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Synergistic interaction between HDAC and MCL-1 inhibitors through downregulation of BCL-XL in multiple myeloma

Anja Schneller,1,2 Niklas Zojer,1 #Arnold Bolomsky,1 #Heinz Ludwig,1*
1 Department of Medicine I, Wilhelminen Cancer Research Institute, Clinic Ottakring, Vienna, Austria
2 Recipient of a DOC Fellowship of the Austrian Academy of Sciences at the Wilhelminen Cancer Research Institute

# These authors contributed equally to this work
*Corresponding author, E-mail: heinz.ludwig@extern.gesundheitsverbund.at

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Author Contributions
A.S. designed and performed experiments and analyzed results. A.B. encouraged the investigation, designed experiments and provided intellectual input and expertise. N.Z. provided supervision and expertise. H.L. provided supervision and secured funding. A.S. and A.B. wrote the manuscript.

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The development of novel therapies is the most important catalyst for the advancement in the treatment of patients with multiple myeloma (MM). Presently, a number of new
therapies including immunotherapeutic drugs, cellular therapies and BH3 mimetics are introduced into clinical practice. This steadily increasing number of effective treatment options makes it practically impossible to compare all available regimens and treatment concepts with each other in different settings. Hence, it becomes increasingly important to obtain a deeper understanding of the mechanism of activity of individual drugs to enable optimal selection of combination partners and treatment sequences in clinical practice1.

In MM, the anti-apoptotic BH3 family member MCL-1 was shown to act as a master regulator of cell survival and resistance to therapy 2,3. Accordingly, several MCL-1 inhibitors, such as S63845, S64315, AMG176 and AZD5991 are currently evaluated in clinical trials 4. However, elevated expression of either BCL-XL or BCL-2 may affect the activity of MCL-1 inhibitors (MCL-1i) 5. This suggests that the simultaneous targeting of multiple anti-apoptotic proteins might significantly enhance the activity of BH3 mimetics and overcome intrinsic as well as acquired drug resistance. In this context, a deregulation of BH3-protein family members by HDAC inhibitors (HDACi) was reported in MM 6 making these compounds attractive combination partners for BH3 mimetics.

Here, we aimed to evaluate synergistic or additive combination approaches for selected BH3 mimetics. We assessed the activity of the pan-HDACi panobinostat or the HDAC6i ricolinostat in combination with inhibitors targeting either BCL-2 (venetoclax), BCL-XL (A-1331952) or MCL-1 (S63845) in a panel of MM cell lines (MM.1S, KMS-12-BM, MOLP-8, U266, SKMM-1, RPMI-8226, OPM-2, NCI-H929). Interestingly, in 6/8 MM cell lines (KMS-12-BM, MM.1S, U266, MOLP-8, NCI-H929, OPM-2) a synergistic or additive effect was observed when combining S63845+HDACi (Figure 1A&D, supplementary Figure 1A-H; not shown). The combination of venetoclax or A-1331952 with either HDACi led to synergistic or additive activity in 3 and 4 cell lines, respectively (Figure 1B&E supplementary Figure 1I-N; not shown).

The observed synergism was confirmed with alternative MCL-1 inhibitors (AZD5991, AMG-701) (not shown) and translated into a significant increase in apoptosis in MM.1S, U266 and KMS-12-BM monoculture experiments using non-lethal concentrations of
S63845, panobinostat and ricolinostat (Figure 1G-I). Similar effects were observed in co-culture experiments with MSCT+ stromal cells (Figure 1J-L). Additional validation experiments confirmed the observed augmentation of the apoptotic signaling cascade including an enhanced release of cytochrome c (Figure 1M), cleavage of caspases 3 and PARP (Figure 1N; supplementary figure 1O&P) in all cell lines analyzed. No cell cycle alterations were observed upon single agent or combinational therapy (supplementary figure 2C-E).

The combination of S63845+HDACi proves to be particularly pronounced in the BCL-XL (co)-dependent MM cells MM1.S and U266, which otherwise would not or only barley respond to single-agent MCL-1 inhibition. Moreover, BCL-XL is not only a major driver of intrinsic but also acquired MCL-1 inhibitor resistance, as well as dual MCL1/BCL2 inhibition. Hence, concurrent BCL-XL inhibition seems to optimally augment the efficacy of MCL-1 inhibitors, but prior clinical trials aiming to directly inhibit BCL-XL failed due to untoward toxicity.

