Development of splice switching antisense oligonucleotides targeting midkine

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BACKGROUND

Midkine is a growth factor/cytokine that is highly expressed during embryonic development followed by down-regulation at birth. However, in many disease settings such as chronic inflammatory diseases, autoimmunity and cancer, midkine expression increases in local tissues with systemic levels elevated 10 to 100-fold. Midkine mediates diverse cell interactions and signaling pathways within the tumour microenvironment, and thereby contributes to metastasis, resistance to immune checkpoint inhibitors and angiogenesis. Drugs targeting midkine represent novel first-in class anti-cancer treatments.

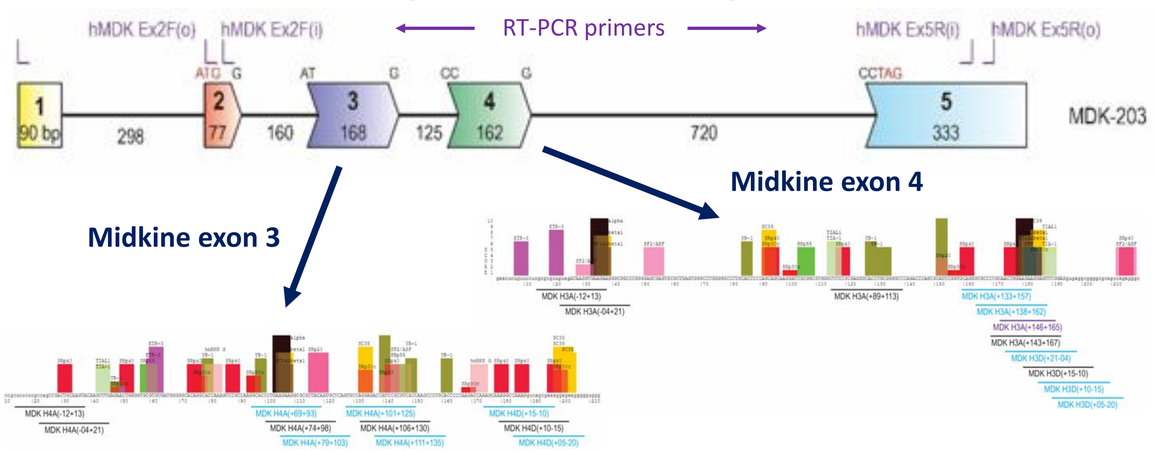
Various biologicals including siRNA, aptamers, peptides and antibodies inhibit midkine in animal models of cancer, autoimmunity and inflammation. Splice switching antisense oligonucleotides (SSOs) are another potential RNA drug that not only reduce the production of target mRNAs, but also generate non-functional proteins by deleting specific exons.

AIM: To develop splice switching oligonucleotides targeting midkine mRNA that elicit alternate splicing resulting in skipping of exons 3 or 4.

METHODS

Guided by SpliceAid to identify splice enhancer binding motifs and consideration of RNA sequence bias constraints, SSOs were designed to predicted splice motifs in exons 3 and 4 of the midkine mRNA and synthesized using 2'OMe-PS chemistry. Exon skipping was assessed by RT-PCR with primers flanking exons 2 and 5 following transfection into midkine-expressing human Huh7 liver cancer and SHSY5Y neuroblastoma cells. Optimisation of lead SSOs was carried out through micro-walking, cocktails of SSOs and PMO oligonucleotide chemistry, followed by Western analysis to detect truncated midkine protein.

