Effects of AS-1763 in Combination with other Targeted Agents for Chronic Lymphocytic Leukemia

Lizbeth Loza [1], Natalia Timofeeva[2], Shady Tantawy[2] , Varsha Gandhi[2]

[1]John P. and Kathrine G. McGovern Medical School at UTHealth

[2]Department of Experimental Therapeutics, University of Texas MD Anderson Cancer Center, Houston Texas

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

Introduction

Bruton's tyrosine kinase (BTK) is an integral receptor in the cell signaling of white blood cells.[1] BTK inhibitors are therapeutics used to interrupt the signaling that promotes the survival and growth of cancerous lymphocytes.[2,3] Despite the effectiveness of BTK inhibitors, there can be drug resistance in the presence of C481S mutant BTKs. [4] AS-1763, a new non-covalent BTK inhibitor has the potential to effectively target both wild-type and mutant BTKs.[4] We conducted experiments of AS-1763 in combination with other therapeutics to determine the presence of synergism and significant biological effects. (Figure 1)

Objectives

Determine whether AS-1763 in combination with other therapeutics affects cell viability and other biological parameters in chronic lymphocytic leukemia (CLL).

Methods

Peripheral blood mononuclear cells (PBMCs) from 11 untreated chronic lymphocytic leukemia (CLL) patients were isolated using Ficoll-Hypaque method. Cells were treated with AS-1763, Venetoclax (BCL-2 inhibitor)[5], AZD5991 (MCL-1 inhibitor)[6], APR-246 (glutathione modulator)[7], and NVP-AUY922 (HSP90 inhibitor)[8]. Cell Titer Glo 2.0 cell viability assay for the drugs alone and their combinations with AS-1763 was performed after 72 hours of incubation. Apoptosis assay using Annexin V/Propidium Iodide staining flow cytometry was done after 24 hours and 72 hours of incubation. Cellular reactive oxygen species (ROS) and mitochondrial superoxide (MitoSOX) were measured in 4 and 24 hours, respectively. GSH-Glo™ luminescent assay was used to measure reduced glutathione levels in PBMCs treated with AS-1763, APR-246, and in combination. Western blot was performed to detect the presence of specific proteins (PARP, HSP90, c-MYC, BTK, p-BTK AKT, MCL-1, Caspase-3, BCL-2, BCL-XL, catalase, SOD1, thioredoxin). Vinculin, B-actin, and SM actin were used as loading controls. Synergy was measured using CompuSyn and Synergy Finder (ZIP, Loewe, HSA, Bliss) software.

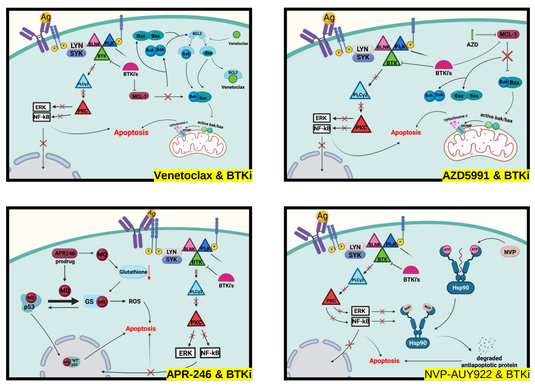


Figure 1. Mechanisms of Drug Interactions with BTK inhibitors.[9] *BTK inhibitors interrupt the signal transduction that occurs when antigen binds the B-cell receptor and prevents entrance of transcription factors into the nucleus, decreasing proliferation and survival. AZD5991 inhibits MCL-1, which antagonizes the association of Bak and Bax, leading to cell growth and proliferation, and thus the opposite effect when treated with AZD5991. BTK inhibitors also decrease MCL-1 protein. APR-246 is a p53/glutathione modulator that aids mutant p53 into behaving like wild-type p53, the protein of a tumor suppressor gene. APR-246 also decreases glutathione levels and increases reactive oxygen species. NVP-AUY922 is an HSP90 inhibitor that prevents HSP90, a chaperone protein, from stopping the degradation of antiapoptotic proteins.*

