Abstract (#A106)

# Investigation of 5-fluorodeoxycytidine with Tetrahydrouridine as a Demethylation Regimen in Solid Tumors

Lihua Wang,[1] Robert J. Kinders,[1] Shivaani Kummar,[2] Sonny Khin,[1] Priya Balasubramanian,[1] Weimin Zhu,[1] Ralph E. Parchment,[1] Edward Newman,[3] Joseph E. Tomaszewski,[4] James H. Doroshow[4]

[1]Laboratory of Human Toxicology and Pharmacology, SAIC-Frederick Inc., NCI-Frederick, Frederick, MD; [2]Developmental Therapeutics Clinic, DCTD, NCI, Bethesda, MD; [3]City of Hope Medical Center National Cancer Center, Duarte, CA; [4]DCTD, NCI, Bethesda, MD

SAIC Frederick

## Introduction

- Methylation-induced silencing of gene promoters is widely documented in numerous cancer types, and the antitumor activity of agents that target this phenomenon has established DNA methyltransferase (DNMT1) as a valid drug target.
- Here, we report changes in pharmacodynemic biomarkers in solid tumor cells during continuous exposure to the combination of 5-fluorodeoxycytidine and tetrahydrouridine (FdC + THU) *in vitro* and *in vivo*. Deoxythymidine was supplemented in media to protect from FdC cytotoxicity and allow sufficient time for demethylation to occur. Decitabine was used as the positive control.
- We tested 4 cell lines for drug effect by measuring:
  1) DNMT1 concentrations in cell
  2) The rate of cell doubling in culture
  3) The methylation status of LINE1 CpG islands
- The mechanism of this combination regimen in tumor cells isolated from paracentesis specimens from patients enrolled on a Phase 1 clinical trial of FdC + THU was confirmed by finding changes in both DNMT1 levels and LINE1 methylation.
- Upregulation of expression of p16 (INK4a), but not GSTP1 or RASSF1, was observed in EJ6 cells treated with decitabine, a DNMT1 inhibitor, using Western blotting.
- Based on our analysis of p16 *in vitro* studies, we developed IFA for circulating tumor cells (CTCs) and implemented this assay in the Phase 1 trial.
- The ongoing Phase 2 trial of this regimen will include measurements of DNMT1 and LINE1 methylation in tumor biopsies and p16 in CTCs to directly assess drug effect on the DNMT1 target.

## Materials and Methods

### Cell Lines, Drug Treatment, and Cell Count

- Bladder cancer cell line EJ6 and breast cancer cell line MDA-MB-231 were grown in DMEM or RPMI 1640 supplemented with 10% FCS at 37˚C with 5% $CO_2$.
- Cells were treated with 100 µM 5-fluoro-2'-deoxycytidine (FdC) (NSC 48006) plus 1 mM tetrahydrouridine (THU) (NSC 112907) and 10 µM thymidine (Affymetrix) for 12 weeks, or treated with decitabine (2'-Deoxy-5-azacytidine) (NSC 127716) for three weeks.
- Cells were counted every week using Nexelom Cellometer Automatic Cell Counter (Nexcelom Bioscience, Lawrence, MA).
- Harvested Cells were harvested every week using Trypsin-EDTA Solution.

### Line-1 methylation assay

- DNA preparation by Easy-DNA kit (Invitrogen).
- Bisulfite conversion of DNA by EZ DNA Methylation kit.
- DNA amplification by PCR using HotStartTaq DNA polymerase and PryoMark Q96 LINE 1 primers.
- Pyrosequencing by PyroMark Q96 MD using PyroMark CpG LINE-1 sequencing primers.

### DNMT expression by ELISA

- Sample preparation: Cell pellets were sonicated in Cell extraction buffer with protease inhibitors. SDS was added to final 1% and boiled. The samples were spun, and the protein estimation was done with BCA.
- Reatic-bind plates 96 wells (Thermo CN 15042) were coated with capture antibody (Anti-dnmt1 2 ug/ml, Abeam) in 100 ul per well coating buffer overnight at 4°C.
- After aspiration, the plate was blocked with 250 ul/well blocking buffer (PBS, 2% BSA) for 1 hour at 37°C.
- After one wash with PBS (pH 7.2, Tween-20, 0.1%), samples were diluted in SuperBlock (Pierce), and 75 ul per well was added into the plate. Samples were then incubated overnight at 4°C.
- After four washes with PBS/Tween, 75 ul of detection antibody (BD, anti-dnmt1 biotinylated antibody, 2 ug/ml in SuperBlock) was incubated for 2 hr at room temperature.
- After washing four times with PBS/Tween, 75 ul of Streptavidin-HRP (Pierce) of 1:4000 diluted in SuperBlock was added to the wells and incubated for 30 min at room temperature.
- Wells were washed four times with PBS/Tween, and 100ul of the substrate (Super signal chemmiluminescence, Thermo) was added to the wells.
- Plate was read on the Tecan plate reader at luminescence. The slope equation was used to determine the concentration of the samples.

