Increscent Therapeutics™

SINGLE LIGAND-MULTIPLE PAYLOAD DELIVERY

Nonconfidential Data Package

March 2018
# Introduction

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# General Procedures
Introduction

Increscent Therapeutics™ is a new technology and intellectual property offering from MPEG LA in the field of therapeutic oligonucleotides. Developed by MPEG LA’s in-house biochemical experts in conjunction with custom laboratory services provided by Axolabs GmbH, Increscent Therapeutics™ provides a versatile technology platform for improving the therapeutic efficacy of oligonucleotide conjugates.

The platform is based on a proprietary system for designing and synthesizing multimeric oligonucleotide conjugates that enable multiple therapeutic payloads to be delivered together in a single compound. Larger conjugates enable dramatically enhanced serum half-life and availability for uptake by target cells and tissue, all with minimal or no increase in typical cytokine markers of cellular toxicity.

The platform may be applied to any therapeutic target addressable by oligonucleotides such as siRNA, saRNA, miRNA, antisense oligonucleotides, and others, and is compatible with a large variety of cell-targeting moieties such as GalNAc, aptamers, peptides, and others.

The patent-protected platform is available for licensing from MPEG LA. For questions and further information, please contact Kristin Neuman, Executive Director, Biotechnology Licensing at kneuman@mpegla.com or +1.914.588.2471.

Discussion

The data in this package represents our work to-date on demonstrating applications for the Increscent™ platform in the field of oligonucleotide therapeutics. While we performed our studies with materials that are well-known in the field so that the results would be easily understood – namely siRNA targeting Factor VII mRNA (“siFVII”) in hepatocytes and a tri-antennary GalNAc ligand – the Increscent platform may be readily translated to other therapeutic oligonucleotides and other cell-targeting moieties.

At the outset, we aimed to demonstrate that a multi-conjugate of two units of siRNA would be more potent in vivo than its monomeric counterpart. In the mouse study presented in Section A, we made three isomeric homo-dimers of siFVII conjugated to GalNAc and tested them against a monomeric siFVII conjugated to GalNAc. In this and all subsequent studies, our siFVII sequences were chemically modified using well understood strategies to enable the siRNA to withstand degradation and to enhance cell penetration without compromising bioactivity. When the knockdown activity was normalized to dose (per unit of siRNA), the activity of the homo-dimers and monomeric control were equivalent. But, when normalized per unit of GalNAc ligand, the homo-dimers outperformed the monomeric control by approximately 2-fold in knockdown potency.
Next, we set out to demonstrate that a multi-conjugate of three units of siRNA, each targeting a different mRNA, would be active \textit{in vivo}. In the mouse study presented in Section B, we made two isomeric GalNAc-conjugated hetero-trimers of siFVII, siApoB, and siTTR and tested them against a pool of monomeric siFVII, siApoB, and siTTR, each conjugated to GalNAc. The potencies of the hetero-trimers were on par with the monomeric pool on a per dose basis.

Working on a hypothesis that larger multi-conjugates will have decreased kidney filtration due to their size, leading to enhanced longevity in serum and greater uptake by target cells, we designed a set of experiments to demonstrate whether large multi-conjugates would produce a significant increase in serum half-life. In Section D, we describe an \textit{in vivo} study of siFVII compounds ranging from monomer to octamer, wherein we discovered that a dramatic increase in serum half-life kicks in with the tetramer and maxes out at the hexamer. The highest increase in serum half-life that we observed was with a hexamer (see Sections C and E), which measured 30 times the serum half-life of a monomer in a mouse study. The same hexamer and monomer were analyzed for cytokine levels, with the hexamer showing minimal to no increase in cytokine markers for cellular toxicity.

Our next round of experiments is underway to compare the knockdown potency of a GalNAc-conjugated siFVII hexamer versus a GalNAc-conjugated siFVII monomer. Data will be available in May of 2018.

In summary, the Increscent™ platform provides opportunities to (1) deliver multiple therapeutic agents in controlled ratios in a single, cell-targeting oligonucleotide conjugate and/or (2) boost activity in cell-targeting oligonucleotide conjugate systems that to-date have not achieved acceptable levels of therapeutic efficacy; and/or (3) lower dosage levels of existing therapeutic oligonucleotide conjugates currently delivered in monomeric form by converting to a multimeric format.
A. Demonstration of Increased Knockdown Per Unit Ligand

