PARSING THE CONTRIBUTIONS OF THE BCL-2 ANTI-APOPTOTIC PROTEINS IN MEDIATING SURVIVAL OF NASOPHARYNGEAL CARCINOMA CELL LINE HK1 USING BCL-2 SELECTIVE INHIBITORS

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Malaysia is ranked number four with highest number of new cases of Nasopharyngeal carcinoma (NPC) for both sexes, in Southeast Asia. The estimated new cases and mortality in Malaysia, for both sexes, is 2,089 and 1,187, respectively in 2018 (GLOBOCAN 2018). Patients diagnosed early with NPC respond well to radio and chemotherapy. The 5-year survival rate for stage I and stage II patients' range from 72 to 90% (Chang *et al*., 2004) but unfortunately this is not the case for patients' who are diagnosed late. The 5-year survival rate for stage III and IV patients are ~55% and 30% respectively, mostly attributed to locoregional recurrence and metastasis (Chang *et al.,* 2004). Prognosis for patients suffering from recurrence or advance disease worsens and therefore they respond poorly to existing treatment modalities. Hence, new treatment options are needed, especially for patients with metastatic disease.

Apoptosis is a form of regulated cell death for tidy disposal of aged, damaged, diseased or otherwise dangerous cells (Mohana-Kumaran *et al*., 2014). Apoptosis can proceed either intrinsically through the mitochondria or extrinsically through the death receptors. Cells initiate the intrinsic apoptosis pathway in response to stress caused by various stimuli such as nutrient deprivation, hypoxia, upregulation of oncogenes e.g. c-myc, viral infection, endoplasmic reticulum stress and DNA damaging agents (Mohana-Kumaran *et al*., 2014). The BCL-2 protein family tightly regulates the intrinsic apoptosis pathway. The family includes both anti-apoptotic and pro-apoptotic proteins. The pro-apoptotic proteins can be further divided into effector proteins BAX and BAK and BH3-only proteins (Adams & Cory, 2018). Upon receiving stress signals, BH3-only proteins either inhibit the

anti-apoptotic proteins or directly activate BAX and BAK. BAX and BAK oligomerize and induce mitochondrial outer membrane permeabilization (MOMP), leading to caspase cascade activation (Adams & Cory, 2018). Anti-apoptotic proteins namely BCL-2, BCL-XL and MCL-1 either sequester their BH3-only protein counterparts or directly bind to BAX and BAK to prevent MOMP and maintain mitochondria outer membrane integrity (Montero & Letai, 2018). The anti-apoptotic proteins are highly expressed in many cancer tissues as a strategy to evade apoptosis.

The BCL-2 selective inhibitors were developed to bind select anti-apoptotic proteins, liberating the pro-apoptotic proteins and inducing apoptosis in cancer cells (Ashkenazi *et al*., 2017). Given that different cell lineages are addicted to different anti-apoptotic proteins for survival, it is crucial to determine which anti-apoptotic proteins that NPC cells are addicted for survival so that the cells can be targeted appropriately. The existence of the BCL-2 selective inhibitors enable a form of chemical BH3 profiling toolkit to dissect the contribution of each anti-apoptotic protein for survival of any given cell population (Leverson *et al*., 2015). Hence, we employed BCL-2 selective inhibitors ABT-199 (Souers *et al*., 2013), WEHI-539 (Lessene *et al*., 2013) and A-1210477 (Leverson *et al*., 2015), which inhibits BCL-2, BCL-XL and MCL-1, respectively, as a chemical toolkit to dissect the contributions of these anti-apoptotic proteins in maintaining survival of NPC cells. ABT-199, which inhibits BCL-2 selectively, obtained FDA approval in April 2016 for treating refractory chronic lymphocytic leukaemia (CLL) with 17p chromosomal deletion. WEHI-539 demonstrates high affinity and selectivity for BCL-XL and kills cells by antagonizing BCL-XL pro-survival activity (Lessene *et al*., 2013). A-1210477 is reported to

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selectively inhibit MCL-1 and disrupt the MCL-1-BIM complex to activate apoptosis in various cancer cells (Leverson *et al*., 2015).