### Immunocytochemistry

- Cell pellets were simultaneously fixed and permeabilized using BD Cytofix/Cytoperm™ kit.
- Cells in suspension were incubated with 1:20 dilution of anti-p16 antibody reagent from Cell Signal (Catalog# 4824) for 1 hr at 4°C.
- After subsequent washes, the cells were incubated with 1:100 dilution Alexa Fluor® conjugated anti-Rabbit IgG (Invitrogen) for 1 hr at 4°C.
- Cells were cytospun onto a slide at 600 rpm for 5 min.
- After air drying the slides, a cover slip was mounted using DAPI-containing mounting media.
- P16 image capture was conducted using Leica 5000 DM with 20x Plan Apo Ph2 (N.A.: 0.75) objective and a Retiga 2000R monochrome CCD camera (QImaging).
- Manual exposure corrections were made based on DAPI intensity.
- The resulting monocaptures (DAPI and p16) were merged into a single RGB image for analysis.

### Western Blotting

- Cell lysates were prepared in Cell Extraction Buffer (Invitrogen) containing protease inhibitor and PMSF.
- 50 µg of protein was loaded per well for all cell lines and separated on 4–20% precast gels by SDS-PAGE.
- Separated proteins were then blotted onto 0.2 um nitrocellulose membranes, blocked with blocking buffer, and probed with antibodies against specific targets.
- Blots were scanned using Odyssey IR Imager (LICOR).

### Patients and Sample Collection

- All enrolled patients and healthy subjects gave informed consent for study inclusion and were enrolled using institutional review board–approved protocols.
- Blood was drawn from metastatic prostate, breast, or other cancer patients at the DCTD, NCI (Bethesda, MD) and other clinical centers (USC, UCD, COH, PENN, UPMC).
- Blood for spiking samples from healthy donors was drawn at NCI-Frederick (Frederick, MD).
- Blood (~8 mL) was collected into CellSave® tubes (Veridex) and processed within 96 hr.
- Thoracentesis and paracentesis clinical patient specimens were received from DCTD.
- Cells were isolated by centrifugation, washed, assessed for viability (>95%), counted, aliquoted, and flash-frozen.

### Cells Fixation, Permeabilization, and Spiking Protocols for CTC Assay Development and Validation

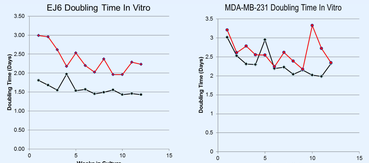
- Epithelial tumor cell lines 293T/17, BT549, and OVCAR4 were grown in DMEM or RPMI 1640 supplemented with 10% FCS at 37°C with 5% $CO_2$.
- Cells were fixed and permeabilized with the one-step BD Cytofix/Cytoperm™ Kit (BD Biosciences).
- Predetermined numbers of cells were suspended in peripheral blood from healthy donors and processed on the CellSearch™ system.

## Materials and Methods Cont.

### CTC Enumeration and Identification of p16-positive CTCs in Patients' Blood

- Each blood sample (7.5 ml) was mixed with sample buffer, centrifuged, and processed using the CellSearch platform (Veridex).
- Serial samples from individual patients were used to determine variability in baseline levels of CTC and changes in CTC and/or p16 in response to treatment.
- Images captured by CellTracks® Analyzer II contained red objects fulfilling predetermined criteria and were automatically presented in gallery format. Final classification of cells was done independently by two operators.
- Cells were classified as CTCs when morphologic features and staining patterns were consistent with those of epithelial cells (CK-PE positive/DAPI positive/CD45-APC negative).
- CTCs had to have a minimum size of at least 4 µm but present with a large heterogeneity in both size and morphology.
- p16-positive CTCs presented with nuclear staining in the green fluorescent channel.