Synthesis of Homo-dimers

Three isomeric homo-dimers of FVII siRNA each carrying a tri-antennary GalNAc ligand were synthesized via a mono-DTME derivative prepared as described in General Procedures. Thus, positional isomer XD-06360 was prepared from two FVII sense strands with the sequences:

\[(\text{C}_{6}\text{SSC}_{6})\text{gcAfaAfgGfuGfcCfaAfcUfcAf}(\text{invdT})(\text{C}_{6}\text{NH}_{2})\]

\[(\text{C}_{6}\text{SSC}_{6})\text{gcAfaAfgGfcGfuGfcCfaAfcUfcAf}(\text{invdT})\]

according to Scheme 1:

\[\text{Scheme 1. Synthesis of GalNAc-FVII-dimer XD06360}\]
Positional isomers XD-06329 and XD 06330 were prepared by similar procedures starting from FVII antisense strand X-18797:

\[
\text{UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C}_6\text{SH)}
\]

according to Scheme 2:

![Scheme 2. Syntheses of GalNAc-FVII-dimers XD-06329 and XD06330](image)

**Determination of Bioactivity of Homo-dimers**

The three GalNAc-conjugated homo-dimeric siFVII isomers (XD-06329, XD-06330 and XD-06360) and a GalNAc-conjugated monomeric siFVII control (XD-06328) were administered to mice subcutaneously at 25 mg/kg or 50 mg/kg in PBS (0.2ml). Group sizes were n=4 mice/group for treated animals and n=5 for saline controls. Blood was collected 1 day prior to treatment and at 1, 3 and 7 days post-treatment, and analyzed for FVII enzyme activity. When normalized to dose per unit of siRNA the activities of the homo-dimers and monomeric control were equivalent. See FIG. 1:
However, the homo-dimers outperformed the monomeric control when normalized per unit of GalNAc. See FIG. 2:
B. Demonstration of Hetero-trimer Activities Per Unit Ligand

Synthesis of Hetero-trimers

GalNAc-conjugated, hetero-trimeric positional isomers of siRNA, each targeting FVII, ApoB, and TTR, were synthesized and tested for biological activity.

Preparation of GalNAc-siFVII-siApoB-siTTR Hetero-trimer with Cleavable Linkages on Alternating Strands (XD-06727)

9 mg (192 nmol) of GalNAc-conjugated, hetero-trimeric siRNA XD-06727 simultaneously targeting FVII, ApoB, and TTR, was prepared in high yield and purity by combining single strands stepwise as depicted in Scheme 3 using the methodology described in General Procedures in the proportions listed in Table 1.

Scheme 3. Synthesis of XD-06727
Each reaction step was monitored by HPLC, which revealed that nearly 100% conversion of the reactants was achieved at each step as shown in FIG. 3

**FIG. 3.** a) Reverse Phase and b) Ion exchange chromatograms of reactions in Scheme 3

(a)

(b)

<table>
<thead>
<tr>
<th>ID</th>
<th>Target</th>
<th>E (L/mol*cm)</th>
<th>1 OD</th>
<th>MW (free Acid)</th>
<th>MW Na salt</th>
<th>Req OD</th>
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<td>TTRs</td>
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<td>7105.6</td>
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<td>FVIIa</td>
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<td></td>
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<td>44144</td>
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<td></td>
</tr>
</tbody>
</table>
Preparation of GalNAc-siFVII-siApoB-siTTR Hetero-trimer with Cleavable Linkages on Sense Strands (XD-06726)

7 mg of GalNAc-conjugated, hetero-trimeric siRNA XD-06726 simultaneously targeting FVII, ApoB and TTR, was prepared in high yield and purity by the procedure depicted in Scheme 4 using the methodology described in General Procedures in the amounts listed in Table 2.

In the opening steps of the reaction, the single-stranded hetero-dimer X19581 with a 5’-amino terminus was prepared using solid phase chemistry and was reacted with 5 equivalents of SPDP (succinimidyl 3-(2-pyridyldithio)propionate).
and after purification by reverse phase HPLC was further reacted with the thiol modified oligonucleotide X18793 (1.2 eq) to yield the single-stranded hetero-trimer X20256 (containing linked sense strands of siFVII, siApoB, and siTTR) in high purity. The sequence of X20256 is shown in Table 3.

Table 3.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence: 5’-3’ for each component monomer</th>
<th>Target/Strand</th>
</tr>
</thead>
</table>

Thereafter, the hetero-trimeric double stranded construct (XD-06726), simultaneously targeting FVII, ApoB and TTR, 7 mg (150 nmol), was prepared by sequentially adding the individual antisense single strands stepwise to the hetero-trimeric sense-strand intermediate (X20256) according to the duplex titration method described in General Procedures.

Table 2. Stoichiometries of Oligomers Used in Synthesis of GalNAc-siFVII-siApoB-siTTR Hetero-trimer (XD-06726).