Based on these results we evaluated whether deregulation of pro- or antiapoptotic Bcl-2 family proteins by HDAC inhibitors explains the observed synergism. Single agent treatment with S63845 monotherapy led to the accumulation of MCL-1 and BCL-XL in MM.1S (Figure 2A) and U266 (supplementary figure 2A), but not in KMS-12-BM cells (supplementary figure 2B). Conversely, combined MCL-1+HDAC inhibition led to the downregulation of BCL-XL and MCL-1 protein levels in all tested cell lines prior to onset of apoptosis (Figure 2B; supplementary figure 2A-B). In addition, a significant increase in BAK activation in KMS-12-BM cells (supplementary figure 1R) was noted. In MM.1S and U266 cell lines BAK is already fully activated by S63845 alone and the combination of MCL-1i and HDACi did not augmented it any further (Figure 2B and supplementary figure 1MQ), suggesting that active BAK is kept under control by alternative anti-apoptotic family members – most likely BCL-XL.

In order to test our assumption that BCL-XL inhibits apoptosis induction by S63845, we transduced MM cell lines with the Tet-on pcW57.1 vector harboring either full length
BCL-X<sub>L</sub> or EGFP (control) and assessed apoptotic cells via flow cytometry. In MM.1S-BCL-X<sub>L</sub> cells, apoptosis significantly decreased upon treatment with S63845+HDACi (Figure 2C left panel) compared to MM.1S-EGFP cells (Figure 2C right panel). Similar findings were obtained in U266 and KMS-12-BM cells (supplementary figure 2F-G). To explore this rescue mechanism further, we examined the binding kinetics of BAK and BIM to BCL-X<sub>L</sub> via co-immunoprecipitations. Upon doxycycline mediated induction of BCL-X<sub>L</sub> or EGFP protein, we treated the cells for 24 h with S63845 alone or in combination with either panobinostat or ricolinostat. Strikingly, BCL-X<sub>L</sub> overexpression in S63845+HDACi treated cells resulted in an increased association between BCL-X<sub>L</sub> and BAK as well as BIM in all investigated cell lines (left panel of figure 2D-G; supplementary figure 2H-I and 3A-D). On the contrary, in EGFP expressing cells, BIM and/or BAK binding to BCL-X<sub>L</sub> strongly decreased upon combined S63845+HDACi exposure as compared to S63845 treatment alone (right panel of figure 2D-G; supplementary figure 2H-I and 3A-D).

Noteworthy, we also observed cell line and combination specific effects such as an exclusive impact of ricolinostat or panobinostat on BIM-BCL-X<sub>L</sub> but not BAK-BCL-X<sub>L</sub> binding, in MM.1S-EGFP and U266-EGFP cells, respectively (right panel of figure 2F-G; right panel of supplementary figure 2H-I). Furthermore, in KMS-12-BM-EGFP cells BIM was not associated with BCL-X<sub>L</sub> (right panel supplementary figure 3C; not shown), as BIM is rather sequestered by BCL-2 (not shown) in line with the MCL-1/BCL-2 dependency of this t(11;14) carrying cell line. In conclusion, these results strengthen a model where BCL-X<sub>L</sub> is capable to sequester BAK and BIM released by S63845, thus prohibiting the onset of the apoptotic signaling cascade.

We next aimed to confirm the impact of panobinostat and ricolinostat on histone and tubulin acetylation as mechanism of synergy with MCL-1 inhibitors. Panobinostat is expected to strongly increase histone acetylation, while ricolinostat, which is selectively targeting HDAC6<sup>13,14</sup>, should increase acetylated alpha-tubulin without altering acetylated histone levels<sup>15</sup>. We treated MM.1S, U266 and KMS-12-BM cells with both HDACi and assessed acetylated and total protein expression levels of α-tubulin and
histone-3 after 24 h. Panobinostat strongly increased acetyl-histone-3 in all three cell lines (Figure 3A, supplementary figure 3E-F). Contrary to our expectations, ricolinostat treatment likewise led to a strong increase in acetyl-histone-3 besides elevating acetyl-alpha-tubulin levels (Figure 3A, supplementary figure 3E-F), indicating that ricolinostat has off-target effects on additional HDAC family members.