### Effect of FdC + THU on EJ6 and MDA-MB-231 Doubling Time *In Vitro*

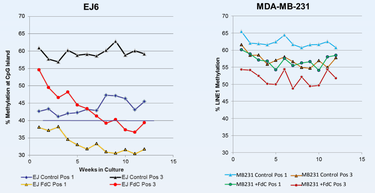



| EJ 6 Control | FdC |
|---|---|
| Mean | 1.58 | 2.36 |
| Standard Error | 0.05 | 0.10 |
| Median | 1.54 | 2.26 |
| Standard Deviation | 0.17 | 0.35 |




| MB-231 Control | FdC |
|---|---|
| Mean | 2.34 | 2.63 |
| Standard Error | 0.10 | 0.10 |
| Median | 2.26 | 2.58 |
| Standard Deviation | 0.34 | 0.35 |

### Effect of FdC + THU on LINE1 Methylation of EJ6 and MDA-MB-231 Cells *In Vitro*







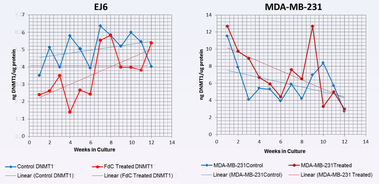
| EJ6 Position 1 | Control | + FdC |
|---|---|---|
| Mean | 43.92 | 33.42 |
| STDEV | 2.08 | 2.88 |
| Mean - 2SDEV | 38.51 | |
| %CV | 4.7 | |

| EJ 6 Position 3 | Control | + FdC |
|---|---|---|
| Mean | 59.45 | 43.42 |
| STDEV | 1.55 | 9.45 |
| Mean - 2 SEV | 56.35 | |
| %CV | 2.6 | |

| MB231 Pos 1 | Control | +FdC |
|---|---|---|
| Mean | 62.24 | 56.97 |
| STDEV | 1.41 | 1.81 |
| Mean - 2 SDEV | 59.43 | |
| % CV | 2.3 | |

| MB231 Pos 3 | Control | + FdC |
|---|---|---|
| Mean | 57.13 | 51.80 |
| STDEV | 1.06 | 2.15 |
| Mean - 2 SDEV | 53.21 | |
| % CV | 3.4 | |

### Effect of FdC + THU on DNMT1 Levels of EJ6 and MDA-MB-231 Cells

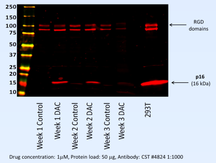




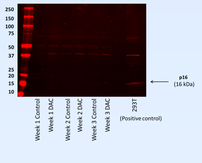


| EJ Cells | Control | Treated |
|---|---|---|
| Mean | 5.01 | 3.63 |
| Standard Deviation | 0.95 | 1.41 |
| Minimum | 3.49 | 1.40 |
| Maximum | 6.35 | 5.84 |
| Sum | 60.15 | 43.60 |
| Count | 12 | 12 |

### DAC Induced Expression of p16 in EJ6 Cells

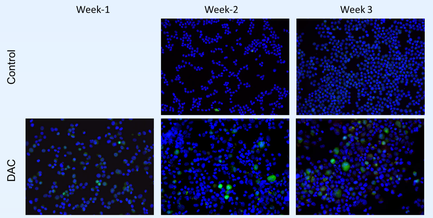



### p16 Expression is not Seen after Decitabine Treatment in MDA-MB-231 Cells




Drug concentration: 1µM, Protein load: 50 µg, Antibody: CST #4824 1:1000

### DAC Induced Expression of p16 in EJ6 Cells




### DNMT1 Protein Concentrations in Patients from the FdC + THU Trial

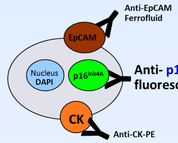
| | DNMT1 Total | |
|---|---|---|
| Sample ID | ng/mL/µg Total Protein | ng/mL/250X10³ cells |
| 30211 | 3.10 | 3.57 |
| 20811 | 0.63 | 0.06 |
| 32211 | 0.41 | 0.48 |
| 32211 | 2.03 | 1.71 |
| Jurkat (10 µg) | 16.80 | ND |
| Jurkat (5 µg) | 8.40 | ND |

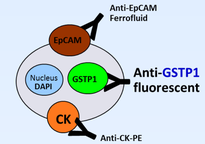
### LINE1 Methylation at CpG Position 1, 2, 3, and 4 in Patient Specimens from the FdC + THU Trial

| | | Methylation (%) | | | | Over all CpGs | |
|---|---|---|---|---|---|---|---|
| Sample ID | Specimen type | Pos. 1 | Pos. 2 | Pos. 3 | Pos. 4 | Mean | STDEV |
| 30211 | Paracentesis Bladder Ca, on cycle 12 | 61.59 | 72.30 | 45.29 | 71.63 | 62.70 | 12.60 |
| 20811 | Thoracentesis Breast Ca, on cycle 3 | 82.62 | 81.82 | 74.31 | 76.76 | 78.88 | 4.00 |
| 32211 | Thoracentesis, left Breast Ca, on cycle 4 | 74.64 | 78.35 | 72.00 | 79.92 | 76.23 | 3.58 |
| 32211 | Thoracentesis, right Breast Ca, on cycle 4 | 75.49 | 83.24 | 78.07 | 85.81 | 80.65 | 4.71 |
| Mean | | 73.59 | 78.93 | 67.42 | 78.53 | 74.62 | 6.05 |
| STDEV | | 8.76 | 4.87 | 14.96 | 5.94 | | |