<table>
<thead>
<tr>
<th>ID</th>
<th>Target</th>
<th>E (L/mol*cm)</th>
<th>Nmol/OD</th>
<th>MW (free Acid)</th>
<th>MW Na salt</th>
<th>Req OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>X20256</td>
<td>FVIIs-ApoBs-TTRs</td>
<td>623900</td>
<td>1.60</td>
<td>22690.8</td>
<td>24075.7</td>
<td>94</td>
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<td>ApoBas</td>
<td>206500</td>
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<td>6762.4</td>
<td>7202.1</td>
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</tr>
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<td>TTRas</td>
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<td>7596.1</td>
<td>8079.7</td>
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</tr>
<tr>
<td>X18795</td>
<td>FVIIs</td>
<td>194800</td>
<td>5.13</td>
<td>6849.4</td>
<td>7289.1</td>
<td>29</td>
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<td>XD-06726</td>
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<td></td>
<td></td>
<td>43898.7</td>
<td>46646.6</td>
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Synthesis of Hetero-tetramer

A GalNAc-conjugated, hetero-tetramer of siRNA, targeting FVII, ApoB, and TTR, was prepared in high yield and purity by the procedure depicted in Scheme 5 using the methodology described in General Procedures in the amounts listed in Table 4; but was not tested for biological activity.

Preparation of GalNAc-siFVII-siApoB-siTTR-siFVII Hetero-tetramer with Cleavable Linkages on Alternating Strands (XD-07140)

Scheme 5. Synthesis of XD-07140

Table 4. Stoichiometries of Oligomers Used in Synthesis of GalNAc-siFVII-siApoB-siTTR Hetero-tetramer (XD-07140).

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<th>ID</th>
<th>Target</th>
<th>E (L/mol*cm)</th>
<th>1 OD</th>
<th>MW (free Acid)</th>
<th>MW Na salt</th>
<th>Req OD</th>
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</thead>
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<tr>
<td>X20336</td>
<td>FVIIs-ApoBs</td>
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<td>2.47</td>
<td>15440.1</td>
<td>16341.4</td>
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</tr>
<tr>
<td>X20366</td>
<td>ApoBs-TTRas</td>
<td>446700</td>
<td>2.24</td>
<td>14748.9</td>
<td>15716.1</td>
<td>5.5</td>
</tr>
<tr>
<td>X22413</td>
<td>TTRs-FVIIs</td>
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<td>2.52</td>
<td>14041.3</td>
<td>14964.5</td>
<td>4.9</td>
</tr>
<tr>
<td>X18795</td>
<td>FVIIas</td>
<td>194800</td>
<td>5.13</td>
<td>6849.4 x2</td>
<td>7289.1 x2</td>
<td>4.8</td>
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<tr>
<td>XD-07140</td>
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<td></td>
<td></td>
<td>57929.1</td>
<td>61600.2</td>
<td></td>
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</table>
Determination of Bioactivity of Hetero-trimers

Hetero-trimers XD-06726 and XD-06727 and a pool of 3 GalNAc-conjugated monomeric siRNAs separately targeting FVII, ApoB, and TTR were each separately administered to mice subcutaneously at a dosage of 50 mg/kg total RNA for the hetero-trimers and 17 mg/kg for each of the monomeric conjugates in PBS (0.1ml).

As a positive control, a lipid nanoparticle (LNP) formulation containing monomeric siRNAs (NPA-741-1) directed against the same targets FVII (XD-00030), ApoB (XD-01078), and TTR (XD-6729) was injected intravenously at 0.5 mg/kg per siRNA.

Group sizes were n=4 mice/treatment group and n=5 for saline controls. Blood was collected 1 day prior to treatment and at 1, 3 and 7 days post-treatment, and serum levels of FVII, ApoB and TTR were measured. mRNA levels from liver lysates at day 7 were also determined. Results are plotted in FIG. 4:

FIG. 4. Serum and mRNA knockdown for FVII, ApoB, and TTR achieved by hetero-trimers XD 06726 and XD-02727, and monomeric GalNAc and LNP controls
C. Demonstration of Increased Serum Half-life of Hexamer vs Monomer

Synthesis of Homo-hexamer

To test the hypothesis that a large multimeric siRNA would have an increased half-life in serum relative to a monomeric equivalent, a homo-hexamer of FVII siRNA (XD-09795) was prepared as described in Scheme 6:

Scheme 6. Preparation of XD-09795

Homo-hexameric sense-strand of FVII siRNA (X-30838) with amino groups at both 3’- and 5’-termini and containing four dCdA cleavable linkers (“—NA—”) and one reductively cleavable DTME linker (“S—CL—S”) was synthesized and purified via the homo-dimer (X30835) and homo-tetramer (X30837). In the opening steps of the synthesis, disulfide group (“S-SR”) was cleaved from both X30833 and X30836 using DTT to give the corresponding 5-thiol derivatives X30834 and X30837 in 97.6% and 91.9% yield, respectively. X30834 (14.9 mg, 986.7 nmol) was then converted to 10.6 mg (700.5 nmol, 71.0%) of the corresponding mono-DTME (“S—CL”) derivative X30835 which was reacted with one equivalent of X30837 to give 4.2 mg (90.7 nmol, 64%) of the single stranded homo-hexamer X30838. 3.8 mg (83 nmol) of hexameric sense strand X30838 and 3.7 mg (502 nmol, 6 mol. equiv) of anti-sense strand X18795 were then annealed to yield 7.5 mg (83.7 nmol) of the corresponding double stranded FVII homo-hexamer XD-09795.
Determination of Half-lives of Homo-hexamer vs Monomer

