An Evaluation of the Presence, Type and Suggestions About the Mechanisms of Drug Interaction Between Venetoclax and GSK595 in Multiple Myeloma

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Abstract Multiple myeloma (MM) is the second most prevalent blood cancer in Singapore. Although the BH3-mimetic Venetoclax is effective against MM, acquired drug resistance occurs due to a shift in the balance between the activities of pro- and anti-apoptotic Bcl-2 family proteins towards survival. Here, we investigate whether the protein arginine methyltransferase 5 (PRMT5) inhibitor GSK3326595 can synergise with Venetoclax by modulating the activities of pro- and anti-apoptotic Bcl-2 family proteins. Using the Zero interaction potency (ZIP) model, we demonstrate the potential synergy between Venetoclax and GSK3326595 on several MM cell lines. Using flow cytometry and gene expression analysis, we also elucidated possible mechanisms of synergy and a seemingly counterintuitive gene expression pattern, which collectively suggest that both drugs likely induce apoptosis at the post-translational level.

Keywords Multiple myeloma (MM) · Venetoclax · Arginine methyltransferase 5 (PRMT5) inhibitor · GSK3326595 · Bcl-2 · Synergise

1 Introduction

Multiple myeloma (MM) is a plasma cell malignancy within the bone marrow that causes numerous complications including cytopenia, lytic bone lesions, renal impairment and hypercalcaemia. Notably, MM is the second most prevalent blood cancer in Singapore, with increasing occurrence in Asia [1]. More importantly, MM patients

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typically cannot recover from MM due to acquired drug resistance, which results in relapse [2].

The BH3-mimetic Venetoclax (ABT-199), which specifically inhibits the anti-apoptotic protein BCL2, is known to be effective against MM but susceptible to acquired drug resistance [2]. BCL2 is one of several anti-apoptotic Bcl-2 family proteins, along with BCL-X_L and MCL1 [3], which inactivate pro-apoptotic proteins of the same family by binding to their BH3 motifs [2]. Death by the intrinsic pathway of apoptosis largely depends on the balance between the activities of both pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Hence, the inhibition of multiple anti-apoptotic Bcl-2 family proteins is needed to trigger apoptosis [4], and shift the balance towards death. Consequently, Venetoclax is only mildly effective against cell lines that have large quantities of both BCL2 and MCL1, with Venetoclax-resistant cells relying on MCL1 for survival [2]. Therefore, to completely eradicate MM cells, it is important to inhibit both BCL2 and MCL1. Additionally, there is a need to discover which drug combinations are synergistic in treating MM, where synergism is defined as multiple drugs having an effect stronger than if individual effects were simply added up, in order to lessen toxicity while being effective. Conversely, antagonism is when the combination is weaker than the sum of individual drugs. Additivity is defined as each drug not affecting the potency of the others [5].

GSK3326595 (GSK595) is a competitive inhibitor of protein arginine methyltransferase 5 (PRMT5), and functions by binding the active site of PRMT5 to displace substrate peptides [6]. PRMT5 depletion has been reported to inhibit growth in various MM cell lines and upregulates p53 activity [7]. This is relevant as p53 has been shown to inhibit MCL1. For instance, p53 activation in acute myeloid leukaemia (AML), leads to upregulation of PUMA (encoded by *BBC3*), BAX and BAK, which bind and inhibit MCL1 [8]. Therefore, we hypothesize that Venetoclax has synergistic effects with GSK595, where GSK595 increases the activity of p53 to reduce the amount of MCL1, which serves to overcome resistance to Venetoclax. Moreover, since p53 also increases the expression of the pro-apoptotic Bcl-2 family proteins PUMA, BAX and BAK, inhibiting PRMT5 to increase the activity of p53 may further shift the balance between pro-and anti-apoptotic Bcl-2 family proteins in favour of apoptosis. In this study, we analysed drug combinations between Venetoclax and GSK595 on the MM cell lines KMS11, KMS12BM and OPM2. We also evaluated apoptosis in OPM2 and changes in the mRNA expression of pro- and anti-apoptotic Bcl-2 family proteins. This paper presents our results on the presence of drug interaction between Venetoclax and GSK595, and proposes a mechanism on how these drugs function together.

