Proceedings

Pre-clinical investigation of inhibition of the DNA damage response as a targetted therapy in myeloproliferative neoplasms shows synergism of ATR inhibitors with standard-of-care treatment †.

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† Presented at the 1st International Electronic Conference on Cancers: Exploiting Cancer Vulnerability by Targetting the DNA Damage Response, Virtual, 1/2/21 to 14/2/21.

Received: 6/12/20; Accepted: 21/12/20; Published: 1/2/21

Abstract: Myeloproliferative neoplasms (MPNs) are a group of haematological malignancies arising from haematopoietic stem cells (HSCs) with acquired driver mutations in *JAK2, MPL* and *CALR*. Current therapies are not selective for the mutant HSC population. Increased replication stress is seen in the presence of mutant *JAK2*, suggesting DNA damage response inhibitors (DDRi) may differentially affect mutant HSCs over wild-type HSCs to restore normal haematopoiesis. Using *JAK2V617F* and *CALR* (del 52) mutant cell lines, we observed that ATR inhibition (ATRi) by AZD6738 and VE-821 significantly reduced viability. The combination of ATRi and hydroxyurea/ JAK1/2 inhibitor - ruxolitinib demonstrated high synergism in both apoptosis induction and proliferation arrest. This study provides preliminary evidence that ATRi combined with standard therapies may be exploited in MPNs harbouring *JAK2* and *CALR* mutations.

Keywords: Myeloproliferative Disorders; Janus Kinase 2; Calreticulin; Ataxia Telangiectasia Mutated Proteins; Janus Kinase Inhibitors; Hydroxyurea

1. Introduction

Myeloproliferative neoplasms (MPNs) are a group of haematological malignancies that arise from haematopoietic stem cells with acquired driver mutations in *JAK2, MPL* and *CALR*. MPNs comprise polycythemia vera (PV), essential thrombocytemia (ET) and myelofibrosis (MF) which are

characterized by aberrant blood cell production resulting in thrombosis, splenomegaly, bone marrow fibrosis and leukemia [1].

Interestingly, genes involved in the DNA double-strand break (DSB) repair pathways of BRCA-dependent homologous recombination repair (HRR) and DNA-dependent protein kinase-mediated nonhomologous end-joining (D-NHEJ), were upregulated in the presence of JAK2(V617F), MPL(W515L), and CALR(del52) frequently observed in MPNs [2]. As JAK2 activation promotes expression of HRR and D-NHEJ genes, it might prevent DNA damage and contribute to chemotherapy resistance [2]. As it was previously reported JAK1/2 inhibitor-ruxolitinib caused downregulation of key members of HRR (BRCA1, RAD51) and D-NHEJ (LIG4) in *JAK2(V617F), MPL(ex10mut)*, and *CALR(del52)*$^{+}$ cell lines, resulting in reduced HRR and D-NHEJ activities [2].

Currently used MPN therapies include hydroxyurea, which induces DNA double-strand breaks (DSB) [3] and ruxolitinib. They are not selective for the disease clone, do not induce complete remission and are limited by frequent side effects. Particularly hydroxyurea therapy is often associated with adverse reactions such as anemia, leukopenia, gastrointestinal symptoms and mucocutaneous toxicity [4]. Hydroxyurea resistance or intolerance occurs in 15 to 24% of patients with PV [4, 5]. Ruxolitinib has been shown to prolong life in patients with myelofibrosis [6], but revealed limited benefit in PV [7] and ET [8] leading to its registration as second-line therapy. All this highlights the need for exploration of new regimens and provides the rationale for evaluation of combination therapies.

DNA damage repair system targeting compounds have been little investigated in haematological malignancies. Tumour reliance on more than one DDR pathway leads to ineffective cell death induction upon single agent use [9]. This provides a rationale to evaluate DDRi in combinations with drugs that may contribute to DSBs formation (hydroxyurea) or indirect DDR pathway inhibition (ruxolitinib). Using *JAK2V617F* and *CALR* (del 52) mutant cell lines, we aimed to determine the effect of single-agent DDRi on cell viability and apoptosis. Moreover, we evaluated the efficacy of DDRi in combination with currently registered therapies - hydroxyurea and ruxolitinib.

2. Materials and Methods

2.1. Cell culture

Cell lines expressing JAK2 (V617F)- HEL and CALR (del52)- MARIMO were seeded at 0.5 x 10^6 cells/ml in RPMI 1640 + GlutaMAX medium supplemented with 10% FBS, 100 units/ml penicillin and 100μg/ml streptomycin. All cells were cultured in a 37°C humidified incubator with 5% CO_2.

