Small-Molecule DNA-Interactive Agents as Alternatives to RNAi or Antisense Technologies: Example of a Novel NFκB Inhibitor

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Thursday 13th September 2012
The Goal

To Selectively Inhibit Gene Transcription with Small Molecules Instead of Macromolecules
Introduction to small-molecule gene regulation.

Drug targets being evaluated with this technology.

Advantages and disadvantages compared to RNAi and Antisense technologies.

Example of a novel potential NF_{κB} inhibitor with significant \textit{in vitro} cytotoxicity and \textit{in vivo} antitumour activity.

HTP Assay to discover novel Transcription Factor Inhibitors (TFIs).
Ligands bind to cell surface receptors and trigger interconnected signalling cascades.

This involves mainly kinases and phosphatases that converge on Transcription Factors (TFs) leading to the recruitment and activation of transcriptional proteins.

TFs are a major control point in gene expression, determining cellular phenotype.

Gene targets: Bcl-xL, myc, cyclin D1, survivin, VEGF

Inflammation, Proliferation & Survival, Migration & Invasion, Angiogenesis, Immune evasion
A general TF either alone or in association with other proteins, such as transcriptional co-activators or co-repressors, will provide the pre-initiation complex (PIC) which controls the recruitment of RNA polymerase II.

A subset of TFs known as “Specific Transcription Factors” bind to DNA some distance from the PIC and provide +ve and –ve control of transcription. They usually only become operational upon dimerization with themselves or another transcription factor, and are the most suitable drug targets (e.g., NFκB, STAT3, HIF-1, etc).
Inhibition of an upstream kinase or phosphatase is likely to affect several TFs, given the intertwined nature of these pathways.

Instead, direct inhibition of the transcription factor should lead to minimal off-target effects, thus potentially affording a more clinically viable therapeutic.
The Challenges of “Drugging” Transcription Factors - Targeting Protein Protein Interactions (PPI)

- For screens in the absence of the DNA: (1) DNA-binding domain of a TF is not always properly folded, (2) PPI may be weaker than when DNA present.
- Lack of distinct binding sites on the protein surfaces.
- Inhibitors may have to be relatively large and bulky to inhibit PPI thus placing them outside of Lipinski's Rules.
- Inhibitors of dimerization are only applicable to those TFs that form homo- or heterodimers, whereas DNA-binding agents could inhibit the function of any TF.
- The structural heterogeneity of proteins necessitates finding novel inhibitors for each new transcription factor target, whereas it might be possible to develop “programmable” elements for DNA-interactive agents.
If the DNA-binding region of the protein is being targeted, there may be a lack of distinct binding sites.

There may be more than one consensus DNA binding sequence for any given TF. Therefore, a highly selective agent designed to one of these sequences may not completely block activity.

Conversely, targeting one of a number of consensus sequences for a given TF may allow the regulation of a subset of genes.

Technology is challenging as variation in DNA is limited to just two purine (adenine and guanine) and two pyrimidine (cytosine and thymine) bases (but 3D-structure may play a role).
# Small-molecule Transcription Factor Inhibitors