2 Materials and Methods

A. *Cell lines*

The cell lines KMS11, KMS12BM and OPM2 were cultured in RPMI 1640 medium with L-Glutamine (Biowest) supplemented with 10% FCS (Biowest).

B. In vitro*proliferation/viability assay*

ABT-199 (Venetoclax; Aobious) and GSK3326595 (GSK595; MedChemExpress) stock solutions were prepared and diluted in sterile-filtered cell culture-grade DMSO (Sigma).

To evaluate cell response to monotherapy or combination therapy, cells were seeded in 96-well white plates (Greiner BioOne) at a density of 6×10^4 cells/ml in 100 μl, treated with either or both compounds at the indicated concentrations, and incubated for 6 days at 37 °C, 5% CO_2 under humidified conditions. Technical duplicates were performed for each independent experiment, using drug concentrations of GSK595 from 0 to 5 μM, and concentrations of venetoclax from 0 to 5 μM. The drugs were mixed at varying ratios in a 6 × 6 checkerboard in a 96-well plate with each drug increasing in concentration along each axis. On day 6 post-treatment, cell viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and the most synergistic concentrations of both compounds and synergy scores were quantified using SynergyFinder using the Zero interaction potency (ZIP) model.

C. *Annexin-V/Hoechst flow cytometry assay*

For analysis of apoptosis by Annexin-V/Hoechst flow cytometry assay, OPM2 cells were seeded in 6-well plates at a seeding density of 2×10^5 cells/ml in 2.9 ml. Each well was treated with either DMSO, GSK595 (0.04 μM), Venetoclax (0.04 μM), or both drugs (0.04 μM each). 2.5×10^5 cells were harvested 4 days post-treatment, washed and resuspended in Annexin-V binding buffer (BD Bioscience). The cells were then labelled with APC-conjugated annexin-V (Thermo Fisher Scientific) and Hoechst 33258 (Thermo Fisher Scientific). Flow cytometric analysis was performed on a FACSAria II (BD Biosciences), and the data analysed using FlowJo 10.1 (Treestar).

D. *RNA extraction*

For gene expression analysis, total RNA was first extracted from cells (treated as indicated above), harvested on day 4 post-treatment, using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. In brief, OPM2 cells (no more than 4 $\times 10^6$ cells) were first lysed and homogenised using a highly denaturing guanidine-thiocyanate-containing buffer to immediately inactivate RNases. The lysate was then homogenised using a QIAshredder spin column. RNeasy spin columns were then used to bind mRNA to a silica-based membrane. On-column DNase digestion was also performed with the RNAse-Free DNase Set (Qiagen) to prevent the carryover of genomic DNA in subsequent qRT-PCR steps. The purified mRNA was finally eluted in 30ul of RNase-free water.

E. *qRT-PCR*

For gene expression analysis, cDNA was synthesized from total RNA by reverse transcription (RT) using the EvoScript Universal cDNA Master (Roche) in accordance with manufacturers' instructions.

Quantitative PCR (qPCR) was performed using the QuantStudio™ 5 Real-Time PCR system (Thermo Fisher Scientific). Taqman™ Fast Universal PCR master mix (Thermo Fisher Scientific) was used with PrimeTime® qPCR Probe Assays (Integrated DNA Technologies) according to manufacturers' instructions. Gene list and PrimeTime® qPCR primers and probes sequences are provided in Appendix Table 1. The thermal cycling parameters used are: 1 cycle of 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Technical duplicates were performed for each independent experiment. Amplification of the housekeeping genes *GAPDH* and *B2M* was conducted for each sample used as an endogenous control and to normalize levels of all target genes.

