

Pre-clinical Development of 4'-thio-2'-deoxycytidine (TdCyd) as a DNA-demethylating Agent for Use in Treating Solid Tissue Tumors

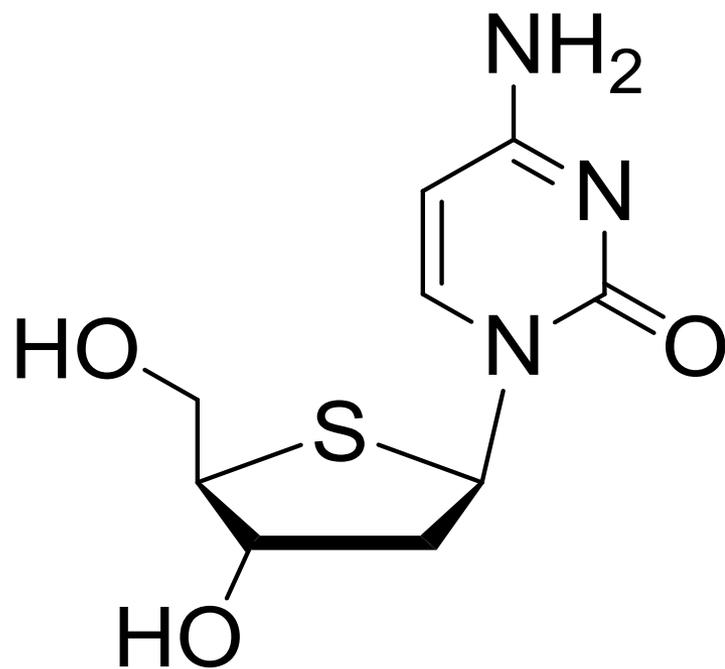
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Introduction

Targeting cancer epigenetic control of cell growth via DNA methylation has been successful in treating hematologic diseases, such as Decitabine (DAC) and Azacitidine for Myelodysplastic Syndrome including Acute Myelomonocytic Leukemia. This success has not extended to solid tissue tumors.

The Division of Cancer Treatment and Diagnosis of NCI has initiated pre-clinical development of 4'-thio-2'-deoxycytidine (TdCyd, NSC764276) as a CpG island demethylating agent for treating solid tumors after promising early results in a lung adenocarcinoma xenograft model (NCI-H23). IP dosing at 4MKG (0.56 MTD) in nu/nu mice on a Q5D x 3 cycle schedule resulted in tumor stasis with acceptable accompanying weight loss in the mice. A DAC-treated control arm treated at MTD resulted in tumor growth delay but not stasis, and a 10% weight loss was noted. Intratumoral levels of DNMT1 were reduced to undetectable levels in xenografts post administration of TdCyd by ELISA and Western Blot assays, but were unaffected by DAC treatment. Mass Spectrometry analysis demonstrated incorporation of both TdCyd and thiothymidine (TdThd) into H23 DNA. In vitro experiments on a selected panel of cancer cell lines confirmed the conversion of TdCyd to the triphosphate.



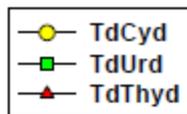
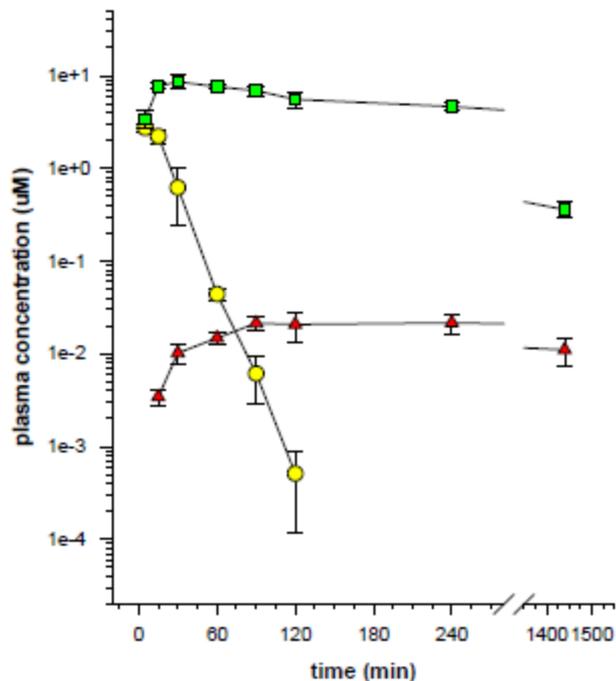
TdCyd
NSC764276

