcripts were generated under *in vitro* conditions with equal efficiencies and cleaved by the cellular extracts at the spacer sequence to generate free siRNA and Rz-255, as shown in Figure 3-18. Lane 1 is the control wt-siRNA-wt-Rz-255 not treated with cellular extract. In lane 2, the chimeric construct was treated with cellular extract and incubated at 37°C for an hour as described in the methodology. The cleaved products were run on a 15% polyacrylamide-7M Urea gel. Lane 2 shows the cleavage of the bi-specific constructs and fragments of expected sizes are detected (Figure 3-18). The 38nt fragment is the Rz RNA produced by the activity of cellular lysate on the 6nt long cleavable linker (represented in green colour in the Figure 3-18). Also, the activity of Dicer enzyme present in the lysate on the shRNA yielded the 21 nt siRNA.
**Figure 3-18: Processing of CXCR4 si-Rz bispecific construct by cellular lysate and analyzed on 15% PAGE**

*In vitro cleavage of CXCR4 RNA by various bi-specific constructs to check the activity of Rz-255*

The various constructs were checked to see if they retain the catalytic activity of the Rz linked to siRNA. For this labeled CXCR4 RNA was incubated with RNA of the bi-specific constructs in 50 mM Tris-HCl, pH 7.5 and 10 mM MgCl₂ at 37°C for 1 hour. The results are shown in Figure 3-19 in which lane 1 is control CXCR4 RNA and in lane 2 the RNA was subjected to the treatment of Rz-255, and it acted as the positive control as the specific cleaved fragments of size 293 and 273 nts were observed. In lane 3 and 5, CXCR4 RNA treated with wt-siRNA-Rz-255 and mt-siRNA-wt-Rz-255, respectively, was loaded and Rz-255 specific cleaved fragments were observed. But when the wt-siRNA-mt-Rz-255 RNA was used to cleave CXCR4 RNA, no cleaved fragments of Rz-255 were seen (lane 4). Similar results were observed when bi-specific construct in which both siRNA and Rz-255 (mt-siRNA-mt-Rz-255) were mutated. No cleaved product specific for Rz-255 were seen when
CXCR4 RNA was incubated with mt-siRNA-mt-Rz-255 (lane 6). This indicated that the constructs which possessed wt-Rz activity, possessed the catalytic activity.

![Image](image_url)

**Lane 1 Control CXCR4 Half transcript**
**Lane 2 CXCR4 Half transcript + Rz-255**
**Lane 3 CXCR4 Half transcript + Wt si-Wt Rz**
**Lane 4 CXCR4 Half transcript + Wt si-Mt Rz**
**Lane 5 CXCR4 Half transcript + Mt si-Wt Rz**
**Lane 6 CXCR4 Half transcript + Mt si-Mt Rz**

**Figure 3-19: Catalytic activity of Ribozyme 255 in various bispecific constructs**

**Various antisense ODNs designed were able to hybridise to their target RNA efficiently**

*In vitro* synthesized α-P<sup>32</sup> labeled CXCR4 RNA was incubated with various antisense ODNs designed, in the presence of 10mM MgCl<sub>2</sub>, 50mM Tris-HCl buffer (pH 7.5) and 1 unit RNAse H enzyme in a final volume of 10μl at 37°C for 1 hr. The autoradiogram is shown in Figure 3-20. Lane 1 depicts the control CXCR4 half transcript, while the subsequent lanes show the cleavage products obtained due to efficient binding of various ODNs to the target RNA. All the four ODNs namely, ODN#4 (lane 2), ODN#3 (lane 3), ODN#2A (lane 4) and ODN#1A (lane 5) are able to effectively bind to the target CXCR4 transcript and produce the cleavage products due to RNAse H activity.
Viral challenge assays

To determine if the co-receptor downregulation resulted in inhibition of HIV-1 entry, DNA-enzyme transfected MAGI cells were co-transfected with p89.6 plasmid producing the X4 tropic HIV virus and analysed for β-galactosidase activity 48 hours post transfection. The level of enzyme correlates positively with the extent of viral replication (Vodicka et al., 1997). The results obtained are represented as bar graphs in Figure 3-21. The Dz-139 results in a 20% inhibition of HIV infection which is further enhanced to nearly 80% with the ODN#1A and ODN#2A separately and together. Also, the viral challenge assay with the Rz-255 yielded similar results (Figure 3-22). Rz-255 alone resulted in 20% inhibition of HIV infection, which is enhanced to 40% when used together with the ODN#3. Also, ODN#3 alone was able to reduce the infection by 20%.

Figure 3-20: RNase H assay to determine the hybridisation efficiency of the oligonucleotides
Figure 3-21: Viral challenge assay to check the efficacy of Dz-139

Figure 3-22: Viral challenge assay to determine the efficacy of Rz-255

Figure 3-23 represents the effect antisense ODN#5 has on the action of the wild type bispecific construct in reducing HIV infection. It is found that alone the wild type construct is able to inhibit the infection by 40% which is increased by 10% on addition of the ODN#5. On the other hand, no increased effect is visualized when an unrelated ODN is used together with the wild type bispecific construct. Also, surprisingly, ODN#5 alone is able to reduce the infection by a good 30%. Further the efficiency of various mutant bispecific constructs in downegulating the infection was compared with the
wild type construct and it was observed that all the constructs were able to reduce infection similarly by nearly 40%. This is represented in Figure 3-24.