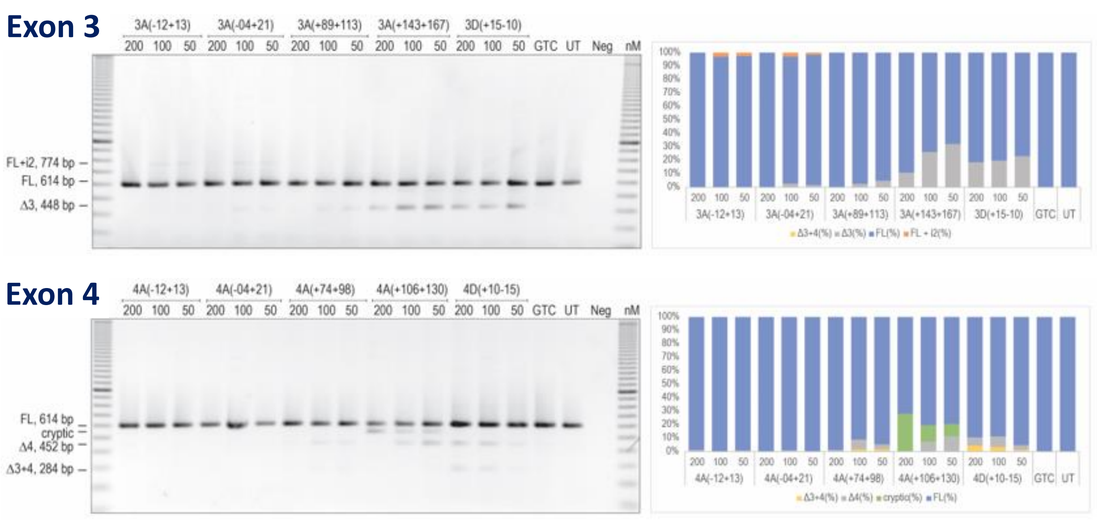
Midkine gene structure showing Exons 1-5



SpliceAid guided design of SSOs targeting midkine exons 3 and 4. The bars indicating likely splice enhancer binding motifs. SSOs used for initial screening are shown in black and micro-walked SSOs in light blue.

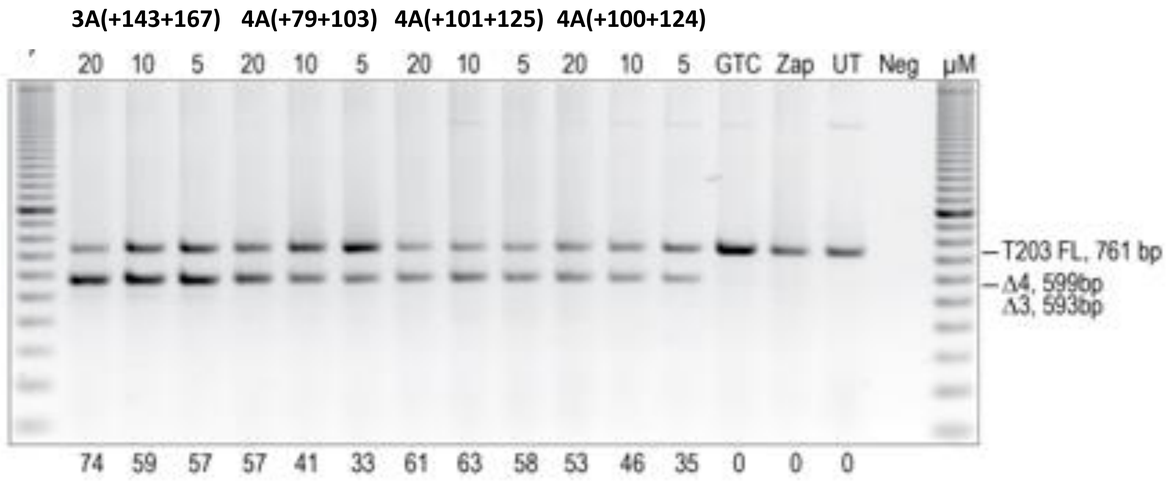
The SSO nomenclature designates the position relative to the splice acceptor and donor sites.
eg **MDK 4A(+101+125)** comprises base pairs 101 to 125 downstream of the start of exon 4.

