Potent efficacy of MCL-1 inhibitor-based therapies in pre-clinical models of mantle cell lymphoma

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Running title

MCL-1 is crucial for maintenance of MCL cells

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Abstract

Apoptosis-regulating BCL-2 family members, which can promote malignant transformation and resistance to therapy, have become prime therapeutic targets, as illustrated by the striking efficacy in certain lymphoid malignancies of the BCL-2-specific inhibitor venetoclax. In other lymphoid malignancies, however, such as the aggressive mantle cell lymphoma (MCL), cell survival might rely instead or also on BCL-2 relative MCL-1. We have explored MCL-1 as a target for killing MCL cells by both genetic and pharmacologic approaches. In several MCL cell lines, *MCL-1* knockout with an inducible CRISPR/Cas9 system triggered spontaneous apoptosis. Accordingly, most MCL cell lines proved sensitive to the specific MCL-1 inhibitor S63845 and MCL-1 inhibition also proved efficacious in an MCL xenograft model. Furthermore, its killing efficacy rose on combination with venetoclax, the BCL-X_L-specific inhibitor A-1331852 or Bruton's tyrosine kinase (BTK) inhibitor ibrutinib, which reduced pro-survival signals. We also tested the MCL-1 inhibitor in primary samples from 13 MCL patients, using CD40L-expressing feeder cells to model their microenvironmental support. Notably, all unstimulated primary MCL samples were very sensitive to S63845, but the CD40L stimulation attenuated their sensitivity. Mass cytometric analysis revealed that the stimulation likely conveyed protection by elevating BCL-X_L and MCL-1. Accordingly, sensitivity of the CD40L-stimulated cells to S63845 was substantially restored by co-treatment with venetoclax, the BCL-X_L-specific inhibitor or ibrutinib. Overall, our findings indicate that MCL-1 is very important for survival of MCL cells and that the MCL-1 inhibitor, both alone and together with ibrutinib, venetoclax or a BCL-X_L inhibitor, offers promise for novel improved MCL therapies.

Introduction

Impaired apoptosis is a cancer hallmark (1), particularly in blood cell malignancies, and strongly affects treatment (2-4). Most cytotoxic cancer therapies act through the intrinsic pathway to apoptosis, which the BCL-2 protein family regulates (2, 3). Whereas several family members promote cell survival, e.g. BCL-2, BCL-X_L, MCL-1 and BFL-1, two other sub-groups instead drive apoptosis: the BCL-2 homology 3 (BH3)-only proteins (e.g. BIM, BID and NOXA) respond to stresses and signal for apoptosis by binding and neutralizing pro-survival relatives, whereas the critical effectors BAX and BAK, once activated, oligomerize and damage the mitochondrial outer membrane (MOM), unleashing the proteolytic cascade that dismantles the cell.

Since pro-survival BCL-2 family members not only promote and maintain transformation but also cause resistance, they represent prime therapeutic targets (2-5). Indeed, 'BH3 mimetic' drugs, which mimic BH3-only proteins by neutralizing certain pro-survival BCL-2 family members, are showing great promise in the clinic, especially for blood cancers. In particular, the BCL-2-selective inhibitor venetoclax (ABT-199) (6) has proven highly effective against chronic lymphocytic leukemia (CLL) (7, 8). Moreover, newly developed BH3 mimetics that selectively target BCL-2 pro-survival relatives, particularly MCL-1, are arousing great interest (9-12), because they may well enhance venetoclax activity and extend BH3 mimetic therapy to diverse malignancies.

Mantle cell lymphoma (MCL), an aggressive non-Hodgkin lymphoma that typically responds only transiently to chemotherapy and remains incurable (13), represents an abnormal proliferation of mature CD5-positive B-cells infiltrating the lymphoid system and frequently also the bone marrow and peripheral blood (14, 15). Its genetic hallmark is the (11;14)(q13;q32) translocation, which induces cyclin D1 overexpression and hence cell cycle deregulation, but full transformation requires additional oncogenic changes, and many contribute to MCL pathogenesis (15, 16). Microenvironmental signals also support MCL growth and augment treatment resistance (17-19).

Targeted therapies for MCL such as the BTK inhibitor ibrutinib, which blocks signals from the B cell antigen receptor, show promise in relapsed and refractory MCL (14). Venetoclax has also shown promise for MCL in early trials, as a single-agent and especially together with ibrutinib (20, 21).

MCL-1 is a particularly promising target for MCL therapy. Its expression in MCL correlates with high-grade morphology and proliferation (22), and NOXA, which specifically binds and blocks MCL-1, is miss-regulated and expressed in MCL (23). Moreover, genetic knockout reveals MCL-1 essential for maintaining several hematological malignancies, including AML (24) and Burkitt lymphoma (25). Finally, recently developed potent and specific MCL-1 inhibitors show remarkable efficacy in cell lines from diverse leukemias and lymphomas (9-12, 26). However, MCL-1 has yet to be systematically assessed as a target for MCL treatment.

Here, we have used both genetic and pharmacological approaches to explore the potential of targeting MCL-1 in MCL. We report that MCL-1 is very important for maintaining survival of MCL cells. We demonstrate sensitivity of both MCL cell lines and primary patient samples to MCL-1 inhibition and identify effective combinations with BH3 mimetics targeting BCL-2 or BCL-X_L, as well as ibrutinib. Although stimuli modeling the tumor microenvironment attenuate sensitivity of the primary cells to MCL-1 inhibition, we show that combination treatment restores efficacy. Our results thus suggest several ways that MCL-1 inhibitors might well advance therapy of this presently incurable malignancy.