TdCyd Overview

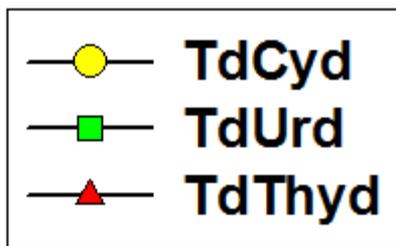
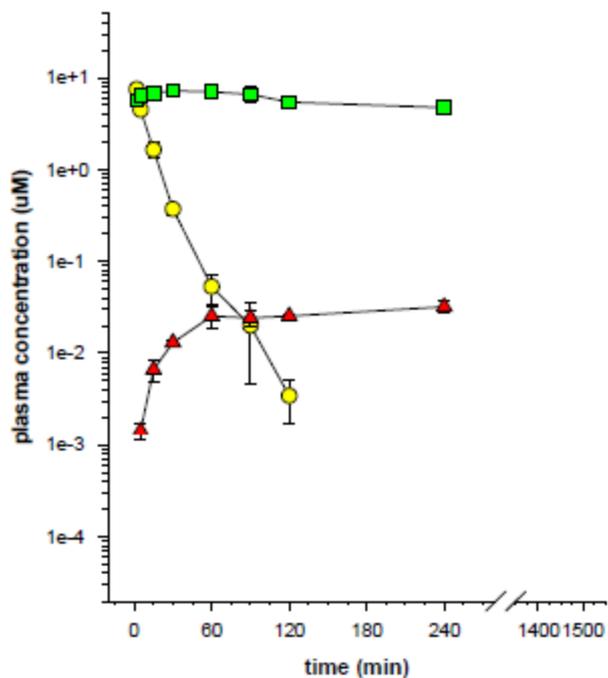
- **4'-Thio-2'-Deoxycytidine (TdCyd) is an agent that depletes DNMT1 both *in vitro* and *in vivo* in tumor cells**
 - Depleting DNMT1 is a useful cancer therapeutic strategy
 - DNMT1 is a maintenance methyltransferase that contributes to the hypermethylation and silencing of tumor suppressor genes
 - DNMT1 also has roles independent of its methyltransferase activity and its knockout causes decreases in cell viability that are preceded by events consistent with activation of DNA damage response.
 - TdCyd is incorporated into DNA far better than decitabine and TdCyd is less toxic than decitabine.
 - **Hypothesis** – Treatment with TdCyd will result in the inhibition of tumor growth due to DNMT1 depletion at doses that are well tolerated in extended schedules.

Pharmacokinetics of 4'-S-deoxyCytidine (NSC764276) in Nude Mice

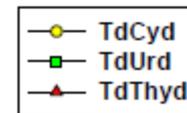
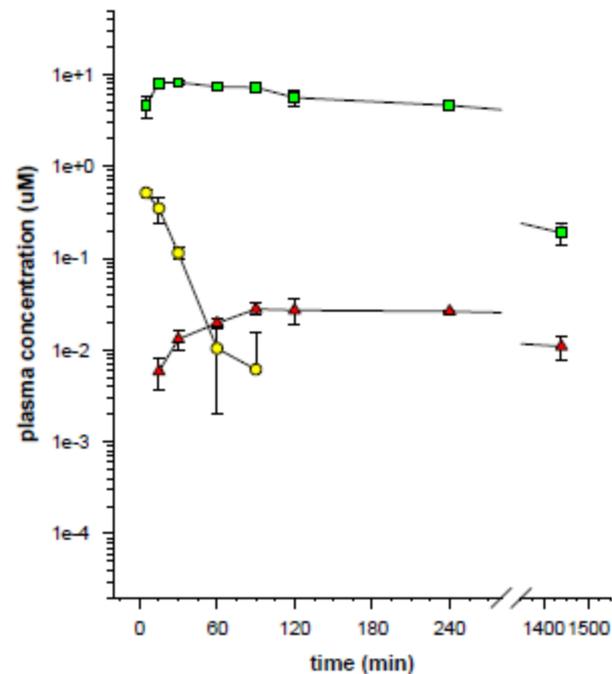
5mg/kg I.P. dosing 764276