2.2. Drugs

Both cell lines were treated with a drug panel comprising hydroxyurea, ruxolitinib, methotrexate, AZD6738 (ATRi), NU7441 (DNA-PKi), Olaparib (PARPi) and VE-821 (ATRi). All drugs were dissolved in 100% DMSO. All treated cells experienced a maximum final 3:1000 dilution of DMSO. Same DMSO dilution containing no drugs was always used as a control. Concentrations used for the experiments were designed based on other studies investigating DDRi [9-12].

2.3. Viability and proliferation assessment

Cells at a density of 2× 10^5/ml were exposed to single drugs and their combinations. After 48-hour exposure proliferation assessment and apoptosis assay was performed. AlamarBlue Cell Viability Reagent (Thermofisher) was used for cell proliferation evaluation according to manufacturer's instruction. AlamarBlue was added to a final dilution of 1:10. The colour was developed after 4 h of incubation and measurement performed on a SoftMax Pro 5.2 plate reader at an excitation wavelength of 570nm and emission wavelength of 600nm. Cell viability was calculated as the ratio of absorbance of treated cells to the absorbance of control cells. Dead Cell Apoptosis Kit (Thermofisher) was used for apoptosis assessment on a BD FACSCalibur, with cells gated on

forward and side scatter, and 10,000 events recorded per condition. Staining with annexin V-FITC and propidium iodide for flow cytometry were used to evaluate the cell death mechanism.

2.4. Statistical analysis

Statistical analysis was performed using GraphpadPrism v8.4.3. Data are represented as mean and standard deviation. Combination index and synergism was evaluated in CompuSyn software. Synergism was defined as a combination index (CI) lower than 1.0 [13].

3. Results

3.1. Single drugs

In *JAK2* and *CALR* mutated cell lines, ATR inhibition by AZD6738 or VE-821, DNA-PKs inhibition by NU7441 and hydroxyurea each reduced viability compared to DMSO control, whereas PARP inhibition by olaparib had a minimal effect. Ruxolitinib alone had a modest effect in the presence of *JAK2V617F* and no effect in *CALR* (del 52) mutated cells (Figure 1).

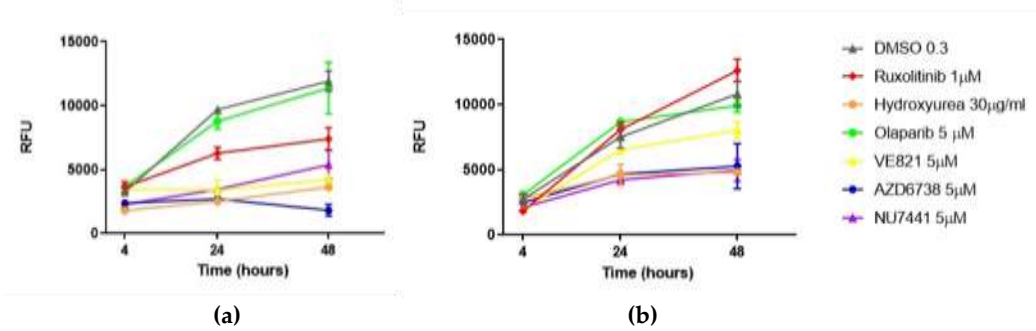
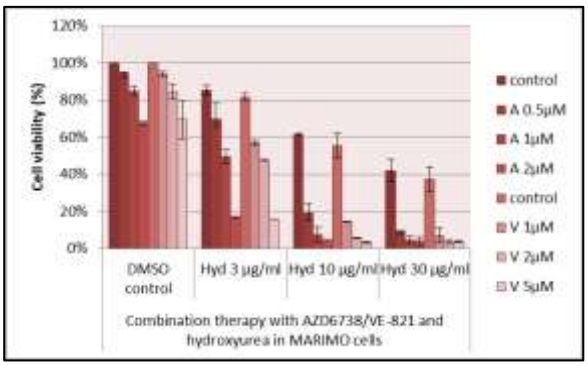


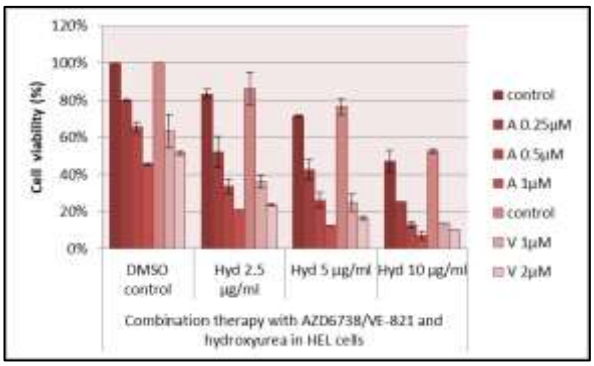
Figure 1. Effect of standard therapies and DDRi on cell viability and proliferation, assessed with AlamarBlue assays, in cell lines representing MPNs. Relative fluorescence unit (RFU) at different time points for (**a**) HEL cells (*JAK2V617F)*; (**b**) MARIMO cells (*CALR del 52*). Error bars show mean and standard deviation of three replicates.