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Small molecule</th>
<th>Research Group</th>
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<tbody>
<tr>
<td>STAT</td>
<td>Flavoperidol</td>
<td>Keith Bible (2006)</td>
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<tr>
<td>HIF</td>
<td>Echinomycin</td>
<td>Giovanni Melilo (2005)</td>
</tr>
<tr>
<td></td>
<td>Polyamides (anti-VEGF)</td>
<td>Peter Dervan (2007)</td>
</tr>
<tr>
<td></td>
<td>Bi-cyclic polyamides (anti-VEGD)</td>
<td>Peter Dervan (2004)</td>
</tr>
<tr>
<td></td>
<td>Polyamide triad (anti-VEGF)</td>
<td>Y Kageyama (2006)</td>
</tr>
<tr>
<td>NFκB</td>
<td>Hairpin polyamide</td>
<td>Peter Dervan (2012)</td>
</tr>
<tr>
<td>AP-1</td>
<td>Polymide (anti-TGFβ1)</td>
<td>K Serie (2006)</td>
</tr>
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<td></td>
<td>Polyamide (anti-LOX-1)</td>
<td>T Sawamura (2008)</td>
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<tr>
<td>Myc</td>
<td>Echinomycin</td>
<td>Giovanni Melilo (2005)</td>
</tr>
<tr>
<td>NF-Y</td>
<td>GWL-78</td>
<td>David Thurston (2008)</td>
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<tr>
<td>EVII</td>
<td>Polyamide</td>
<td>Archibald Perkins (2011)</td>
</tr>
<tr>
<td>CREB</td>
<td>Platinum (II) complexes</td>
<td>Chi-Ming Che (2011)</td>
</tr>
<tr>
<td>Pit-1, Brn-3</td>
<td>DB293</td>
<td>David Wilson (2008)</td>
</tr>
</tbody>
</table>
Examples of Small-molecule TF Inhibitors

S3I-201 (Stat3, PPI)

Flavoperidol (Stat3, PDI)

Topotecan (HIF-1, PPI)

Hairpin Polyamide (HIF-1, NFκB, PDI)

Resveratrol (AP-1, PPI)

T-5224 (AP-1, PPI)
Small-molecule TF Inhibitors

Mycro1 (c-Myc, PDI)

Mycmycin-1 (c-Myc, PDI)

DB293 (POU, PDI)

Pt(C^N^N)(CN-L)] + (PDI)
NFkB, AP-1 , Sp-1, EGR-1 TF Inhibitor

Lefmycin-1 (PDI)
LEF-1 TF Inhibitor
G-quadruplex DNA Targeting TF Inhibitors

TMPyP4

Quarfloxin

c-Myc Inhibitor

c-Myc Inhibitor, Phase II Clinical Trial
Quadruplex Targeted Agents as Selective Modulators of Gene Expression

Model for the activation and repression of gene transcription involving G-quadruplex structure in the promoter region of C-Myc.

Siddiqui-Jain A et al. PNAS 2002;99:11593-11598
Search for Drug-Like G-quadruplex Ligands

- Most G-quadruplex targeting ligands are non-drug-like
- Only 1 molecule in clinical trail till date

Figure 3. Drug-likeness of the 13 identified G-quadruplex-targeting ligands showing adherence to the Lipinski Rule of Five.
Principle of RNAi
Advantages and Disadvantages of Small-Molecule Approach Compared to RNAi and Antisense Technologies

Advantages

- Low molecular weight, stable and easy to synthesize (lower cost?).
- Potentially orally available with good ADMET (i.e., within Lipinski’s Rules etc.).
- Potentially able to block transcription at source rather than effect on gene product (could be more efficient at gene silencing).

Disadvantages

- Achieving or approaching 100% selectivity for unique DNA sequences is challenging.
- May be an inherent limit to selectivity based on fact that many TFs recognize and bind to more than one DNA sequence (i.e., four binding sites known for NFκB)
- Very challenging to identify potent (i.e., sub μM) PPI inhibitors.
Development of a Novel Potential NFκB Inhibitor

Why Target NFκB?

- Plays crucial role in initiation and progression of pancreatic adenocarcinoma for which there is a significant clinical need for new therapeutic agents.

- Also valid target for many haematological malignancies.
A number of important Transcription Factor consensus recognition sites are relatively GC-Rich:

**NFκB**  GGGRNNYYCC (R = Purine, Y = Pyrimidine, N = Any Base)
**EGR:**  GCGGGGGGCG
**AP-1**  GACATTGC
**C-Myc**  CACGTG
**CREB**  TGACGTCA
**STAT3**  TTCCGGGGA
**HIF-1**  [A/G]CGTG

However, most known sequence-selective DNA-binding agents are AT-targeting (e.g., Netropsin, Distamycin).
Search for GC-Selective Building Blocks

Most non-covalent minor-groove binders have a similar scaffold containing 5-membered heterocycles (e.g., pyrrole, imidazole) as building blocks.