5mg/kg I.V. dosing 764276



5mg/kg P.O. dosing 764276

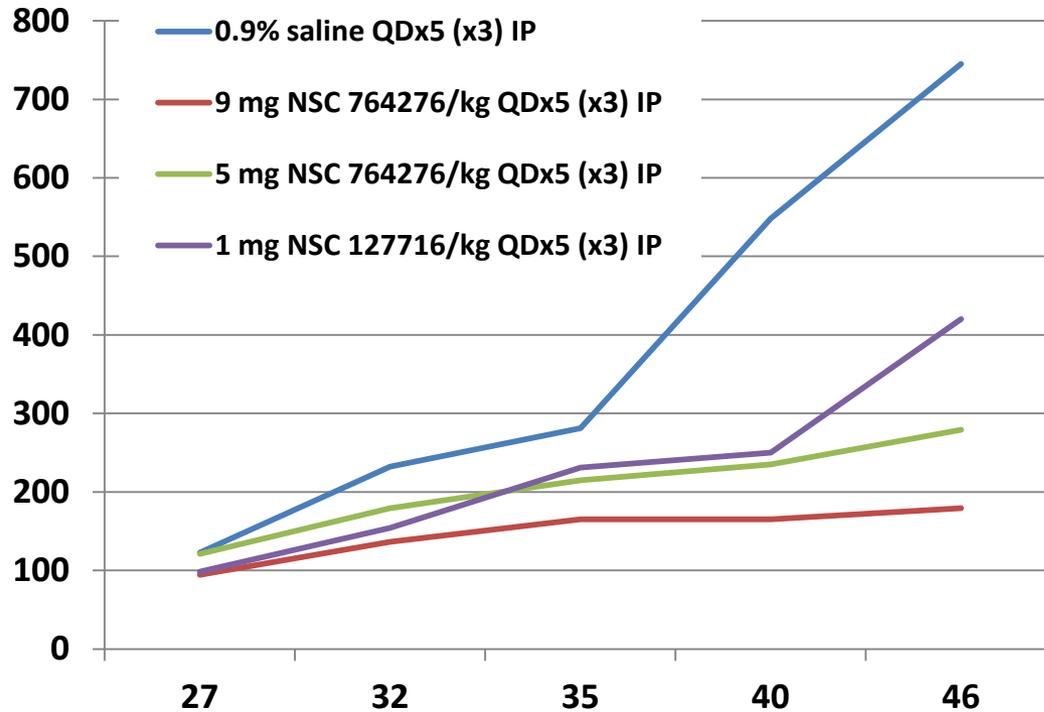


Pharmacokinetics of 4'-S-deoxyCytidine (NSC764276) in Nude Mice

Legend

- Plasma samples were prepared for analysis by precipitating proteins with 3 volumes of acetonitrile containing 0.75 μ M Gemcitabine (ISTD). The samples were centrifuged, the supernate collected, dried and reconstituted in PBS. Aliquots of the reconstituted samples were then analyzed by HPLC/HR-MS. Concentrations were determined by comparison to plasma samples spiked with known amounts of the thio-deoxypyrimidines.

Inhibition of NCI-H23 Xenograft growth by TdCyd at 3 Dosing Levels



METHODS:

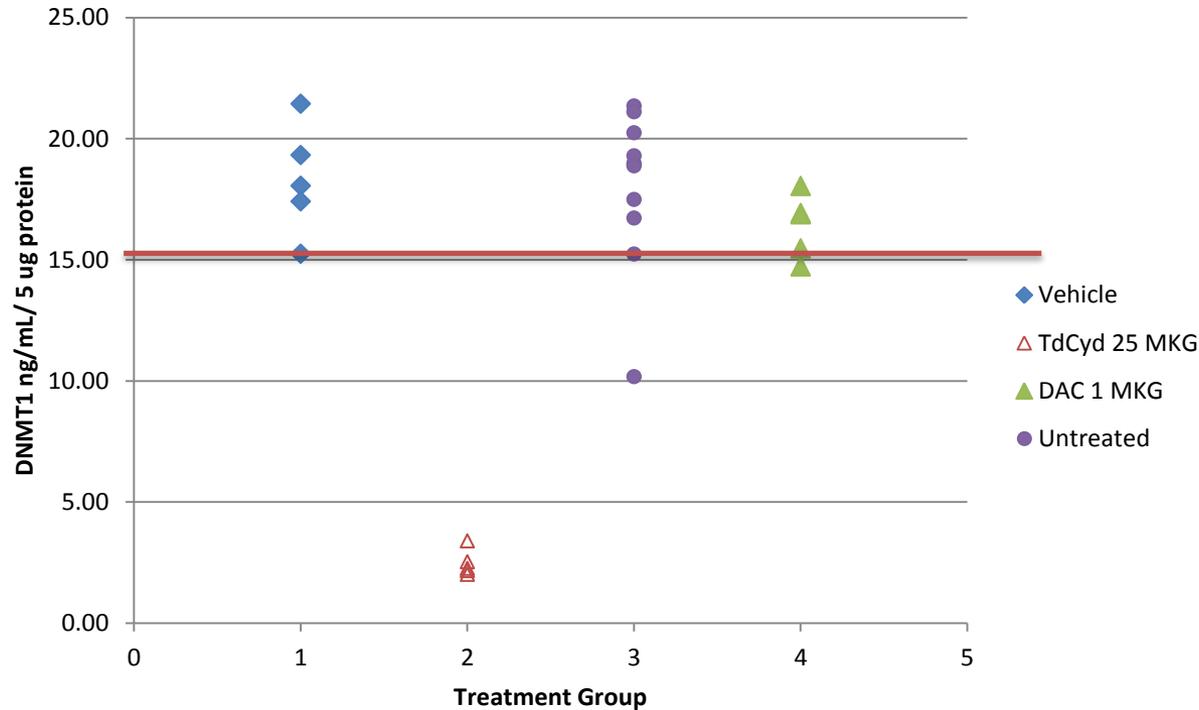
1×10^7 NCI-H23 human lung tumor cells were implanted into the subcutaneous tissues of the flank of female athymic nu/nuNcr mice. The mice were randomized 4 groups when the tumors reached a median of 115 mg in size. The treatment groups included:

