Inhibition of DNA methyltransferase by RX-3117 (fluorocyclopentenylcytosine) leads to upregulation of hypomethylated targets

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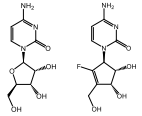
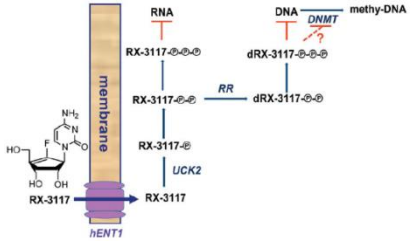
INTRODUCTION

- RX-3117 (fluorocyclopentenylcytosine) is a novel cytidine analog[1]
- RX-3117 resembles azacytidine (aza-CR) and aza--deoxycytidine (aza-CdR)
- RX-3117 is incorporated into RNA and DNA
- 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
- 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
- The rate of deamination of RX-3117 is much slower than gemcitabine
- A Phase 0 study has shown an excellent oral bioavailability of RX-3117
- RX-3117 currently undergoes Phase 1 evaluation
- The maximal tolerated dose is higher than 2,000 mg/day
- Currently both UCK2 and DNMT1 are being evaluated as potential biomarkers

AIMS OF THE STUDY

Does RX-3117 treatment affect:
•Expression and activity of DNMT1?
•DNA methylation?
•The function of proteins for which the gene is known to be regulated by methylation:

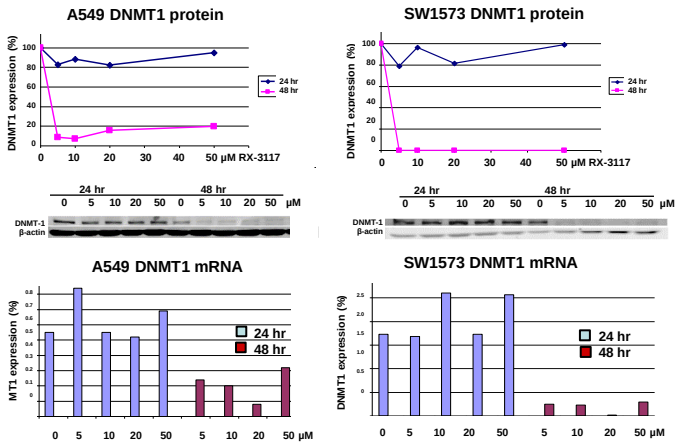
- **Proton-coupled folate transporter (PCFT): transports folic acid, methotrexate (MTX) and pemetrexed (PMX) at pH 5.5 and 7.4, and the gene is highly methylated[4,5]**
- **E-cadherin, an adhesion molecule**
- **p16INK a tumor suppressor protein**
- **O-6 Methylguanine DNA methyltransferase (MGMT), a DNA repair gene**

References

1. Choi W.J., et al,, J. Med. Chem. 55 (2012) 4521-4525.
2. Peters G.J., et al., Invest New Drugs 31 (2013) 1444-1457.
3. Yang M.Y., et al., Anticancer Research 34 (2014) 6951-6959.
4. Diop-Bove N.K., et al., MCT 8 (2009) 2424-31.
5. Gonen N., et al., BBRC 376 (2008) 787-92.
6. Jansen G., et al., JBC 273 (1998) 30189-30198.

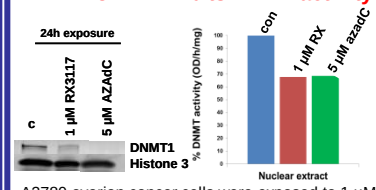
RESULTS

RX-3117 down-regulates DNMT1 protein and gene expression



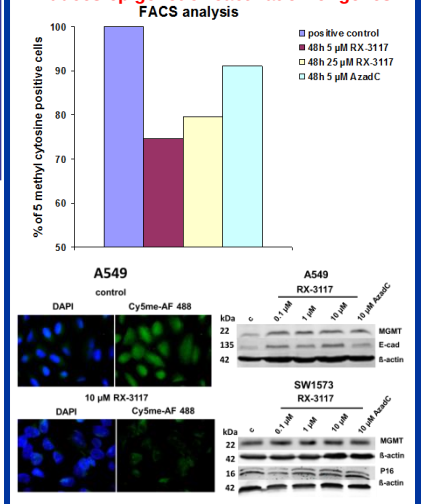
A549 and SW1573 cells were exposed to 5, 10, 20 and 50 μM RX-3117 for 24 or 48 hr. Cells were harvested and protein expression was measured using western blotting (upper panel). RNA was isolated and gene expression was measured using RT-PCR (lower panel)

RX-3117 inhibits DNMT activity



A2780 ovarian cancer cells were exposed to 1 μM RX-3117 or 5 μM aza-dC for 24 hr. Nuclear extracts were isolated and DNMT1 expression was measured by western blot and activity by a commercial kit as described in the Methods.