Key Objectives

Develop minor-groove binders which can better tolerate GC sequences and/or have a preference for GC over AT.

Develop these into GC sequence-targeting Transcription Factor Inhibitors

Biaryl building blocks
Spans two base pairs
Incorporation of biaryl building block makes the molecule less curved: Possibility of GC tolerance? Phenyl group offers additional Van der Waals interaction

- 4 Library types.
- All possible $p$ & $m$ combinations were used.
- Microwave assisted Suzuki reaction was used to make the biaryl building blocks.
- 265 compounds purified using catch and release SPE procedure.

Fluorescent Intercalator Displacement (FID) Assay

Addition of ethidium bromide

Nonspecific intercalation results in fluorescence

Addition of a DNA binding compound

DNA affinity is measured as a decrease in relative fluorescence indicating binding with displacement of ethidium bromide.

EtBr intercalates dsDNA

Drug displaces EtBr

1,024 possible 5-base pair sequences

from Acc Chem Res; 2004; 37(1), 61-9
Identification of MPB as GC-Targeting Building Block

Top 25 Sequences – FID Assay

Most preferred sequences for Py-MPB-Py and Py-MPB-Im:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GC-Targeting Building Block</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCCGGG</td>
<td>GGGGCC</td>
</tr>
<tr>
<td>TGGCCC</td>
<td>GGCCCC</td>
</tr>
<tr>
<td>CCTCCC</td>
<td>GGGGCT</td>
</tr>
<tr>
<td>TCCGGG</td>
<td>GGGGCC</td>
</tr>
<tr>
<td>TGGCCC</td>
<td>GGCCCC</td>
</tr>
<tr>
<td>CCTCCC</td>
<td>GGGGCT</td>
</tr>
</tbody>
</table>

NFκB consensus sequence: GGGRNNYCC (R=Purine, Y=Pyrimidine)
PBDs are naturally-occurring sequence-selective minor-groove binders that form a covalent aminal linkage between their C11-position and the N2 of guanine in the minor groove of DNA.

Biaryl building blocks, with an emphasis on the MPB motif, were coupled to a PBD capping unit.

MPB building blocks were either directly linked to the PBD via a C4-linker or separated by pyrrole/imidazole heterocycles.
PBD-MPB Conjugates – Typical Synthetic Route

1. K₂CO₃/DMF → MeO, 88%
2. HNO₃/Ac₂O → MeO, 72%
3. KMNO₄, Acetone/Water reflux → 85%
4. Oxalyl chloride → MeO, 82%
5. H₂/Pd-C → MeO, 91%
6. Allylchloroformate → MeO, 78%
7. TEMPO/BAIB → 84%
8. DHP/PTSA → MeO, 79%
9. NaOH/Dioxane/H₂O → 94%
10. EDCI/DMAP → MeO, 88%
11. NaOH/Dioxane/H₂O → 91%
12. (A)
13. Cl₃COCl, Ether → Cl₃CO, 95%
14. NBS, THF → Cl₃CO, 95%
15. MeONa, MeOH → H₂CO, 88%
16. (PPh₃)₂Pd, K₂CO₃, Ethanol/Toluene/Water 9:3:1, 91%
17. 4M HCl in Dioxane → 91%
18. (B, MPB)
19. Tetrakis Pd/Pyrrolidine → Triphenyl phosphine, 91%
20. (KMR-28-39)
Evaluating Interaction of PBD-MPBs with DNA Sequences Using HPLC/MS

PBD-MPB conjugates react much faster with GC-rich sequences compared to AT-rich sequences.