Results

Lowering MCL-1 genetically induces spontaneous cell death in MCL cell lines

To establish whether MCL-1 is crucial for MCL cell survival, we imposed acute MCL-1 knockout by transducing MCL cell lines Mino, Jeko1, Rec1 and Granta519 with a lentivirus-based doxycycline-inducible CRISPR/Cas9 system targeting *MCL1* (27) and assessed the impact of MCL-1 loss on single-cell clones by inducing *MCL1*-sgRNA expression. Remarkably, simply lowering MCL-1 protein levels (Fig. 1A), which left expression of other pro-survival proteins unaffected (Fig. S1A), triggered significant spontaneous apoptosis in Mino, Jeko1 and Rec1 clones (Figure 1B and Fig. S1B), but only minimally affected viability of Granta519 clones, probably at least in part because Granta519 cells markedly overexpress BCL-2 (28) (See below.) Thus, three of four MCL cell lines required their normal MCL-1 level for continued survival.

MCL cell lines are sensitive to MCL-1 inhibitor S63845

As targeting *MCL-1* genetically established its importance for MCL cell survival, we explored pharmacological MCL-1 inhibition by treating five MCL cell lines (Mino, Jeko1, Rec1, Granta519 and Z138) with the recently described potent and specific MCL-1 inhibitor S63845 (9). As expected, Granta519 was resistant, but the other four lines responded, with LC50s $\leq$ 0.7 μM (Fig. 1C). Their sensitivity was independent of p53 status (Mino: mutant p53; Jeko1: p53 loss; Rec1, Granta519 and Z138: WT p53 (29-31)). Because BH3 mimetic drugs act downstream of p53, sensitivity is typically independent of p53 status (2, 32). Indeed, Mino cells, despite mutant p53, were the most sensitive to MCL-1 inhibition (LC50: 301 nM).

The response to BH3 mimetics sometimes correlates with the relative expression levels of the major pro-survival proteins (2, 3). Overall, however, neither the levels of MCL-1, BCL-2 or BCL-X_L (Fig. 1D) nor the ratio MCL-1/BCL-2, MCL-1/BCL-X_L or MCL-1/(BCL-2+BCL-X_L) (Fig. S1C) correlated with sensitivity to S63845. Nevertheless, Granta519's high BCL-2 (28) likely contributes to resistance to MCL-1 inhibition. We also examined expression of BH3-only proteins BIM and NOXA (Fig. 1D). Only two of the five MCL lines expressed BIM, in accord with loss of its expression in many MCL cell lines (33, 34). Neither BIM expression nor NOXA levels correlated with the sensitivity of the lines.

Like other BH3 mimetics, S63845 acts by binding the canonical groove of MCL-1, thereby freeing BH3-only proteins to induce cell death (2-4, 9). To confirm its mechanism of action in MCL cells, we tested its impact on binding of NOXA and BIM to MCL-1 in Mino and Rec1 cells by co-immunoprecipitation (Fig. 1E). Although only the Rec1 cells expressed BIM, both BIM and NOXA can bind to MCL-1 (Fig. 1E, right panel). Indeed, all the NOXA in Rec1 co-immunoprecipitated with MCL-1 (compare lanes 13 and 14). As expected (9), bound S63845 stabilized MCL-1 and increased MCL-1 levels, particularly in Mino cells (compare lanes 1 and 2 with 3 and 4). Notably, S63845 strongly reduced the NOXA and BIM bound to MCL-1 (Fig. 1E, right panel, compare NOXA in lane 18 with 19 and 20 and BIM in lane 22 with 23 and 24). Thus, S63845 efficiently displaces BH3-only proteins from MCL-1, allowing them to attack other family members and unleash apoptosis (2-4).

To test S63845 activity in an *in vivo* MCL model, we injected Mino cells subcutaneously into NSG mice and treated them twice weekly with S63845. Notably, MCL-1 inhibition delayed

tumor growth (Fig. 1F) and significantly extended median mouse survival from 20.5 to 24.5 days (19.5 %) (Fig 1G).

Combining S63845 with other BH3 mimetics increases efficacy

Because non-targeted pro-survival family members can limit the sensitivity of tumor cells to targeted BH3 mimetics (2-4), we explored whether co-targeting BCL-2 or BCL-X_L, or both, enhanced sensitivity to S63845. Indeed, co-targeting BCL-2 with venetoclax (6), or BCL-X_L with A-1331852 (35), proved very effective (Fig. 2A and Fig. S2A). For example, with both Jeko1 and Rec1 cells, either venetoclax or A-1331852 greatly increased sensitivity to S63845, even though each had almost no effect as single agents (Fig. 2A). Even targeting both BCL-2 and BCL-X_L, without also targeting MCL-1, only modestly reduced viability of Jeko1 or Rec1. By contrast, Mino and Z138 were highly sensitive to BCL-X_L inhibition alone (Fig. 2A and S2A). As expected, the S63845-resistant and BCL-2-overexpressing (28) Granta519 responded more to venetoclax alone than S63845 alone, and targeting BCL-X_L or MCL-1 as well further decreased its viability (Fig. S2A).