- 1) 0.9% Saline QDx5 (x3) IP
- 2) 9 mg NSC 764276/kg QDx5 (x3) IP
- 3) 5 mg NSC 764276/kg QDx5 (x3) IP
- 4) 1 mg NSC 127716/kg QDx5(x3) IP

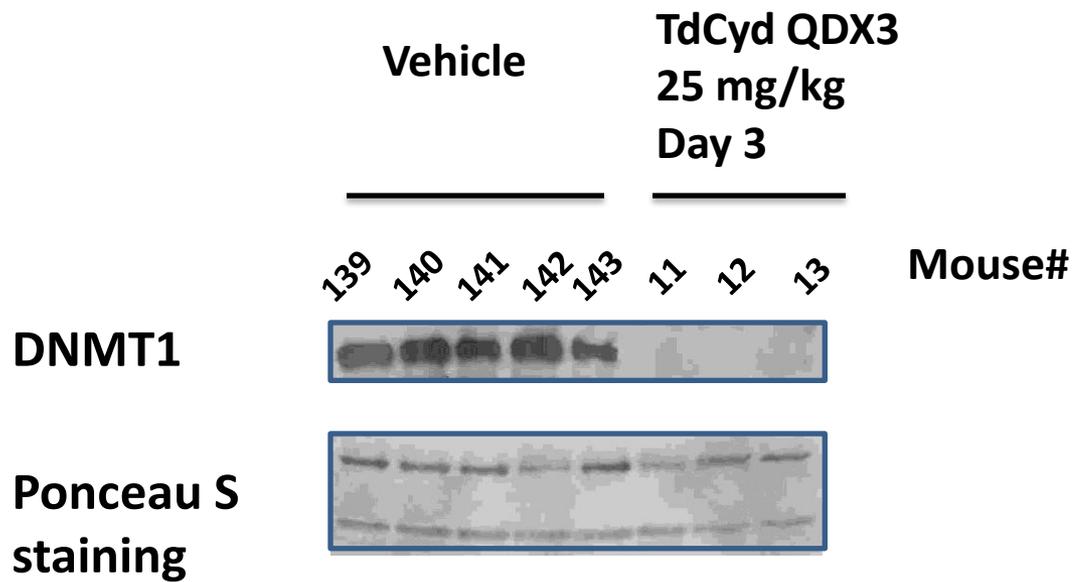
Tumor and body weights were monitored until tumors were harvested for PD studies on day 46 (4 hr post-dose). The 9 mg NSC 764276/kg and the 1 mg NSC 127716/kg doses exceeded tolerability and further studies at these doses were not pursued.

