

SUPPRESSION OF KRAS-G12D AND BRAF-V600E ONCOGENE TRANSCRIPTION WITH PNA CONJUGATES

Jeffrey H. Rothman, Gary K. Schwartz, Oliver Surriga

Columbia University Medical Center and Memorial Sloan Kettering Cancer Center
New York, NY USA

DISCLOSURE SLIDE

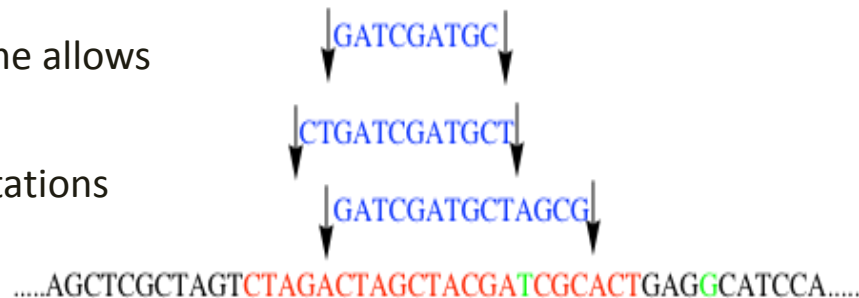
Research support from Fortress Biotech and Celgene
MTA with Calico

Crux of the Strategy

- . **Pursue this strategy**
 - . Limitations of current therapies
 - . Relatively non-specific target choices inherent in their designs
 - . Facilitate a strategy that is even more specific
- . **Target tumor-specific gene sequences *directly* -such as oncogenes**
 - . Defining of tumorigenesis
 - . Distinct from normal cells
 - . Offer a unique target

Targeting Genes with Complementary Oligonucleotides -Advantages

- Therapeutic design merely dictated by the complementary target gene sequence (canonical)
- Opportunity to 'drug the un-druggable' (gene: tumor specific mutation, functional, and necessary for cell survival)
- An 18mer sequence length statistically unique for targeting within genome
- Obstruction of transcription is the goal**; this relies merely upon the physical chemistry of complementary binding of gene target by oligonucleotide
- No reliance upon cellular processing or other mechanisms
 - Many choices within gene target frame allows optimization
 - Suppression not affected by new mutations outside target sequence



Complementary PNA Oligomer

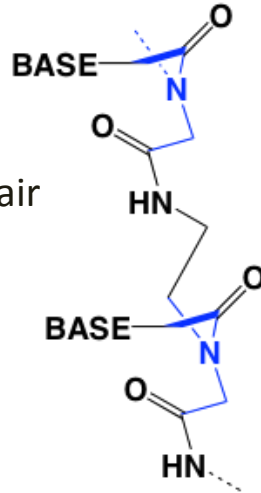
Peptide nucleic acid (PNA) oligomers are DNA analogues which are capable of binding gene sequences 1000-fold more avidly than complementary native DNA by strand invasion.

Invades duplex DNA and forms a significantly more stable hetero-duplex with the complementary strand by **displacing the analogous native strand**.

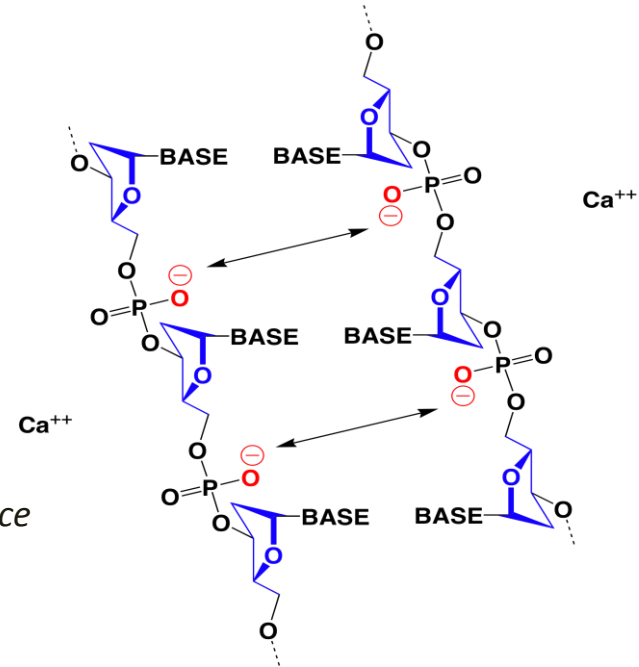


-Very sensitive to single base pair mismatch

-Resistant to degradation



Nielsen, P.G., et al., *Science* 1991, 245, 1497-1500.



How can these PNA oligomers be delivered into the cell?

- Make nuclear and cell membrane permeable by conjugation to delivery peptides
 - From nature
 - HIV TAT-encoded peptide
 - NLS from SV40 Large Tag
 - Penetratin
 - MPS (membrane permeable sequence)
 - Mastoparan
 - Mimetic
 - Cationic
 - Hydrophobic
 - Very accessible / Reliable
 - **Plasmids**
 - **Promoters**
 - Limited accessibility / Unreliable
 - **Transcribed chromosomal regions**
- Confluent automated solid phase peptide synthesis (SPPS)

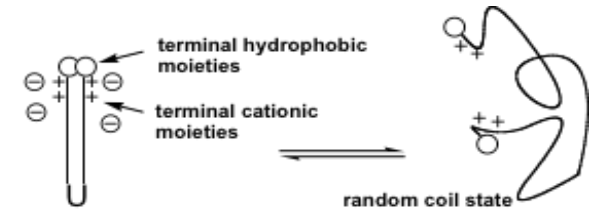
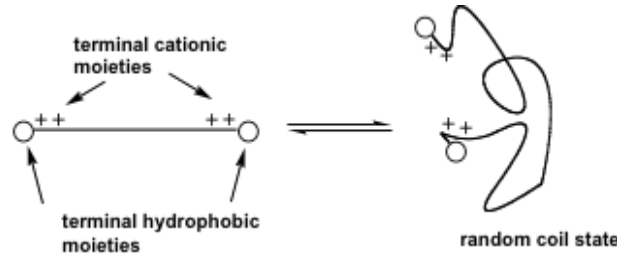
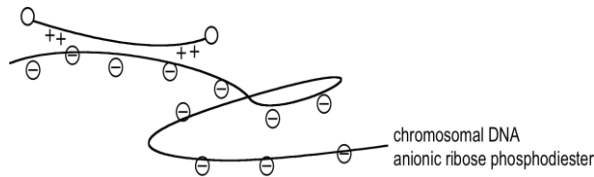
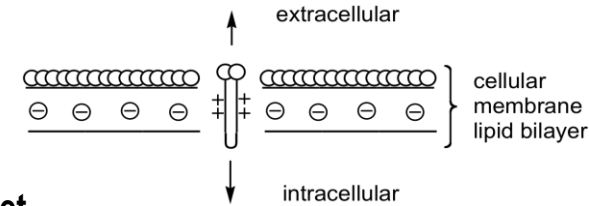