The preliminary work presented here focused on testing the sensitivity of NPC cell line HK1 (Epstein-Barr virus (EBV) negative cell line) to the aforementioned BCL-2 selective inhibitors alone and as combinations *in vitro*. The HK1 cell line was easy to grow and the cost to maintain these cells in culture was inexpensive, hence the HK1 cells were employed in this pilot study. Approximately 3000 HK1 cells were seeded in 96-well plates in RPMI supplemented with 10% *(v/v)* heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and were left to attach for 6-7 hours. Cells were treated with single agent ABT-199 (LKT Laboratories Inc., St Paul, MN, USA), WEHI-539 (MedChemExpress, Monmouth Junction, NJ, USA) and A-1210477 (Selleckchem, Houston, TX, USA) in a dilution series of 2-fold concentration steps. Assays were terminated when untreated wells were still sub-confluent. Cells were lysed and stained with with SyBr Green I dye (Thermo Fisher Scientific, Waltham, MA, USA), and quantified by fluorescence (485 nm excitation, 530 nm emission) using the CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). Sensitivity of HK1 cells to drug combinations was measured by testing a fixed concentration of WEHI-539 (4 μM) and a fixed concentration of A-1210477 (4 μM) with increasing concentrations of ABT-199 (dilution series of 2-fold concentration steps). All drug sensitivity assays were conducted 4 times (n=4) and average IC_{50} values were calculated from the experimental data. In the plots, the y-axis represents cell proliferation, with cell proliferation of the untreated controls representing 100%. The x-axis was formatted to have a base 10 logarithmic scale but the drug concentrations used were not log-transformed prior to plotting of the graphs. Drug interactions were determined using the Chou and Talalay method (Chou, 2006). The CompuSyn 1.0 software (ComboSyn Inc. NJ, USA) was used to calculate the combination index values, which is a measure of synergy.

In order to determine the contributions of BCL-2, BCL-XL and MCL-1 in mediating HK1 cell survival, the cells were treated with single agent ABT-199, WEHI-539 and A-1210477 for 72 hours. The HK1 cells were insensitive to single agent treatment of ABT-199 (Figure 1(a) open circle and Table 1) indicating that the cells do not solely depend on BCL-2 for survival. Similarly, another study which tested Bcl-2 oligodeoxynucleotide antisense (Bcl-2-ASO) (TriLink Biotechnologies) in combination with radiotherapy on another NPC cell line, C666-1 and C15 NPC xenografts, the drug only demonstrated an additive effect in these models. The

limited outcome of this study was attributed to poor drug penetration and expression of other anti-apoptotic proteins namely BCL-XL and BFL-1 (Yip *et al.,* 2005), indicating BCL-2 alone does not dictate cell survival. The HK1 cells were also resistant to single agent treatment of A-1210477 (Figure 1(a) open triangle and Table 1) and WEHI-539 (Figure 1(a) open box and Table 1) indicating that the cells were neither BCL-XL nor MCL-1 dependent. A number of other studies have demonstrated that inhibition of one anti-apoptotic protein may be compensated by upregulation of other anti-apoptotic proteins. For example, inhibition of BCL-2/BCL-XL led to MCL-1 expression (Yecies *et al*., 2010; Lucas *et al*., 2012) or BCL-XL expression (Vogler *et al*., 2009, Morales *et al*., 2011) as a compensatory survival mechanism. This compensatory mechanism may explain the insensitivity of the HK1 cells to inhibition of a single anti-apoptotic protein as other anti-apoptotic proteins may compensate for the loss of the inhibited one.