3.2. Drug combinations

Combinations of DDRi with currently registered drugs - ruxolitinib and hydroxyurea revealed promising synergistic toxicity. The combination of both different ATRi (AZD6738/ VE-821) and hydroxyurea demonstrated consistent high synergism in both cell lines in terms of cell viability reduction (Figure 2a and 2b). For the combination of 20μg/ml hydroxyurea and 1μM AZD6738, the CI was 0.2 in HEL cells and 0.1 in MARIMO cells. Synergistic toxicity was also observed for ruxolitinib and ATR inhibitor combination, but only in *JAK2* mutated cell line, where the CI was 0.3 for the combination of 1μM ruxolitinib and 2μM AZD6738 (Figure 2c).

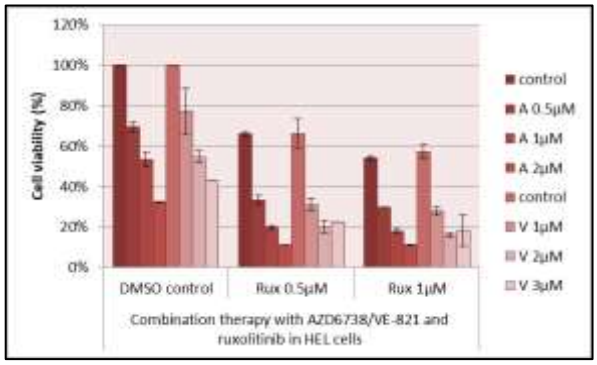


(**a**)



(**b**)

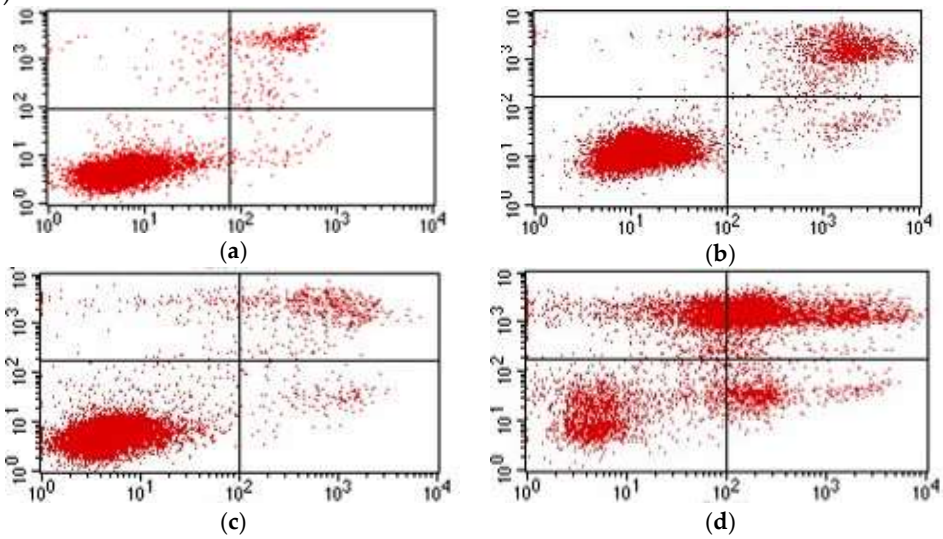
Figure 2. Cell viability in HEL cells (**a**) and MARIMO cells (**b**) measured by AlamarBlue assay after 48-hour exposure to combinations of hydroxyurea (Hyd) with ATRi- AZD6738 (A) or VE-821 (V), and in HEL cells (**c**) after exposure to ruxolitinib (Rux) with ATRi. Error bars show mean and standard deviation of two replicates.



(**c**)

3.3. Apoptosis

The effects seen on AlamarBlue assays could be a consequence of decreased cell proliferation or increased cell death. To investigate these alternatives, the percentage of live cells following drug treatment was measured for MARIMO cells using flow cytometry and annexinV-FITC and propidium iodide staining. The percentage of live cells following 0.1% DMSO treatment was 94% (Figure 3a), and was minimally affected by single-agent hydroxyurea 10μg/ml (83%) (Figure 3b), AZD6738 2μM (92%) (Figure 3c) or VE-821 5μM (91%) (Figure 3e). Combining hydroxyurea and ATRi produced a substantial reduction in the percentage of live cells, to 19% for 10μg/ml hydroxyurea with 2μM AZD6738 (Figure 3d), and 24% for 10μg/ml hydroxyurea with 5μM VE-821 (Figure 3f).