Vanguard experiment using this motif complementary to BRAF V600E – poor results

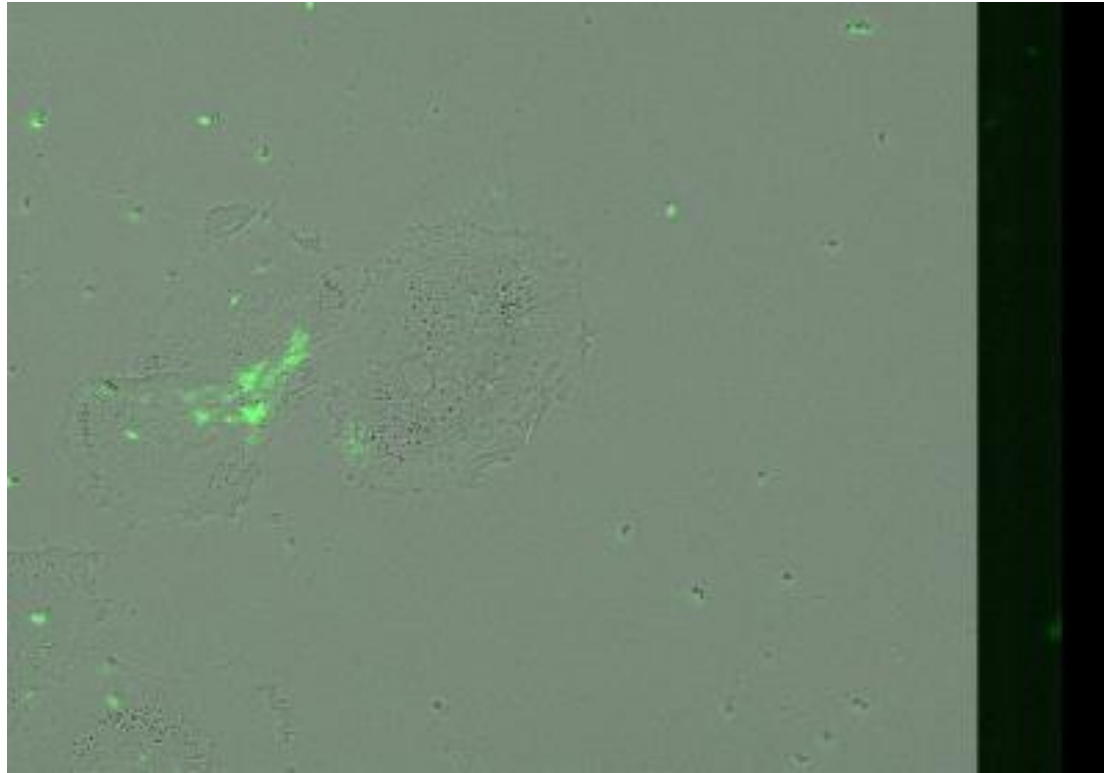
What Improvements and Modifications can be made?



- Improve PNA-peptide conjugate delivery by creating a configuration more amenable to phospholipid bilayer insertion
 - Better stabilize these conjugates within the cellular membranes
 - Their equilibrium within the cell and nucleus is more quickly achieved
- Better stabilize the PNA toward the chromosomal target
 - Statistical challenge for the PNA complement finding its chromosomal target
 - Ensure that the PNA conjugate remains within the chromosomal binding arena
 - Reduce entropic obstacles toward strand-invading helix initiation

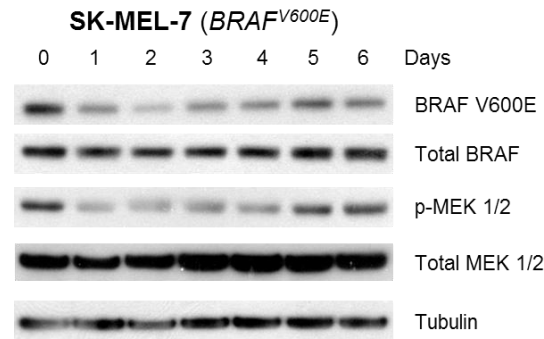
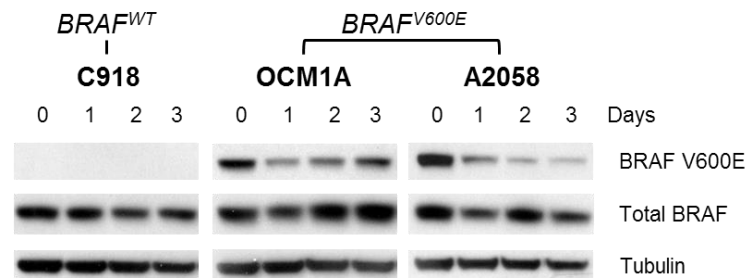
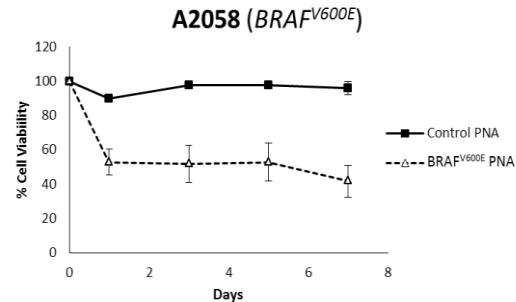
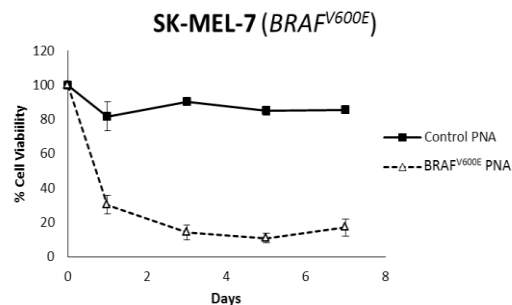
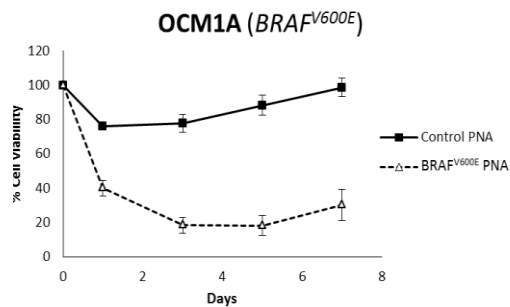
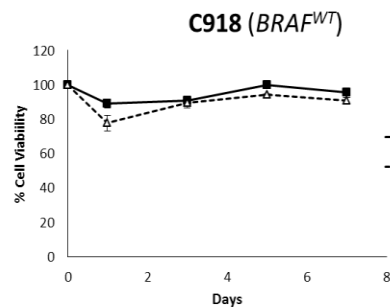