K M Rahman et al. J. AM. CHEM. SOC. 2009, 131, 13756–13766
FRET Melting Study: Evidence of Sequence Preference of PBD-MPBs

PBD-MPB conjugates showed greater stabilisation potential with GC-rich sequences.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DTmAT</th>
<th>ΔTmGC</th>
<th>ΔTmGC/ΔTmAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBD-MPB</td>
<td>0.3</td>
<td>3.9</td>
<td>13.00</td>
</tr>
<tr>
<td>PBD-MPB-Py</td>
<td>2.1</td>
<td>3.1</td>
<td>1.48</td>
</tr>
<tr>
<td>PBD-MPB-Im</td>
<td>0.5</td>
<td>2.8</td>
<td>5.60</td>
</tr>
<tr>
<td>PBD-Py-MPB</td>
<td>5</td>
<td>1.4</td>
<td>0.28</td>
</tr>
<tr>
<td>PBD-Im-MPB</td>
<td>6</td>
<td>6.2</td>
<td>1.03</td>
</tr>
<tr>
<td>PBD-MPB-MPB</td>
<td>0.8</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>(GW 78) PBD-Py-Py</td>
<td>7.4</td>
<td>1.1</td>
<td>0.15</td>
</tr>
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</table>
PBD-MPB conjugates showed better accommodation and higher number of possible H-bond interactions with GC-rich sequences.

**Further Evidence of GC Preference**

### Footprintng - Higher tolerance for GC-rich Sequences

5′ - GGATCCCGGGATATCAAGCTATATAGCTATAGATCTAGAATTCCGGAACCGCGTGTTAAACGTTAACCGGTAACCT
3′ - CCTAGGGCCCTATAGCTATATACCGCGGTTTTAAATCGATATCTAGATCTTTAAGGCCCTTGCGCACAATTGCAATTGCCATGGA

AGGCCCTGCACTCGCGCATGCTAGCGCTTAAGTAGTACTAGTGCAACGTGGCCATGGATCC - 3′
TCCGACGTCGACGGTACGATCGCGAATTCATGATACGTGCACCGGTACCTAGG
Two lead molecules identified (KMR-28-35, KMR-28-39)

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (nanomolar)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A431</td>
</tr>
<tr>
<td>KMR-28-35</td>
<td>0.0056</td>
</tr>
<tr>
<td>KMR-28-39</td>
<td>0.018</td>
</tr>
</tbody>
</table>

In vitro Cytotoxicity: Total 53 molecules screened
MTD of Lead Compounds

(A) 125ug/kg/mouse/day
275ug/kg/mouse/day
400ug/kg/mouse/day

(B) 300ug/kg/mouse/day
KMR-28-39 showed significant dose dependent activity in *in vivo* xenograft of ER negative Breast cancer MDA MB 231 in Mouse model.
KMR-28-39 activity in MIA PaCa2 Xenograft

- KMR-28-39 has Significant Activity in \textit{in vivo} xenograft of Pancreatic Cancer in Mouse Model
- No systemic toxicity was observed
Effect on Downstream Gene Expression

BCL 2 Expression: MIA PaCa2 Tumour

A. Control Tumour Tissue

B. Treated Tumour Tissue

B. % Stained Area vs. Control and Treated Conditions
Transcription Factor Plate Array Assay

% change in TF activity

- NFAT
- EGR
- NFkB
- SMAD
- OCT--4

KMR-28-39
PBD-MPB hybrids significantly inhibited NFκB regulatory proteins IKKα and IKKβ even at 0.1 nM after both 4 hours and 24 hours exposure.
Interaction of KMR-28-39 with NFκB Cognate DNA Sequence

5’-GGGGGTCGCC-3’

5’-GGGACAGCCC-3’
Interaction of KMR-28-39 with NFκB Protein
**What’s next?**

### Stage 1A

- (a) Synthesis of 5g non-GMP batch of KMR-28-29
- (b) Safety toxicology in two species (rat and dog)
- (c) PK study in mice
- (d) Establish MTD in mice
- (e) Further xenograft studies (dose-scheduling etc.)
- (f) MOA studies (confirm NFkB inhibitory activity)

