NOVEL SYNTHETIC THIENOPYRIDINE DERIVATIVES INDUCE TUBULIN DISRUPTION AND CELL CYCLE ARREST IN MALIGNANT B CELLS WITH LEVEL OF ACTIVITY RELATED TO THEIR CHEMICAL STRUCTURE

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Introduction

- Treatment for B-cell malignancies often involves the use of Microtubule Targeting Agents (MTA) as part of combination therapy[1]
- MTA drugs including vincristine and vinblastine have been hugely successful but toxicity and development of drug resistance can limit their effectiveness
- We have synthesised 10 novel compounds (thienopyridines), each with a modified chemical structure (Figure 1), that we have tested as potential new MTAs.
- Thienopyridines, commonly used as anti-thrombotic therapy, have recently been identified as potential anti-cancer agents, with PLCγ, Jak2 and Lck suggested as direct targets, while mitotic spindle inhibition via microtubule de-stabilisation has also been hypothesised[2,3].
- Investigation into the MTA activity of thienopyridines may open up future therapeutic use of these compounds.

Aims

To investigate the anti-proliferative and microtubule-disrupting properties of ten novel thienopyridine compounds, in B-cell Lymphoma cells.

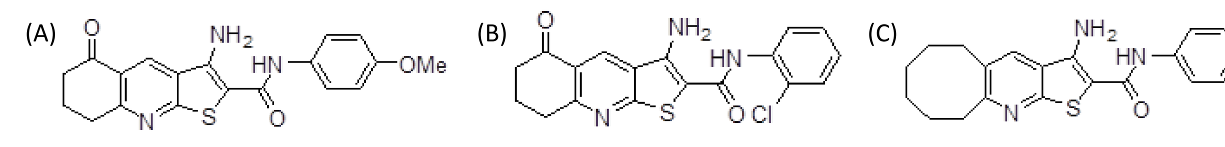

Figure 1: The chemical structures of three novel thienopyridine compounds (A) DJ0109, (B) DJ0171 and (C) DJ0199.