Cellular localization of modified fluorescein-labeled PNA-Peptide Conjugate

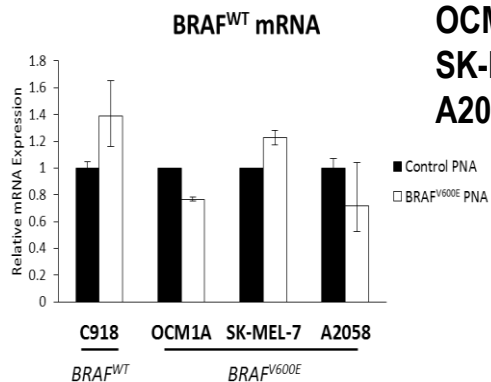
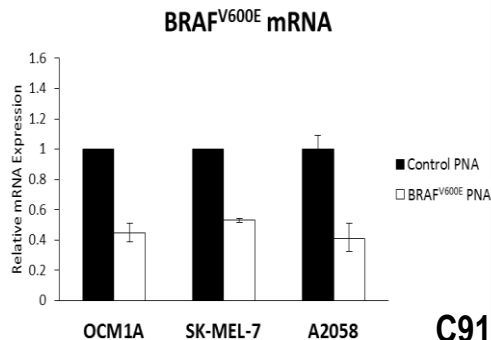


Time Course for Growth Inhibition and *BRAF*^{V600E} Suppression

BRAF^{V600}-complementary PNA 300 nM

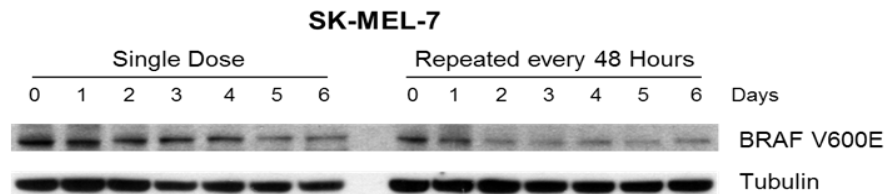
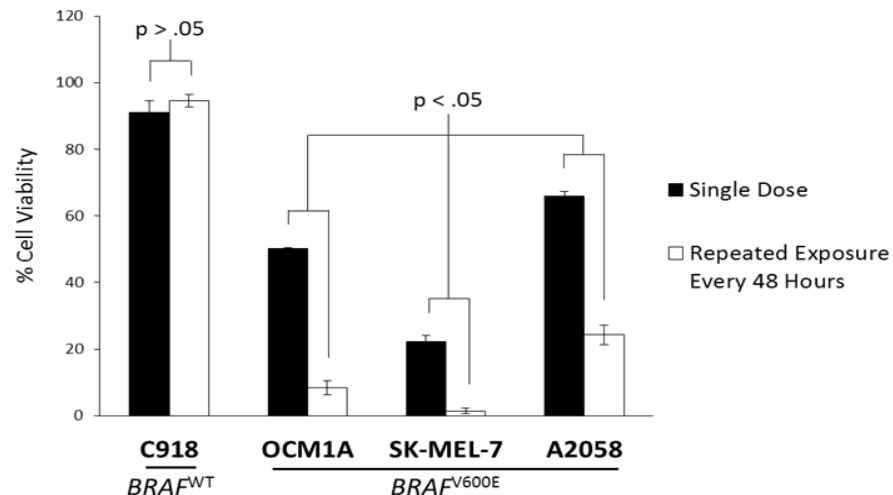


BRAF mRNA Suppression by *BRAF*^{V600E}-complementary or control PNA

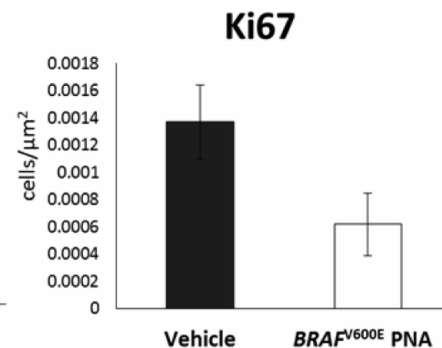
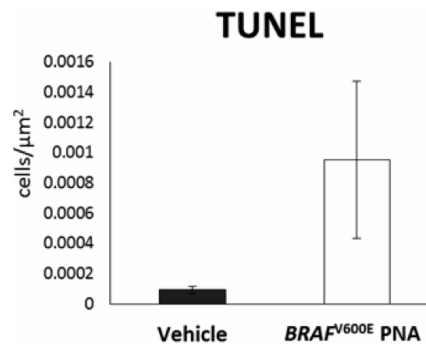
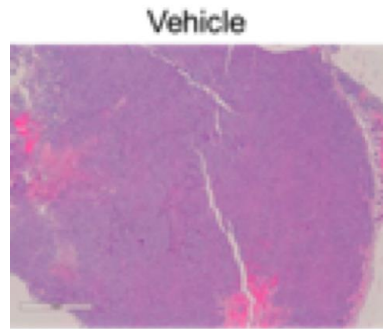
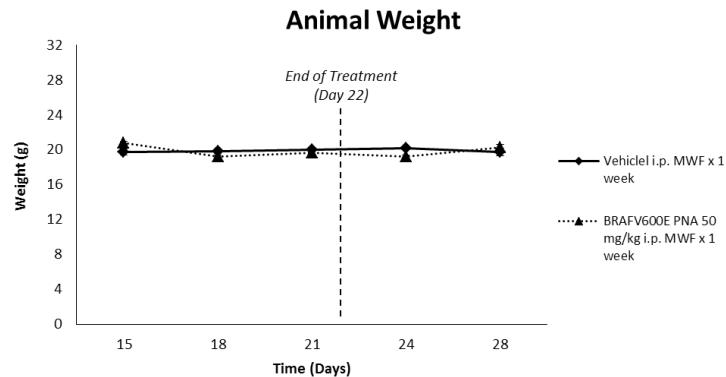
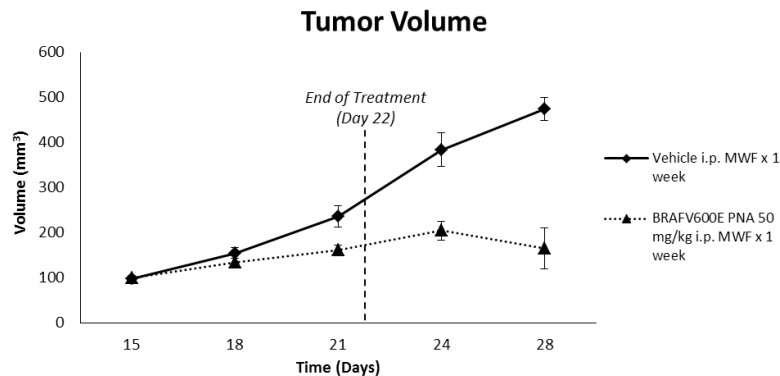