The serum half-life of the homo-hexamer XD-09795 and a matching monomeric control XD-09794 was determined by iv bolus injection of the test materials at a concentration of 1ng/ml in x1 PBS via tail vein into 3 cohorts of 4 C57/BL6N female mice aged approx. 11 weeks per cohort. Dosage was 20mg/kg for both FVII monomer and FVII homo-hexamer. Blood samples were drawn at 5, 30, 60, and 120 minutes. The concentration of FVII siRNA at these time points was determined as described in General Procedures and plotted as shown in FIG. 5.

FIG. 5
D. Determination of the Size Range for Increased Serum Half-life of Homo-Multimers

Synthesis of Homo-multimers

To determine the size at which the increase in serum half-life commenced and ended, a series of homo-multimers of an siRNA directed against FVII mRNA were prepared via the methodologies described in General Procedures.

Thus, the following sequences:

FVII sense: 5’-gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)-3’
FVII anti-sense: 5’-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3’

were linked via the endonuclease cleavable linkers dCdA and the reductively cleavable linker DTME to form 2, 3, 4, 5, 6, 7, and 8-mers as follows:

Synthesis of FVII Monomer XD-09794

Monomeric sense strand X18789 of FVII siRNA with amino function at the 5’-terminus was synthesized and purified. Yield, 48.3 mg, 6.694 mmol, 18.6%. The corresponding antisense
strand X18795 was likewise synthesized to yield 46.3mg, 6.35 mmol, 31.9%. 5.35 mg (747.3 nmol) of sense strand X18789 and 5.45 mg (747.3 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (747.4 nmol) of the corresponding double-stranded FVII monomer XD-09794.

**Synthesis of FVII Dimer XD-10635**

![Synthesis of homo-Dimer of FVII siRNA](image)

\[
\text{X30833} = 5'\{C_6SC_4\}\text{gfaAfgfGfuGfcFfaAfcUfcAf(invdT)dCdAgaAfaAfgfGfcGfuGfcFfaAfcUfcAf(invdT)(C_4NH}_2\}\text{-3'}
\]

\[
\text{X18795} = 5'\text{-UfsGfaGfuUfgGfcAfcGfcFfuGfuscu-3'}
\]

Homo-dimeric sense-strand of FVII siRNA X30833 with amino and di-sulfide groups at the 3’- and 5’- termini, respectively, and containing a dCdA cleavable linker (–NA–) was synthesized and purified. Yield, 35.8 mg, 6.694 mmol, 18.6%.

5.51 mg (362.6 nmol) of homo-dimeric sense strand X30833 and 5.29 mg (725.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (362.6 nmol) of the corresponding double stranded FVII homo-dimer XD-10635.
Synthesis of FVII Trimer XD-10636

Synthesis of homo-Trimer of FVII siRNA

- Sense strand ex synthesizer
- Annealed with 3 mol equiv anti-sense strand

Homo-trimeric sense-strand of FVII siRNA X34003 with amino and di-sulfide groups at the 3'- and 5'- termini, respectively, and containing two dCdA cleavable linkers (---NA--) was synthesized and purified. Yield, 19.6 mg (857.9 nmol, 19.3%).

5.16 mg (225.5 nmol) of homo-trimeric sense strand X34003 and 4.93 mg (676.5 nmol) of anti-sense strand X18795 were then annealed to yield 10.1 mg (225.5 nmol) of the corresponding double stranded FVII homo-trimer XD-10636.
Synthesis of FVII Tetramer XD-10637

Synthesis of homo-Tetramer of FVII siRNA

- Sense strand ex synthesizer
- Annealed with 4 mol equiv anti-sense strand

Homo-tetrameric sense-strand of FVII siRNA X30836 with amino and di-sulfide groups at the 3'- and 5'-termini, respectively, and containing three dCdA cleavable linkers (--NA--) was synthesized and purified. Yield, 53.1 mg (1734.5 nmol, 13%).