Accordingly, we aimed to investigate whether the synergism of ricolinostat and S63845 is facilitated via HDAC6 inhibition or via the epigenetic off-target effect. To this end, we performed viability assays with MM.1S and U266 cells transduced with a Tet-on miR-E plasmid harboring a control shRNA (Renilla), or HDAC6 shRNA. HDAC6 knockdown was confirmed 48 h after induction with 0.3µg/ml doxycycline (Figure 3B, supplementary figure 3G). However, S63845 significantly decreased cell viability regardless of the presence of an HDAC6 knockdown (Figure 3C-D, supplementary figure 3H-J). These results suggest that the synergism between S63845 and ricolinostat is due to the unspecific epigenetic effect of ricolinostat.

To validate our findings in primary MM cells we treated CD138-selected MM cells ex vivo for 20 h with single-agent HDACi and S63845 as well as the corresponding combinations before evaluating apoptosis induction. This demonstrated an increase of apoptotic cells upon combination treatment with S63845+HDACi in three out of four samples tested, whereas the magnitude was highly variable (Figure 3E-H). Unfortunately, we were not able to collect sufficient cell material to establish a link between Bcl-2 family dependencies and combination activity. However, we investigated whether the synergism accompanied by a downregulation of BCL-X_L in MM patient samples ex vivo. For this purpose, we treated patient samples for 10 h with S63845 alone or in combination with HDACi and determined BCL-X_L protein expression. In both analyzed patient samples, downregulation of BCL-X_L protein expression was observed in the range of MM cell lines (approx. 30% vs. S63845 single-agent treatment) upon S63845 combination with either panobinostat or ricolinostat, respectively. (Figure 3I-J). This suggests that the combination of MCL-1 inhibitors with HDACi is capable to tackle both, MCL-1 and BCL-X_L, in MM patient cells. However, our findings need to be
confirmed in enlarged patient cohorts and advanced *in vivo* models (i.e. carrying humanized MCL-1) \(^4\) to better evaluate the clinical potential of our results as well as to define patient stratification markers.

In conclusion, our findings support a model where BCL-\(X_L\) sequesters BAK/BIM released in response to MCL-1 inhibition, particularly in tumor clones with baseline BCL-\(X_L\) functionality such as MM.1S and U266 cells. By combining MCL-1i with HDACi, BCL-\(X_L\) protein is downregulated, leading to unrestrained BAK activation and initiation of the apoptotic signaling cascade (*Figure 3K*). Previous efforts to pharmacologically target BCL-\(X_L\) failed due to its role in megakaryopoiesis \(^{12,11}\). Hence, our findings point towards an alternative opportunity to indirectly tackle BCL-\(X_L\)/MCL-1 co-dependent MM cells by combining Mcl-i1 and HDACi and highlight the importance of exploring various options of apoptosis induction for designing new treatment concepts for clinical evaluation.
References


Figure legends

Figure 1.
Concurrent MCL-1 and HDAC inhibition synergistically kills MM cells in vitro via apoptosis induction. Cell viability of MM1.S cells 96 h after treatment with S63845 (A)(D), Venetoclax (B)(E), A-1331952 (C)(F) alone or in combination with either panobinostat (A-C) or ricolinostat (D-F). Results are presented relative to 0.1% DMSO control. Combination index (CI) was calculated and stated as range. CI values of < 0.8, 0.8–1.2, and >1.2 were interpreted as synergistic, additive, and antagonistic drug activity, respectively. Apoptosis induction in MM.1S, U266 and KMS-12-BM cells was assessed by 7AAD/Annexin V staining 72 h after treatment in absence (G-I) or presence of MSCT+ stromal cells (J-L). (M) Cytochrome c release assay 24 h after treatment initiation at the indicated concentrations. One representative experiment of 2 is shown. (N) Assessment of cleaved caspase 3 and cleaved PARP via flow cytometry was performed 48 or 72 h post treatment induction, respectively. Error bars indicate standard deviation of the mean (SDM) of triplicate experiments. Differences between groups were calculated with one-way ANOVA, corrected for multiple comparison with Bonferroni-Holm correction, where **** denotes $P <0.0001$, ** denotes $P<0.001$ and * denotes $P<0.05$