Results

AS-1763 showed mild cytotoxic effect in 72 hours measured by Cell Titer Glo 2.0 assay with IC50 of 29 μM and by Annexin V/PI flow cytometry. (Figure 2, 5)

According to CompuSyn software analysis, the effect of AS-1763 and Venetoclax was mostly synergistic, additive with APR-246, and no strong evidence of synergy was found with AZD5991 and NVP-AUY922. (Figure 2 A-D)

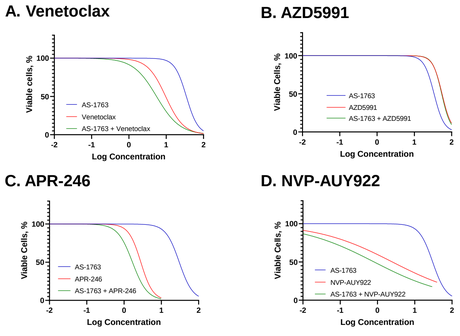


Table 1. CI values

Combo	ED50	ED75	ED90	ED95
AS+Ven	1	0.89	0.75	0.67
AS+AZD	1.4	1.4	1.45	1.47
AS+APR	1	1	0.98	0.95
AS+NVP	1	0.46	0.74	1.29

Figure 2. Dose response curves of AS-1763 alone and in combinations. *Slope curves for cell viability in varying concentrations of the drugs.*

Table 1. Combination index (CI) for effective dose (ED) 50, 75, 90, 95. *Values depicting synergistic (CI < 1), additive (CI = 1), and antagonistic (CI > 1) effects of drugs in combination with AS-1763.*

Using Synergy Finder (ZIP, Loewe, HSA, Bliss) software, with matrix plating of Venetoclax and APR-246, AS-1763 0.5-30 *μM* potentiates low doses of both Venetoclax and APR-246. (Figure 3, 4)

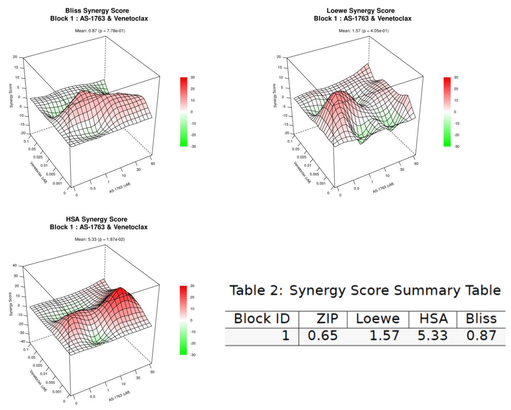


Table 2: Synergy Score Summary Table

Block ID	ZIP	Loewe	HSA	Bliss
1	0.65	1.57	5.33	0.87

Figure 3. Venetoclax and AS-1763 combination Matrix. *Surface plot representing synergy score of Venetoclax and AS-1763 0.5-30 μM.*

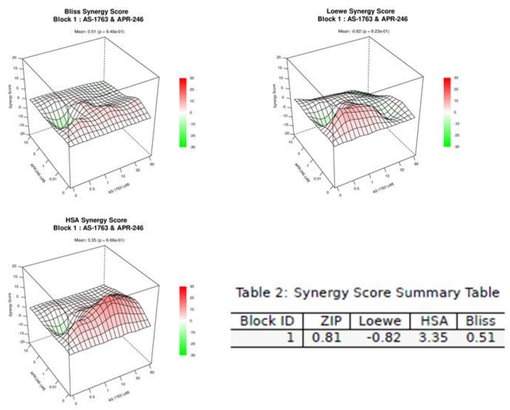


Table 2: Synergy Score Summary Table

Block ID	ZIP	Loewe	HSA	Bliss
1	0.81	-0.82	3.35	0.51

Figure 4. APR-246 and AS-1763 combination Matrix *Surface plot representing synergy score of APR-246 and AS-1763 0.5-30 μM.*

AZD5991 and NVP-AUY922 in combination with AS-1763 increased levels of apoptosis in Annexin/PI. QVD (pan-caspase inhibitor) rescues the toxicity. (Figure 5)