5.53 mg (180.8 nmol) of homo-tetrameric sense strand X30836 and 5.27 mg (723.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (180.8 nmol) of the corresponding double stranded FVII homo-tetramer XD-10637.
Synthesis of FVII Pentamer XD-10638

Synthesis of homo-Pentamer of FVII siRNA

- Sense strand ex synthesizer
- Annealed with 5 mol equiv anti-sense strand

Homo-pentameric sense-strand of FVII siRNA X34004 with amino and di-sulfide groups at the 3'- and 5'- termini, respectively, and containing four dCdA cleavable linkers (--NA--) was synthesized and purified. Yield, 35.9 mg (938 nmol, 10.6%).

5.53 mg (144.5 nmol) of homo-pentameric sense strand X34004 and 5.27 mg (723.2 nmol) of anti-sense X18795 were then annealed to yield 10.8 mg (144.5 nmol) of the corresponding double stranded FVII homo-pentamer XD-10638.
Synthesis of FVII Hexamer XD-10639

Synthesis of homo-Hexamer of FVII siRNA

- Sense strand ex synthesizer
- Annealed with 6 mol equiv anti-sense strand


X18795 = 5’-UfsGfaGfuUfgGfcAfcGfuUfuGfcusu-3’

Homo-hexameric sense-strand of FVII siRNA X34005 with amino and di-sulfide groups at the 3’- and 5’- termini, respectively, and containing five dCdA cleavable linkers (---NA--) was synthesized and purified. Yield, 21.4 mg (466.1 nmol, 5.3%).

5.15 mg (144.5 nmol) of homo-pentameric sense strand X34005 and 4.89 mg (723.2 nmol) of anti-sense X18795 were then annealed to yield 10.04 mg (111.9 nmol) of the corresponding double stranded FVII homo-hexamer XD-10639.
Synthesis of FVII Heptamer XD-10640

Homo-heptameric sense-strand of FVII siRNA X-34009 with amino groups at both 3’- and 5’-termini and containing five dCdA cleavable linkers (---NA-->) and one reductively cleavable DTME linker (S—CL—S) was synthesized and purified via the single-stranded homo-dimer X30833 and homo-pentamer X34004 prepared above. Disulfide group was cleaved from X30833 and X34004 using DTT to give the corresponding 5-thiol derivatives X30834 (28.3 mg, 1877.9 nmol, 86.7%) and X34006 (21.8 mg, 572.2 nmol), respectively. Using the procedure described above X30834 was then converted to the corresponding mono-DTME (S—CL) derivative X30835 (22.6 mg, 1465.2 nmol, 78.1%). 8.8 mg (572.2 nmol) of X30835 was reacted with X34006 (21.8mg, 572.2 nmol) to give the single-stranded homo-heptamer X34009 (8.96 mg, 167.3 nmol, 29.2%). 5.53 mg, (103.3 nmol) of homo-heptameric sense strand X34009 and 5.27 mg (723.1 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (103.3 nmol) of the corresponding double stranded FVII homo-heptamer XD-10640.
Synthesis of FVII Octamer XD-10641

Homo-octameric sense-strand of FVII siRNA X34010 with amino groups at both the 3’- and 5’-termini and containing six dCdA cleavable linkers (---NA--) and one reductively cleavable DTME linker (5′-CL-5′) was synthesized and purified via the single-stranded homo-dimer X30833 and homo-hexamer X34005 prepared above. Disulfide group was cleaved from X-34005 using DTT to give the corresponding 5'-thiol derivative X34007 (11.5mg, 251nmol, 99.7%), which was reacted with the previously obtained mono-DTME homo-dimer derivative X30835 (3.85mg, 250.2nmol) to give the single-stranded homo-octamer X34010 (5.2mg, 85.0nmol, 34.0%). 4.92 mg (80.33 nmol) of homo-octameric sense strand X-34010 and 4.68 mg (642.4 nmol) of anti-sense strand X18795 were then annealed to yield 9.6 mg (80.3 nmol) of the corresponding double stranded FVII homo-octamer XD-10641.
Determination of Serum Half-lives of the Homo-multimers

The serum half-lives of the homo-multimers XD-10635, XD-10636, XD-10637, XD-10638, XD-10639, XD-10640, and XD-10641 and the corresponding monomer XD-09794 were determined by iv bolus injection of test material at a concentration of 1 ng/ml in x1 PBS via tail vein into 3 cohorts of 4 C57/BL6N female mice aged approx. 11 weeks per cohort. Dosage was 20mg/kg for both FVII monomer and FVII multimers and blood samples were drawn at 5, 30, 60, 120 and 360 minutes.