3 Results

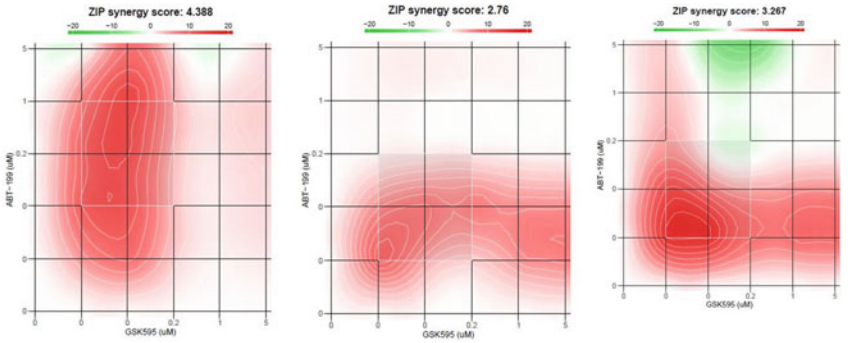
A. *Interaction between Venetoclax and GSK595*

To identify the type and presence of drug interaction between Venetoclax and GSK595, we exposed several MM cell lines (KMS11, KMS12BM and OPM2) to increasing concentrations of either drug combined at different ratios. We then assessed potential drug interactions using the ZIP model, where delta scores (δ) >0, <0 or =0 indicate synergy, antagonism or a lack of interaction, respectively [9].

The interaction between Venetoclax and GSK595 are generally synergistic in the cell lines tested; with average synergy scores of 3.27, 2.76 and 4.39, and the most synergistic areas having scores of 7.39, 4.26 and 8.09 for OPM2, KMS12BM and KMS11 respectively. For KMS11 and KMS12BM, we observed synergy along a single axis (Fig. 1a, b). In contrast, we observed synergy along both axes for OPM2. Synergy in OPM2 was most pronounced at lower concentrations of both Venetoclax and GSK595, while antagonism was seen at higher concentrations of Venetoclax and moderate concentrations of GSK595 (Fig. 1c). Overall, the evidence suggests that Venetoclax and GSK595 are likely to produce a synergistic effect, with OPM2 displaying a wider variety of concentration ratios which are synergistic.

B. *Monotherapy and Combination Therapy*

To confirm the presence of drug synergy in OPM2, we compared the degree of apoptosis between monotherapy and combination therapy. Based on the ZIP model data (Fig.1), we chose drug concentrations which produced significant synergism. Consistent with previous reports where tumor cell killing by GKS595 was generally



a: Synergy scores at each concentration ratio as measured by ZIP model in SynergyFinder in KMS11 when treated with 0 to 5 μM of Venetoclax and GSK595 for 6 days as measured by CellTiter-Glo cell viability assay

b: Synergy scores at each concentration ratio as measured by ZIP model in SynergyFinder in KMS12BM when treated with 0 to 5 μM of Venetoclax and GSK595 for 6 days as measured by CellTiter-Glo cell viability assay

c: Synergy scores at each concentration ratio as measured by ZIP model in SynergyFinder in OPM2 when treated with 0 to 5 μM of Venetoclax and GSK595 for 6 days as measured by CellTiter-Glo cell viability assay.

Fig. 1 ZIP synergy score maps of MM cell lines to venetoclax and GSK595

observed after 6 days [6, 7], we observed no apparent change in the proportion of apoptotic cells (Annexin V^+ $Hoechst^-$) in GSK595-treated OPM2 cells compared to DMSO-treated controls 4 days post-treatment (Fig. 2). In contrast, we observed a twofold increase in the proportion of apoptotic OPM2 cells upon Venetoclax treatment compared to DMSO-treated controls 4 days post-treatment (Fig. 2); which is consistent with the rapid cell killing action of Venetoclax previously documented [2]. With combination treatment, the proportion of apoptotic OPM2 cells was very similar to that of Venetoclax monotherapy (Fig. 2). Therefore, these results suggest that Venetoclax contributes to early eradication of MM cells, and that the observed synergy with GSK595 could occur after 4 days of treatment.