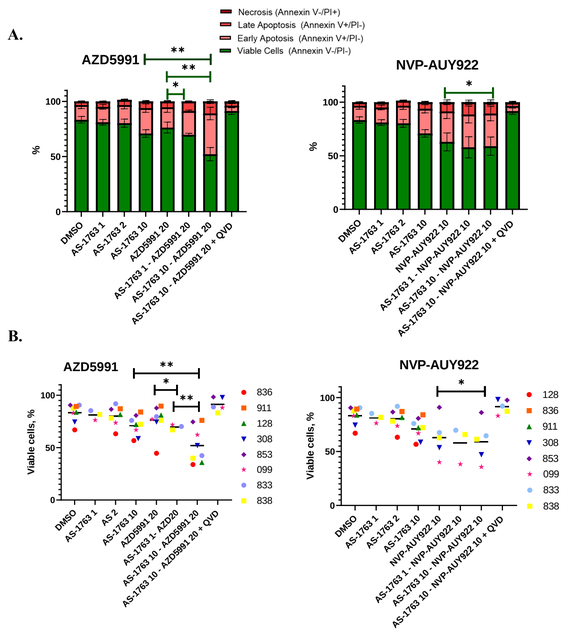


Figure 5. Apoptosis measured using Annexin V/Propidium Iodide Staining after 24 hours of drug treatment. *A. Percentage of viable cells (green), early apoptosis (pink), late apoptosis (red), and necrosis (deep red) for AZD5991, NVP-AUY922, and their combination with AS-1763. B. Plot graphs of viable cells in individual CLL patients with AZD5991, NVP-AUY922, and their combination with AS-1763.*

Venetoclax 1-10 nM combined with AS-1763 1 μM had a mean increase in apoptosis of 22.33% (range 20.69% - 24.78%) compared to Venetoclax alone.

AZD5991 10-30 nM combined with AS-1763 1 μM had a mean increase in apoptosis of 28.66% (range 20.21% - 34.51%) compared to AZD5991 alone.

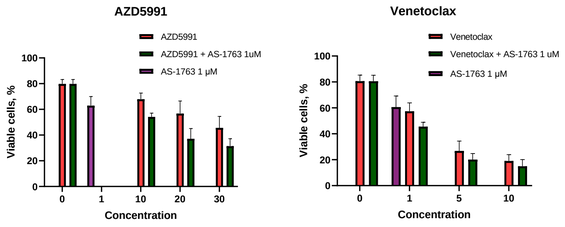


Figure 6. Apoptosis measured using Annexin V/Propidium Iodide Staining after 72 hours of drug treatment. *Bar graphs displaying viable cells in individual CLL patients after treatment with AZD5991, Venetoclax, and their combination with AS-1763. AS-1763 was added at hour 0 of plating while AZD5991 and Venetoclax were added 48 hours after plating.*

AS-1763 was found to increase ROS production starting at 1 μM. However, no increase of ROS was detected with APR-246 and its combination with AS-1763 compared to AS-1763 alone. (Figure 7) Although there is a decrease in SOD1 protein expression in some patient samples with the treatment of AS-1763, the activity of SOD1 is unknown, and thus, further study is required. (Figure 8)

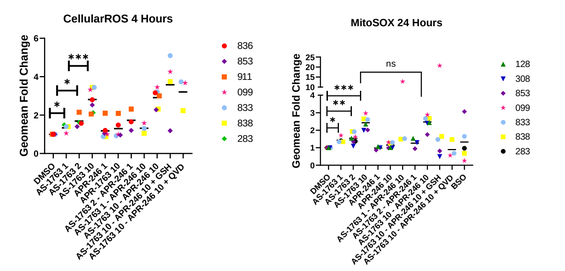


Figure 7. Geomean fold change of reactive oxygen species in CellularROS and superoxide in MitoSOX in 4 hours and 24 hours, respectively. *Plot graphs of the geomean fold change in individual CLL patients treated with AS-1763, APR-246, and in combination.*