Notably, checkerboard titrations (Fig. S2B) and BLISS analysis (36, 37) in Mino and Jeko1 cells revealed that combining S63845 with venetoclax (Fig. 2B) or A-1331852 (Fig. S2C) is highly synergistic across a range of concentrations.

Next, we explored whether combining S63845 with the clinically-approved venetoclax also increased efficacy in our xenograft model. NSG mice harboring Mino xenografts were treated with venetoclax alone or venetoclax plus S63845. Like treatment with S63845 alone (Fig. 1G), venetoclax alone produced a short albeit significant increase in mouse survival from 26 to 28 days (7.7 %) (Fig. 2C, left panel). Importantly, mice treated with venetoclax plus S63845 had a highly significant and prolonged extension in survival from 29.5 to 47.5 days (a 61% increase) (Fig. 2C, right panel). Their protracted survival, long after cessation of treatment, highlights the great potential of combining these BH3 mimetics in the clinic.

Pre-treating MCL cell lines with ibrutinib sensitizes them to S63845 by down-regulating pro-survival BCL-2 relatives

The great efficacy of targeting both BTK and BCL-2 in patients with relapsed or refractory MCL (21) prompted us to test whether pre-treatment with ibrutinib, as in the clinical schedule, also enhanced S63845 efficacy in MCL cells. Indeed, although ibrutinib alone had

little or no effect on MCL cell viability, it sensitized Mino, Jeko1 and Rec1 cells to S63845 (Fig. 3A), reducing their LC50 for S63845 by 2.7-fold to 4.3-fold (Fig. S3A). Consistent with the ibrutinib-resistance of Granta519 and Z138 (38, 39), their sensitivity to S63845 or venetoclax was unaffected (Fig. 3A and S3B). Interestingly, pre-treatment with ibrutinib only modestly increased sensitivity to venetoclax for Rec1 cells and not significantly for Mino or Jeko1 (Fig. 3A). Hence, ibrutinib plus MCL-1 inhibition could well prove effective in MCL patients even if the ibrutinib-venetoclax combination fails.

To investigate how ibrutinib sensitizes the MCL cells to S63845, we first tested how it affected expression of the pro-survival proteins in the ibrutinib-sensitive and ibrutinib-resistant lines (Fig. 3B). Ibrutinib reduced MCL-1 levels in Mino and Jeko1, and BCL-X_L in Mino and Rec1 cells, whereas BCL-2 and BCL-W levels changed very little in all three lines (Fig. 3B). Interestingly, BFL-1, which engages pro-apoptotic relatives similarly to MCL-1 (40, 41) and mediates chemoresistance in diverse lymphoma models (42, 43), was reduced by ibrutinib in all three ibrutinib-sensitive lines, although its basal level was low compared to Granta519 cells (Fig. 3B). As expected, pro-survival protein expression did not drop in the ibrutinib-resistant cells (Fig. 3B). Indeed, ibrutinib even increased BFL-1 and BCL-W in Granta519, probably contributing to its resistance.

We also assessed how ibrutinib affected six pro-apoptotic BCL-2 family members (Fig. 3B). As discussed above, only two cell lines expressed BIM, and ibrutinib did not affect its level. Ibrutinib actually reduced NOXA expression in Mino cells; this is not unexpected, because BCR signaling affects NOXA expression (23). No line showed major changes for BH3-only BAD and PUMA or effector BAK. Interestingly, BAX increased in both ibrutinib-resistant Granta519 and Z138.

In summary, the reduced expression of certain pro-survival BCL-2 relatives evoked by ibrutinib in the ibrutinib-sensitive cell lines probably largely accounts for their heightened sensitivity to MCL-1 plus BTK inhibition.

Primary MCL cells are sensitive to MCL-1 inhibition, but CD40L stimulation attenuates their sensitivity

To extend the cell line results to a more clinically relevant setting, we tested S63845 on fresh or cryopreserved primary MCL samples from peripheral blood (PB) and/or bone marrow

(BM) The 14 samples from 13 patients included 10 taken at diagnosis while 4 were from a relapse or refractory (R/RF) stage (Fig. 4, Fig. S4 and Table 1).

Microenvironmental signals can activate pro-survival pathways in MCL cells that reduce their sensitivity to targeted therapies, including venetoclax (18, 19). To mimic and assess potential effects of a lymphoid microenvironment, we co-cultured the primary cells on apoptosis-deficient Bax/Bak knockout MEFs expressing human CD40L and provided a cytokine cocktail (19) (CK) containing IGF-1, BAFF, IL-6 and IL-10. This support milieu, designed to support primary MCL cells *ex vivo,* induces a molecular profile in MCL cells mimicking that which they exhibit within lymphoid organs (19). To assess how this support affected S63845 responses, we compared viability of the unstimulated cells and those stimulated with CD40L plus CK upon treatment with increasing S63845. Since the primary samples included normal cells, we identified the tumor cells by co-staining the treated samples for both CD5 and CD19 (Fig. S4A). Table 1 shows the percent tumor cells in PB and BM for each MCL sample.