Figure 3-23: Viral challenge assay to check the efficacy of Wtsi-wtRz and its ODN

Figure 3-24: Viral challenge assay to check the efficacy of various bispecific constructs

Discussion

Several antiviral approaches against HIV-1 have earlier been described but none of them seem to provide long term protection as escape mutants eventually emerge. HIV-1 is known to accumulate mutations rapidly and
therefore any antiviral strategy that is based on a single target in the HIV-1 genome is not likely to be very useful. Since HIV-1 infection depends on the successful interaction with its trimeric HIV-1 envelope with HIV-1 coreceptors (mainly CXCR4 and CCR5), they constitute promising targets for antiviral intervention. Also, many groups have targeted CCR5, there is a need to address the other co-receptor, CXCR4 which is utilized by X4 tropic viruses.

There have been some reports earlier on the down modulation of CXCR4 coreceptors (Anderson and Akkina, 2005; Basu et al., 2000) but almost all of these studies reported very poor in vitro cleavage activity by Rz or Dz on a truncated CXCR4 RNA and the surface levels of CXCR4 remained moderately low. We reasoned that one of the reasons for sub-optimal cleavage could be the secondary structures near the catalytic Dz cleavage site that could be melted or relaxed by designing specific ODNs very close to the Dz. We designed Dz-139 and several ODNs were screened that could augment the Dz mediated cleavage of the target RNA. We observed that ODNs immediately upstream and downstream of the Dz’s hybridizing arms powerfully augmented the cleavage of both full length and truncated CXCR4 RNA. ODNs with no gap (ODN#1A and ODN#2A) worked about 40% better than ODNs with one nucleotide gap (ODN#1 and ODN#2), in causing augmentation of cleavage, probably because they were able to melt the secondary structures more efficiently or facilitated more efficient hybridization between the substrate and enzyme.

Most interesting results were obtained when Dz and ODNs were introduced into a mammalian cell. Dz alone caused a potent reduction in the levels of CXCR4 RNA by RT-PCR techniques. In the presence of Dz + ODN # 1A, almost complete knock down effect was observed. It is also likely that ODNs facilitated the RNAse-H mediated decay of the substrate RNA. The most
remarkable effect of this combined treatment on down regulation of surface CXCR4 expression was observed in human T cells (Jurkat) which is known to be an excellent host for HIV-1 replication. In the presence of Dz alone, about 50% reduction in the surface expression was repeatedly observed. This is very close to all the previous observations with siRNAs or Rzs. But the surface levels were down-modulated to more than 80% with ODN #1A and with the combined treatment of both ODNs (ODN #1A & 2A); more than 85% surface reduction was observed in CXCR4. Also, interesting are the results obtained when Rz-255 was used with the upstream and downstream ODNs, namely ODN#3 and ODN#4. Although Rz-255 was not very efficient in cleaving CXCR4 transcript, its efficiency increased tremendously with the use of ODN#3 and ODN#4. Remarkable results were obtained with the Rz-255 and the ODN#3 and ODN#4 when intracellular studies were carried out. Both the ODNs were able to reduce the expression of CXCR4 effectively but the upstream ODN#3 may have better hybridizing potential as compared to ODN#4. Both the Dz-139 and Rz-255 were also able to inhibit HIV infection. The most significant finding was the inhibition of HIV infection by Dz-139 and its antisense ODNs to nearly 80% as determined by viral challenge assay. This is the first time when such marked reduction in HIV infection has been achieved by employing catalytic and non-catalytic DNA.

In the present work a novel bispecific construct was also made, where a siRNA and a Rz were combined together to synergise the effect of both of the antiviral approaches. The potency of the approach could also be explained on the basis that once the target RNA is cleaved by either of the two antiviral approaches (siRNA or Rz), it may open up secondary structures at the target sites. This may facilitate more efficient hybridization between the target RNA and Rz or siRNA. The wild type bispecific construct was able to reduce the cell surface expression of CXCR4 by 50% similar to that obtained by using the
construct with mutant Rz-255. The bispecific mutant construct in which the siRNA is mutated, could inhibit 30% cell surface expression of CXCR4. Therefore, the siRNA contributed more potently in reducing the target gene expression in comparison to Rz-255. Studies from our own group (Unwalla et al., 2006), using two different kinds of catalytic nucleic acids (Rzs and 10-23 catalytic DNA-enzymes) and others (Goila and Banerjea, 1998; Kurreck et al., 2002) have clearly established that some sites are more accessible to either RNA or DNA based catalytic nucleic acids. So, it can be argued that the Rz was not very effective due to poor availability of its target site. Also, it is noteworthy that the siRNA target site selected involved the ‘AUG’ of the target RNA which is usually accessible towards siRNA mediated down regulation. The ability of the wild type bispecific construct was enhanced further by employing ODN#5 just downstream to the siRNA. Viral inhibition of nearly 40% was achieved with the wildtype bispecific construct. Surprisingly, the mutant bispecific constructs were also able to significantly inhibit HIV infection, measured by β-gal activity. Perhaps, these four point mutant siRNA were able to induce the miRNA pathway to inhibit gene expression.

In summary, we presented evidence that significant enhancement of Dz and Rz mediated cleavage could be obtained by including specific ODNs. When introduced into cells, they resulted in potent knock down of the target gene, in our case, one of the most important HIV-1 coreceptors. The intracellular effectiveness of these ODNs and Dzs could be improved significantly by using chemically modified (stabilized) nucleotides (Kurreck et al., 2003). Also, we were able to emphasise the importance of using multiple nucleic acid approach in one construct, where either of the approaches downregulates the target, here CXCR4 gene and also opens up the RNA structure so that antisense molecules can further enhance the effect of these approaches.
individually. Recently, Gupta et al., in 2008 published a similar study, where the X gene of Hepatitis was targeted with a bispecific construct and ODN yielding very gratifying results. These chimeric constructs can be delivered to the desired cell via lentiviral vectors or other vectors for achieving specific gene suppression.