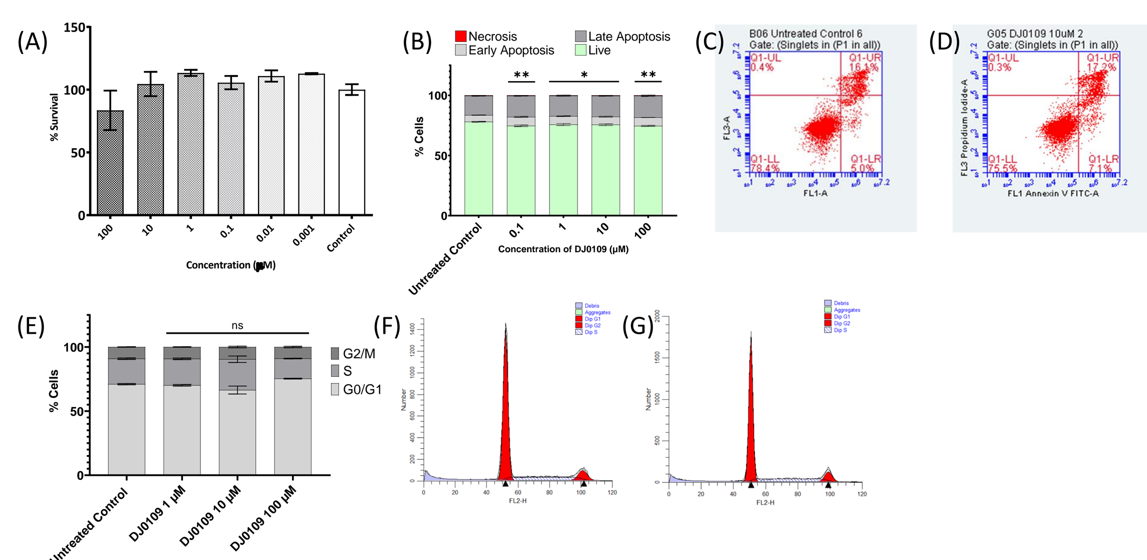

Figure 2 Cell viability assays for DAUDI cells treated with various concentrations of Thienopyridine DJ0109 for 48 hours as determined by MTS assay (A) and Annexin V/Propidium Iodine Flow Cytometry (B), and Cell Cycle Analysis with Propidium Iodine Flow Cytometry (E). Data are presented as mean ±SEM of n=3 (untreated control n=6). For MTS (A) data have been normalised against 100 % survival for un-treated cells, and 0 % survival for cells treated with 50 % ethanol for 24 h. For Annexin V/Propidium Iodide (B) and Cell Cycle (E) data are presented as mean ±SEM of n=3.

Representative dot plots for Annexin V/PI (C-D) are presented where Early Apoptosis (Lower Right Quadrant (LR)) = Annexin V+/PI-, Late Apoptosis (Upper Right Quadrant (UR)) = Annexin V+/PI+, Live Cell (Lower Left Quadrant (LL)) = Annexin V-/PI-, Necrosis (Upper Left Quadrant (UR)) = Annexin V-/PI+.

Representative Histograms for Cell Cycle Untreated Control (F) and treatment with 10 µM DJ0109 (G).

Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test * represents < 0.05, ** represents < 0.01, *** represents < 0.001.

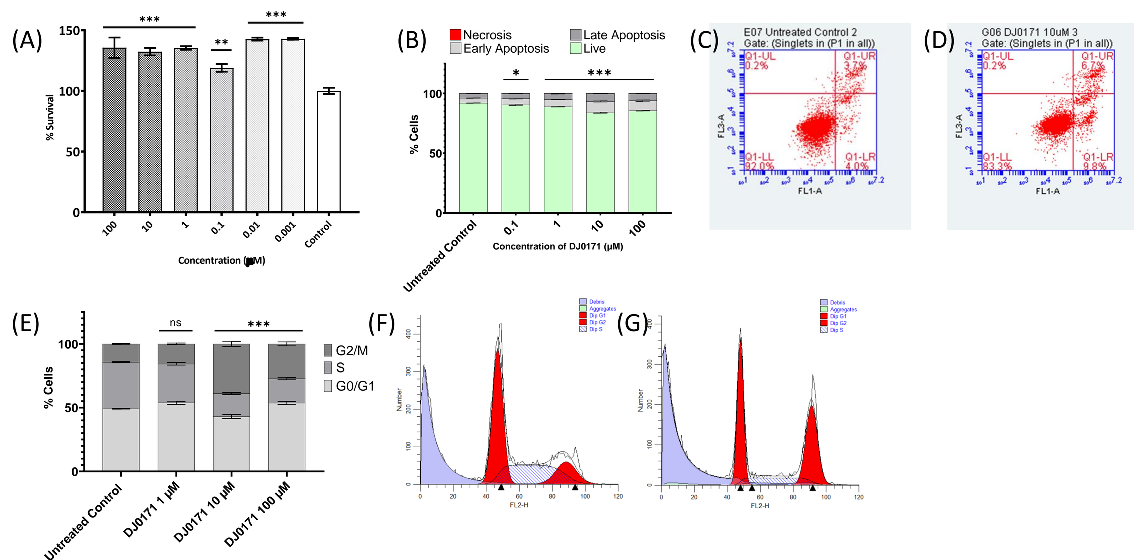

Figure 3 Cell viability assays for DAUDI cells treated with various concentrations of Thienopyridine DJ0171 for 48 hours as determined by MTS assay (A) and Annexin V/Propidium Iodine Flow Cytometry (B), and Cell Cycle Analysis with Propidium Iodine Flow Cytometry (E). Data are presented as mean ±SEM of n=3 (untreated control n=6). For MTS (A) data have been normalised against 100 % survival for un-treated cells, and 0 % survival for cells treated with 50 % ethanol for 24 h. For Annexin V/Propidium Iodide (B) and Cell Cycle (E) data are presented as mean ±SEM of n=3.