Notably, all 14 unstimulated primary MCL samples, whether from PB or BM, and whether fresh or frozen, proved very sensitive to MCL-1 inhibition, with LC50s from 19 to 679 nM (Fig. 4A and Table 1). Interestingly, however, the stimulated primary samples were more resistant, giving LC50s from 0.23 μM to over 10 μM (Fig. 4A and Table 1); the sensitivity reductions ranged from ~3-fold to ~500-fold (Fig. 4B). Previous cryopreservation of 10 of the 14 primary samples did not notably affect their sensitivity to S63845, with or without stimulation (Fig. S4B). Also, BM-infiltrating and PB-derived samples exhibited similar sensitivity to S63845, either with or without stimulation (Fig. 4C and S4C). Of note, CD40L conveyed most of the protection, as CD40L plus CK did not potentiate the effects of CD40L alone in the three tested primary samples (Fig. S4D).

Interestingly, on comparing tumor cells from patients at diagnosis (Dx) with those at relapsed/refractory stage (R/RF) (Table 1), we found that both groups showed similar sensitivity to S63845 when unstimulated, whether derived from PB or BM (Fig. 4D). However, when stimulated with CD40L plus CK, the R/RF samples remained significantly more sensitive to MCL-1 inhibition than the Dx samples (Fig. 4D), indicating that the R/RF tumors had become less responsive to microenvironmental signals. Hence, MCL patients

with relapse/refractory disease might have deeper responses to MCL-1 inhibitors than other MCL patients.

These *ex-vivo* findings suggest that circulating PB-derived and BM-infiltrating MCL cells are very sensitive to MCL-1 inhibition, but signals from the lymph node microenvironment *in vivo* probably reduce their sensitivity.

CD40L stimulation induces a strong pro-survival signal in primary MCL cells

To explore how CD40L mediates resistance to S63845 in primary MCL cells, we used mass cytometry (CyTOF) and diverse antibodies against extra- and intra-cellular targets to simultaneously monitor, at the single cell level, how the stimulation affects cellular signaling, cell-cycle status and expression of pro-survival proteins in three patient samples (#30, #265, #292) with different tumor content (Fig. 4E, Fig. S5 and Table 1).

Stimulation with CD40L plus CK for 72 hours induced a strong pro-proliferative and pro-survival signal in all three primary samples. The several-fold increased phosphorylation of ribosomal protein S6 indicates mTOR pathway induction, and the augmented cell cycle hallmarks CDK4, Cyclin D1 and phospho-RB, which rose ~3- to 7-fold, indicate increased cell division (Fig. 4E and S5). Elevated NFκB-pathway activation, which is stimulated by CD40L in MCL cells and promotes pro-survival signals (19, 44), is evident from the increased IκBα (up 2- to 7-fold), which is first degraded to allow NFκB expression but then induced by NFκB in an autoregulatory loop (45). Interestingly, the stimulated primary cells also up-regulated chemokine receptor CXCR4 (Fig. 4E), the receptor for chemokine CXCL12, which stromal cells constitutively secrete and is critical for lymphoma cell homing to a supportive environment (17, 46).

Pertinently, stimulation markedly up-regulated pro-survival BCL-2 family proteins (Fig. 4E and S5). Although BCL-2 remained unchanged, in all three patient samples BCL-X_L rose 10- to 28-fold and MCL-1 ~3-fold, confirming that CD40L induces a strong pro-survival signal in primary MCL cells (18, 19). Mass cytometry at different times of stimulation showed signaling pathways, exemplified by S6 and RB phosphorylation, were activated by 8 hours of stimulation (Fig. S5). As expected, IκB up-regulation was delayed and only observed at 72

hours (Fig. S5). The pro-survival proteins were induced after 24 hours but higher after 72 hours (Fig. S5).

The increased BCL-X_L and MCL-1 probably mediates the resistance of the stimulated primary MCL cells to killing by MCL-1 inhibition, perhaps because these two pro-survival proteins are the principal guards on pro-apoptotic BAK (47). The potential role of BFL-1 and BCL-W could not be assessed due to lack of an antibody suitable for CyTOF.

Combination treatment restores sensitivity of CD40L-stimulated cells to S63845

The strong stimulation of pro-survival signals in primary MCL cells by CD40L (Fig. 4E and S5) suggested that co-targeting BCL-2 or BCL-X_L might restore their sensitivity to S63845. Therefore, we treated the stimulated cells with S63845, venetoclax or A-1331852, alone and in combination (Fig. 5A). Whereas only one primary MCL sample showed substantial killing by the BCL-X_L inhibitor alone (blue bar, Fig. 5A), five of 14 stimulated primary samples remained relatively sensitive to S63845 alone (grey bar) or venetoclax alone (red bar). Notably, all samples from patients with relapsed/refectory disease were amongst the five samples which retained <70% viability following S63845 treatment (Fig. 5A). Interestingly, these samples were also still sensitive to venetoclax, highlighting that both MCL-1 and BCL-2 seem major vulnerabilities in this normally aggressive subset of MCL.

Intriguingly, as with the MCL cell lines (Fig. 2A), combining S63845 with venetoclax or A-1331852 strongly increased killing in all CD40L-stimulated primary MCL cells (Fig. 5A). The BCL-X_L inhibitor restored considerable sensitivity to S63845. Targeting both BCL-2 and BCL-X_L also enhanced killing in most stimulated primary samples, similarly to S63845 plus venetoclax, but often less than S63845 plus A-1331852. Combining all three BH3 mimetics obliterated almost all tumor cells in all samples. Thus, co-targeting MCL-1 with BCL-2 or BCL-X_L can effectively kill MCL cells protected by microenvironmental support.