TdCyd potently depletes DNMT1 in NCI H23 Xenografts

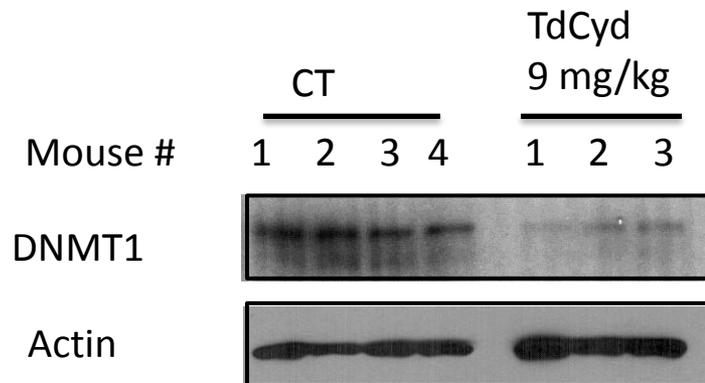
DNMT1 Knockdown by TdCyd vs Decitabine in NCI-H23 Xenograft (IV, QDx5 on Day 3)



DNMT1 trapped on DNA-coated capture plate and probed with a DNMT-1 specific antibody



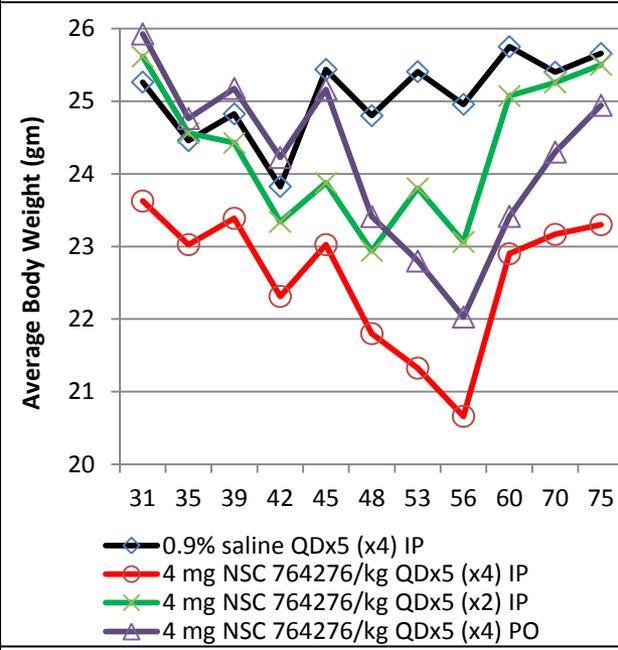
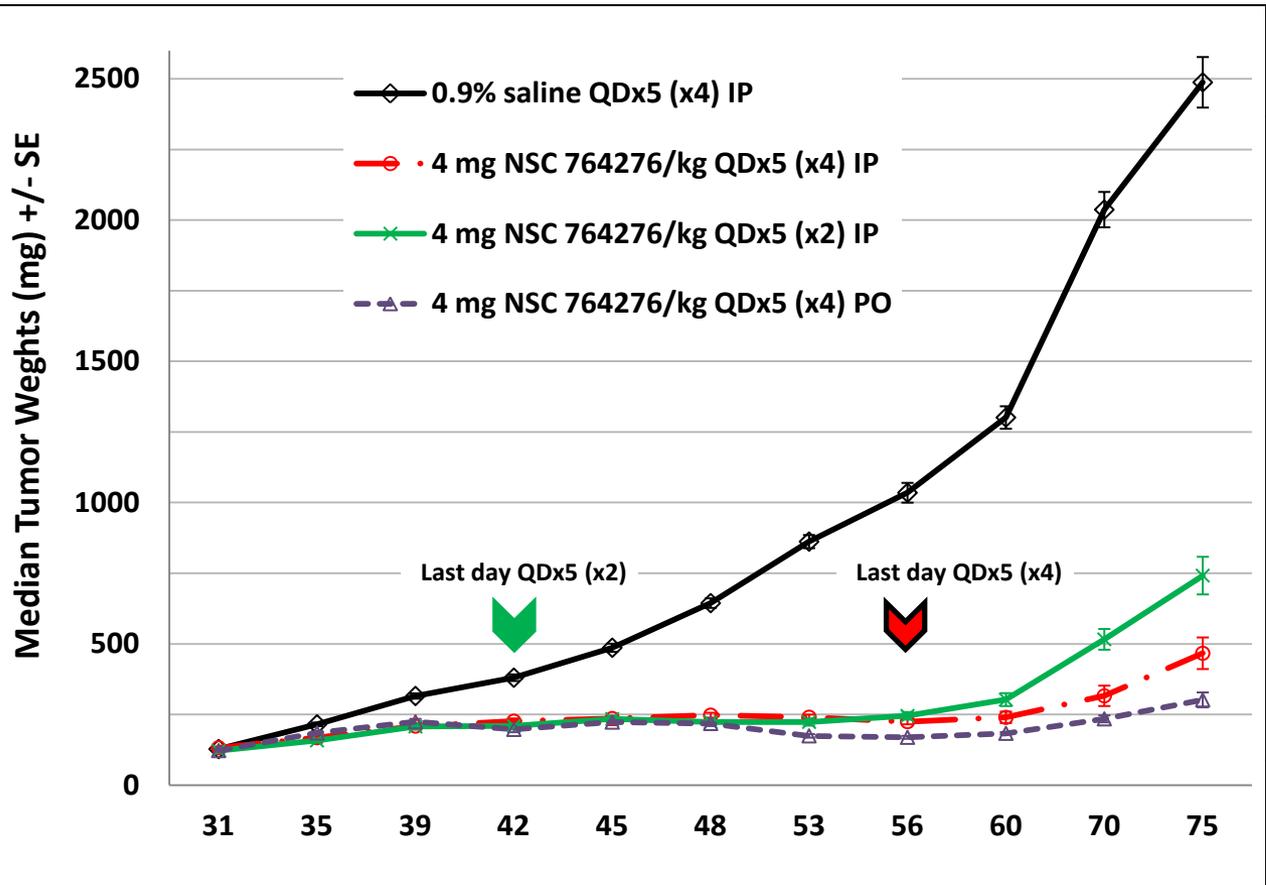
Western blot performed on NCI H23 nu/nu Xenografts from BTB model