Figure 2.
BCL-XL overexpression sequesters BIM and BAK and impairs S63845 mediated apoptosis induction. (A) Immunoblot analysis of the indicated Bcl-2 family members was performed 24 h post treatment initiation. One representative experiment of three independent biological replicates is shown. (B) BAK activation in MM.1S was determined by staining with antibodies against its active form. (C) MM.1S cells transduced with pcW57.1 EGFP (left panel) or pcW57.1 BCL-XL (right panel) were treated for 24 h with 0.5µg/ml doxycycline to induce protein overexpression and afterwards exposed to the indicated treatments. Apoptotic cells were assessed 24 h post treatment induction. Results indicate the mean +/- SDM of three independent experiments. Differences between groups were calculated with one-way ANOVA,
corrected for multiple comparison with Bonferroni-Holm correction, where **** denotes $P < 0.0001$, ** denotes $P < 0.001$ and * denotes $P < 0.05$ (D) – (G) Co-immunoprecipitation experiments in MM.1S cells transduced with either pcW57.1-EGFP (right panels) or pcW57.1-BCL-XL (left panels) were performed after 24h pretreatment with 0.5$\mu$g/ml doxycycline to induce protein overexpression and subsequent drug exposure for 24 h.

**Figure 3.**

**Ricolinostat promotes the activity of S63845 independent of HDAC6 inhibition.** (A) MM.1S cells were either treated with panobinostat or ricolinostat for 24 h, then whole-cell lysates were blotted for the indicated proteins. (B) single cell clones of MM.1S cells harboring a Tet-on miR-E vector expressing either a shRNA targeting Renilla (control) or an shRNA targeting HDAC6 were exposed to 0.3 $\mu$g/ml doxycycline for 48 h and whole-cell lysates were blotted for the indicated proteins. Western blots are representative for three independent experiments. (C)(D) Cells were pretreated with doxycycline for 48 h and viability was assessed after an additional 48 h treatment with increasing concentrations of S63845 as indicated in the figure. Results show the mean +/- SD of three independent experiments performed in triplicates. (E)-(J) CD138 purified plasma cells sorted from patients with multiple myeloma were exposed to S63845, panobinostat or ricolinostat the respective combinations for 20 h (E)-(H) or 10 h (I)-(J). (E)-(H) Apoptotic cells were determined via Annexin V / 7AAD positive staining. (I)-(J) Whole-cell lysates of primary MM cells were blotted for the indicated proteins. Short-term exposure of primary patient samples was chosen to avoid spontaneous cell death. (K) Proposed model of the underlying mechanism of the observed synergism between MCL-1i and HDACi in MM. In S63845 treated cells BCL-XL is capable to sequester BAK, hence inhibiting the apoptotic signaling cascade resulting in diminished MM cell death. In combination with HDACi, BCL-XL protein is downregulated and thereby BAK released by MCL-1i can be activated, oligomerize and insert into the MOMP which in turn releases cytochrome c, activates initiator as well as effector caspases, leading to PARP cleavage and cell death.
**MM.1S Panobinostat + Ricolinostat**

- α-Tubulin 52 kDa
- GAPDH 37 kDa
- Histone-3 17 kDa

**MM.1S Tet-on miR-E Renilla**

- Viability in %
- S63845 [nM] 0, 62.5, 125, 250, 500

**MM.1S Tet-on miR-E shRNA**

- Viability in %
- S63845 [nM] 0, 62.5, 125, 250, 500

**Patient #1**

- 7AAD/Annexin V + cells in %
- S63845 [65nM], Panobinostat [8nM], Ricolinostat [1.7μM]

**Patient #2**

- 7AAD/Annexin V + cells in %
- S63845 [65nM], Panobinostat [8nM], Ricolinostat [1.7μM]

**Patient #3**

- 7AAD/Annexin V + cells in %
- S63845 [65nM], Panobinostat [8nM], Ricolinostat [1.7μM]

**Patient #4**

- 7AAD/Annexin V + cells in %
- S63845 [65nM], Panobinostat [8nM], Ricolinostat [1.7μM]