C918: BRAF WT
 OCM1A: BRAF V600E / WT
 SK-MEL7: BRAF V600E / WT
 A2058: BRAF V600E / WT

Repeated BRAF^{V600E}-complementary PNA re-exposure leads to improved inhibition of cell proliferation

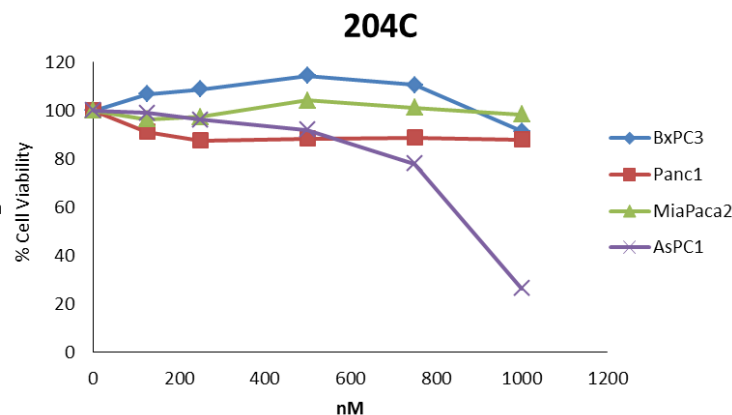
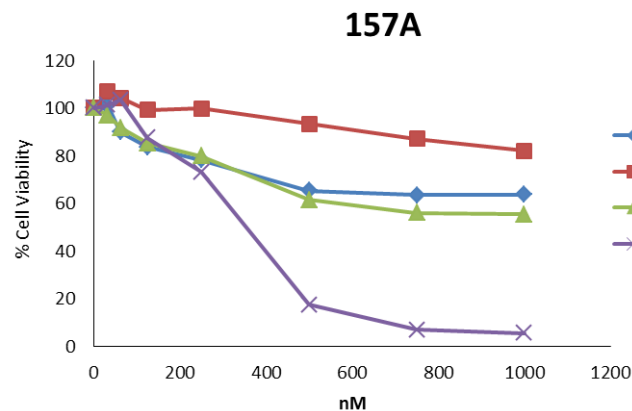


Follow-up study: In vivo Growth Suppression of *BRAF*^{V600E} Xenografts by *BRAF*^{V600E} –complementary PNAs



PNA Oligomer Conjugates Suppress KRAS G12D Transcription

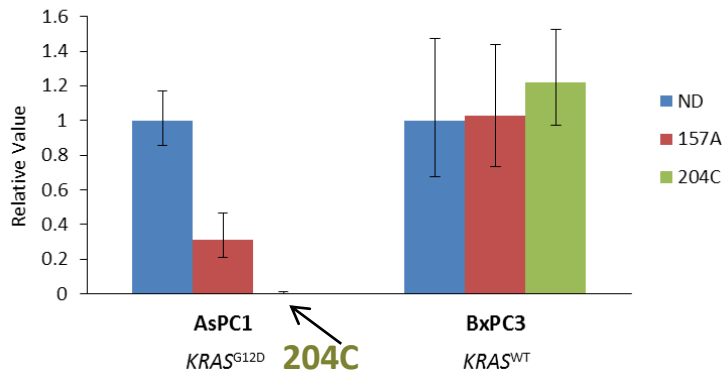
7-day *In Vitro* Growth Suppression of $KRAS^{G12D}$ Mutant Human Pancreatic Cells by $KRAS^{G12D}$ -complementary PNA Conjugate



Human Pancreatic Cell Lines

BxPC3 KRAS WT
Panc1 KRAS WT/G12D
MiaPaca2 KRAS G12C
AsPC1 KRAS G12D

KRAS mRNA



*Panc1 is not dependent
Upon KRAS G12D as
WT is up-regulated*

Singh, A., et al., *Cancer Cell*
2009, 15, 489-500.

**204C completely and specifically
obstructs KRAS G12D
transcription**

KRAS Allele-specific Transcription Suppression in Single-source Cell Lines

Cell Viability

Measured after 7 days exposure to KRAS G12D-complementary PNA oligomer conjugates