Screening SSOs by RT-PCR



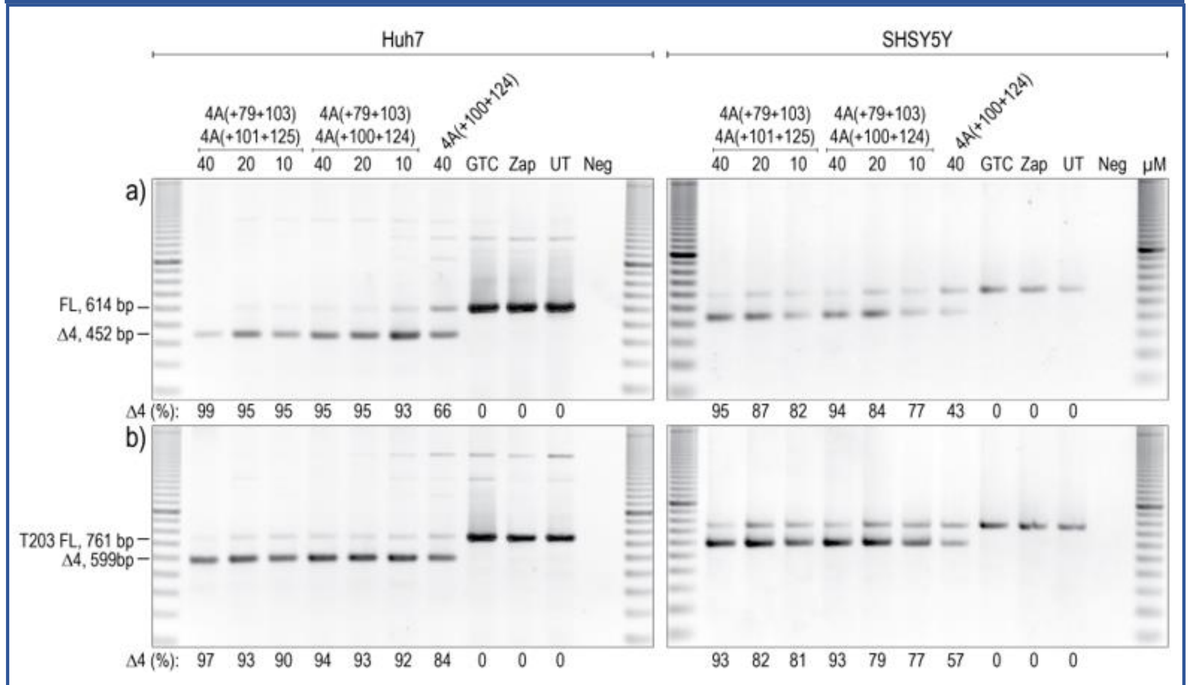
Evaluation of exon skipping in Huh7 cells. mRNA from cells transfected with Exon 3 and 4 2'OMe-PS SSOs was analysed by RT-PCR followed by agarose gel electrophoresis. The mRNA with exons deleted is show as **Δ3** or **Δ4** relative to full length **(FL)** midkine mRNA and is quantified by densitometry. Maximal exon skipping ranges from 30% for exon 3 to 10% for exon 4, with several SSOs showing no activity.

Optimization of SSOs



Microwalking and PMO chemistry improved exon skipping. Minor changes in position by microwalking identified lead SSOs with enhanced production of midkine mRNA with exon 3 and 4 deleted. SSOs with phosphorodiamidate morpholino oligomer (PMO) chemistry from Gene Tools improved exon skipping with up to 74% or 63% efficiency for exon 3 or 4 SSOs respectively.

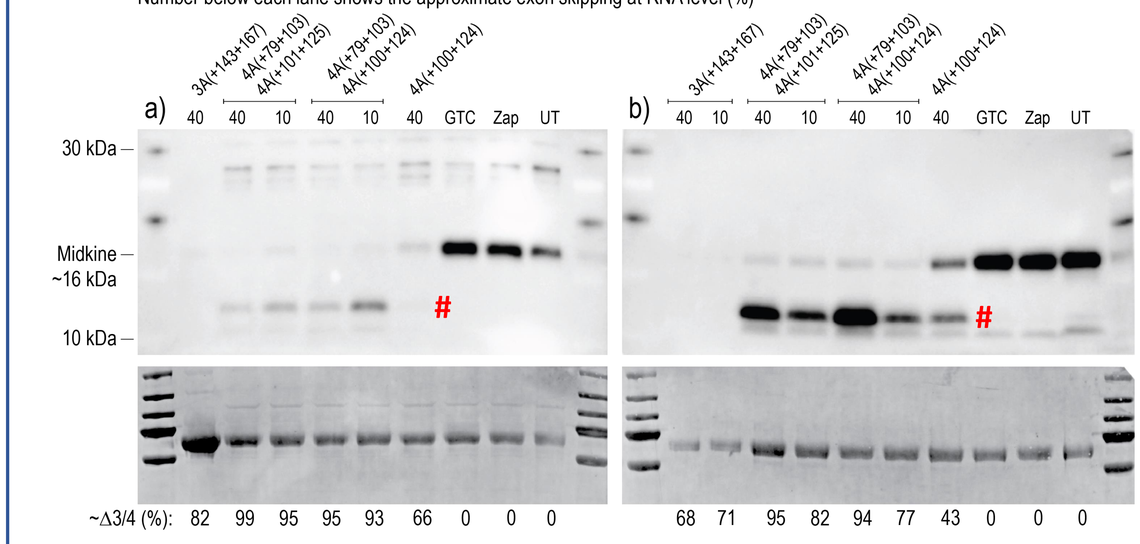
SSO cocktails improve exon skipping



Evaluation of exon 3 and exon 4 SSO combinations. Over 90% exon skipping could be achieved when two SSOs with PMO chemistry were transfected into Huh7 or SHSY5Y cells. A corresponding decrease in full length midkine mRNA was apparent