(**a**) (**b**) (**c**) (**d**)

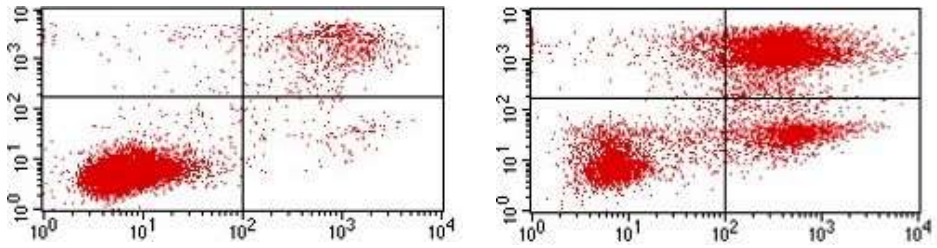


Figure 3. The combination of hydroxyurea and ATRi leads to a substantial synergistic increase in the apoptosis induction in MARIMO cells after 48-hour exposure. (**a**) DMSO 0.1% control (**b**) 10μg/mL hydroxyurea (**c**) 2μM AZD6738 (**d**) Combined 2μM AZD6738 and 10μg/ml hydroxyurea (**e**) 5μM VE-821 (**f**) Combined 5μM VE-821 and 10μg/ml hydroxyurea. X axis- annexinV-FITC, Y axis- propidium iodide.

4. Discussion

Investigation of DDRi paves a promising avenue for new therapies in hematological malignancies [14]. In this study we evaluated our hypothesis that DDRi might target leukemic cells in the presence of mutated *JAK2* and *CALR*, and might enhance the efficacy of currently used drugs. ATRi AZD6738 and VE-821 showed effects as single agents. ATRi demonstrated a synergistic effect in combination with currently approved drugs- hydroxyurea and ruxolitinib. Synergism between ATRi and chemotherapeutics has already been investigated in AML cell lines and solid tumours [10, 15]. Interestingly, AZD6738 was previously evaluated in chronic lymphocytic leukemia (CLL) in vitro and in vivo study and reported to show high efficacy in p53-null or ATM-null CLL cells, but not in wild type cells [9].

Synergistic toxicity of hydroxyurea and ATRi combination observed in our study can be explained by genotoxic effect of both chemotherapeutic and ATRi. As hydroxyurea targets ribonucleotide reductase consequently it promotes chromosome fragility and DNA DSBs [16]. As ATRi leads to impairment of DNA single- and double-stand breaks' repair, accumulation of breaks consequently causes apoptosis. Moreover, combination therapies including ATRi and hydroxyurea might be of clinical value as hydroxyurea resistance/ intolerance occurs relatively frequently [5].

Olaparib was ineffective when used in monotherapy. However, combination of ruxolitinib and olaparib has been already demonstrated to synergistically affect primary MPN cells harbouring JAK2 or CALR mutations [2]. Finally, ruxolitinib treatment showed efficacy only in cells with *JAK2V617F*, not in *CALR* mutated ones. Consequently, its synergism with ATRi was revealed only in the presence of *JAK2* mutation.

5. Conclusions

To conclude, DDRi reduces viability in cells expressing the driver mutations seen in MPNs. ATRi constitute promising therapeutic approach in hematological malignancies characterized by increased reliance on DNA damage repair pathways activity. Most notably, ATRi exhibit a synergistic effect with the current standard-of-care treatment hydroxyurea and ruxolitinib to reduce cell viability by inducing apoptosis. This study provides preliminary evidence that ATRi combined with standard therapies may be exploited in MPNs harbouring *JAK2* and *CALR* mutations. Further preclinical investigation is warranted.

Author Contributions: Conceptualization, AS, ST, HB, AC, MZ, IH,EC; methodology, AS, ST, AC, HB; formal analysis, AS, ST; investigation, AS; resources, ST, HB, AC, MZ, IH; data curation, AS, ST; writing—original draft preparation, AS; writing—review and editing, ST, HB, AC, MZ, IH,EC; visualization, AS, ST; supervision, ST, AC, HB, MZ; project administration, ST; funding acquisition, ST, AC, HB, IH, MZ. All authors have read and agreed to the published version of the manuscript. **Funding:** This research was funded by the White Rose University Consortium Collaboration Fund and supported by the National Institute for Health Research.

Acknowledgements: Thank you to S. Clarke in the University of Sheffield Flow Cytometry core facility technical support for the flow cytometry experiments. Thank you to N. Tawfiq, S. Lawson, J. Down, G. Stewart and M. A. Garcia for help with experimental techniques. MARIMO cells were a kind gift from Professor Hitoshi Kiyoi of Nagoya University, via Stuart Scott of UKNEQUAS leucocyte Immunophenotyping.

Conflicts of Interest: The authors declare no conflict of interest.

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