| | | Methylation (%) | | | | Over all CpGs | |
|---|---|---|---|---|---|---|---|
| Sample ID | Specimen type | Pos. 1 | Pos. 2 | Pos. 3 | Pos. 4 | Mean | STDEV |
| PBMC1 | Healthy donor | 90.04 | 78.19 | 79.13 | 89.43 | 84.20 | 6.41 |
| PBMC2 | Healthy donor | 83.95 | 79.78 | 79.36 | 95.23 | 84.58 | 7.40 |
| PBMC3 | Healthy donor | 82.65 | 73.78 | 81.18 | 90.40 | 82.00 | 6.81 |
| PBMC4 | Healthy donor | 84.76 | 78.36 | 77.81 | 94.31 | 83.81 | 7.68 |
| PBMC5 | Healthy donor | 79.78 | 79.60 | 72.01 | 83.39 | 78.70 | 4.79 |
| PBMC6 | Healthy donor | 79.71 | 78.36 | 75.23 | 81.11 | 78.60 | 2.51 |
| PBMC7 | Healthy donor | 85.44 | 81.76 | 74.76 | 87.76 | 82.43 | 5.68 |
| PBMC8 | Healthy donor | 82.98 | 78.50 | 78.14 | 84.03 | 80.91 | 3.03 |
| Mean | | 83.66 | 78.54 | 77.20 | 88.21 | 81.90 | 5.04 |
| STDEV | | 3.32 | 2.26 | 2.98 | 5.13 | | |

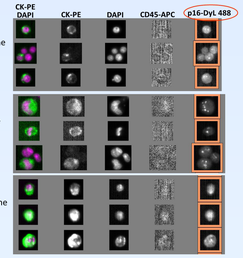
### *Assay Development and Feasibility*



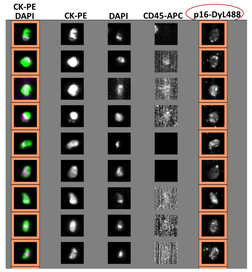




Reference: LHTP003.8.1.1
http://dctd.cancer.gov/ResearchResources/biomarkers/CTCs.htm

### Comparison of p16 Images by CellSearch System in Three Cancer Cell Lines

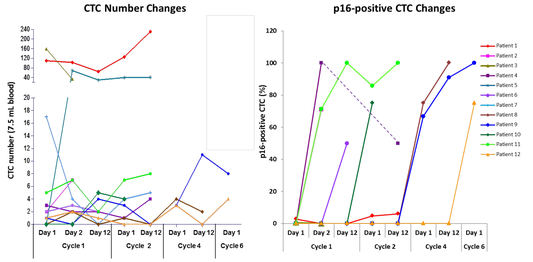



### Detection of p16$^{Ink4A}$-positive CTC in Patient Samples from FdC + THU Trial by CellSearch System

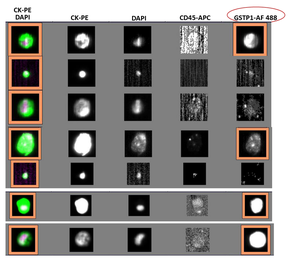



Cycle 4, Day 12

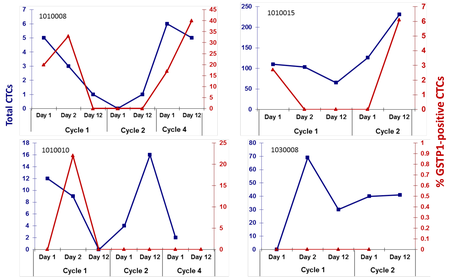
### Changes in CTC Number and p16$^{Ink4A}$ -positive CTC during Treatment with FdC + THU in Evaluable Patients




### Detection of GSTP1-positive CTC in Patient Samples from FdC + THU Trial by CellSearch System




### Changes in CTC Number and GSTP1-positive CTC during Treatment with FdC + THU in Evaluable Patients




## Conclusions

1. FdC + THU modify tumor cell growth by inhibiting DNMT1 and decreasing LINE1 promoter methylation. The effect was seen after two weeks exposure in culture.
2. DNMT1 and LINE1 methylation changes were detected in ascitic fluid from a patient with the FdC + THU treatment protocol.
3. Upregulation of expression of P16$^{INK4}$, but not GSTP1 or RASSF1 could be measured in patients under the FdC treatment by an IFA of CTCs.
4. The IFA for P16 regulation in CTC used in the Phase I trial will be used in a planned Phase II trial of an oral formulation.

### Acknowledgment

This Research has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E and NCI RAID Project #266 Grant.