**Patient #5**

- Relative BCL-XL expression
- BCL-XL
- GAPDH 37 kDa

**Patient #6**

- Relative BCL-XL expression
- BCL-XL
- GAPDH 37 kDa

**K**

- HDACi
- BCL2, BAX, MCL1

- BCLXL sequesters freed BIM and BAK

- Caspase 3 activation
- Caspase 9 activation

- BAK activation

- + HDACi

- Apoptosis
Figure legends

Supplement figure 1.
Concurrent MCL-1 and HDAC inhibition synergistically kills MM cells *in vitro*. Cell viability of indicated human MM cell lines 96h after treatment with S63845 (A)-(H) and venetoclax (I)-(N) alone or in combination with either panobinostat (A),(C),(E),(G),(I),(K),(M) or ricolinostat (B),(D),(F),(H),(J),(L),(N). Results are presented relative to 0.1% DMSO control. Combination index (CI) values of < 0.8, 0.8–1.2, and >1.2 were interpreted as synergistic, additive, and antagonistic drug activity, respectively. (O)-(P) Assessment of cleaved caspase 3 and cleaved PARP was performed via flow cytometry 48 or 72 hours post treatment induction, respectively. (Q)-(R) BAK activation was determined by staining with antibodies against its active form. Graphs show the mean +/- standard deviation of the mean (SDM) of triplicate experiments. Differences between groups were calculated with one-way ANOVA, corrected for multiple comparison with Bonferroni-Holm correction, where **** denotes \( P < 0.0001 \), ** denotes \( P < 0.001 \) and * denotes \( P < 0.05 \)

Supplement figure 2.
S63845 in combination with HDACi downregulates BCL-X\(_L\) and BCL-X\(_L\) overexpression reduces apoptosis by sequestering BIM and BAK. (A-B) Immunoblot analysis of the indicated Bcl-2 family members was performed 24 h post treatment initiation. One representative experiment of three independent biological replicates is shown. (C-E) Cell cycle analysis was performed 24h after treatment initiation with either single-agent or combination treatment of S63845 and HDACi. Differences between groups were calculated with three-way ANOVA, corrected for multiple comparison with Bonferroni-Holm correction. (FG) MM cells transduced with pcW57.1 EGFP (left panel) or pcW57.1 BCL-X\(_L\) (right panel) were treated for 24h with 0.5 µg/ml doxycycline to induce protein overexpression and afterwards exposed to the indicated treatments. Apoptotic cells were assessed 24 hours post treatment induction. Results indicate the mean +/- SDM of three independent experiments. Differences between groups were calculated with one-way ANOVA, corrected for multiple comparison with Bonferroni-Holm correction, where **** denotes \( P < 0.0001 \), ** denotes
P<0.001 and * denotes P<0.05 (H–I) Co-immunoprecipitation experiments in U266 cells transduced with either pcW57.1-EGFP (right panels) or pcW57.1-BCL-XL (left panels) were performed after 24h pretreatment with 0.5µg/ml doxycycline to induce protein overexpression and subsequent drug exposure for 24 hours.

**Supplement figure 3.**

**Ricolinostat promotes the activity of S63845 independent of HDAC6 inhibition.**

(A–D) Co-immunoprecipitation experiments in MM cells transduced with either pcW57.1-EGFP (right panels) or pcW57.1-BCL-XL (left panels) were performed after 24 h pretreatment with 0.5 µg/ml doxycycline to induce protein overexpression and subsequent drug exposure for 24 hours. (E) KMS-12-BM and (F) U266 cells were either treated with panobinostat or ricolinostat for 24h, then whole-cell lysates were blotted for the indicated proteins. (G) Single cell clones of U266 cells harboring a Tet-on miR-E vector expressing either a shRNA targeting Renilla (control) or two different shRNAs targeting HDAC6 were exposed to 0.3 µg/ml doxycycline for 48 h and whole-cell lysates were blotted for the indicated proteins. Western blots are representative for three independent experiments. (H–J) U266 cells were pretreated with doxycycline for 48 h and viability was assessed after an additional 48 h treatment with increasing concentrations of S63845 as indicated in the figure. Results show the mean +/- SD of three independent experiments performed in triplicates.
Supplement figure 1.
Supplement figure 3.

A U266 pcW57.1 BCL-XL

B U266 pcW57.1 BCL-XL

C KMS-12-BM pcW57.1 BCL-XL

D KMS-12-BM pcW57.1 BCL-XL

E KMS-12-BM

F U266

G U266 Tet-on miR-E

H U266 Tet-on miR-E Renilla

I U266 Tet-on miR-E shRNA #2

J U266 Tet-on miR-E shRNA #3