RX-3117 inhibits DNA methylation and induces epigenetic reactivation of genes



A549 cells were exposed to RX-3117 or aza-dC and global methylation was measured using FACS (upper panel) or immunofluorescence (middle panel) with an antibody against 5-methyl-cytosine. Control cells were set at 100% (upper panel).

The lower panel shows the expression of MGMT, E-cadherin and p16INK4 after exposure to RX-3117 and aza-dC

METHODS

Cell lines:
- CCRF-CEM cells and its MTX resistant variant CEM-MTX, characterized by a deficiency of the reduced folate carrier (RFC)[6]. The PCFT gene in CEM cells is highly methylated[5]
- CEM cells are cultured in RPMI medium with 10% fetal bovine serum (FBS)
- A549 and SW1573 non-small cell lung cancer (NSCLC) and A2780 ovarian cancer cell lines, which are cultured in DMEM medium with 10% FBS

Western Blots, immunhistochemistry and RT-PCR
- DNMT1 protein expression was measured by Western Blotting after exposure to RX-3117 for 24 or 48 hr
- DNMT1 RNA expression was measured by real-time PCR after 24 and 48 hr exposure to RX-3117
- DNMT enzyme activity was measured in isolated nuclei after exposure 1 μM RX-3117 or 5 μM aza-CdR using a DNA methyltransferase assay kit provided by EpiGentek using the ability of a CpG dinding domain to bind to methylated DNA.
- In A549 cells the effect of 5 μM RX-3117 on overall methylation was measured with a specific antibody against 5-methyl-cytosine
- Bands on Western blots were visualized using appropriate InfraRedDye using an Odyssey InfraRed imager.

Methotrexate (MTX) transport
MTX transport was measured using radiolabellled MTX in CEM wild type and CEM-MTX cell lines:
- CEM cells have a high RFC activity; CEM-MTX are completely deficient in RFC-mediated transport
- CEM cells have a highly methylated PCFT transporter and a very low PCFT mediated transport[5]
- MTX transport at pH 7.4 is predominantly RFC mediated and less than 2% by PCFT
- Folic acid was used to inhibit PCFT mediated transport
- L-leucovorin (L-LV) was added to completely inhibit RFC medited transport
- CEM and CEM-MTX cells were exposed to 29.6 μM RX-3117 and to 0,19 μM aza-CdR as a positive control
- MTX transport was measured after 24 hr to the drugs in a 3 minutes uptake assay using 2 μM [3',5,'7-^{3}H]-MTX.

Statistics were done using the Student's t-test.

CONCLUSIONS

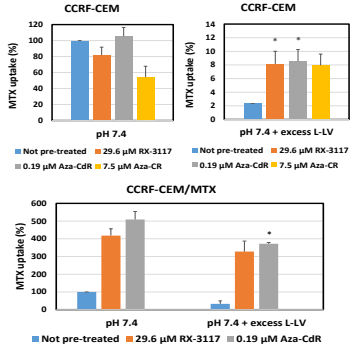
- **RX-3117 downregulates DNMT1 protein and RNA expression**
- **RX-3117 decreases DNA methylation**
- **RX-3117 mediated hypomethylation increases:**
  - **expression of MGMT and E-cadherin**
  - **PCFT mediated transport of MTX**

RX-3117 is a new epigenetic modulator

RESULTS

RX-3117 upregulates PCFT mediated transport of MTX

	CEM	CEM/MTX
pH level	MTX uptake (pmol/min/1*10^7 cells)	
pH 5.5	1.11 ± 0.14	0.49 ± 0.13
pH 5.5 + 1 mM FA	0.66 ± 0.11	0.51 ± 0.21
pH 7.4	2.8 ± 0.52	0.03 ± 0.02
pH 7.4 + 1 mM L-LV	0.046 ± 0.01	0.01 ± 0.01



Effect of 24 hr exposure to RX-3117 on PCFT mediated transport of MTX. Folic acid (FA) was added to inhibit PCFT and L-LV to inhibit RFC mediated MTX transport. Aza-CdR and Aza-CR were included as a positive control

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