Short Communication

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Selective BH3-mimetics targeting BCL-2, BCL-X_L or MCL-1 induce severe mitochondrial perturbations

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Abstract: Induction of apoptosis by selective BH3-mimetics is currently investigated as a novel strategy for cancer treatment. Here, we report that selective BH3-mimetics induce apoptosis in a variety of hematological malignancies. Apoptosis is accompanied by severe mitochondrial toxicities upstream of caspase activation. Specifically, the selective BH3-mimetics ABT-199, A-1331852 and S63845, which target BCL-2, BCL-X_L and MCL-1, respectively, induce comparable ultrastructural changes including mitochondrial swelling, a decrease of mitochondrial matrix density and severe loss of cristae structure. These shared effects on mitochondrial morphology indicate a similar function of these anti-apoptotic BCL-2 proteins in maintaining mitochondrial integrity and function.

Keywords: apoptosis; BCL-2 proteins; BH3-mimetics; mitochondria.

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Due to their central role in regulating apoptosis, the anti-apoptotic BCL-2 proteins are promising targets for the development of novel anticancer therapeutics (Adams and Cory, 2007). To this end, several so-called BH3-mimetics have been developed that bind with nanomolar affinities to the hydrophobic groove of anti-apoptotic BCL-2 proteins (Vogler et al., 2009). By binding and inhibiting anti-apoptotic BCL-2 proteins like BCL-2, BCL-X_L and MCL-1, BH3-mimetics can induce the activation and oligomerization of BAX and/or BAK, which form pores in the outer mitochondrial membrane. Although the precise molecular nature of these pores is still not fully characterized, it is well reported that activation of BAX and/or BAK is sufficient to facilitate the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol and hence induce the activation of caspases and apoptosis (Westphal et al., 2014). An alternative way for cytochrome *c* to escape from the mitochondrial intermembrane space involves the opening of the permeability transition pore (PTP) in the inner mitochondrial membrane, which results in mitochondrial swelling, loss of cristae structure, rupture of the outer mitochondrial membrane and subsequent release of pro-apoptotic factors like cytochrome *c* (Giorgio et al., 2018).

We previously reported that treatment of chronic lymphocytic leukemia cells with either ABT-199 or ABT-737, a BH3-mimetic inhibiting BCL-2 and BCL-X_L, causes apoptosis accompanied by mitochondrial swelling, loss of cristae structure and rupture of the outer mitochondrial membrane, features more commonly associated with the opening of the PTP in the inner mitochondrial membrane than with BAX/BAK pore formation in the outer mitochondrial membrane (Vogler et al., 2008, 2013). In this study, we utilized specific and selective inhibitors of either BCL-2 (ABT-199) (Souers et al., 2013), BCL-X_L (A-1331852) (Leverson et al., 2015) or MCL-1 (S63845) (Kotschy et al., 2016) to investigate the effects of BH3-mimetics on mitochondrial morphology during apoptosis in hematological malignancies. Treatment with low concentrations of ABT-199 induces rapid apoptosis in RIVA (diffuse large B-cell lymphoma, DLBCL) as well as MAVER-1 (mantle

cell lymphoma) cells as demonstrated by externalization of phosphatidylserine (PS) in the cell membrane of the apoptotic cells (Figure 1A). In line with an activation of the intrinsic apoptotic pathway, exposure to BH3-mimetics also induced simultaneous loss of mitochondrial membrane potential (Figure 1B). To investigate whether exposure to ABT-199 also resulted in ultrastructural changes, upstream and/or independent of caspase activation and subsequent cleavage of cellular targets, we treated RIVA and MAVER-1 with ABT-199 in the presence of the caspase inhibitor z-VAD.fmk before performing transmission electron microscopy. For these experiments, z-VAD.fmk was included as we had previously shown cell death induced by ABT-199 to be caspase-mediated (Vogler et al., 2008), and in these experiments were aiming to investigate only cellular effects that were induced upstream of caspase activation. As anticipated, in comparison to untreated control cells, cells treated with ABT-199 in the presence of z-VAD.fmk exhibit no overall change in nuclear or cellular morphology (data not shown). Mitochondria in the untreated control cells possess intact outer and inner membranes with well-defined cristae, characteristic of healthy mitochondria with structural and functional integrity (Figure 1C and D). In contrast, cells treated with ABT-199 exhibit strikingly swollen mitochondria, with a significant loss of mitochondrial matrix density and a ruptured outer mitochondrial membrane, suggesting that inhibition of BCL-2 resulted in marked mitochondrial ultrastructural changes, upstream and/or independent of the other hallmarks of apoptosis (Figure 1E and F).