The great efficacy in MCL patients of ibrutinib plus venetoclax (21), and the enhanced killing in MCL cell lines on combining ibrutinib with BH3 mimetics (Fig. 3A), prompted us to test if ibrutinib pre-treatment restores sensitivity of CD40L-stimulated primary cells to S63845, venetoclax or A-1331852 (Fig. 5B). Ibrutinib alone did not reduce their viability but did partially re-sensitize most of the 11 primary samples tested to S63845 and to venetoclax (Fig. 5B, left and middle panels, respectively); overall killing of the primary cells increased significantly, albeit less than in the cell lines. In some primary samples, ibrutinib also

enhanced sensitivity to A-1331852 (Fig. 5B, right panel). Thus, ibrutinib plus MCL-1 inhibition could prove a very promising alternative to ibrutinib plus venetoclax.

MCL-1 inhibitor is effective in venetoclax-resistant primary MCL cells

To determine if MCL-1 inhibition might benefit venetoclax-resistant patients, we tested S63845 on a primary MCL sample from a patient who had developed resistance to sequential treatment with ibrutinib and venetoclax, due to loss of chromosome 9p and SMARCA4, which up-regulated BCL-X_L (48). Agarwal and coworkers showed that this sample, which was completely resistant to venetoclax and ibrutinib *in vitro*, still responded to BCL-X_L inhibition (their Fig. 4A) (48). Importantly, we found that this sample also responded to MCL-1 inhibition, with a sensitivity similar to that reported with A-1331852 (Fig. 6A). Furthermore, in primary cells from an MCL patient (#292) who became relatively resistant to venetoclax by an unknown mechanism, S63845 alone was more effective than venetoclax or A-1331852 alone (Fig. 6B). Also, S63845 plus venetoclax induced substantial killing, even at very low doses (100 nM) that lacked single agent activity (Fig 6C). Hence, when venetoclax fails, targeting MCL-1 may well still succeed, and combining venetoclax with MCL-1 inhibition in such patients might well further increase efficacy.

Discussion

The recent emergence of selective and potent MCL-1 inhibitors (9-12, 49) has attracted great interest, because genetic tools have shown that sustained growth of several blood cancers, including AML (24), Burkitt lymphoma (25) and multiple myeloma (50), requires MCL-1, and pre-clinical studies have shown efficacy of MCL-1-specific inhibitors on cell lines from these malignancies (9-12, 49, 51). By reducing MCL-1 levels with an inducible CRISPR/Cas9 system (27), we showed MCL-1 to be crucial for maintaining several MCL cell lines with different p53 status (Fig. 1A, 1B and S1B), which also proved sensitive to pharmacologic inhibition of MCL-1 with the selective and potent S63845 (9) (Fig. 1C). Its activity in an MCL xenograft model (Fig. 1F and 1G) further highlighted its potential for treating MCL.

Significantly, primary MCL cells were also sensitive to MCL-1 inhibition. PB-derived circulating or BM-infiltrating lymphoma cells from MCL patients were very sensitive *ex vivo* to S63845 (Fig. 4A and 4B, Table 1). *In vivo*, however, the tumor cells mainly reside in a supportive microenvironment of other immune and stromal cells (17). Recent work

demonstrated that mimicking the microenvironment by culturing primary MCL cells on CD40L-expressing stromal cells, plus cytokine support, recapitulated molecular signatures of MCL cells in the lymph node (19). Significantly, these cells showed elevated BCL-2 pro-survival family members and increased resistance to different drugs, including venetoclax (18, 19). We found that CD40L stimulation also rendered primary MCL cells more refractory to MCL-1 inhibition (Fig. 4A and 4B, Table 1), probably by increasing BCL-X_L and MCL-1 (Fig. 4E and S5). The up-regulated BCL-X_L in such stimulated MCL cells can attenuate their responses to venetoclax and other drugs (18, 19). Pertinently, Agarwal et al recently reported that genomic alterations in MCL elevating BCL-X_L expression mediate resistance to venetoclax (48). Interestingly, our data suggests that tumor cells from patients with relapsed/refractory disease are less protected by microenvironmental support signals (Fig. 4D and Fig. 5A), and hence that S63845 or venetoclax monotherapy could be very effective in these patients, who usually have a dismal prognosis. Indeed, venetoclax monotherapy has produced impressive responses in relapsed or refractory MCL patients (20, 52).

Since the relative levels of MCL-1, BCL-2 and BCL-X_L are major determinants of cancer cell responses to therapies, including BH3 mimetics (2, 3), co-targeting more than one of them can enhance efficacy (18, 26, 53). Indeed, co-targeting BCL-2 or BCL-X_L increased sensitivity to MCL-1 inhibition with both MCL cell lines (Fig. 2A, 2B and S2) and CD40L-stimulated primary MCL cells (Fig. 5A). The striking synergy in the stimulated primary MCL cells of targeting MCL-1 plus BCL-X_L suggests that this combination may well be an efficacious way to kill lymphoma cells protected by the microenvironment. Whether this combination will have an adequate therapeutic window is unclear, however, because inhibiting BCL-X_L can kill platelets (7, 54).