C. *mRNA Levels of Pro- and Anti-apoptotic Bcl-2 Family Members*

To understand the molecular mechanisms underlying synergism between Venetoclax and GSK595, we next performed gene expression analysis to evaluate the mRNA levels of pro- and anti-apoptotic Bcl-2 family members. Unexpectedly, we found that the mRNA of pro-apoptotic Bcl-2 members decreased while the mRNA of anti-apoptotic members increased. Specifically, *BAK1* and *BBC3*, which encode pro-apoptotic BAK and PUMA proteins respectively, showed a notable decrease upon combination therapy (Fig. 3). Similarly, *BAK1* and *BBC3* expression decreased in response to Venetoclax; but remained relatively unchanged with GSK595 treatment (Fig. 3). The expression of *BAX*, which encodes the pro-apoptotic protein BAX, remained unchanged under all treatment conditions compared to the DMSO-treated control (Fig. 3). Meanwhile, the mRNA of anti-apoptotic Bcl-2 members increased to different extents. *BCL2* expression increased after GSK595 treatment and combination therapy, while *BCL2L1* (encodes for BCL-X_L) and *MCL1* expression did not change appreciably during treatment (Fig. 3). Conversely, we observed a consistent and obvious increase in *PRMT5* expression after any treatment; where the largest increase was seen upon combination therapy (Fig. 3). Taken together, our data suggests that the synergistic cell killing effect of the Venetoclax-GSK595

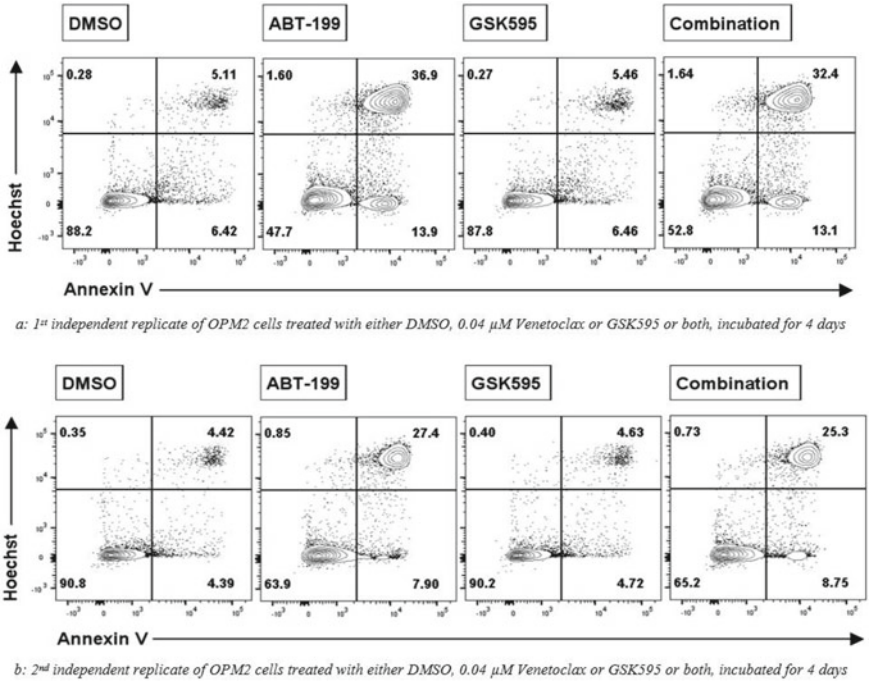


Fig. 2 Flow cytometry assays on OPM2. The bottom left, bottom right and top right gates indicate live, apoptotic and dead cells respectively