The serum samples were digested with proteinase K and a specific complementary Atto425 Peptide Nucleic Acid-fluorescent probe was hybridized to the antisense strand. Subsequent AEX-HPLC analysis enabled discrimination of intact antisense strand from metabolites leading to high specificity of the method. Only values for the intact parent compound are reported in Fig. 6

![Smooth Line Scatter Plot of FVII siRNA levels in serum for FVII monomer and multimers over time](image-url)

**FIG. 6.** Smooth Line Scatter Plot of FVII siRNA levels in serum for FVII monomer and multimers over time
From these data the concentrations of the FVII multimers at each of the specific timepoints can be compared:

**FIG. 7.** Bar Chart of FVII siRNA levels in serum for FVII monomer and multimers at 5 mins

**FIG. 8.** Bar Chart of FVII siRNA levels in serum for FVII monomer and multimers at 30 mins
FIG. 9. Bar Chart of FVII siRNA levels in serum for FVII monomer and multimers at 60 mins

FIG. 10. Bar Chart of FVII siRNA levels in serum for FVII monomer and multimers at 120 mins

The area under each of the half-life curves in FIG. 6 serves as a proxy for bioavailability, both in absolute units (FIG. 11) and normalized to monomer (Table 5 and FIG. 12).
FIG. 11. Bar Chart of Total FVII siRNA levels in serum (Area Under the Curve) for FVII multimers

TABLE 5. Area Under The Curve – i) absolute numbers in minutes * ng / mL; and ii) normalized to monomer

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<th>Monomer</th>
<th>Dimer</th>
<th>3-mer</th>
<th>4-mer</th>
<th>5-mer</th>
<th>6-mer</th>
<th>7-mer</th>
<th>8-mer</th>
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<td>24.635</td>
<td>7.3257</td>
<td>6.7969</td>
<td>6.9315</td>
</tr>
<tr>
<td>360</td>
<td>6.6</td>
<td>625</td>
<td>20</td>
<td>86</td>
<td>23.0</td>
<td>50.5</td>
<td>77</td>
<td>115</td>
</tr>
<tr>
<td>Total AUC (minute * ng / mL)</td>
<td>621727</td>
<td>1604630</td>
<td>2715490</td>
<td>7271583</td>
<td>10689448</td>
<td>13420917</td>
<td>11862813</td>
<td>13384888</td>
</tr>
<tr>
<td>AUC, Normalized to Monomer</td>
<td>1.0</td>
<td>2.6</td>
<td>4.4</td>
<td>11.7</td>
<td>17.2</td>
<td>21.5</td>
<td>19.1</td>
<td>21.5</td>
</tr>
</tbody>
</table>
Calculation of time taken for multimers to reach same FVII siRNA concentration as monomer at 5 minutes

Because the FVII concentration of the monomer was already significantly less than 50% of that injected at the first sample time (5 minutes), the times taken for the serum FVII levels of the multimers to equal that of the monomer at 5 minutes were also calculated using the following equation:

\[ Y = (Y_0 - \text{Plateau}) \times \exp(-K \times X) + \text{Plateau} \]

wherein plateau was set at the concentration of monomer at 5 minutes (34245 ng/ml) (Fig. 13 and Table 6).
TABLE 6. Calculated times for homo-multimers to reach concentration of FVII monomer at 5 minutes

<table>
<thead>
<tr>
<th>Formula</th>
<th>Monomer</th>
<th>Dimer</th>
<th>3-mer</th>
<th>4-mer</th>
<th>5-mer</th>
<th>6-mer</th>
<th>7-mer</th>
<th>8-mer</th>
</tr>
</thead>
<tbody>
<tr>
<td>34245 = (231173 - 0) * e^(-kx) + 0, where x is minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34245 = 231173 * e^(-kx)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.14813453 = e^(-kx)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ln(0.14813453) = -kx</td>
<td>0.3819</td>
<td>0.1862</td>
<td>0.06203</td>
<td>0.03487</td>
<td>0.02683</td>
<td>0.01996</td>
<td>0.02015</td>
<td>0.019</td>
</tr>
<tr>
<td>Time (min)</td>
<td>-1 909025779</td>
<td>5.0</td>
<td>10.1</td>
<td>23.3</td>
<td>54.8</td>
<td>71.2</td>
<td>95.7</td>
<td>94.8</td>
</tr>
</tbody>
</table>

FIG. 13. Bar Chart of time taken for multimers to reach same FVII siRNA concentration as monomer at 5 minutes
E. Demonstration of Minimal Increase in Toxicity of Homo-hexamer vs Monomer

Separately, blood samples from cohorts treated with monomer XD-09794 and homo-hexamer XD-09795 were analyzed for cytokine levels at the various time points. The analysis was performed on a MSD U-Plex platform and levels of GM-CSF, IFN-gamma, IL-1beta, IL-2, IL-4, IL-6, IL-10, IL-12p70, KC-GRO, TNF-alpha determined. No significant difference in toxicity between monomer and hexamer was observed.

