Evaluation of a Novel Compound for Anticancer Therapy

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1. Background

- DNA double strand breaks (DSBs) are a highly cytotoxic form of DNA damage that can form naturally during DNA replication
- DSBs signal via Ataxia-telangiectasia and Rad3-related (ATR) for cell cycle arrest, and primarily via homologous recombination repair (HRR) for repair
- BRCA1/BRCA2 are key components of HRR and are commonly mutated in cancer
- When HRR is defective, e.g. in BRCA-deficient cells, a back-up pathway called microhomology-mediated end joining (MMEJ) is used

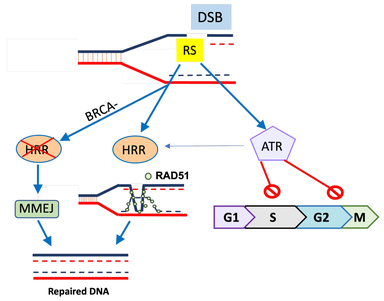


Figure 1: Endogenous DSB repair. Replication stress (RS) caused by DSB signals to ATR and HRR for cell cycle arrest and DSB repair respectively. ATR also signals to HRR. BRCA-deficient cells are defective in HRR so instead use MMEJ to repair DNA

2. Aim and Hypothesis

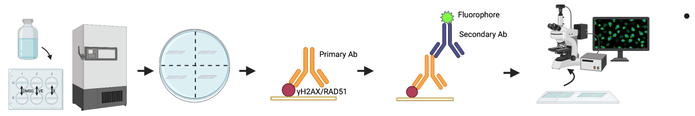
Aim: To evaluate the **cytotoxicity** of a novel anti-cancer drug (**Compound X**), that targets the MMEJ pathway, as a **single agent** and in **combination** with a known ATR inhibitor (**VE-821**) on **wildtype** (WT) and **BRCA2 deficient** (B2-) human colorectal adenocarcinoma (**DLD1**) cells

Hypothesis:
- MMEJ inhibitors cause DNA damage accumulation which activates ATR
- MMEJ inhibition preferentially kills cells lacking HRR (B2-)
- ATR inhibitors are synergistic with MMEJ inhibition to kill cells

3. Methods

A. Measurement of DNA Damage by Immunofluorescence

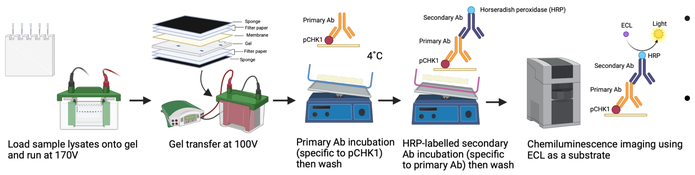
Following cell culture, seeding and drugging…



- Used fluorescent antibodies to γH2AX and RAD51 for determination of DNA damage levels and HRR function respectively

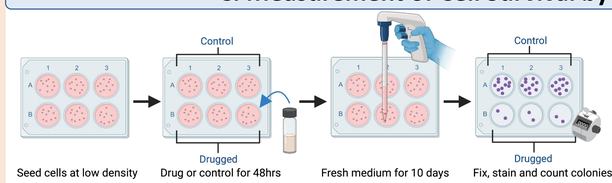
B. Measurement of ATR Activation by Western Blotting

Following cell culture, seeding, drugging and harvesting then sample lysate preparation….



- ATR signals to cell cycle checkpoints via CHK1 phosphorylation at serine 345
- Looked at pCHK1 (phosphorylated CHK1) mean pixel values to determine cellular ATR activity

C. Measurement of Cell Survival by Colony Formation



- Single agent and combination clonogenic assays were carried out

Images created with BioRender.com

4. Results

A. Compound X increases DNA damage and repair by HRR

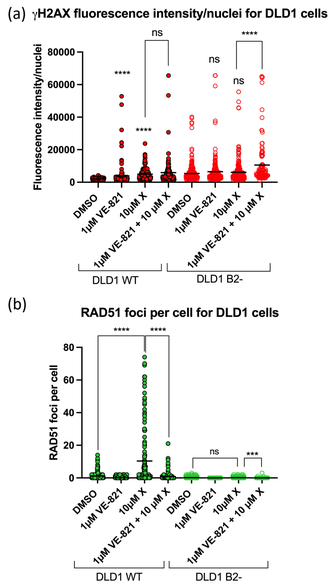


Figure 2: γH2AX fluorescence intensity/nuclei (a) and RAD51 foci per cell (b) for DLD1 WT and B2- cells. **** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05, ns = no significant difference. Significance determined using Mann-Whitney U test