To investigate the effect of BCL-X_L inhibition on mitochondrial structural changes, RCK8 (DLBCL) and K562 (chronic myeloid leukemia) cells that primarily rely on BCL-X_L for survival were chosen (Figure 2A and B). RCK8 cells exhibit a mixed mitochondrial morphology, with some cells displaying dark mitochondria indicative of

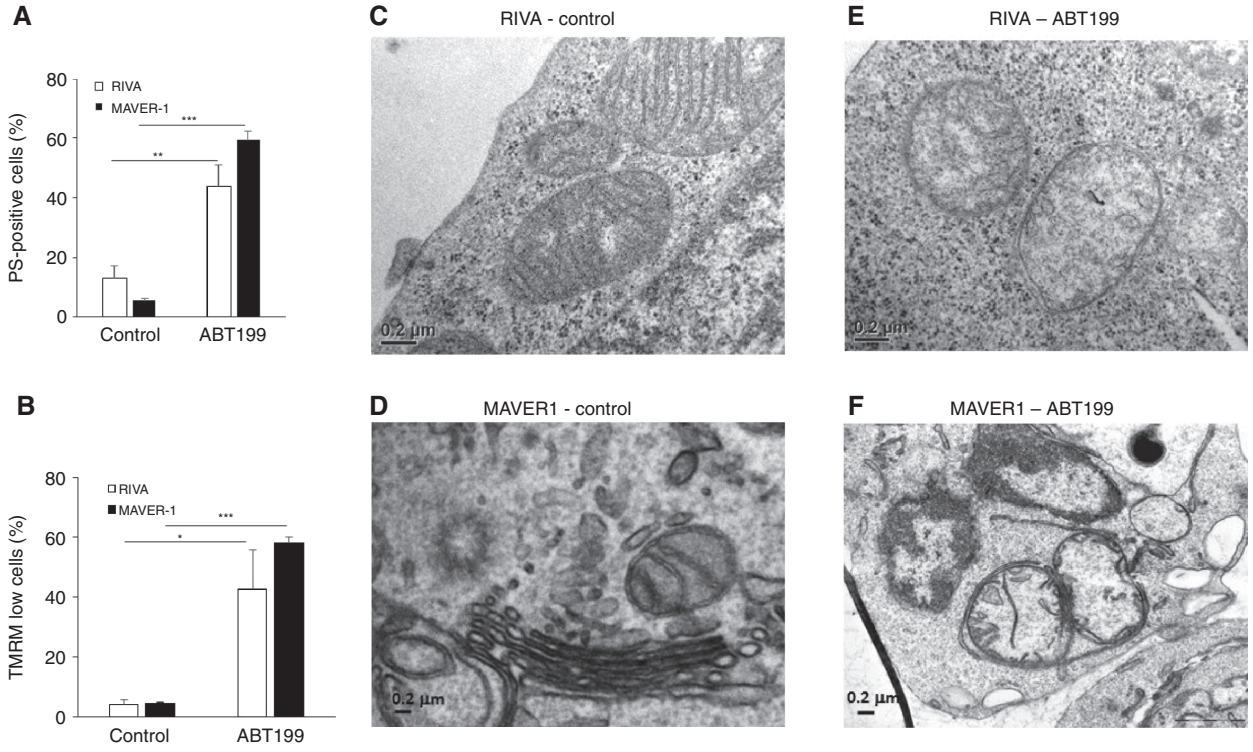

Figure 1: Mitochondrial perturbations induced by ABT-199 in BCL-2 dependent cells.
(A and B) RIVA and MAVER-1 cells were exposed to 10 nM ABT-199 (Selleck Chemicals, Houston, TX, USA) for 4 h before analysis of apoptosis by PS exposure and binding of AnnexinV-FITC followed by flow cytometry (A) or loss of mitochondrial membrane potential (B) as measured by staining with 50 nM tetramethylrhodamine methyl ester (TMRM, Sigma, Deisenhofen, Germany) and flow cytometry. Flow cytometry was performed using FACSCanto II (BD Biosciences, Heidelberg, Germany). Data presented are mean plus standard deviation (n = 3 for RIVA and n = 4 for MAVER-1). Asterisks indicate statistical significance as analyzed using Student's *t*-test with two-tailed distribution, two-sample, unequal variance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (C and E) RIVA cells were either left untreated (C) or exposed to 10 nM ABT-199 for 4 h in the presence of 25 µM z-VAD.fmk (Bachem, Heidelberg, Germany) (E) before fixation in 2.5% glutaraldehyde and processing for electron microscopy. (D and F) MAVER-1 cells were either left untreated (D) or exposed to 10 nM ABT-199 for 2 h in the presence of z-VAD.fmk (25 µM) (F) before fixation in 2.5% glutaraldehyde and processing for electron microscopy.