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Representative Histograms for Cell Cycle Untreated Control (F) and treatment with 10 µM DJ0171 (G).

Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test * represents < 0.05, ** represents < 0.01, *** represents < 0.001.

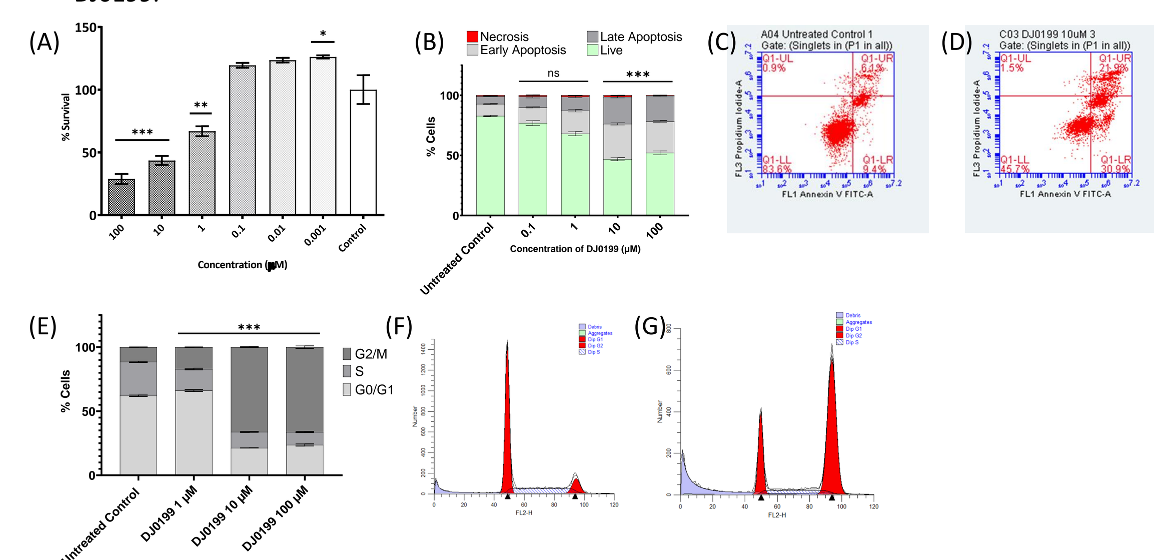

Figure 4 Cell viability assays for DAUDI cells treated with various concentrations of Thienopyridine DJ0199 for 48 hours as determined by MTS assay (A) and Annexin V/Propidium Iodine Flow Cytometry (B), and Cell Cycle Analysis with Propidium Iodine Flow Cytometry (E). Data are presented as mean ±SEM of n=3 (untreated control n=6). For MTS (A) data have been normalised against 100 % survival for un-treated cells, and 0 % survival for cells treated with 50 % ethanol for 24 h. For Annexin V/Propidium Iodide (B) and Cell Cycle (E) data are presented as mean ±SEM of n=3.

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Representative Histograms for Cell Cycle Untreated Control (F) and treatment with 10 µM DJ0199 (G).

Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test * represents < 0.05, ** represents < 0.01, *** represents < 0.001.