γH2AX
- Single agent Compound X (sX) and single agent VE-821 (sVE-821) increased DNA damage in WT cells
- Didn't see expected increase in DNA damage between sX and combination in WT cells
- Higher levels of DNA damage in B2- cells than in WT cells

RAD51
- sX significantly increased RAD51 foci in WT cells, indicating increased reliance on HRR
- sVE-821 decreased HRR function in WT cells
- VE-821 + X combination led to significant decrease in HRR function compared to sX in WT cells
- No RAD51 foci seen in B2- cells (HRR-defective)

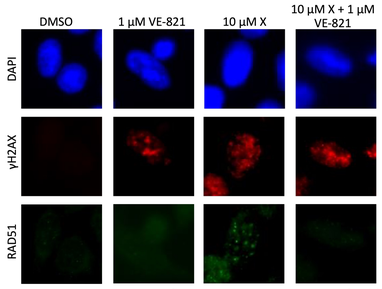
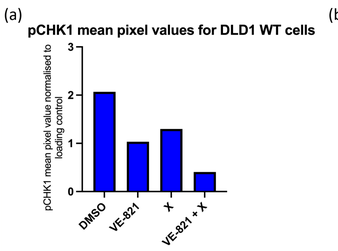


Figure 3: DLD1 WT immunofluorescence microscopy images for different treatments. Visualised using Leica DM6 microscope

B. Compound X did not activate ATR



Would have expected sX to increase ATR activity in WT cells as it significantly increased DNA damage levels – this should signal to ATR

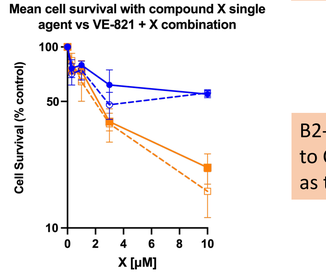
Figure 4: Mean pCHK1 mean pixel values for WT (a) and B2- (b) cells. Calculated from results of two western blot repeats using Vinculin and Ponceau S as loading controls

- sVE-821 decreased ATR activity in WT and B2- cells
- sX decreased ATR activity in WT and B2- cells
- VE-821 + X decreased ATR activity compared to sX in WT and B2- cells

C. Compound X preferentially kills HRR-defective cells and has modest synergy with ATR inhibitor

VE-821 LC50 (μM)
WT = 1.14
B2- = 1.09

VE-821 LC50 ≈ 1 μM for B2- and WT cells so this concentration was used in combination experiments



B2- cells were more sensitive to Compound X than WT cells as they rely on MMEJ

Figure 6: Mean cell survivals with Compound X single agent vs VE-821 + X combination. Colony counts from three single agent X and VE-821 + X combination clonogenic assay repeats with DLD1 WT and B2- cells were used to calculate mean cell survivals (% control) at different Compound X concentrations and determine mean LC50 values. Standard error of the means shown

Table 1: **Table showing potential sensitisation**. Mean LC50 and cell survival at 3 μM X values determined from three DLD1 WT and B2- combination clonogenic assay repeats. Standard error of the means shown

	WT cells	B2- cells
LC50 X (μM)	9.03 ± 0.73	2.24 ± 0.33
LC50 X (μM) + 1 μM VE-821	9.18 ± 0.24	2.06 ± 0.74
Fold potentiation	0.98	1.09
% survival at 3 μM X	61.67 ± 13.04	38.69 ± 4.31
% survival at 3 μM X + 1 μM VE-821	47.98 ± 8.16	37.69 ± 7.88
Fold potentiation	1.29	1.03

- VE-821 only sensitised WT cells to sX at concentrations < 3 μM
- Would expect greater sensitisation for WT cells as HRR can occur with sX but cannot with combination

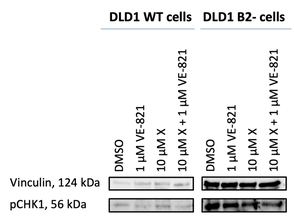


Figure 5: Blot from N=1 western blot repeat. pCHK1 levels determined using vinculin as loading control

5. Conclusions

- Compound X **preferentially killed BRCA2-deficient cells** and **increased DNA damage** in wildtype (WT) cells
- Unexpectedly, Compound X **didn't activate ATR** and was **only synergistic with ATR inhibition at low concentrations**
- **Novel finding** - Compound X **upregulated homologous recombination repair** (HRR) in WT cells, suggesting HRR is a backup pathway for microhomology mediated end-joining
- *Further studies are required to understand the underlying mechanisms behind these findings*