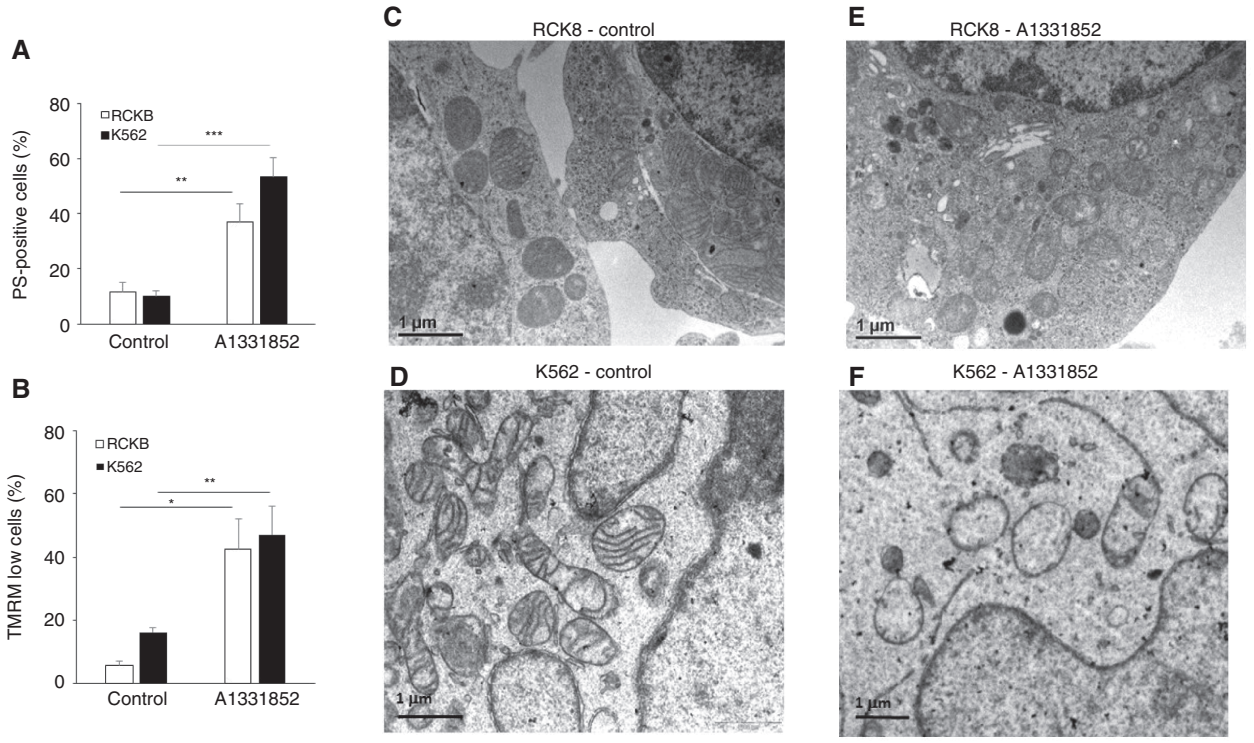

Figure 2: Mitochondrial perturbations induced by A-1331852 in BCL-X_L dependent cells.
(A and B) RCK8 and K562 cells were exposed to A-1331852 (Selleck Chemicals) (3 nM for RCK8 and 10 nM for K562 cells) for before analysis of apoptosis by PS exposure and binding of AnnexinV-FITC (A) or loss of mitochondrial membrane potential (B) as measured by staining with TMRM and flow cytometry. Data presented are mean plus standard deviation (n = 4). Asterisks indicate statistical significance as analyzed using Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) (C and E) RCK8 cells were either left untreated (C) or exposed to 3 nM A-1331852 for 4 h in the presence of z-VAD.fmk (25 µM) (E). (D and F) K562 cells were either left untreated (D) or exposed to 10 nM A-1331852 for 2 h in the presence of z-VAD.fmk (25 µM) (F) before fixation in 2.5% glutaraldehyde and processing for electron microscopy.

higher matrix density, and other cells displaying larger and brighter mitochondria with less dense mitochondrial matrix and regular cristae structures (Figure 2C). In cells exposed to A-1331852, the mitochondrial cristae structure is largely lost and mitochondria appear smaller, indicative of mitochondrial fragmentation or fission. Rupture of the outer mitochondrial membrane is also evident in some swollen mitochondria (Figure 2E). Similarly, the well-defined mitochondrial cristae in the untreated K562 cells are completely lost following exposure to A-1331852, indicative of severe mitochondrial toxicity (Figure 2D and F). Interestingly, BCL-X_L has recently been described to maintain a functional mitochondrial network, and BCL-X_L knockout murine embryonic fibroblasts displayed fragmented mitochondria as assessed by fluorescence microscopy (Pfeiffer et al., 2017).