We next investigated the sensitivity of the HK1 cells to co-inhibition of BCL-2 and MCL-1. Cells were treated with increasing concentrations of ABT-199 (0-32 μM) in the absence and presence of a fixed dose of A-1210477 at 4 μM for 72 hours (Figure 1(b). Cells however, did not respond to this combination (IC_{50}: 25.4 ± 1.3 μM; Figure 1(b) open triangle) indicating that other anti-apoptotic proteins involved in mediating cell survival. Moving forward, WEHI-539 (BCL-XL inhibitor) was added to the earlier combination. The cells were treated with increasing concentrations of ABT-199 (0-32 μM) in the absence and presence of fixed concentration of A-1210477 (4 μM) and WEHI-539 (4 μM) for 72 hours. Combination of all three inhibitors inhibited HK1 cell proliferation in a dose-dependent manner (IC_{50}: 0.53 μM; Figure 1(c) open diamond) highlighting the collaborative effect of BCL-2, BCL-XL and MCL-1 in maintaining HK1 cell survival. To determine if the drugs were acting synergistically, we used the Chou and Talalay method to calculate a combination index (CI) (Chou, 2006). CI is a measure of synergy where values < 1 indicate synergy. Combining all three drugs was synergistic in the HK1 cells (Table 2). Similar to this study, a number of studies have reported on the participation of several anti-apoptotic proteins in maintaining cell survival rather than a single anti-apoptotic protein. For example, anti-apoptotic proteins BCL-2, MCL-1 and BFL-1/A1 were reported to collectively safeguard the survival of T cells, B cells and dendritic cells (Carrington *et al*., 2017). In another study co-inhibition of both BCL-2 and BCL-XL were required to inhibit survival of a number of small cell lung cancer cells (SCLCs) indicating that
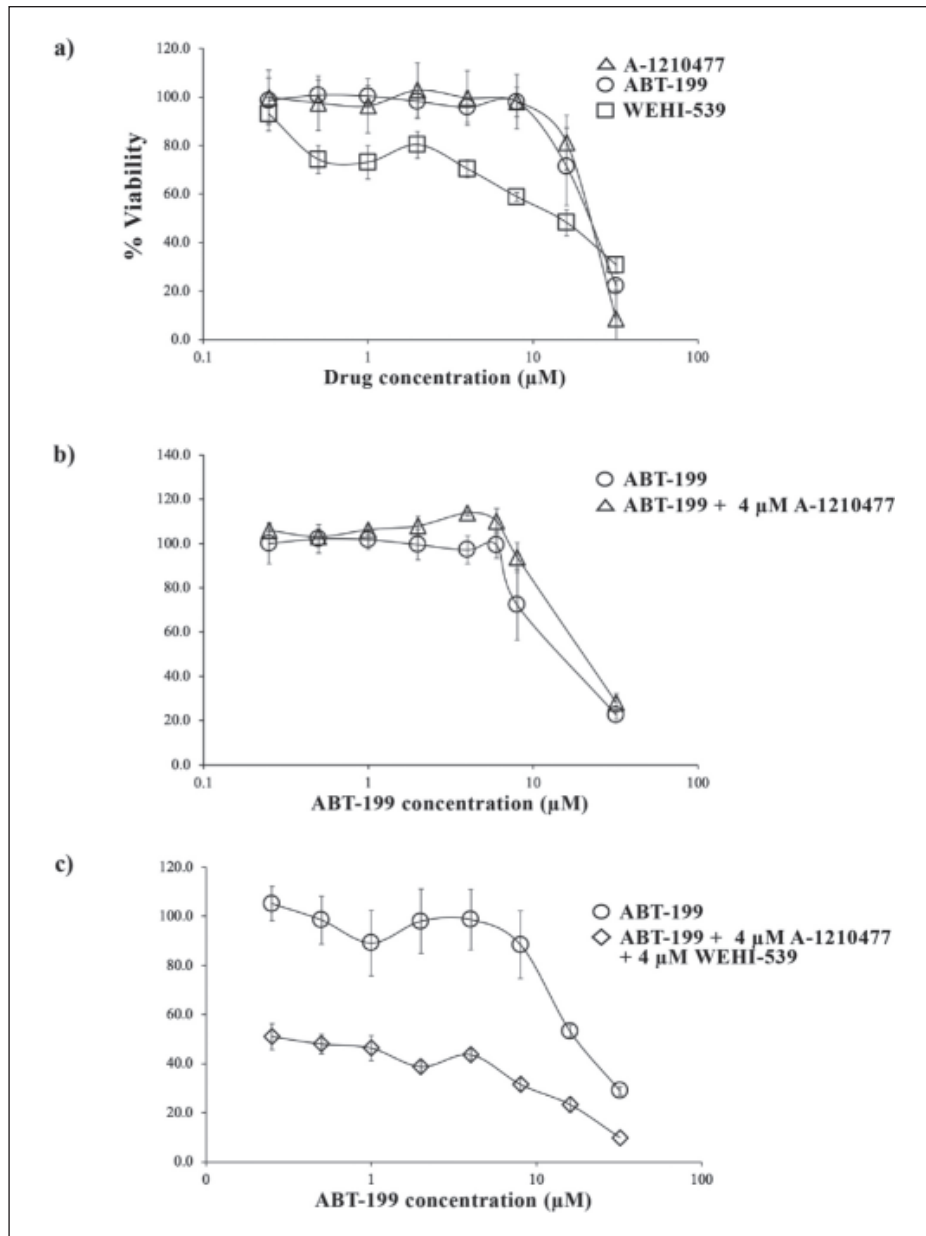
Fig. 1. HK1 cells were resistant to single agent treatment of the BCL-2 selective inhibitors and co-inhibition of BCL-2 and MCL-1 but responded to combination of all three inhibitors. HK1 cells were treated with **a)** single agent ABT-199 (0-32 μM), A-1210477 (0-32 μM) and WEHI-539 (0-32 mM) for 72 hours; **b)** ABT-199 (0-32 μM) in the absence and presence of A-1210477 (4 μM) for 72 hours; **c)** ABT-199 (0-32 μM) in the absence and presence of A-1210477 (4 μM) and WEHI-539 (4 μM) for 72 hours. Cell proliferation was assessed using the SyBr Green I assay. Points represent mean ± SEM of four repeats. All drug sensitivity assays were conducted four times (n=4) and average IC$_{50}$ values were calculated from the experimental data.