MEF Cell lines –courtesy NCI RAS Initiative*

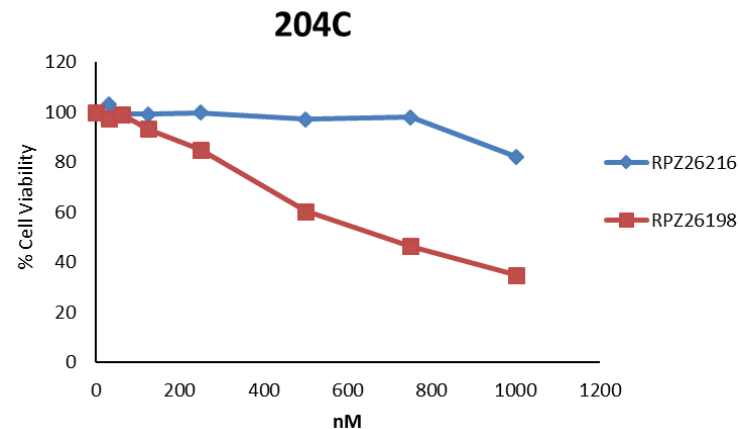
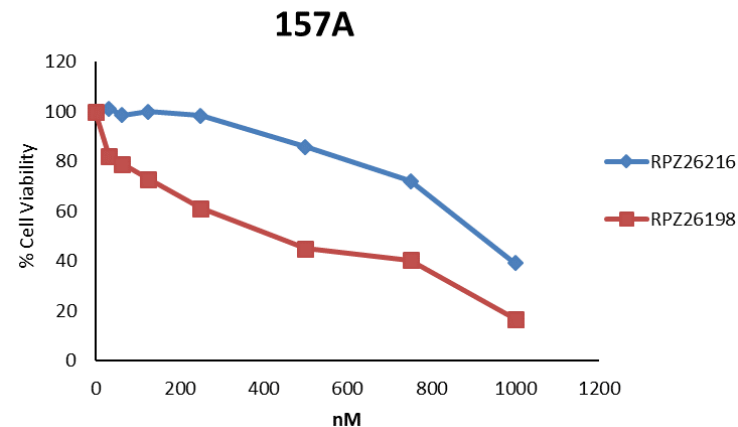
RPZ26216 KRAS WT

RPZ26198 KRAS G12D

Cell lines are derived from single source Mouse Embryonic Fibroblasts expressing inserted specific human KRAS alleles.

This offers well-controlled comparisons.

**NCI RAS Initiative currently creating primers for PCR experiment for us as this is only evidence of specific G12D transcription suppression that they have encountered to date.*



Conclusions

- ♦ Specific complementary PNA oligomers can be delivered to gene targets and effectively suppress transcription of genes that drive tumor growth
- ♦ In vivo, this results in growth suppression and tumor necrosis
- ♦ This strategy can be applied to any target with a known gene sequence (e.g. EWS-FLI1 translocation) / range of oncogenic drivers
- ♦ This can lead to a novel therapeutic approach in the developing of genetically targeted drug therapies
- ♦ **FUTURE CONSIDERATIONS:**
 - ♦ ***Consider targeting mutated genes essential for cellular function***
 - Often found in tumor cells
 - Also offers a unique target / mutations offer a 'handle' by which they can be identified / suppressed

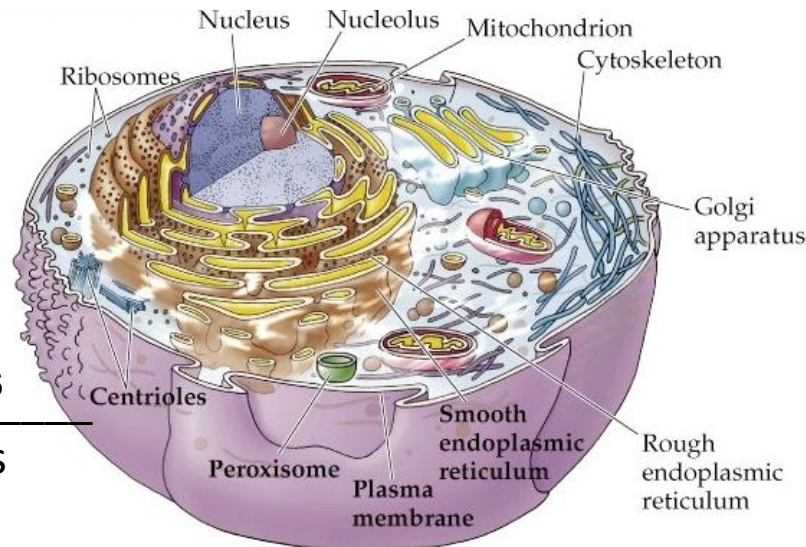
Finding the target is statistically problematic

- Binding target is only a *single* gene sequence per cell with often limited accessibility ($\sim 1 \text{ uM}$)
- Possibilities for binding to multitude of non-specific sites within a cell hampers target binding by sheer overwhelming number ($\sim 20 \text{ M}$)

$$dG = dH - TdS = -kT \ln(Z)$$

$$Z = \frac{\sum N_i \exp[-E_i/kT]}{\sum N_k \exp[-E_k/kT]} \quad \frac{\text{on-target binding states}}{\text{all possible states}}$$

- Also represents the entropy to be overcome to bind its gene target



where $k \gg i$

How can we improve the odds?

Better binding through helical stabilization

Zimm-Bragg statistical helical model

Statistical Weights

- C-coil configuration
- H-helical configuration
- σ -nucleation coefficient

Dimer sequence	Statistical weight
...CC...	1
...CH...	σs
...HC...	σs
...HH...	σs^2

$$\mathcal{Z} = (0, 1) \cdot \left\{ \prod_{j=1}^N \mathbf{W}_j \right\} \cdot (1, 1) \quad \mathbf{W}_j = \begin{bmatrix} s_j & 1 \\ \sigma_j s_j & 1 \end{bmatrix} \quad \begin{array}{l} \sigma \ll 1 < s \\ s = \frac{[H]}{[C]} \end{array}$$

Full partition function for a chain of N residues

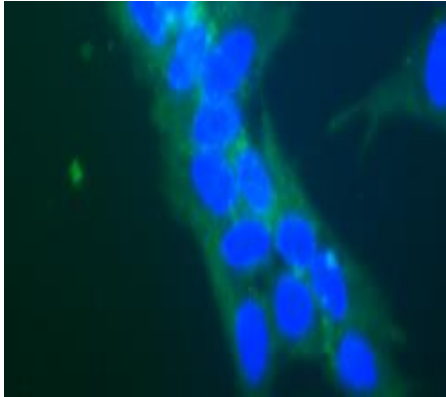
Only nearest-neighbor interactions considered in model

Transfer matrix of j th residue equals the matrix of statistical weights for the state transition

