RX-3117 promotes epigenetic effects in cancer cells through enhanced degradation of DNMT1

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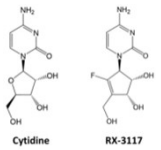
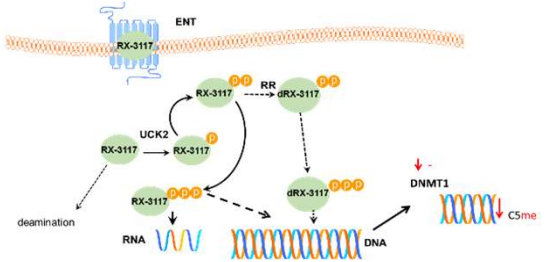
INTRODUCTION

- RX-3117 (fluorocyclopentenylcytosine) is a novel cytidine analog[1]
- RX-3117 inhibits DNA methylation similar to azacytidine (aza-CR) and aza--deoxycytidine (aza-CdR)
- RX-3117 is incorporated into RNA and DNA[2]
- RX-3117 is active in cell lines and tumors resistant to gemcitabine[2,3]



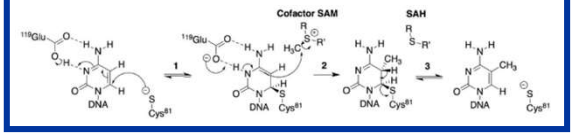


- RX-3117 is taken up by the human equilibrative nucleoside transporter (hENT) and activated by uridine-cytidine kinase 2 (UCK2) to RX-3117-MP[4,5]
- RX-3117 downregulates DNA methyltransferase 1 (DNMT1)[1,2]
- DNMT1 is responsible for maintaining methylation in newly synthesized DNA in the S-phase and methylates cytosine residues in hemimethylated DNA
- RX-3117 currently undergoes Phase IIa evaluation in advanced bladder cancer and also in combination with Abraxane in newly diagnosed pancreatic cancer

AIM OF THE STUDY

How does RX-3117 mediate down regulation of DNMT1?

Mechanism of DNA methylation
- Cysteine in DNMT attacks cytidine and a covalent DNA-enzyme complex is formed
- S-adenosyl-L-methionine (SAM) donates its methyl group to cytidine
- Proten elimination and release of cysteine



References

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2.Peters G.J., et al. Invest New Drugs, 31; 1444-1457, 2013
3.Yang M.Y et al. Anticancer Research, 34; 6951-6959, 2014
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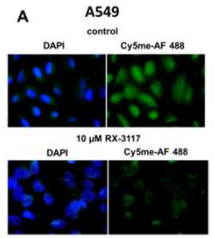
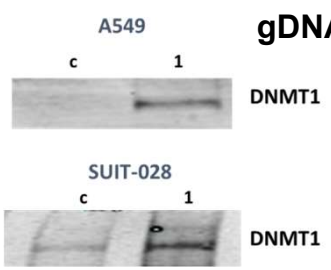
RESULTS

RX-3117 down-regulates total DNA methylation and traps DNA-DNMT1

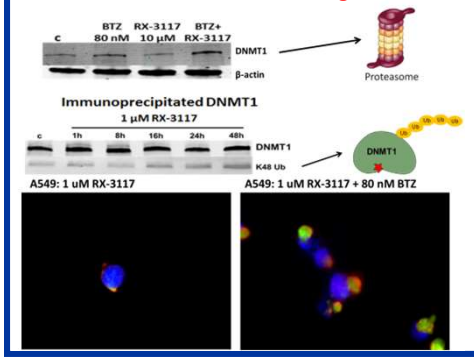


A549 cells were exposed to RX-3117. Global methylation was measured using immunofluorescence with an antibody against 5-methyl-cytosine.