Next, we investigated the effects of a specific and potent inhibitor of MCL-1, S63845, whose derivative, MIK665 is currently being investigated in clinical trials for the treatment of multiple myeloma and acute myeloid leukemia (NCT02979366 and NCT02992483). Using SUDHL10 DLBCL cells that depend primarily on MCL-1 for survival, we observe a rapid induction of intrinsic apoptosis upon exposure to S63845 accompanied by a loss of mitochondrial membrane potential (Figure 3A and B). SUDHL10 cells contain large clearly defined bright mitochondria with regular cristae structures. Exposure to S63845 induces a prominent loss of cristae structures and rupture of the outer mitochondrial membranes in some affected mitochondria. Inhibition of MCL-1 by S63845 also induces more fragmented, smaller mitochondria with vesicular structures rather than the well-defined cristae structures, in line with our previous report on mitochondrial fragmentation induced by the related MCL-1 inhibitor, A-1210477 (Milani et al., 2017). Interestingly, a similar mitochondrial phenotype was previously reported in MCL-1 knockout murine embryonic fibroblasts (Perciavalle et al., 2012). In that study, MCL-1 deletion induced the appearance of punctate mitochondria, the loss of the tubular mitochondrial network as well as ultrastructural defects including defective cristae harboring balloon-like, vesicular structures.