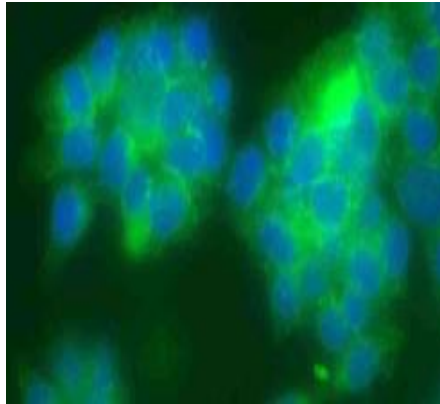


Evaluate Delivery Peptide Modifications to Maximize Cellular Diffusion

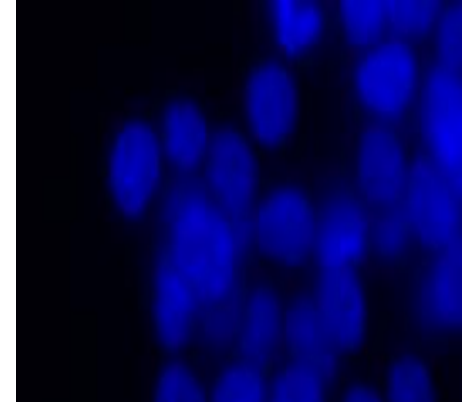
Fluor-PNA-NLS



Fluor-PNA-NLS-cationic modified
Peptide motifs



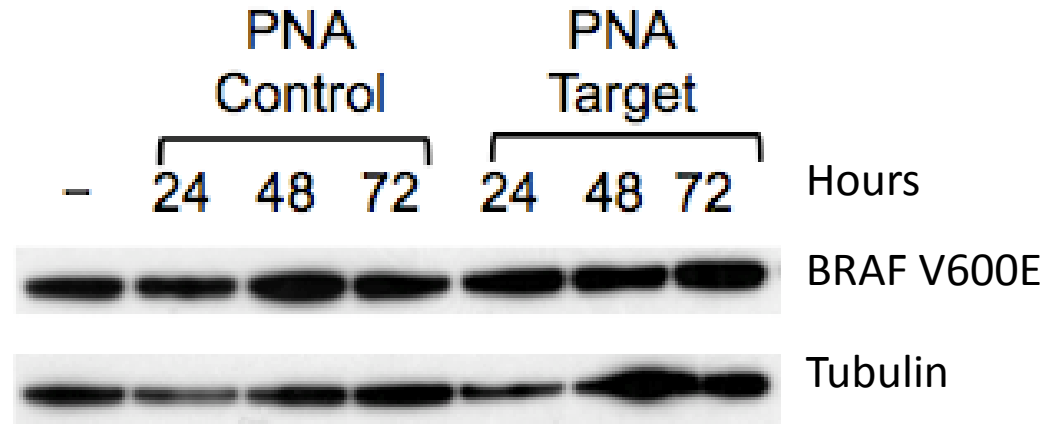
Fluor-PNA-polyglutamate
(anionic motif)



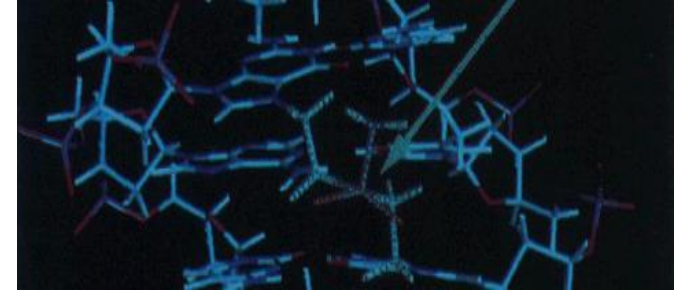
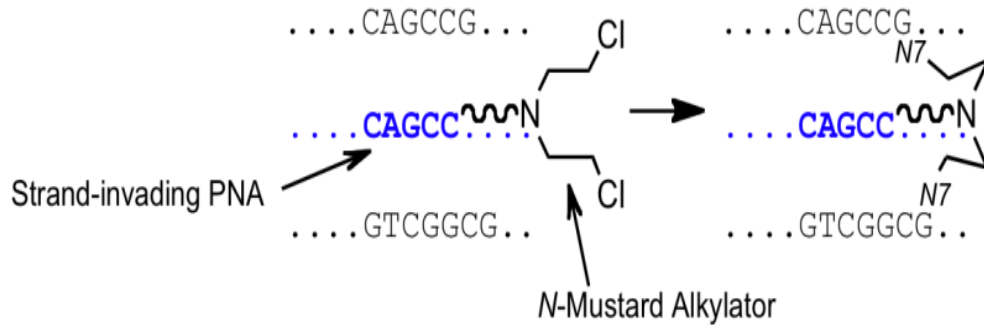
HCT 116 colon cancer cells exposed to (green) fluorescein-labeled PNA oligomer/delivery-peptide conjugate, after being washed, where (blue) Hoechst 3342 denotes nuclei; cytoplasm shows green fluorescence and denoted nuclei changes Hoechst 3342 to blue-green

Vanguard Experiments: chromosomal gene transcription suppression

- ♦ Aim to suppress the transcribed region overlapping the $BRAF^{V600E}$ mutation in OCM1A melanoma cell lines with $BRAF^{V600E}$ -complementary PNA-delivery peptide of standard design
- ♦ $BRAF^{V600E}$ protein *not* noticeably suppressed
- ♦ *What modifications may be employed to improve efficacy*



Irreversible attachment of PNA-peptide to target sequence for Transcription Obstruction