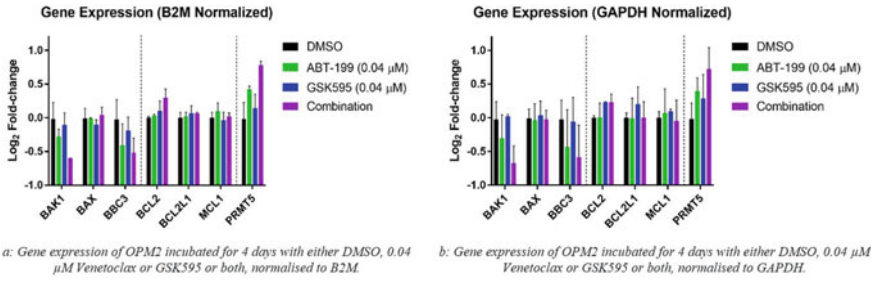


Fig. 3 Gene expression profiles of *BAK1, BAX, BBC3, BCL2, BCL2L1, MCL1, PRMT5* normalized to *B2M* or *GAPDH*. Results shown are the average (±SEM) of two independent experiments

combination in MM cells is unlikely to involve regulation of Bcl-2 family proteins at the transcriptional level.

4 Discussion

MM cells are highly dependent on anti-apoptotic Bcl2 family proteins to evade apoptosis [2], and the current drug Venetoclax targets the anti-apoptotic protein BCL2 to induce cell death via the intrinsic pathway of apoptosis [4]. However, Venetoclax can be resisted by cells that overexpress MCL1 [2]. Fortunately, MCL1 can be inhibited by p53 activation, which leads to transcriptional upregulation of pro-apoptotic Bcl-2 family proteins that inhibit MCL1 [8]. Thus, activating p53 is a potential strategy to overcome Venetoclax resistance by tipping the balance between pro- and anti-apoptotic proteins towards cell death. Hence, we evaluated the effects of Venetoclax in combination with the PRMT5 inhibitor GSK595, which competitively inhibits PRMT5 to increase the activity of p53 [6, 7], to check for the presence of any interactions, especially synergistic effects to minimise toxicity and maximise efficacy in vivo.

In this study, we discovered synergy between Venetoclax and GSK595 in MM cells. Interestingly, our observations in OPM2 are inconsistent with previous reports, which suggest that OPM2 is relatively insensitive to Venetoclax [2]. In addition, the presence of a p53 mutation in OPM2 has been proposed to render OPM2 resistant to another PRMT5 competitive inhibitor functioning similar to GSK595, EPZ015666 [7]. Hence, either drug can only be expected to have minimal effect on OPM2. Instead, Venetoclax and GSK595 showed evident growth inhibition activity against OPM2 individually, and to a greater extent, in combination 6 days post-treatment based on the CellTiter-Glo cell viability assay (Fig. 1). Therefore, while Venetoclax and GSK595 generally appear to elicit a synergistic effect on MM cell lines, the results for OPM2 should be interpreted with caution.

We chose to further investigate Venetoclax-GSK595 synergism in OPM2 as it showed a wider range of synergistic concentration ratios than the other cell lines tested. We found that while Venetoclax substantially elevated the proportion of apoptotic cells, GSK595 resulted in no apparent change in the proportion of apoptotic cells, and contributed little towards apoptotic cell killing in combination with Venetoclax at least at 4 days post-treatment (Fig. 2). One possible interpretation is that a time point of 4 days post-treatment for the apoptosis assay was too early and insufficient for the effect of GSK595 to manifest. Unlike Venetoclax, which acts by directly inhibiting BCL-2 [2], GSK595 inhibits PRMT5 to reduce arginine methylation and promote p53 activation [6, 7], which subsequently leads to transcriptional upregulation of pro-apoptotic Bcl-2 family proteins [8]. As cellular processes involving changes to pre-existing proteins take a shorter time to be effected than processes that need new proteins to be transcribed and then translated [10], the time required for GSK595 to initiate apoptosis may be greater than 4 days. This may explain why a synergistic effect could be observed 6 days post-treatment (CellTiter-Glo cell viability assay, Fig. 1) but not at 4 days post-treatment with the apoptosis assay (Fig. 2). As such, we propose that further investigation should be performed on days 2 and 6 to account for the potential time-dependent effects of drug synergism. Taken together, the type of interaction between GSK595 and venetoclax requires further clarification.