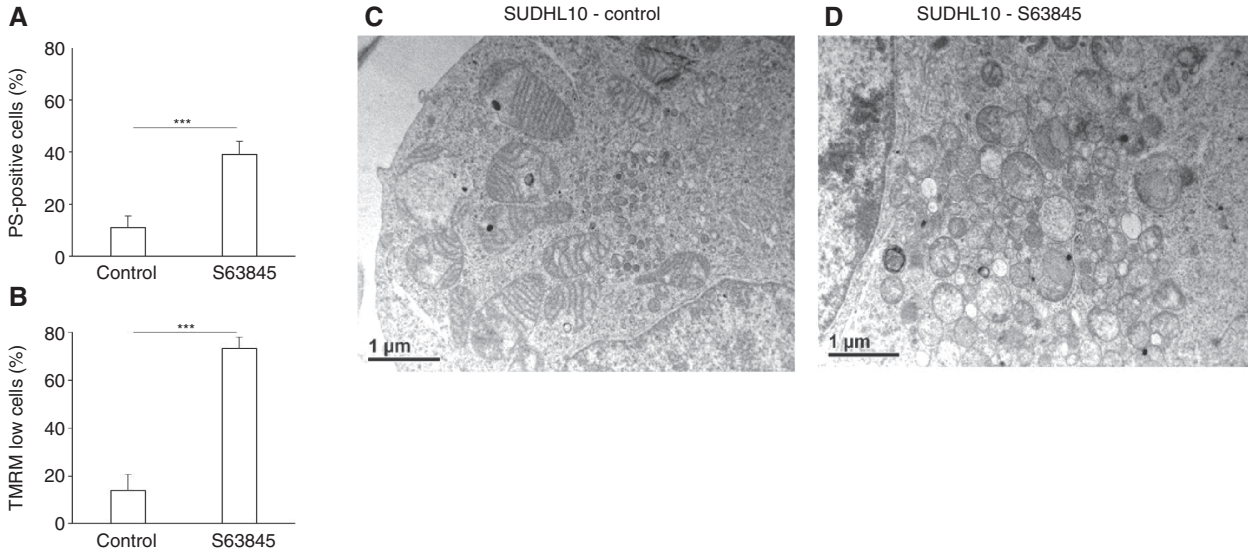

Figure 3: Mitochondrial perturbations induced by inhibition of MCL-1.
(A and B) SUDHL10 cells were exposed to 100 nM S63845 (Active Biochemicals, Bonn, Germany) for 4 h before analysis of apoptosis by PS exposure and binding of AnnexinV-FITC (A) or loss of mitochondrial membrane potential (B) as measured by staining with TMRM and flow cytometry. Data presented are mean plus standard deviation (n = 4). Asterisks indicate statistical significance as analyzed using Student's *t*-test (***$p < 0.001$). (C and D) SUDHL10 cells were either left untreated (C) or exposed to 100 nM S63845 for 4 h in the presence of z-VAD.fmk (25 µM) (D) before fixation in 2.5% glutaraldehyde and processing for electron microscopy.

Although mitochondria are involved in apoptotic and necrotic forms of cell death, the molecular characteristics of apoptosis and necrosis are very different. While classical apoptosis involves the formation of a BAX/BAK mediated pore in the outer mitochondrial membrane, necrotic cell death is facilitated by opening of the PTP, a calcium-dependent channel connecting inner and outer mitochondrial membranes (Kroemer et al., 2007). Upon PTP opening, small solutes and water move osmotically into the mitochondrial matrix, thus causing swelling and rupture of the outer mitochondrial membrane (Bernardi et al., 2006). Our data showing swelling of mitochondria upon exposure to selective BH3-mimetics indicate that BCL-2, BCL-X_L and MCL-1 all play a role in the regulation of PTP opening. This conclusion is supported by the finding that also BAX and BAK can be involved in the formation of the PTP (Karch et al., 2013), thus providing a molecular link between the anti-apoptotic BCL-2 proteins and PTP opening.

In addition to swelling we observe a prominent loss of cristae structures upon exposure to BH3-mimetics. A large fraction of mitochondrial cytochrome *c* is located and sequestered in the cristae, and hence efficient release of cytochrome *c* requires the opening of cristae structures (Scorrano et al., 2002). Opening of mitochondrial cristae junctions is regulated by the dynamin-related protein optic atrophy 1 (OPA1) (Frezza et al., 2006). Interestingly, several reports have linked the BCL-2 family to cristae opening, where BH3-only proteins and/or active BAX/BAK are required to facilitate OPA1 mediated cristae opening and subsequent cytochrome *c* release (Yamaguchi et al., 2008; Landes et al., 2010).

Taken together, we describe a common function of the anti-apoptotic BCL-2 proteins BCL-2, BCL-X_L and MCL-1 in maintaining mitochondrial integrity and cristae structures, which can be antagonized by using selective BH3-mimetics and occurs during BH3-mimetic induced apoptosis in malignant hematological cells.

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Conflict of interest statement: The authors have no conflict of interest to declare.

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