Methods

- DAUDI cells were cultured in the presence of novel thienopyridines DJ0109, DJ0171 and DJ0199 at a range of concentrations (100 µM to 1 nM) for 48 h
- **MTS assay** was used to assess biochemical activity as an indicator of viability post-treatment.
- Apoptosis/necrosis was assessed using **Annexin V-FITC/Propidiuim Iodide flow cytometry assay.**
- **Cell cycle analysis** was assessed by flow cytometry using single staining with propidium iodide following fixation and permeabilisation using 70% ethanol, and RNA depletion using RNASe A. Analysis was performed using BD Cell Quest Pro software.
- **Tubulin Assay** was performed to assess the direct effects of thienopyridines on Tubulin polymerisation. Thienopyridines at 10 µM concentration were mixed with porcine Tubulin and heated to 37 °C with absorbance at 340 nm read every 30 seconds for 1 hour using a plate reader. Paclitaxel = positive stabilisation control. Nocodazole = negative stabilisation control G-PEM = untreated control.
- **Confocal Microscopy** was used to assess the effects on tubulin. Cells were fixed in 4% paraformaldehyde, permeabilised in 1% Triton X-100, blocked with 5 % Normal Goat Serum then stained with α-Tubulin (DM1A) Mouse mAb (Alexa Fluor® 488 Conjugate) antibody and DAPI nucleic acid stain. Imaged at x630 resolution using Leica TCS SPE confocal microscope.
- **Western Blotting** for target proteins relating to BCR-signalling (PLCγ2 + P- PLCγ2 (Y759)) and cell cycle control (Cyclin B1, cdc-2 + P-cdc2 (Y15)) was conducted on lysates from DAUDI cells treated for 48 h with 10 µM of thienopyridines. BCR signalling cascade was activated with 1 minute of stimulation using anti-IgM prior to cell lysis with RIPA buffer.

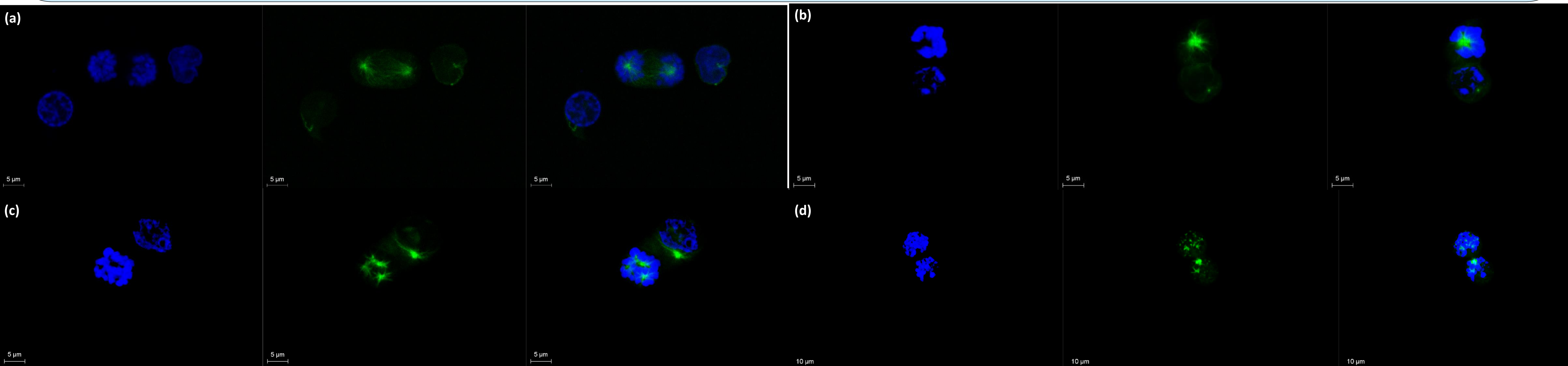

Figure 5: Fluorescent Confocal Microscopy micrographs of duel stained DAUDI cells during mitosis at x63 magnification following treatment with (a) 10 % RPMI Media; (b) 10 µM DJ0109; (c) 10 µM DJ0171 and (d) 10 µM DJ0199 for 48 h. (a) Cell at mitosis, with α-Tubulin spindle fibres and chromosomes visible and presence of two polar centrioles; (b) Two cells, with presence of α-Tubulin spindle; (c) two cells, one at mitosis showing multipolar arrangement of α-Tubulin fibres; (d) Two cells, one at mitosis showing diffuse α-Tubulin . Blue = DAPI, Green = α-Tubulin Alexa Fluor 488, Blue + Green = Overlay