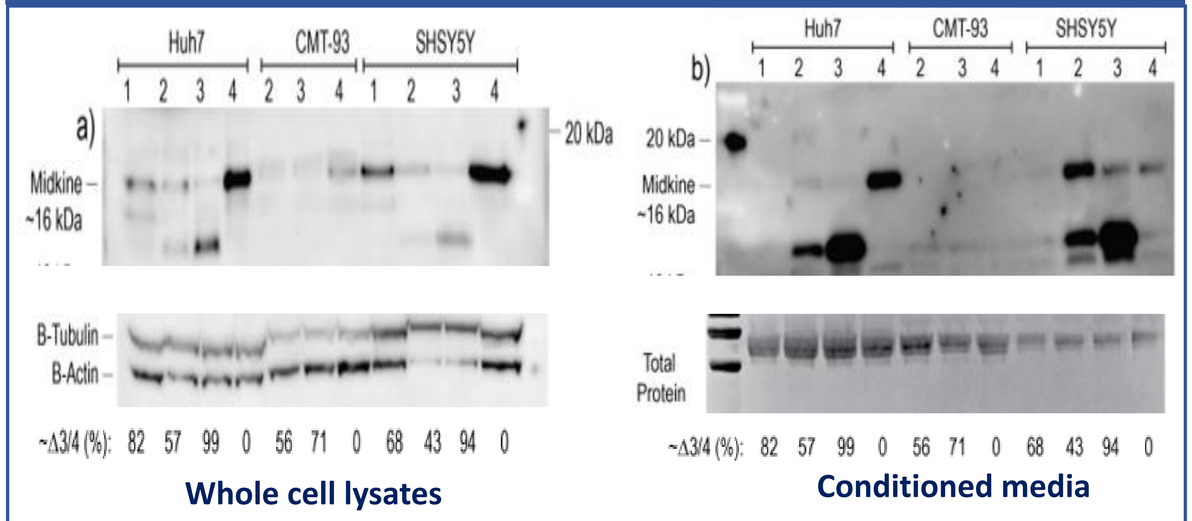
Detection of truncated midkine protein

a) Huh7 and b) SHSY5Y, 600,000 cells/treatment, Neon transfection with PMOs,
Media changed to serum free at 48 hr, collected at 72 hr
12% Bis-Tris mini gel, MOPs buffer, iBlot 7 min transfer
MDK-PA5109951 1:1000, O/N at 4°C - 15s, 5sum
Total protein Coomassie stain - Cy7
Number below each lane shows the approximate exon skipping at RNA level (%)



Western analysis of midkine protein. Conditioned media from Huh7 or SHSY5Y cells was collected and midkine protein detected using a specific antibody (Invitrogen/Thermo Fisher). Minimal full length midkine protein was produced while a band **(#)** corresponding to a potential shorter form of midkine was evident with exon 4 SSOs. No truncated midkine was evident for exon 3 SSOs. In the SHSY5Y cells there was an apparent association between the degree of exon skipping at the mRNA level and abundance of the truncated midkine protein. The lower panels show total protein staining as loading control

Truncated midkine protein is predominantly secreted



Comparison of truncated midkine protein in media and cell lysates. Conditioned media and cell lysates were collected from human Huh7 liver, SHSY5Y neuroblastoma or mouse colorectal CMT-93 cancer cells and full length or truncated midkine protein detected by Westerns. **Lane 1** = Exon 3 SSO 3A(+143+167); **Lane 2** = Exon 4 SSO 4A(+100+124); **Lane 3** Exon 4 cocktail 4A(+79+103) & 4A(+100+124); **Lane 4 GTC control**. Lower amounts of truncated midkine protein are retained within cells relative to secreted.

CONCLUSIONS

- Candidate splice switching antisense oligonucleotides targeting exons 3 and 4 of midkine mRNA were identified.
- Optimisation of lead SSOs through microwalking, cocktails of SSOs and PMO chemistry resulted in >90% exon skipped MDK mRNA in human cancer cells.
- A corresponding increase in truncated midkine protein secreted from cells was evident.