Combining the MCL-1 inhibitor with venetoclax might prove more feasible as it was nearly as effective as the combination with the BCL-X_L inhibitor in most stimulated primary MCL cells (Fig. 5A), and it markedly extended mouse survival (by 61%) in our MCL xenograft model (Fig. 2C), long after treatment had ceased. This combination is an exciting treatment option to kill MCL tumor cells that prove refractory to the MCL-1 inhibitor alone, such as certain MCL cells nurtured by the lymphoid environment. Interestingly, another recent pre-clinical study suggests that inhibiting both BCL-2 and MCL-1 could be effective even in patients with relapsed MCL and adverse cytogenetics (26). Studies in AML (10, 11, 49) and multiple myeloma (55) further highlight the potential of this combination and suggest it may

well have a therapeutic window. Hence, our findings with MCL could well prove relevant to diverse blood cell malignancies.

Combining a BH3 mimetic with a different targeted therapy can overcome resistance and treatment failure, as exemplified for MCL by the impressive efficacy of venetoclax plus ibrutinib (21). Our findings suggest that ibrutinib plus an MCL-1 inhibitor may prove even more effective. Ibrutinib pre-treatment strongly sensitized several MCL cell lines to S63845, but only slightly increased killing by venetoclax (Fig. 3A). This probably reflects different effects of ibrutinib on pro-survival BCL-2 family members. Pertinently, MCL cell lines sensitized by ibrutinib had down-regulated MCL-1 and/or BCL-X_L, whereas BCL-2 expression was unaffected (Fig. 3B). Furthermore, BFL-1, a close pro-survival relative of MCL-1 that engages the same pro-apoptotic family members (40, 41), was reduced in ibrutinib-sensitive cells (Fig. 3B). Hence, lower BFL-1 may well boost the sensitization by ibrutinib to S63845, particularly since BFL-1 is implicated in chemoresistance in other lymphoma models (42, 43) and its mRNA is overexpressed in MCL cells (56, 57). Interestingly, besides very high BCL-2 levels, Granta519 cells also exhibited the highest BFL-1 expression levels in the MCL cell line panel (Fig. 3B), implicating BFL-1 in their resistance to MCL-1 inhibition (Fig. 1) and highlighting the potential of targeting BFL-1.

Importantly, even in our highly stimulated *ex-vivo* co-culture system, ibrutinib sensitized most primary MCL samples to S63845 and probably a smaller proportion to venetoclax or the BCL-X_L inhibitor (Fig. 5B). As well as directly inhibiting pro-survival signals from the B-cell antigen receptor in MCL cells, ibrutinib aids combination treatment *in-vivo* by reducing expression on MCL cells of chemokine receptor CXCR4, which directs MCL cells to supportive microenvironments (46). Interestingly, CD40L plus CK stimulation of primary MCL cells strongly induced CXCR4 (Fig. 4E and S5), revealing a positive feedback loop between the tumor cells and their niche. By reducing CXCR4 expression, ibrutinib increases circulating MCL cells *in vivo* (18, 58), and the resulting deprivation of support signals renders them the MCL cells more vulnerable to venetoclax (18). Since unstimulated circulating PB-derived MCL cells are highly sensitive to MCL-1 inhibition (Fig. 4A and 4B, Table 1), ibrutinib plus MCL-1 inhibition may have even greater synergy *in vivo*.

Another therapeutic strategy is targeting pro-survival pathways activated by cytokine stimulation. We found that CD40L-stimulated primary MCL cells have activated the NFκB

pathway (Fig. 4E and S5), which can up-regulate BCL-X_L (Fig. 4E) (18, 19). Hence, inhibiting that pathway, perhaps using anti-CD20 obinutuzumab (19), should enhance killing of MCL cells by BH3 mimetics.

Recent clinical studies using venetoclax have shown that targeting pro-survival proteins can enhance MCL therapy (20, 21), and our findings indicate that MCL-1 represents an exciting additional target. S63845 killed MCL cells very efficiently, both as a single agent but especially together with other targeted therapies, including other BH3 mimetics and ibrutinib. MCL-1 inhibitor-based therapy may even aid patients resistant to venetoclax (Fig. 6). Although the MCL-1 dependence of normal cardiomyocytes, hepatocytes and neurons (59-61) raises safety concerns for MCL-1 inhibitors, recent studies using humanized *MCL1* mice suggest that these inhibitors should have a therapeutic window (10, 51), and clinical trials are evaluating the safety of three different MCL-1 inhibitors. The striking efficacy of MCL-1 inhibitors in diverse pre-clinical cancer models (9-12) indicates that MCL-1 represents a major vulnerability in multiple cancer types, as well as MCL.

Materials and Methods

Cell lines and primary MCL cells

MCL cell lines Jeko1 and Rec1 were kindly provided by Heiko van der Kuip (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany), and Mino, Granta519 and Z138 from ATCC. All cell lines were authenticated in September 2019 using the GenePrint 10 System (Promega) and routinely checked for Mycoplasma using the MycoAlert™ mycoplasma detection kit (Lonza).

All primary MCL samples were unseparated mononuclear cells (MNCs), including both normal and tumor cells, isolated by Ficoll-Paque separation from peripheral blood or bone marrow aspirates. Samples came from the Cancer Collaborative Biobank (CCB), Brisbane, Australia or the Royal Melbourne Hospital or Peter MacCallum Cancer Center, Melbourne, Australia. All patients gave informed consent and the local ethics committee approved their use.

Culture of MCL cell lines and primary MCL cells are detailed in Supplementary Information on Experimental Procedures.

Xenograft model

Experiments with mice followed our institute's Animal Ethics Committee guidelines. Mino cells were injected subcutaneously into the right flank of NOD-SCID-γIL2-/- (NSG) mice (see Supplementary Information).