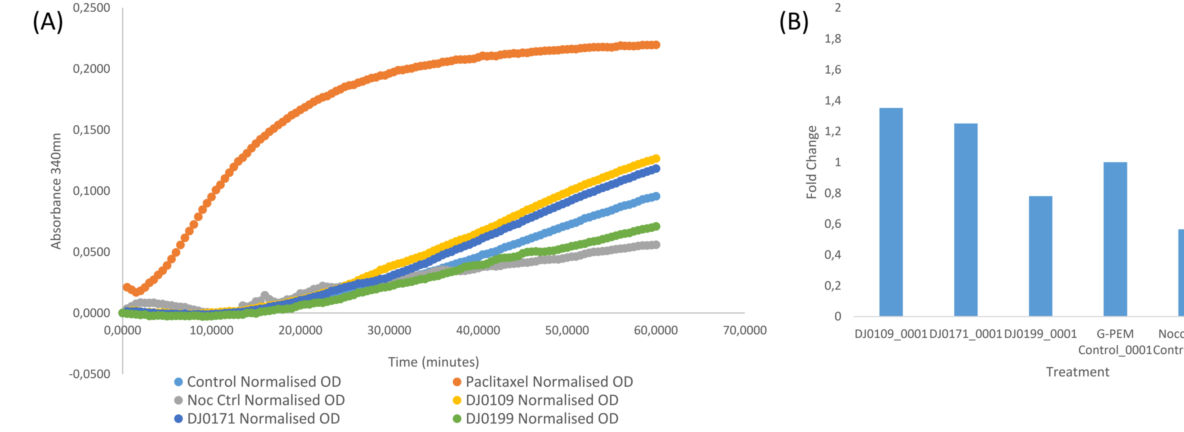

Figure 6: Tubulin Polymerisation Kinetic Assay (A) and Fold Change of average rate of reaction against untreated (G-PEM) control (B).
Ice cold 100 µL Porcine Tubulin in G-PEM buffer was added to 10 µL of 10x concentration Thienopyridine in DMSO (final concentration 10 µM), G-PEM buffer (Untreated control), 10 x concentration of Paclitaxel (final concentration 10 µM, Positive Control) and 10 x concentration of Nocodazole (final concentration 10 µM, Negative Control) on a pre-heated (37°C) 96-well plate with absorbance at 340 nm measure every 30 seconds for 60 minutes.
Thienopyridine Treatments (10 µM DJ0109, DJ0171, DJ0199 in DMSO); Paclitaxel positive tubulin control (10 µM in DMSO); Nocodazole negative tubulin control (10 µM in DMSO); G-PEM Control (80 mM PIPES pH 6.9, 2 mM MgCl2, 0.5mM EGTA, 1 mM GTP in dH_2O)