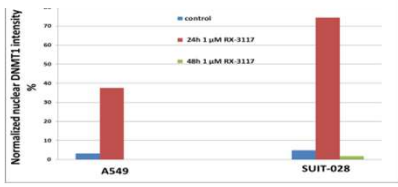
A549 and SUIT-028 cells were exposed to 1 μM RX-3117. DNA was isolated and stained for DNMT1

The proteasome inhibitor bortezomib prevents RX-3117 mediated downregulation of DNMT1



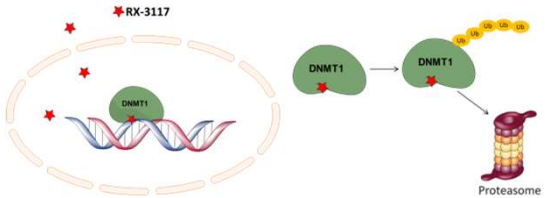
A549 cells were treated with 1 or 10 μM RX-3117 in the presence or absence of 80 nM bortezomib (BTZ) for 24 hr (upper blot), at the times indicated (middle blot) or 48 hr (lower figure). Cytosolic DNMT1 and the K48 ubiquitin tag K48 were visualized by Western blotting. The lower figure visualizes the ubiquitination using confocal microscopy. Green: DNMT1, blue, nucleus and red is proteasome.

Nuclear trapping of DNMT1 upon RX-3117 treatment

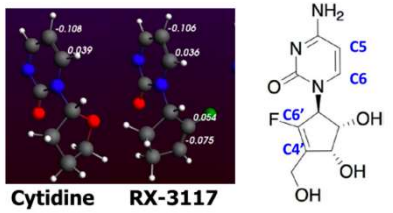


A549 and SUIT-028 cells were treated with 1 μM RX-3117 and nuclear trapping was analyszed using Image stream FACS analysis.

Proposed mechanism of DNMT1 downregulation



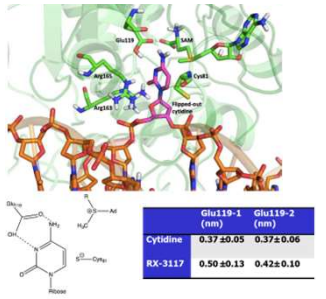
Quantum Modeling (QM) of DNMT1: *VDD charge analysis of RX-3117



	C5	C6	C4'	C6'
Cytidine	-0.108	0.039	*	*
RX-3117	-0.106	0.036	-0.075	0.054

Electrophilicity of the C5-C6 double bond. No significant difference in the VDD charge analysis for the C5-C6 bond were found (C6: 0.039 and 0.036; C5: -0.108 and -0.106)
*VDD, Voronoi Deformation Density

Molecular modeling (MD) of RX-3117 interaction with DNMT1



Base flipping of cytidine in DNMT1 (left) and the distance of cytidine and RX-3117 to key residues in DNMT1. The distance for cytidine between C6-Cys81 (0.44 nm) and C5-SAM (0.38 nm) were not different for RX-3117.

METHODS

Cell lines:
- Non-small cell lung cancer (NSCLC) A549 cells and pancreatic ductal adeno carcinoma (PDAC) SUIT-028 cells are cultured in DMEM supplemented with 10% fetal bovine serum (FBS)

DNMT1 binding to DNA and the effect of RX-3117
- DNMT1 protein expression was measured by Western Blotting after exposure to 1 μM RX-3117 for 24 hr
- DNMT1 binding to DNA was determined using immunostaining of DNMT1-DNA and ImageStream FACS
- Bands on Western blots were visualized using appropriate InfraRedDye using an Odyssey InfraRed imager.

DNMT1 ubiquitination
- DNMT1 ubiquitination was studied after exposure of the cells to 1 μM RX-3117 for 24-48 hr using the ubiquitin tag K48 . Inhibition of ubiquitination was achieved by exposure to 80 nM of the proteasome inhibitor bortezomib.
- Confocal Microscopy was used to study translocalisation of the nucleus to the cytosol

Molecular Modeling (MD) and Quantum Modeling (QM)

CONCLUSIONS

- **RX-3117 downregulates DNMT1 protein**
- **RX-3117 causes epigenetic changes in the cell by trapping DNMT1 to DNA**
- **DNMT1 is then translocated to the cytosol to the proteasome for degradation**
- **MD and QM leave the possibility open for a nucleophilic attack at C6 and of trapping by affecting the methyl transfer or leaving Cys81**

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