An important part of our findings was the gene expression analysis. While the proportion of live cells always decreased after drug treatment (Fig. 2), there was a counterintuitive change in the ratio of pro- to anti-apoptotic gene expression. Thus, it is likely that the observed cell death is not regulated at the transcriptional or post-transcriptional level, but rather at the post-translational level. Indeed, Venetoclax directly inhibits BCL2 [2], while GSK595 directly inhibits PRMT5 [6]. Since these inhibitory activities occur at the protein level, the mRNA levels of *BCL2* and *PRMT5* would not be expected to decrease. Interestingly, the pro-apoptotic p53 target genes *BAK1*, *BBC3* and *BAX* remained relatively unchanged despite GSK595 treatment, which has been reported to activate p53 [6]. However, this is consistent with previous reports that OPM2 cannot upregulate these genes via p53 due to a mutation in codon 175 of p53 [7], which causes the DNA-binding surface to lose its conformation, resulting in non-functional p53 [11]. Although Venetoclax appears to decrease the expression of pro-apoptotic Bcl-2 members *BAK1* and *BBC3*, antagonism is unlikely to occur as studies conducted in AML cells resistant to p53 activation have shown that Venetoclax is able to significantly counteract resistance to p53 activation [8]. Therefore, further tests involving protein quantification and activity must be carried out to confirm if regulation occurs at the post-translational level to determine the mechanism of any interaction between Venetoclax and GSK595 in MM cells.

5 Conclusion

In conclusion, our data suggests that the interaction between GSK595 and Venetoclax is potentially synergistic, although further tests are required to exclude an additive effect. Moreover, our data suggests that both drugs likely regulate cell death at the post-translational level, rather than at the transcriptional or post-transcriptional level. Importantly, we have not fully elucidated whether the drug synergy between Venetoclax and GSK595 is applicable across MM, or whether the synergistic effect is limited to specific MM subtypes (e.g. with functional p53). Therefore, future work should explore drug interactions between both drugs using a wider panel of cell lines, as well as analyze protein levels in addition to mRNA levels, to further clarify the mechanisms involved.

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Appendix

Table 1 Gene list, PrimeTime® qPCR primers and probes sequences

Gene	*BAK1*
Probe	5′-/56-FAM/TCAGAGTTC/ZEN/CAGACCATGTTGCAGC/3IABkFQ/-3’
Primer 1	5′-CGACATCAACCGACGCTAT-3’
Primer 2	5′-CAGAAGAGCCACCACACG-3’
Gene	*BAX*
Probe	5′-/56-FAM/TTTCCGAGT/ZEN/GGCAGCTGACATGTT/3IABkFQ/-3’
Primer 1	5′-AGTTGAAGTTGCCGTCAGAA-3’
Primer 2	5′-GGAGCTGCAGAGGATGATTG-3’
Gene	*BBC3*
Probe	5′-/56-FAM/TGCTCCTCT/ZEN/TGTCTCCGCCG/3IABkFQ/-3’
Primer 1	5′-ACGACCTCAACGCACAGTA-3’
Primer 2	5′-CACCTAATTGGGCTCCATCT-3’
Gene	*BCL2*
Probe	5′/56-FAM/CAGGATAAC/ZEN/GGAGGCTGGGATGC/3IABkFQ/-3’
Primer 1	5′-GATGACTGAGTACCTGAACCG-3’
Primer 2	5′-AGCCAGGAGAAATCAAACAGAG-3’
Gene	*BCL2L1*
Probe	5′-/56-FAM/AAGTATCCC/ZEN/AGCCGCCGTTCTC/3IABkFQ/-3’
Primer 1	5′-GCCACTTACCTGAATGACCAC-3’
Primer 2	5′-GCATTGTTCCCATAGAGTTCCA-3’
Gene	*MCL1*
Probe	5′-/56-FAM/TCCACAAAC/ZEN/CCATCCCAGCCTC/3IABkFQ/-3’
Primer 1	5′-CATTAGCAGAAAGTATCACAGACG-3’
Primer 2	5′-ACATTCCTGATGCCACCTT-3’
Gene	*PRMT5*
Probe	5′-/56-FAM/CATCGCCAG/ZEN/AAACGCACACAGAT/3IABkFQ/-3’
Primer 1	5′-GTTTCCCATCCTCTTCCCTAT-3’
Primer 2	5′-CCCACTCATACCACACCTTC-3’
Gene	*GAPDH*
Probe	5′-/56-FAM/AAGGTCGGA/ZEN/GTCAACGGATTTGGTC/3IABkFQ/-3’
Primer 1	5′-ACATCGCTCAGACACCATG-3’
Primer 2	5′-TGTAGTTGAGGTCAATGAAGGG-3’
Gene	*B2M*
Probe	5′-/56-FAM/CCTGCCGTG/ZEN/TGAACCATGTGACT/3IABkFQ/-3’
Primer 1	5′-GGACTGGTCTTTCTATCTCTTGT-3’
Primer 2	5′-ACCTCCATGATGCTGCTTAC-3’