Western blot of CEM Xenograft extracts, performed at SRI
 TdCyd was administered IV to female nu/nu mice, 9 MKG QDx9

Left Panel: NCI-H23 (Lung Adenocarcinoma) Xenograft growth inhibition by Tdcyd, comparing IP to PO administration

Right Panel: Nude mouse weight loss during TdCyd treatment



Legend for Xenograft Model Growth Inhibition panels

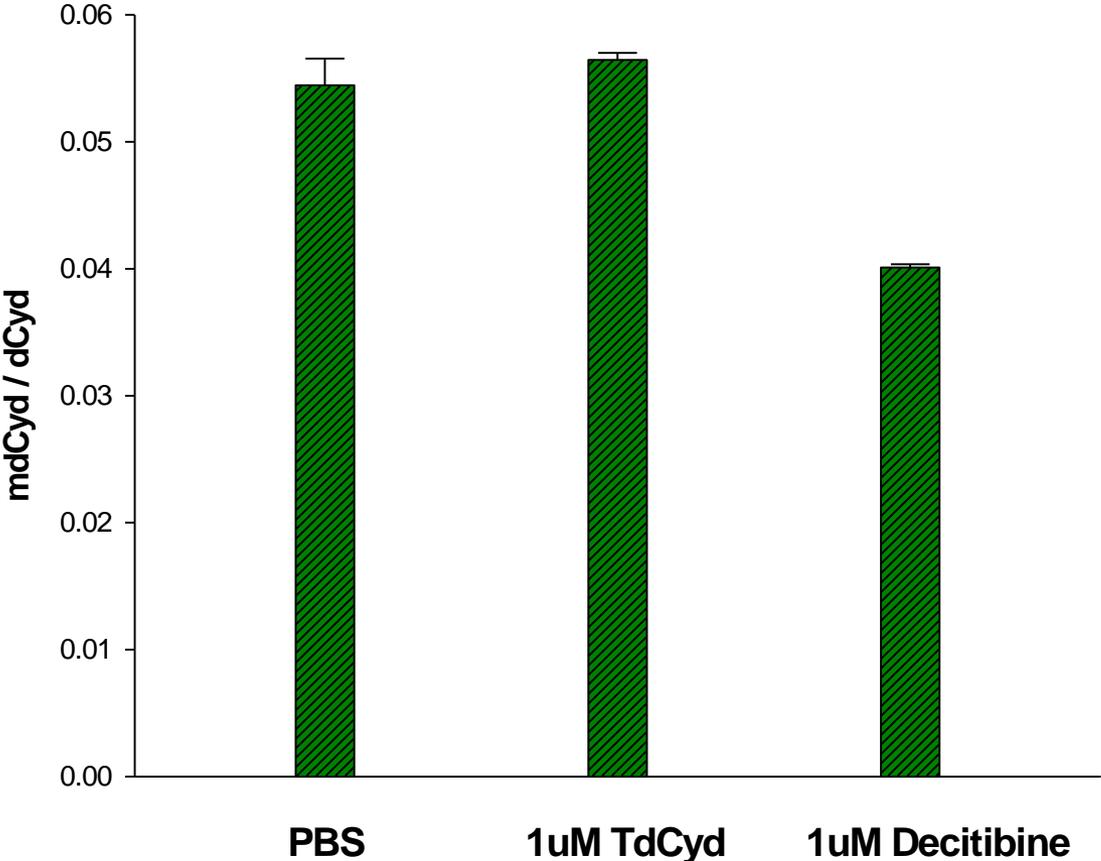
- **METHODS:**
- **1x10⁷ NCI-H23 human lung tumor cells** were implanted into the subcutaneous tissues of the flank of female athymic nu/nuNcr mice. The mice were randomized 8 groups when the tumors reached a median of 125 mg in size. The treatment groups included:
- 1) 0.9% Saline QDx5 (x4) IP
- 2) 4 mg NSC 764276/kg QDx5 (x4) IP
- 3) 4 mg NSC 764276/kg QDx5 (x2) IP
- 4) 4 mg NSC 764276/kg QDx5 (x4) PO
- Tumors and body weights were monitored 2-3X/week for the duration of the study.