Mass cytometry

Mass cytometric analysis was similar to that described (62). Supplementary Information details protocols, reagents and data processing.

Knockout of MCL-1

MCL-1 knockout was induced using an inducible lentiviral guide RNA (sgRNA) platform (27).

Immunoblotting, Immunoprecipitation, Quantification and Statistical Analysis

Analyses used standard techniques, as detailed in Supplementary Information.

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Author Contributions

MAD, AWR and JMA designed research; MAD, CET, RT, LG and PL performed experiments; MAD, CET, MJH, DHG, AWR and JMA analyzed data; and MAD and JMA wrote the paper.

Conflict of Interest Disclosures

All authors are employees of the Walter and Eliza Hall Institute, which receives milestone and royalty payments related to venetoclax. MJH, GLK and AWR have received research funding from Servier.

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Figure Legends

Fig. 1. Genetic and pharmacologic targeting of MCL-1 induces cell death in MCL cell lines. (A) and (B) Acute MCL-1 knockout induces spontaneous cell death in MCL cell lines. Mino, Jeko1, Rec1 and Granta519 cells were transduced with a lentiviral-doxycycline-inducible CRISPR/Cas9 system targeting *MCL1* (27) and single-cell clones tested for MCL-1 protein 48 hours after doxycycline (dox)-induced *MCL1*-sgRNA expression. **(A)** The reduction in MCL-1 protein in three single-cell clones, assessed by immunoblotting. **(B)** Cell viability, +/- dox induction, of the three single cell clones shown in (A), which exhibited less MCL-1 upon sgRNA expression. Cell viability was measured by AnnexinV-APC staining and flow cytometry. Data are means ± SEM of the three single clones in (A), which were tested in 2 independent experiments. Statistical difference was analyzed by two-tailed unpaired *t*-tests (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ****: $P < 0.0001$) **(C)** MCL cell lines are sensitive to MCL-1 inhibitor S63845. Dose-response curves and LC50 values of the indicated lines treated for 24 h with increasing S63845 are shown. Cell viability was measured as above. **(D)** Immunoblots of the indicated BCL-2 family proteins in MCL cell lines, representative of at least 2 independent experiments. **(E)** To kill MCL cells, S63845 displaces pro-apoptotic proteins from MCL-1. MCL-1 was immunoprecipitated from MCL cell lines Mino and Rec1, which had been treated with S63845 (4 h) or left untreated. Binding of NOXA and BIM to MCL-1 was tested by immunoblotting unbound and immunoprecipitated fractions. The immunoblots are representative of at least 2 independent experiments. **(F)** and **(G)** S63845 shows activity in Mino xenografted mice. **(F)** Tumor volume at 20 days post-transplant (data are means ± SEM). **(G)** Kaplan–Meier survival curves showing overall mouse survival. NSG mice were injected with Mino cells and treated on indicated days with vehicle or 25 mg/kg S63845 (twice weekly). Mice were euthanized when tumor volume reached 0.5 cm^3. Statistical difference was analyzed by two-tailed unpaired *t*-test in (F) and Log-rank (Mantel-Cox) test in (G).

Fig. 2. MCL cell lines are sensitized to S63845 by other BH3 mimetics. (A) The increased efficacy of combining MCL-1 inhibitor S63845 with other BH3 mimetics, shown by 24-h treatment of the indicated MCL cell lines with S63845 alone (Mino, 250 nM; others, 500 nM) or together with BCL-2-specific venetoclax or BCL-X_L-specific A-1331852 (each 500 nM). Cell viability was measured as in Fig. 1. All data are means ± SEM of at least three independent experiments. Statistical difference was analyzed by one-way ANOVA, Tuckey's multiple comparisons tests (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$;

****: $P < 0.0001$). **(B)** MCL cell lines Mino and Jeko1 treated with increasing S63845 and venetoclax for 24 h were subjected to viability assays using TMRE (as shown in Fig. S2B) followed by BLISS score analysis. BLISS values >0.0 indicate synergy between the two drugs at the indicated concentrations. All data are means of two independent experiments. **(C)** Combining S63845 with venetoclax is highly effective in Mino xenografted mice. Kaplan–Meier survival curves showing overall survival of NSG mice injected with Mino cells and treated on indicated days with 100 mg/kg venetoclax alone (left panel) or co-treated with 75 mg/kg venetoclax (red arrows) and 25 mg/kg S63845 (grey arrows) (right panel). Mice were euthanized when tumor volume reached 0.5 cm^3. Statistical difference was analyzed by Log-rank (Mantel-Cox) test. In the combination treatment arm, two mice that developed a tumor-unrelated illness were euthanized and censored from the data.

Fig. 3. BTK inhibition sensitizes MCL cell lines to S63845 by reducing pro-survival protein expression. (A) Pre-treatment with ibrutinib strongly sensitizes MCL cell lines to MCL-1 inhibition. The indicated cells were pre-treated (or not) with 1 μM ibrutinib for 24 h, then with 500 nM of S63845, venetoclax or A-1331852 (Mino: 250 nM S63845) for another 24 h. Cell viability was measured as in Fig. 1. All data are means ± SEM of at least three independent experiments. Statistical difference was analyzed by one-way ANOVA, Tuckey's multiple comparisons tests (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$). **(B)** Ibrutinib reduces levels of certain pro-survival BCL-2 family proteins. The indicated MCL cell lines were treated with 1 μM ibrutinib or left untreated for 24 h and immunoblotted to reveal changed levels of BCL-2 family proteins. Immunoblots were quantified by densitometry and normalized to the HSP70 level before ratios of ibrutinib-treated to untreated were calculated. The blots are representative of at least 2 independent experiments.