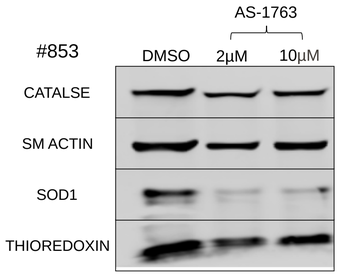
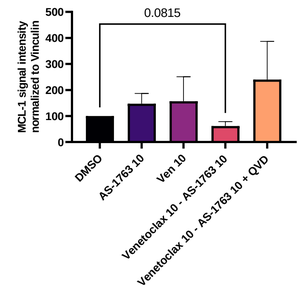


Figure 8. Impact of AS-1763 treatment on antioxidants. *AS-1763 treatment resulted in decrease of superoxide dismutase 1 (SOD1) expression in CLL patient #853.*

There is a trend in decrease of myeloid cell leukemia 1 protein (MCL-1) for AS-1763 10 μM and Venetoclax 10 nM combination-treated cells compared to DMSO, but statistical significance was not reached. (Figure 9)



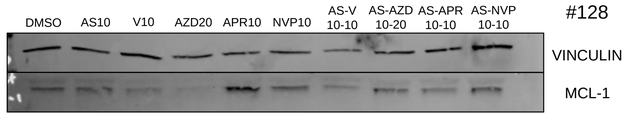


Figure 9. Western blot of MCL-1 protein. *Impaired expression of myeloid cell leukemia 1 protein observed in CLL patient #128 with treatment of AS-1763 10 μM and Venetoclax 10 nM in comparison to DMSO. Vinculin used as a loading control.*

There was no statistically significant decrease in reduced glutathione concentration in cells treated with a combination of AS-1763 and APR-246.

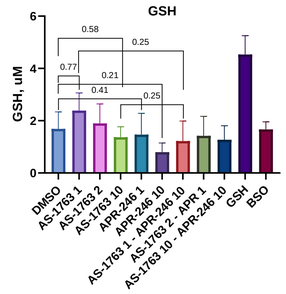


Figure. 10 GSH-Glo™ Luminescent Assay for the level of GSH. *Bar graph depicting reduced glutathione levels in CLL patient cells treated with AS-1763, APR-246, and in combination.*

Conclusions

AS-1763 has shown moderate toxicity when used alone. However, AS-1763 was found to enhance the effects of low doses of Venetocax and APR-246. Additionally, AS-1763 increased the rate of apoptosis in cells treated with AZD5991 and NVP-AUY922. When cells were treated with Venetoclax, AZD5991, APR-246, and NVP-AUY92 in combination with AS-1763, the addition of QVD rescued the cells. This suggests that caspase activation may be involved in the mechanism of apoptosis targeted by these therapeutics. AS-1763 was also found to increase both cellular and mitochondrial reactive oxygen species at a dose of 1 μM. This implies that oxidative stress may also play a role in the drug's mechanism of action. However, AS-1763 does not seem to have an impact on glutathione levels, so other antioxidants and their activities may be involved. Treatment with AS-1763 decreased SOD1 protein, an enzyme involved in scavenging ROS. The mechanism of SOD1 decrease remains unknown, requiring further study.

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[9]Figure 1 images created with BioRender.com

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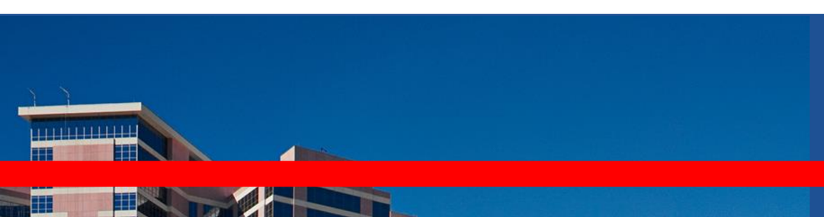