References

1. "Home > Cancer Information > About Cancer > Multiple Myeloma." National University Cancer Institute Singapore NCIS. https://www.ncis.com.sg/Cancer-Information/About-Cancer/Pages/Multiple-Myeloma.aspx
2. E.A. Punnoose *et al.*, "Expression profile of BCL-2, BCL-XL, and MCL-1 predicts pharmacological response to the BCL-2 selective antagonist Venetoclax in multiple myeloma models," in *Molecular Cancer Therapeutics,* vol.15, no. 5, pp.1132–1144, Apr.2016. [Online]. Available: doi: https://doi.org/10.1158/1535-7163.MCT-15-0730
3. A. Shamas-Din, J. Kale, B. Leber and D.W. Andrews, "Mechanisms of action of Bcl-2 family proteins," in *Cold Spring Harbour Perspectives in Biology*, vol.5, no. 4, Apr.2013. [Online]. Available: doi: https://doi.org/10.1101/cshperspect.a008714
4. Alberts *et al.* "Cell Death," In *Molecular Biology of The Cell*, New York, United States: Garland Science, Taylor and Francis Group, LLC, 2015, ch.18, pp.1026–1027
5. T. Pemovska, J.W. Bigenzahn and G. Superti-Furga, "Recent advances in combinatorial drug screening and synergy scoring," in *Current Opinion in Pharmacology,* vol.42, pp.102–110, Oct.2018, [Online]. Available: https://doi.org/10.1016/j.coph.2018.07.008
6. Gerhart *et al.*, "Activation of the p53-MDM4 regulatory axis defines the anti-tumour response to PRMT5 inhibition through its role in regulating cellular splicing," in *Sci. Rep.,* vol.8, Jun.2018. [Online]. Available: https://doi.org/10.1038/s41598-018-28002-y
7. Gullà *et al.*, "Protein arginine methyltransferase 5 has prognostic relevance and is a druggable target in multiple myeloma" in *Leukemia*, vol.32, pp.996–1002, Apr.2018. [Online]. Available: doi:https://doi.org/10.1038/leu.2017.334
8. Pan *et al.*, "Synthetic lethality of combined Bcl-2 inhibition and p53 activation in AML: mechanisms and superior antileukemic efficacy," in *Cancer cell*, vol.32, no.6, pp.748–760.e6, Dec.2017. [Online]. Available: doi:https://doi.org/10.1016/j.ccell.2017.11.003
9. B. Yadav, K. Wennerberg, T. Aittokallio and J. Tang, "Searching for drug synergy in complex dose–response landscapes using an Interaction Potency Model" in *Computational and structural biotechnology journal*, vol.13, pp.504–513, Sep.2015. [Online]. Available: doi:https://doi.org/10.1016/j.csbj.2015.09.001
10. Alberts *et al.*. "Cell signalling," In *Molecular Biology of The Cell*, New York, United States: Garland Science, Taylor and Francis Group, LLC, 2015, ch.15, pp. 825
11. Liu, X.Y.; Pestka, S.; Shi, Y.F., "Can p53 act as a biomarker in cancer management and therapy?" In *Recent Advances in Cancer Research and Therapy*, Amsterdam, Netherlands: Elsevier: Amsterdam, 2012, ch.9.2