Results: Tumor growth delays were seen in all experimental treatments. Treatment with NSC 764276 produced complete tumor stasis given in 2 cycles or 4 cycles IP or 4 cycles PO. All treatments were associated with some body weight loss which recovered quickly following cessation of treatment. All treated groups were statistically significantly ($p < 0.05$) smaller than the vehicle control group start on day 39 and continuing through the entire experimental period.

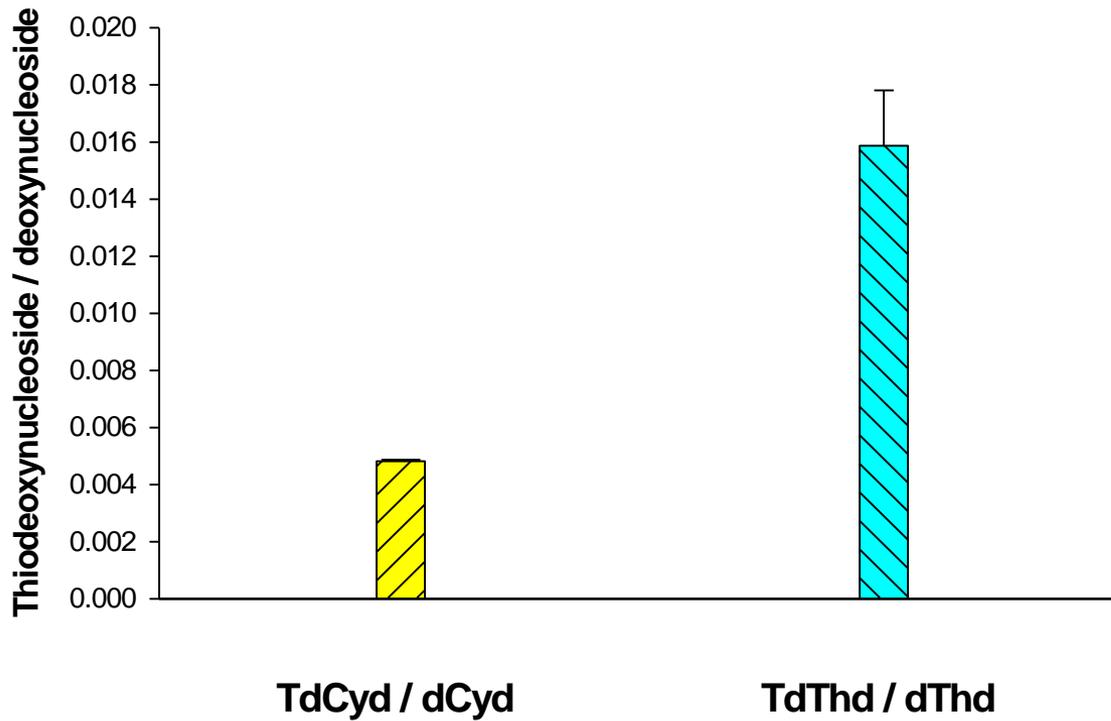
In Vitro Experiments Demonstrate Incorporation of TdCyd and its metabolite thio-deoxythymidine into NCI-H23 DNA

this is the title for the following 2 graphs

DNA Global Methylation Status
NCI-H23 Cells treated for 24hr



NCI-H23 DNA Incorporation of Thiothionucleosides
Following Treatment with 1 μ M TdCyd for 24hr