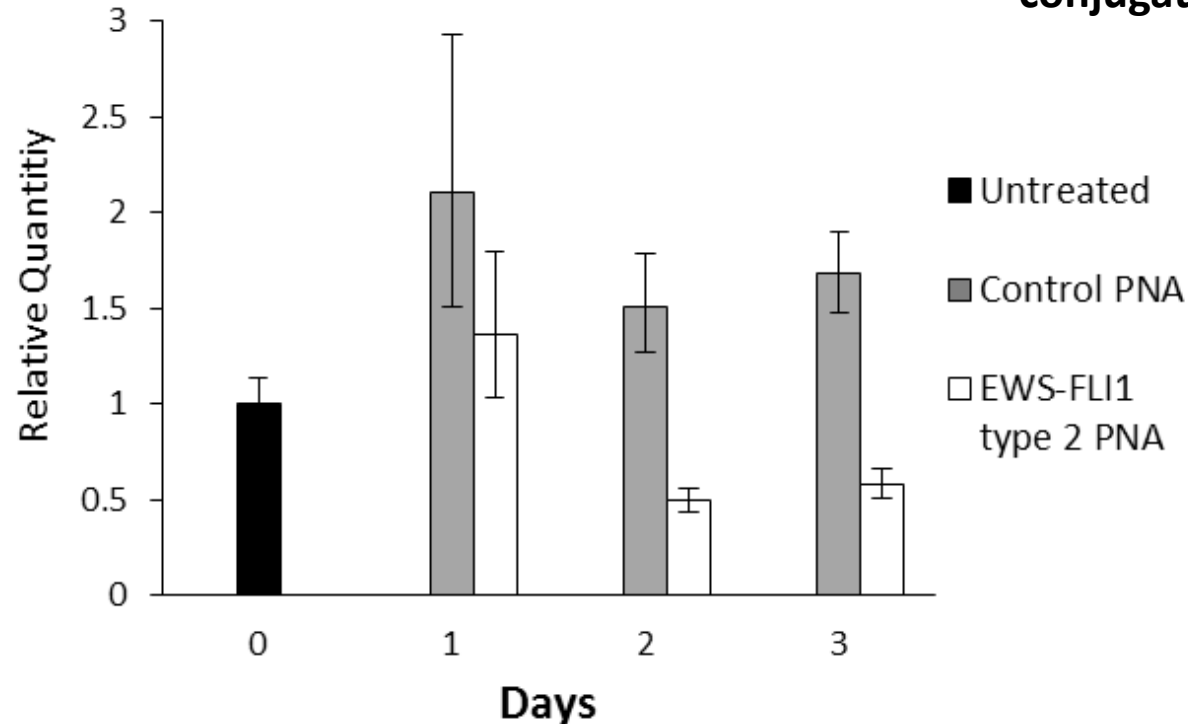


PNA oligomer terminally derivatized with a nitrogen mustard alkylating the target gene sequence at proximal *N7* guanines

EWS-FLI1 (Type 2) mRNA Suppression by a PNA Conjugate Specific to *EWS-FLI1* (Type 2) in CHP100 Cells

EWS-FLI1 type 2 mRNA

(750 nM PNA conjugate / one dose)



EWS-FLI1 (Type 2) Protein Suppression by *EWS-FLI1* (Type 2)-Complementary PNA in CHP100 Cells

750 nM, 48 hours

Ctrl: Control PNA

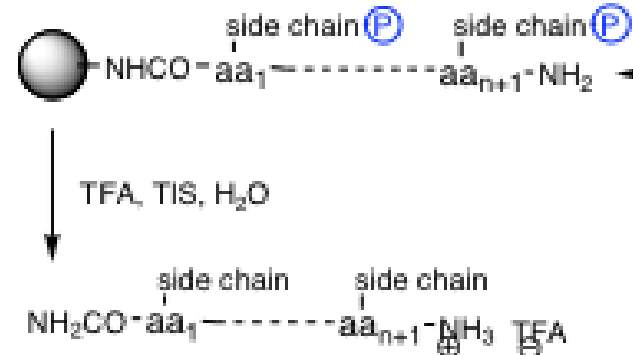
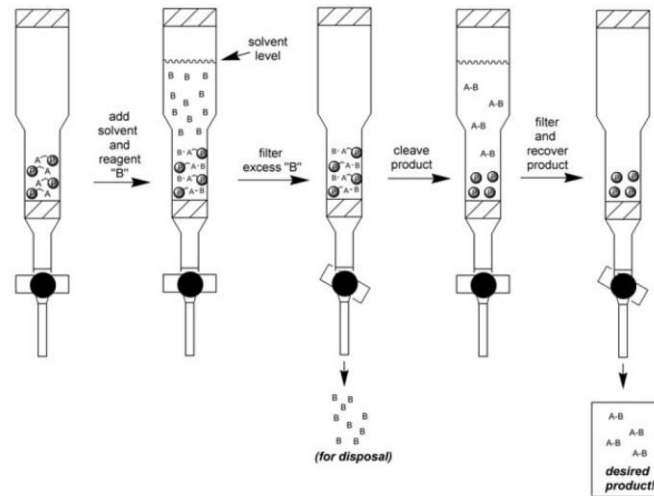
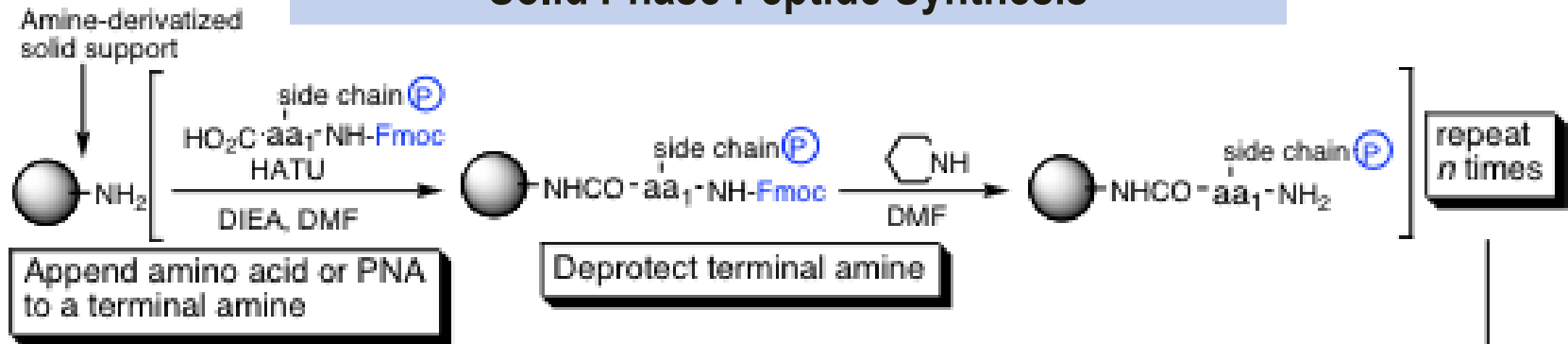
Comp: EWS-FLI1 type 2-complementary PNA



*Decrease in GLI-1 expression only in the *EWS-FLI1* type 2 cell line, CHP100

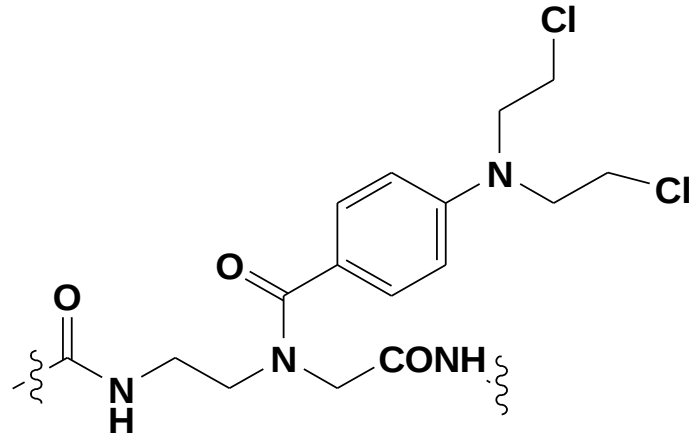
Elspeth Beauchamp, et al., GLI1 Is a Direct Transcriptional Target of EWS-FLI1 Oncoprotein, *J. Biol. Chem.*, **2009** Apr 3; 284(14): 9074–9082.

