Mutagenesis of Triplex Targeted Crosslinks

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Experimental systems for analysis of DNA repair and mutagenesis in mammalian cells

Treatment of cells with damaging agent-random modification

Survival
strand breakage
immunofluorescence
mutagenesis across a marker gene

Plasmids- site specific modification

survival
mutagenesis
inhibition of transcription

covalent modification of a specific chromosomal site
GENE TARGETING

• Find and bind a target sequence in living mammalian cells
  – Cells must survive

• Influence Transcription
• Change genomic sequence

• Introduce DNA damage

Sequence recognition in chromosomal DNA

  denatured: single strand
  major groove
  minor groove
Two Modes of Triple Helix Formation

Parallel Triplets

Antiparallel Triplets
DNA Triple Helix

Pyrimidine motif

• homopurine:homopyrimidine sequences in duplex DNA
  - Abundant in mammalian genomes
  - Common in promoters and introns

• Inherent property of nucleic acids

• Triplex formation: kiss and zip

• Very stringent with respect to sequence
Challenges to Bioactivity of Triplex Forming Oligonucleotides

**Oligonucleotide:duplex**

- Poor activity of Pyrimidine motif TFOs in physiological pH.
- Nuclease sensitivity of oligonucleotides
- Conformational constraint required for triplex formation
- Charge repulsion between TFO and target duplex
  - Low Mg**++** in cell

**Genomic targeting**

- Chromatin-target access
Adoption of 3’-endo (North) Conformation by 2’-O-modified Nucleosides

Some Salient Features about 2’-O-(2-Aminoethyl) Nucleotides:

* C-3’-endo conformation
* Confers nuclease stability
* Extra H-bond with phosphate

Angew. Chem. Int. Ed. 1998, 37, 1288-1291
NMR Structure: 2’-O-(2-Aminoethyl) TFOs
TTTCTCTTTTTTTCTTTCT-pso TFO

XbaI

5’ TTTTCATTTTCTCTTTTTTCTTCTTCT TAGa atgt

HPRT
5’-Pso-2’-O-(2-Aminoethyl) Chimeric TFO

\[ \text{Pso} = \text{structure} \]

\[ \text{Pso-C}_6\text{TC}^{\text{Me}}\text{TTC}^{\text{Me}}\text{TTTTTT-O-} \]

\[ \text{Pso-C}_6\text{TC}^{\text{Me}}\text{TTC}^{\text{Me}}\text{TTTTTT-O-} \]

\[ \text{Pso-C}_6\text{TC}^{\text{Me}}\text{TTC}^{\text{Me}}\text{TTTTTT-O-T}_{\text{AE}}\text{T} \]
Thioguanine resistant colonies following treatment with pso-TFO
TFOs with a cluster of aminoethoxy residues are bioactive

\[ \texttt{TTCTTTTTTTTCTTCT- pso TFO} \]

Bioactivity requires clustered AE residues
A single mismatch is inactivating

![Mutation Frequency (%)](image)

Triplexes are much less stable in cells than in vitro

Triplexes are unwound by chromosomal helicases and translocases
Bioactivity

Thermal stability

\[ \text{tTTCTcttttttctttct-pso TFO} \]

\[ T_m \text{ does not distinguish active and inactive TFOs} \]
AE cluster supports faster association (kiss)
Association rate appears important for bioactivity
Why would Association Rate Matter?

Triplexes are stable in vitro

If, after formation, triplexes were stable in cells, then waiting longer would be useful.

If they are not stable in cells then the association step becomes very important

Enzymes elute triplexes
  – Helicases
  – Translocases
  – Chromatin remodeling complexes
Can the biology of the cell influence target access?

Triplex- Compatible with Chromatin Structure?

Crystal structure of the Nucleosome Core (2.8 Å)
Influence of Cell Cycle on TFO-Pso or Pso Activity

Cell Cycle Phase

TFO-Pso

Pso

HPRT (%)
Targeted Crosslinking in vivo in S phase cells

The genomic restriction fragment with the targeted crosslink is denaturation resistant
Targeted Crosslinking *in vivo* in S phase cells

**XbaI**  
Exon 5

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TTTCTCTTTTTTTCT  TCTA  G a atgt
          ......A T ........
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Restriction Resistance demonstrates physical presence of targeted crosslink.
Efficient Gene Targeting requires an effective reagent and an available target

Glazer lab found enhanced targeting in permeabilized cells following induction of transcription in target region

We find enhanced targeting activity in S phase cells relative to quiescent cells

Target access is a critical feature of gene targeting. Cellular treatments that influence chromatin structure may effect target access.
DNA Interstrand Crosslinks

Reaction of DNA and oxidation products formed during normal cellular metabolism
- lipid peroxidation products
- chemotherapy

Cytotoxic lesions
- more toxic than classical monoadducts (UV, Benzopyrene)
- two log differential for unrepaired XL vs monoadducts

Absolute Blocks to Replication and Transcription
- Failure to resolve blocked or stalled forks results in rearrangements, chromosome abnormalities, genomic instability

cannot be accumulated in proliferating cells
Crosslink Repair

More complex than repair of single strand adducts

Requires release of crosslinked strands and sequential gap filling, excision, and gap filling

Base Substitutions and Double Strand Breaks can be formed during crosslink repair

Crosslink hypersensitivity a feature of cells deficient in two groups of genes:

ERCC1/XPF

Recombinational repair:

BRCA1, BRCA2, Rad51, Rad51 paralogues (XRCC2, XRCC3)

essential for integrity of replication process
Mutagenic endpoints of the psoralen-TFO crosslinks

Mutagenesis by free psoralen in mammalian cells is dominated by base substitutions, few deletions

Mutagenesis by triplex-psoralen crosslinks in supF plasmids largely base substitutions as well as deletions
Deletion Mutagenesis of Chromosomal Pso-TFO Crosslinks

XbaI Exon 5

TTTCTCTTTTTTCT TCT A Gaatgt

......A T.........
Base Substitution Mutagenesis of Targeted Crosslinks

XbaI Exon 5

TTTCTCTTTTTTCT TCT A Gaatgt

......A T...............
Events and Endpoints in TFO-Crosslink Metabolism

**RECOGNITION**

- Single-strand incision
  - Incisions on template strand for transcription or leading strand synthesis

- Dual-strand incision
  - Targeted XL 25%
  - BASE SUBSTITUTION 5%
  - DELETION 0.2%

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DELETION 0.2%
Section on Gene Targeting

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