Analysis of Thio-Deoxypyrimidines

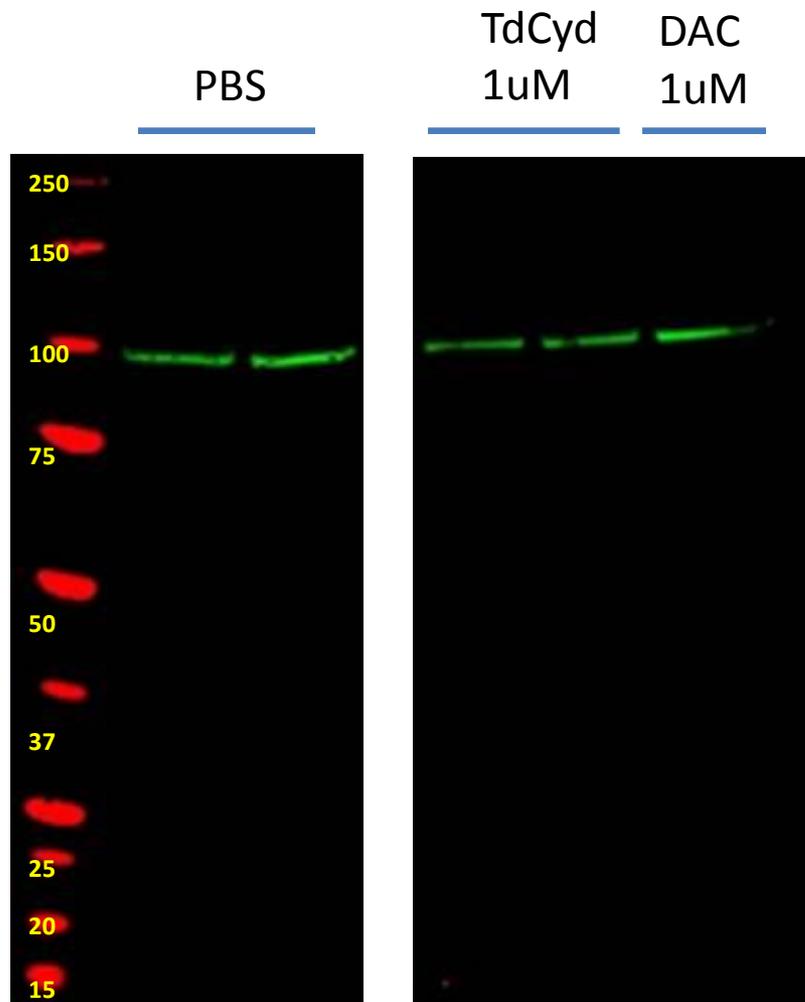
DNA from cell cultures was collected and purified through the use of spin column kits (Quiagen). Briefly, cells after treatment had the media removed, were washed with PBS, collected after trypsinization, counted, and aliquots containing $1-3 \times 10^6$ cells were used for analysis. After isolation of the purified DNA, the DNA was digested by the actions of phosphodiesterase and alkaline phosphatase for at least 4hr. Aliquots of the resulting solutions were analyzed directly by HPLC/HR-MS. Relative response rates of the individual deoxynucleosides were determined from standards to determine incorporation levels.

[1] Global methylation of DNA (i.e., the ratio of methyl-dC to dC in a digest of all DNA):

- [a] unaffected by TdCyd under the conditions of this experiment. This finding is consistent with our experiments in which TdCyd treatment has shown no effect on global methylation.
- [b] decitabine is a powerful modifier, producing a nearly 2-fold decrease in this experiment. This finding is consistent with many experiments and across multiple labs.

[2] TdC is incorporated into DNA, and increases during 24 hours to 96 hours, reaching about 12% substitution for endogenous dC.

~~TdT incorporation into DNA is lower than TdC, consistent with diminished de-amination in the presence of THU.~~



NCI- H23 96 hours in vitro continuous exposure shows no effect of Decitibine or TdCyd on DNMT3a levels

Primary: DNMT3a, (BD) 1 ug/ml
 Secondary: Li-Cor IRDye 800CW Goat anti-Ms (Licor) 0.1 ug/ml
 * All loaded at 20 mcg/well

Summary

- TdCyd is effective against both CEM and H23 (lung adenocarcinoma) Xenografts (nude mice, IV) and is better tolerated in mice than decitabine
- TdCyd and its metabolite thiothymidine are both incorporated into DNA
- Oral formulation of TdCyd shows adequate bioavailability
- TdCyd knocks down DNMT1 levels in NCI-H23 xenografts, while Decitabine does not
- Decitabine modifies CpG island methylation in H23 Xenograft DNA but TdCyd does not
- Neither compound affects DNMT3a levels in NCI H23 cells in vitro

Acknowledgements

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