Solid Phase Peptide Synthesis



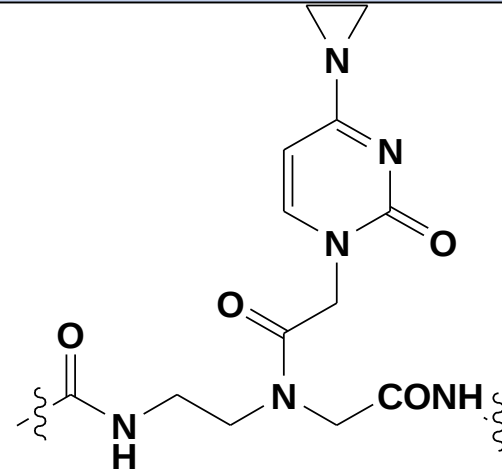
Finally Cleave Peptide from Solid Support and Deprotect side chains

Irreversible attachment of PNA-peptide to target sequence for Transcription Obstruction with *N*-mustard Alkylators



Abasic PNA derivatized with Phenylacetic Mustard (PAM)

Phenylacetic mustard would be best placed at the termini to alkylate proximal *N7* guanine as this is not easily accommodated within the plane of the nucleobase

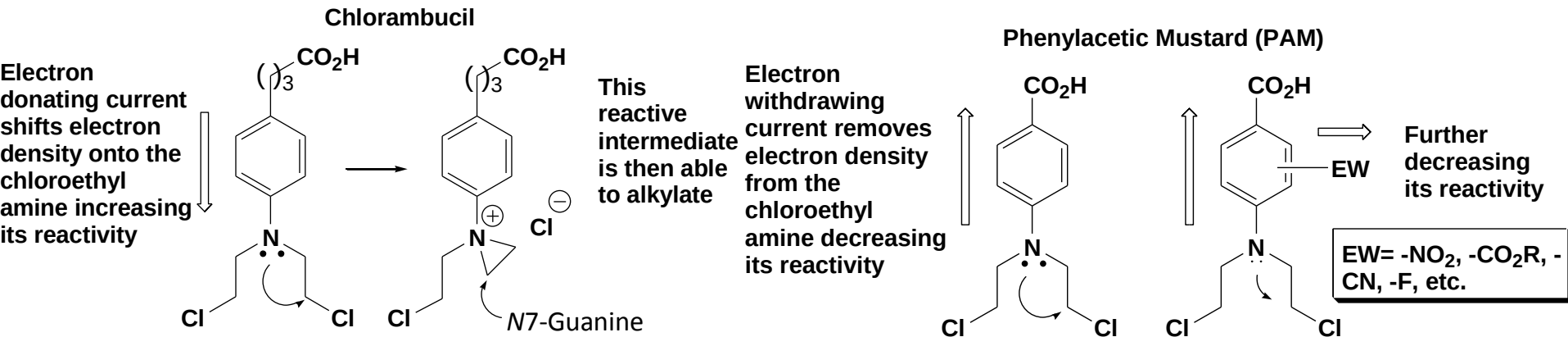


Abasic PNA derivatized with 1,2 Ethancytosine

The 1,2-ethancytosine and its acyclic analogue would fit well within the nucleobase plane; these will H-bond and then alkylate an opposing cytosine.

BETTER

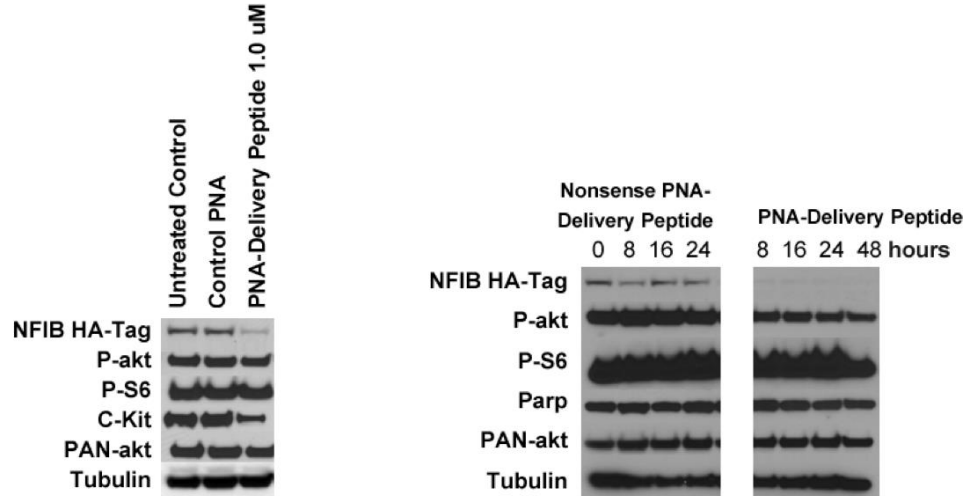
Higher Specificity with Attenuated *N*-mustard Alkylators



- Alkylating moieties being held fixed proximally to its nucleophilic target upon the PNA binding its target gene sequence, would effectively increase reactant concentrations near 20-M.
- Reactivity is fortified by a statistically increased interaction, thus obviating the need for such strong and non-specific alkylator reactivity and improving specificity.
- Any possible intramolecular alkylations would be reduced
- Ortho and further substitution of phenylacetic mustard (PAM) with electron withdrawing moieties would attenuate aziridinium formation and hence its reactivity

Vanguard Experiments: extra-chromosomal gene transcription suppression

- *MYB-NFIB* translocation. easily accessible within a transfected plasmid



- Suppression of *MYB-NFIB* was evident within 8hrs of exposure to its complementary PNA-delivery peptide
- Downstream C-Kit signaling decreased
- Nonsense sequence (control) PNA-peptide shows no effect
- *Next evaluate effectiveness toward suppression of chromosomal gene*