Key: Hexamer XD-09795  Monomer XD-09794
IL-6

IL-4

IL-10

IL-6 Concentration (pg/mL)

IL-4 Concentration (pg/mL)

IL-10 Concentration (pg/mL)

Time (Min)
**TNF-α**

- **X-axis**: Time (Min)
- **Y-axis**: TNF-alpha Concentration (pg/mL)

**IFN-γ**

- **X-axis**: Time (Min)
- **Y-axis**: IFN-gamma Serum Concentration (pg/mL)
General Procedures

Single Chain Oligonucleotide Synthesis

Oligoribonucleotides were assembled on ABI 394 and 3900 synthesizers (Applied Biosystems) at the 10 µmol scale, or on an Oligopilot 10 synthesizer at 28 µmol scale, using phosphoramidite chemistry. Solid supports were polystyrene loaded with 2'-deoxythymidine (Glen Research, Sterling, Virginia, USA), or controlled pore glass (CPG, 520Å, with a loading of 75 µmol/g, obtained from Prime Synthesis, Aston, PA, USA). Ancillary synthesis reagents, DNA-, 2'-O-Methyl RNA-, and 2'-deoxy-2'-fluoro-RNA phosphoramidites were obtained from SAFC Proligo (Hamburg, Germany). Specifically, 5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite monomers of 2'-O-methyl-uridine (2'-OMe-U), 4-N-acetyl-2'-O-methyl-cytidine (2'-OMe-C^Ac), 6-N-benzoyl-2'-O-methyl-adenosine (2'-OMe-A^bz) and 2-N-isobutyrguanosine (2'-OMe-G^iBu) were used to build the oligomer sequences. 2'-Fluoro modifications were introduced employing the corresponding phosphoramidites carrying the same nucleobase protecting groups as the 2'-OMe RNA building blocks. Coupling time for all phosphoramidites (70 mM in Acetonitrile) was 3 min employing 5-Ethylthio-1H-tetrazole (ETT, 0.5 M in Acetonitrile) as activator. Phosphorothioate linkages were introduced using 50 mM 3-((Dimethylamino-methyliden)amino)-3H-1,2,4-dithiazole-3-thione (DDTT, AM Chemicals, Oceanside, California, USA) in a 1:1 (v/v) mixture of pyridine and Acetonitrile.

Upon completion of the solid phase synthesis, including removal of the DMT group (“DMT off synthesis”), oligonucleotides were cleaved from the solid support and deprotected using a 1:1 mixture consisting of aqueous methylamine (41%) and concentrated aqueous ammonia (32%) for 3 hours at 25°C according to published methods. Wincott, F. et al: Synthesis, deprotection, analysis and purification of RNA and ribozymes. Nucleic Acids Res, 23: 2677-2684 (1995).

Subsequently, crude oligomers were purified by anionic exchange HPLC using a column packed with Source Q15 (GE Healthcare) and an AKTA Explorer system (GE Healthcare). Buffer A was 10 mM sodium perchlorate, 20 mM Tris, 1 mM EDTA, pH 7.4 (Fluka, Buchs, Switzerland) in 20% aqueous acetonitrile and buffer B was the same as buffer A with 500 mM sodium perchlorate. A gradient of 22% B to 42% B within 32 column volumes (CV) was employed. UV traces at 280 nm were recorded. Appropriate fractions were pooled and precipitated with 3M NaOAc, pH=5.2 and 70% ethanol. Pellets were collected by centrifugation. Alternatively, desalting was carried out using Sephadex HiPrep columns (GE Healthcare) according to the manufacturer’s recommendations.

Oligonucleotides were reconstituted in water and identity of the oligonucleotides was confirmed by electrospray ionization mass spectrometry (ESI-MS). Purity was assessed by analytical anion-exchange HPLC.

5’-aminohexyl linkers were introduced employing the TFA-protected hexylamino-linker phosphoramidite (Sigma-Aldrich, SAFC, Hamburg, Germany). 3’-hexylamino-linkers were
introduced using a phtalimido protected hexylamino-linker immobilized on CPG (Prime Synthesis, Aston, PA, USA). Deprotection and purification was performed as above.

**Generation of Thiol-terminated siRNA**

3’- or 5’-terminal thiol groups were introduced via 1-O-Dimethoxytrityl-hexyl-disulfide,1’-[2-cyanoethyl]-(N,N-diisopropyl)]-phosphoramidite linker (NucleoSyn, Olivet Cedex, France). After deprotection and purification as above, each disulfide containing oligomer was reduced using Dithiothreitol (DTT) (0.1 M DTT stock solution (Sigma-Aldrich Chemie GmbH, Munich, Germany, #646563) in Triethylammonium bicarbonate buffer (TEABc, 0.1M, pH 8.5, Sigma, #90360). The oligonucleotide was dissolved in TEABc buffer (100mM, pH 8.5) to yield a 1 mM solution. To accomplish the disulfide reduction a 50-100 fold molar DTT excess was added to the oligonucleotide solution. The progress of the reduction was monitored by analytical AEX HPLC on a Dionex DNA Pac 200 column (4x 250 mm) obtained from Thermo Fisher. The reduced material, i.e. the corresponding thiol (C6SH), elutes prior to the starting material. After completion of the reaction, excess reagent is removed by size exclusion chromatography using a HiPrep column from GE Healthcare and water as eluent. Subsequently, the oligonucleotide is precipitated using 3 M NaOAc (pH 5.2) and ethanol and stored at minus 20 °C.