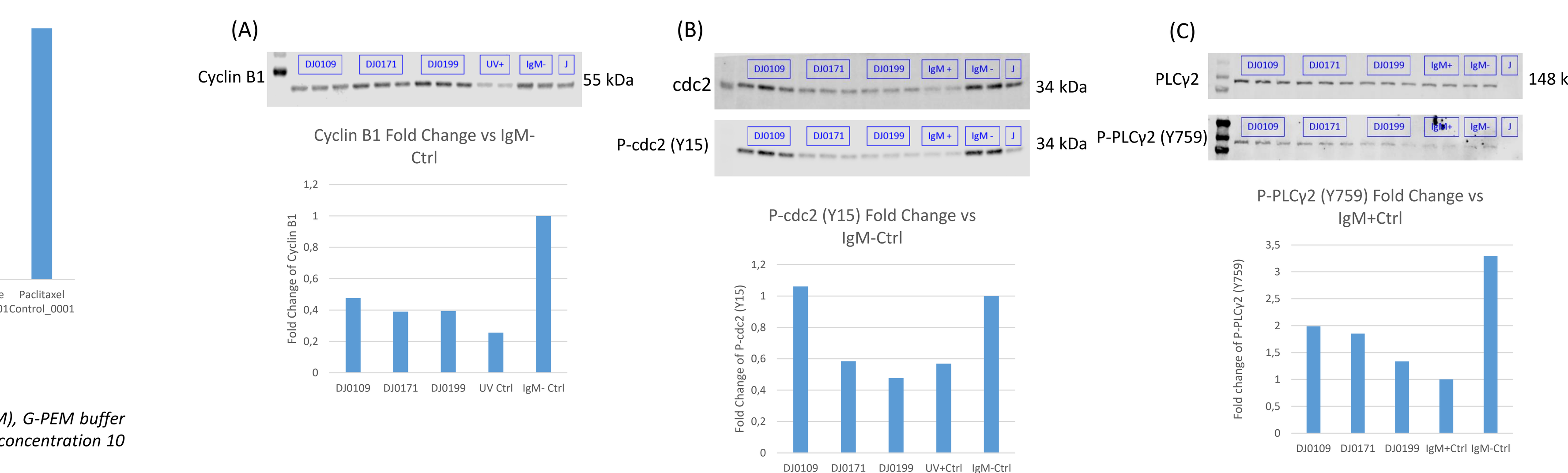

Figure 7: Western Blot Analysis of Thienopyridine-treated DAUDI cell lysates for proteins Cyclin B1 (A), phospho-cdc2 (Y15) (B) and phospho-PLCγ2 (Y759) (C).
Equal volumes of DAUDI protein lysates (8 µg) were loaded per lane of a 10 % Bis/Acrylamide gel and separated by SDS-PAGE electrophoresis and wet transfer to Millepore Immobilon-FL PVDF membrane. Target proteins were detected using CST Cyclin B1 1:1000 (Rabbit), CST cdc2 1:1000 (Mouse), CST P-cdc2 (Y15) 1:1000 (Rabbit), ABCAM PLCγ2 1:2000 (Mouse), CST P-PLCγ2 1:1000 (Rabbit) primary antibodies and Li-Cor IRDye 800CW Goat anti-Mouse IgG 1:15000 and Li-Cor IRDye 680RD Goat anti-Rabbit IgG 1:15000 secondary antibodies. Band densitometry was analysed using Li-Cor Image Studio Lite software with Cyclin B1 normalised to Total Protein Stain and P-cdc2/P-PLCγ2 normalised to pan-protein. Fold change relative to control samples was calculated using Microsoft Excel.

Results

- Across MTS (Figure 1-3 A), AVPI (Figure 1-3 B-D) and Cell Cycle (Figure 1-3 E-F) assays, DJ0199 showed greater activity compared to the other compounds, inducing a significant reduction in cell viability, an increase in early and late apoptosis and a shift towards G2/M at 10 µM and 100 µM concentrations. Necrosis was not induced by these compounds when assessed using AVPI.
- DJ0171 did not show a reduction on cell viability by MTS assay, but was found to exhibit a similar but weaker activity to DJ0199 when all other assays were considered. Increased G2/M arrest was also seen following treatment with DJ0171 at 10 µM and 100 µM.
- DJ0109 and DJ0171 showed a stabilising effect on tubulin polymerisation while DJ0199 showed a destabilising effect at 10 µM dosage (Figure 6) and this was reflected in the patterns of microtubule disruption via confocal microscopy (Figure 5).
- All three compounds showed reduced Cyclin B1 expression compared to IgM negative control on western blot analysis (Figure 7), while DJ0171 and DJ0199 showed reduced inhibitory phosphorylation of cdc2 at Tyrosine 15. All compounds showed an increase in the activating phosphorylation of PLCγ2 at Tyrosine 759 relative to untreated control lysates.

Conclusions

- Novel thienopyridine derivatives have anti-mitotic activity in malignant B-cells - Potential for use in the treatment of mature B-cell malignancies.
- Cell cycle results mirror those in breast cancer cell lines, also highlighting cell cycle arrest at the G2/M checkpoint[3]. The observed patterns of inhibition in relation to chemical structure are similar to those previously published for these compounds[2, 3].Future work aims to clarify their effect on PLCγ2 and the mechanism by which this may result in the observed effect on microtubule assembly.

References

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