Table 1. IC$_{50}$ values of single agent treatment of HK1 cells with ABT-199, A-1210477 and WEHI-539 for 72 hours

Drugs	IC$_{50}$ ± SEM (μM)
ABT-199	20.5 ± 3.1
A-1210477	21.5 ± 0.3
WEHI-539	14.7 ± 2.7

some SCLC cells depend on both these proteins for survival (Leverson *et al.*, 2015).

Collectively, our data demonstrate that NPC cell line HK1 neither depend solely on BCL-2 nor BCL-XL nor MCL-1 for survival implying functional redundancy in the BCL-2 anti-apoptotic proteins. Inhibition of all three anti-apoptotic proteins, BCL-2-BCL-XL-MCL-1 was required to

Table 2. The synergistic drug effects of combination of ABT-199, A-1210477 and WEHI-539 in the HK1 cell line. Cells were treated with increasing concentrations of ABT-199 in the absence and presence of A-1210477 (4 µM) and WEHI-539 (4 µM)

ABT-199 [µM]	A-1210477 [µM]	WEHI-539 [µM]	CI	Drug Combination Interaction
0.25			0.328	Synergism
0.5			0.302	Synergism
1	4	4	0.299	Strong synergism
2			0.248	Strong synergism
4			0.355	Synergism
8			0.289	Strong synergism

Note: Combination index (CI) values were calculated using CompuSyn 1.0 (ComboSyn Inc., NJ, USA). [ ] indicates drug concentration; CI, combination index; values between 0.1–0.3 indicate strong synergy and values between 0.3–0.7 indicate synergy.

inhibit cell proliferation of the HK1 cells suggesting that all three proteins mediate cell survival. We acknowledge that our study has limitations. We present results generated from only one NPC cell line, to parse the contributions of the anti-apoptotic proteins for NPC cell survival. Future experiments will include additional NPC cell lines (Epstein-Barr virus (EBV) positive cell lines) to confirm findings reported in this study. Given that there are growing evidences that solid tumours respond more effectively to co-inhibition of BCL-XL and MCL-1 (Baranski *et al.*, 2015; Leverson *et al.*, 2015; de Jong, van *et al.*, 2016; Lee *et al.*, 2019), future studies will focus on testing the sensitivity of NPC cell lines to co-inhibition of these two proteins and findings will be compared to sensitivity of cells to co-inhibition of BCL-2 and MCL-1. Moreover, the contribution of anti-apoptotic protein BFL-1 for NPC cell survival will also be interrogated. However, with the findings put forward we believe that drugs targeting combinations of BCL-2 anti-apoptotic proteins could be promising treatment approach for NPC management in the future.

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REFERENCES

Adams, J.M. & Cory, S. (2018). The BCL-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death Differ*, **25**(1): 27-36.