**General Procedure for Preparation of Mono-DTME Oligomer**

Thiol modified oligonucleotide was dissolved in 300 mM NaOAc (pH 5.2) containing 25% acetonitrile to give a 20 OD/mL solution. 40 equivalents dithiobismaleimidoethane (DTME, Thermo Fisher, # 22335) were dissolved in acetonitrile to furnish a 15.6 mM solution. The DTME solution was added to the oligonucleotide-containing solution and agitated at 25 °C on a Thermomixer (Eppendorf, Hamburg, Germany). Progress of the reaction was monitored by analytical AEX HPLC using a Dionex DNA Pac200 column (4x 250 mm). Depending on the required purity level, excess DTME is either removed by size exclusion HPLC using a HiPrep column (GE Healthcare) or the crude reaction mixture is purified by preparative AEX HPLC using a column packed with Source 15 Q resin commercially available from GE Healthcare.

**General Procedure for Preparation of Dimer via DTME Functionality**

The DTME modified oligonucleotide prepared according to the procedure in Example 2 was reacted with another oligonucleotide equipped with a thiol linker. This reaction could either be carried out on the single stranded sequence or after prior annealing of the complementary oligonucleotide of one of the reaction partners. Consequently, if desired, the DTME modified oligonucleotide was reacted with the thiol modified oligonucleotide directly, or was annealed with its complementary strand and the resulting duplex reacted with the thiol modified oligonucleotide. Alternatively, the thiol modified oligonucleotide was annealed with its complementary strand and this duplex reacted with the DTME modified single strand. In all cases the reaction was carried out in aqueous solution in the presence of 300 mM NaOAc (pH 5.2).
General Procedure for Annealing of Single Stranded RNAs (ssRNAs) to Form Double Stranded RNA (dsRNA)

dsRNAs were generated from RNA single strands by mixing a slight excess of the required complementarity antisense strand(s) relative to sense strand and annealing in 20 mM NaCl/4 mM sodium phosphate pH 6.8 buffer. Successful duplex formation was confirmed by native size exclusion HPLC using a Superdex 75 column (10 x 300 mm) from GE Healthcare. Samples were stored frozen until use.

In the sequences described herein, upper case letters “A”, “C”, “G” and “U” represent RNA nucleotides. Lower case letters “c”, “g”, “a”, and “u” represent 2-O-methyl-modified nucleotides; “s” represents phosphorothioate; and “dT” represents deoxythymidine residues; upper case letters A, C, G, U followed by “f” indicate 2-fluoro nucleotides; “(SHC6)” represents a thiohexyl linker; “(DTME)” represents the cleavable homobifunctional crosslinker dithiobismaleimidoethane; “C6NH2” and “C6NH” are used interchangeably to represent the aminohexyl linker; “C6SSC6” represents the dihexyldisulfide linker; and “InvdT” means inverted thymidine.

General Procedure to Generate Multimeric siRNAs by Sequential Annealing

Preparation of multimeric siRNAs via stepwise annealing was performed in water and utilized stepwise addition of complementary strands. No heating/cooling of the solution was required. After each addition, an aliquot of the annealing solution was removed and monitored for duplex formation using analytical RP HPLC under native conditions (20°C). The required amounts to combine equimolar amounts of complementary single strands were calculated based on the extinction coefficients for the individual single strands computed by the nearest neighbor method. If the analytical RP HPLC trace showed excess single strand, additional amounts of the corresponding complementary strand were added to force duplex formation (“duplex titration”).

Duplex titration was monitored using a Dionex Ultimate 3000 HPLC system equipped with an XBridge C18 Oligo BEH (2.5 µm; 2.1x50 mm, Waters) column equilibrated to 20°C. The diagnostic wavelength was 260 nm. Buffer A was 100 mM hexafluoro-isopropanol (HFIP), 16.3 mM triethylamine (TEA) containing 1 % methanol. Buffer B had the same composition except MeOH was 95 %. A gradient from 5 % to 70 % buffer B in 30 minutes was applied at a flow rate of 250 µL/min. The two complementary strands were run independently to establish retention times. Then, the aliquot containing the duplex solution was analyzed and compared to the retention times of the constituent single strands. In case the duplex solution showed a significant amount of single strand the corresponding complementary strand was added to the duplex solution.