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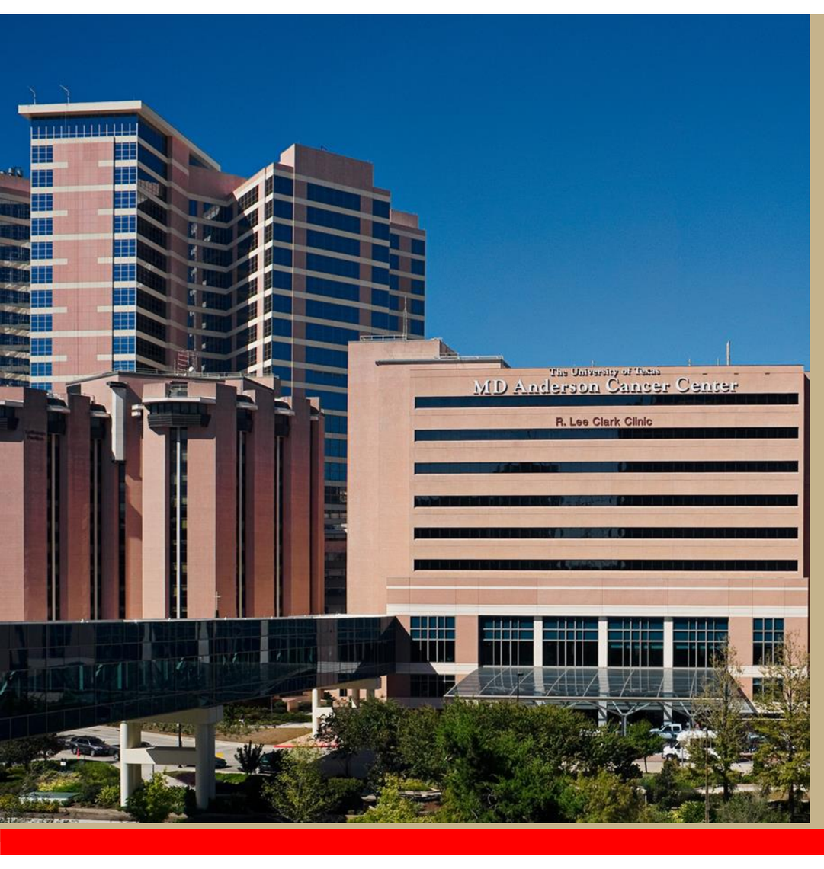

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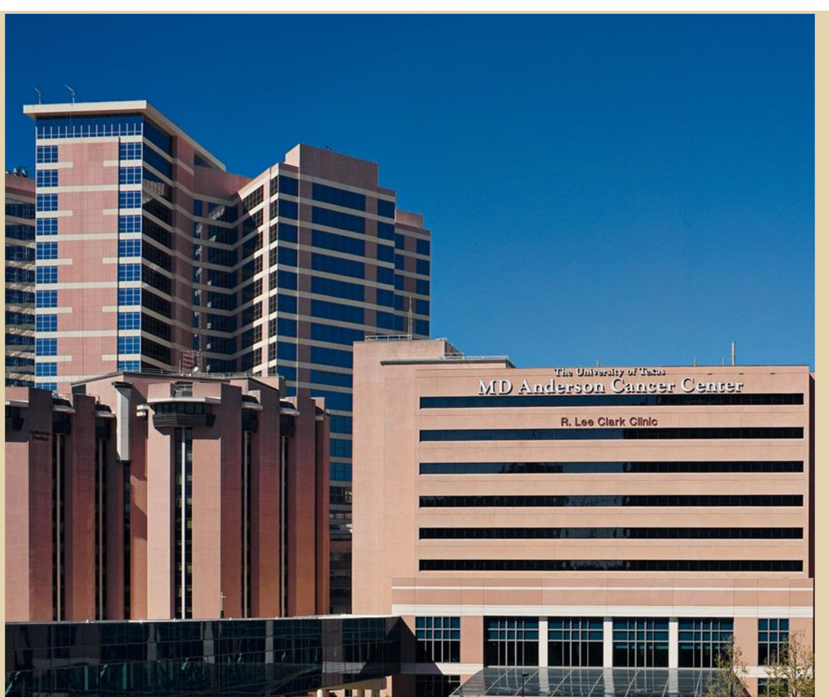

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