Ashkenazi, A., Fairbrother, W.J., Leverson, J.D. & Souers, A.J. (2017). From basic apoptosis discoveries to advanced selective BCL-2 family inhibitors. *Nature Reviews Drug Discovery*, **16**(4): 273.

Baranski, Z., de Jong, Y., Ilkova, T., Peterse, E.F., Cleton-Jansen, A.M., van de Water, B. & Danen, E.H. (2015). Pharmacological inhibition of Bcl-xL sensitizes osteosarcoma to doxorubicin. *Oncotarget*, **6**(34): 36113.

Carrington, E.M., Zhan, Y., Brady, J.L., Zhang, J.G., Sutherland, R.M., Anstee, N.S. & Huntington, N.D. (2017). Anti-apoptotic proteins BCL-2, MCL-1 and A1 summate collectively to maintain survival of immune cell populations both *in vitro* and *in vivo*. *Cell Death & Differentiation*, **24**(5): 878-888.

Chou, T.C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*, **58**(3): 621-681.

de Jong, Y., van Maldegem, A.M., Marino-Enriquez, A., de Jong, D., Suijker, J., Briaire-de Bruijn, I.H. & Fletcher, J.A. (2016). Inhibition of Bcl-2 family members sensitizes mesenchymal chondrosarcoma to conventional chemotherapy: report on a novel mesenchymal chondrosarcoma cell line. *Lab Invest*, **96**(10): 1128-1137.

Lee, E.F., Harris, T.J., Tran, S., Evangelista, M., Arulananda, S., John, T. & Segal, D. (2019). BCL-XL and MCL-1 are the key BCL-2 family proteins in melanoma cell survival. *Cell Death Dis*, **10**(5): 1-14.

Lessene, G., Czabotar, P.E., Sleebs, B.E., Zobel, K., Lowes, K.N., Adams, J.M. & Flygare, J.A. (2013). Structure-guided design of a selective BCL-X L inhibitor. *Nature Chemical Biology*, **9**(6): 390-397.

Leverson, J.D., Phillips, D.C., Mitten, M.J., Boghaert, E.R., Diaz, D., Tahir, S.K. & Lowes, K.N. (2015). Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Science Translational Medicine,* **7**(279): 279ra240.

Leverson, J.D., Zhang, H., Chen, J., Tahir, S.K., Phillips, D.C., Xue, J. & Kovar, P. (2015). Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death & Disease*, **6**(1): e1590-e1590.

Lucas, K.M., Mohana-Kumaran, N., Lau, D., Zhang, X.D., Hersey, P., Huang, D.C. & Allen, J.D. (2012). Modulation of NOXA and MCL-1 as a strategy for sensitizing melanoma cells to the BH3-mimetic ABT-737. *Clinical Cancer Research*, **18**(3): 783-795.

Mohana-Kumaran, N., Hill, D.S., Allen, J.D. & Haass, N.K. (2014). Targeting the intrinsic apoptosis pathway as a strategy for melanoma therapy. *Pigment Cell & Melanoma Research*, **27**(4): 525-539.

Montero, J. & Letai, A. (2018). Why do BCL-2 inhibitors work and where should we use them in the clinic? *Cell Death Differ*, **25**(1): 56-64.

Morales, A.A., Kurtoglu, M., Matulis, S.M., Liu, J., Siefker, D., Gutman, D.M. & Boise, L.H. (2011). Distribution of Bim determines Mcl-1 dependence or codependence with Bcl-xL/Bcl-2 in Mcl-1 – expressing myeloma cells. *Blood, The Journal of the American Society of Hematology*, **118**(5): 1329-1339.

Souers, A.J., Leverson, J.D., Boghaert, E.R., Ackler, S.L., Catron, N.D., Chen, J. & Huang, D.C. (2013). ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nature Medicine*, **19**(2): 202.

Vogler, M., Butterworth, M., Majid, A., Walewska, R.J., Sun, X.M., Dyer, M.J. & Cohen, G.M. (2009). Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. *Blood, The Journal of the American Society of Hematology*, **113**(18): 4403-4413.

Yecies, D., Carlson, N.E., Deng, J. & Letai, A. (2010). Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood, The Journal of the American Society of Hematology*, **115**(16): 3304-3313.