### Stage 1B

- Unfavourable Toxicological/PK profile
- Second Round of Lead Optimisation

### Stage 2

- Go
  - Good Toxicology and PK
  - Confirmed xenograft activity

#### Phase I Trials

- (a) Preparation for Phase I
- (b) Synthesis of 5-10g GMP
- (c) IV Formulation

#### End of Project

- No Go
  - Poor Toxicology and/or PK
  - Problems relating to xenograft activity
It may prove possible to develop small-molecule Transcription Factor Inhibitors (TFIs) to selectively down-regulate specific genes (or sets of genes) for use in a number of therapeutic areas including oncology.

Such agents could have advantages over RNAi and antisense approaches in being orally available with good ADMET, stable and relatively cheap to synthesize.

Molecules that block the Protein-DNA interaction (rather than the Protein-Protein Interaction) may have advantages of being (1) more-easily obtained (PPIs are highly problematic), and (2) more-precise control (i.e., different DNA sequences for same TF).

Overall, TFIs may have an advantage over agents such as kinase inhibitors that inhibit up-stream, as these targets may ultimately influence more than one transcription factor.
Acknowledgements

Professor David Thurston
Professor Barbara Pedley
Professor Keith Fox
Dr Colin James
Dr Maria dela-Fuente
Paul Jackson

Funding
Cancer Research UK
AICR
Commonwealth Fund
END
High Throughput Screening (HTS) to Discover Novel TFIs

Time Resolved FRET Technology
Targeting Other Transcription Factors

Screening Cascade to Discover TF Inhibitors

- Data Obtained from Preliminary Screening Assay
- Molecular Modelling
- Secondary Screening
  - FRET Melting
  - TF target screening
  - HPLC-MS
  - In vitro cytotoxicity
- Cellular Experiments
  - RT PCR
  - Western blot - upstream and downstream targets
  - TF activation assays
  - Luciferase Reporter Assay

Design and Synthesis

- SAR

Lead Compounds

In vivo Human Tumour Xenograft Studies

Feedback to design stage
Alnylam RSV Drug Fails in Phase IIb: Company to Weigh Options with Regulators

May 31, 2012

By Doug Macaron

In another setback to its respiratory syncytial virus program, Alnylam Pharmaceuticals announced this week that its RSV therapeutic candidate ALN-RSV01 failed to meet the primary endpoint in a phase IIb trial.

Company officials said during a conference call that no decision on the fate of the drug will be made until they meet with US and European regulators later this year. However, Alnylam CEO John Maraganore indicated that the termination of the program altogether is a possibility.

If Alnylam chooses this route, ALN-RSV01 will join a growing list of RNAi candidates that the company has dropped over the years, including a follow-up RSV treatment, a flu therapy, and a drug for wet age-related macular degeneration.

Alnylam began developing the RSV treatment in 2005, and later found a partner for the drug in Cubist Pharmaceuticals. However, in 2009, Alnylam reported phase II data showing that while the drug was safe and well tolerated, hints of efficacy were “exploratory” at best given the small size of the study.

In the trial, 24 lung transplant patients with confirmed RSV infections were randomized to receive inhaled ALN-RSV01 or placebo once daily for three consecutive days. The drug was shown to significantly decrease the incidence of new or progressive bronchiolitis obliterans syndrome, or BOS, a non-reversible obstructive lung disease, at the 80-mg dose level.

In this issue of Gene Silencing News:

- Alnylam RSV Drug Fails in Phase IIb; Company to Weigh Options with Regulators
- As Traverse Files for Bankruptcy, Co-Founder Seeks Partnership for Delivery Technology
- NIH Awards More Than $1M to Fund Three microRNA-Related Grants in May
- RXi Cleared to Begin Phase I Testing of Anti-Scarring Drug