Fig. 4. CD40L + CK-stimulation attenuates sensitivity of primary MCL cells to S63845 by inducing a strong pro-survival signal. (A) Representative dose-response curves of previously cryopreserved (#20 and #42-2) or fresh (#250) primary MCL samples. Primary cells from bone marrow (BM) or peripheral blood (PB) of MCL patients were treated with increasing S63845 either immediately after processing (unstimulated) or after 24 h stimulation on CD40L-expressing feeder cells and a cytokine cocktail (CK) containing IGF-1, BAFF, IL-6 and IL-10. After 24 h of S63845 treatment, the cells were harvested and cell death in the $CD5^+CD19^+$ tumor cells analyzed by CD5/CD19/AnnexinV staining and flow

cytometry (see Fig. S4A). **(B)** Stimulation of primary MCL cells with CD40L plus CK strongly increased the LC50 for S63845 in all BM and PB-derived samples. **(C)** BM- and PB-derived samples show similar sensitivity to S63845 before and after stimulation with CD40L plus CK. **(D)** Primary samples from patients with relapsed/refractory (R/RF) disease remain more sensitive to S63845 after stimulation with CD40L plus CK than samples from patients at diagnosis (DX). Statistical difference in (C) and (D) was analyzed by two-tailed paired *t*-tests (ns: not significant, *p, 0.05). **(E)** Mass cytometric (CyTOF) analysis of how CD40L- plus CK-stimulation affects intracellular signaling (pS6, IκBα, CXCR4), cell cycle (CDK4, Cyclin D1, pRB) and expression of pro-survival BCL-2 proteins (BCL-xL, MCL-1, BCL-2) in $CD5^+CD19^+$ cells from primary MCL samples #265 (fresh) and #30 (cryopreserved). **(**See Fig. S5 for a third sample.) After staining for viability with cisplatin and fixing with paraformaldehyde, cells were barcoded using 20-plex palladium barcoding, then stained with cell surface antibodies (CD5, CD19) to mark MCL cells before permeabilizing them and staining with antibodies to intracellular antigens. After staining with a 125-nm $^{191}Ir/^{193}Ir$ DNA intercalator, cells were analyzed using a Helios mass cytometer. Histograms of $CD5^+CD19^+$ single cells are displayed.

Fig. 5. Sensitivity of CD40L-stimulated MCL cells to MCL-1 inhibitor-based combination treatments. (A) Concomitant targeting of different pro-survival BCL-2 family proteins efficiently kills stimulated primary MCL cells. PB-derived MCL cells from patients at diagnosis (Dx) or at relapse/refractory (R/RF) stage were stimulated with CD40L plus CK for 24 h and then treated with S63845 alone (500 nM) or together with BCL-2-specific venetoclax or BCL-X_L-specific A-1331852 (each 500 nM) for 24 h. Cell death induction in the $CD5^+CD19^+$ tumor cell population was analyzed by CD5/CD19/AnnexinV staining and flow cytometry. The color-coded bars indicate the mean viability of the different treatments, normalized to the untreated control. Red symbols mark the three samples analyzed by mass cytometry in Fig. 4 or Fig. S5. **(B)** Ibrutinib treatment partly re-sensitizes stimulated primary cells to S63845. 11 of the 14 PB-derived MCL samples, which were stimulated with CD40L plus CK for 24 h, were then left untreated or treated with 1 μM ibrutinib for 24 h before treatment with 500 nM S63845 (left panel), venetoclax (middle panel) or A-1331852 (right panel) for another 24 hours. Cell death induction was analyzed as in (A). Bars represent means of all samples. Statistical difference in (A) and (B) was analyzed by paired one-way ANOVA, Tuckey's multiple comparisons tests; the respective p-values are indicated.

Fig. 6. Venetoclax-resistant primary cells still respond to MCL-1 inhibitor-based therapy. (A) Primary cells from a patient who became resistant to venetoclax plus ibrutinib were treated for 24 h with increasing concentrations of S63845 (grey line). Cell death induction was measured by CD5/CD19/PI staining and flow cytometry. For comparison, the dose response of this sample treated with venetoclax (dotted red), A-1331852 (dotted blue) or ibrutinib (dotted black) for 24 h, as published by Agarwal et al (48) (their Fig. 4A), is shown. **(B)** Dose response curve of primary cells from a patient (#292) who developed resistance to venetoclax; the cells were treated for 24 h with increasing S63845 (grey line), venetoclax (red line) or A-1331852 (blue line). Cell death induction was measured as in Fig. 5. **(C)** Venetoclax-resistant cells (from patient #292 as in (B)) were treated with S63845 alone (100 nM) or together with venetoclax or BCL-X_L-specific A-1331852 (each 100 nM) for 24 h. Cell death induction in the $CD5^+CD19^+$ tumor cells was analyzed by CD5/CD19/AnnexinV staining and flow cytometry. As patient material was limited, these experiments could be performed only once.

Tables

Table 1: Summary of primary MCL samples and their sensitivity to S63845 in presence or absence of CD40L plus CK-stimulation.

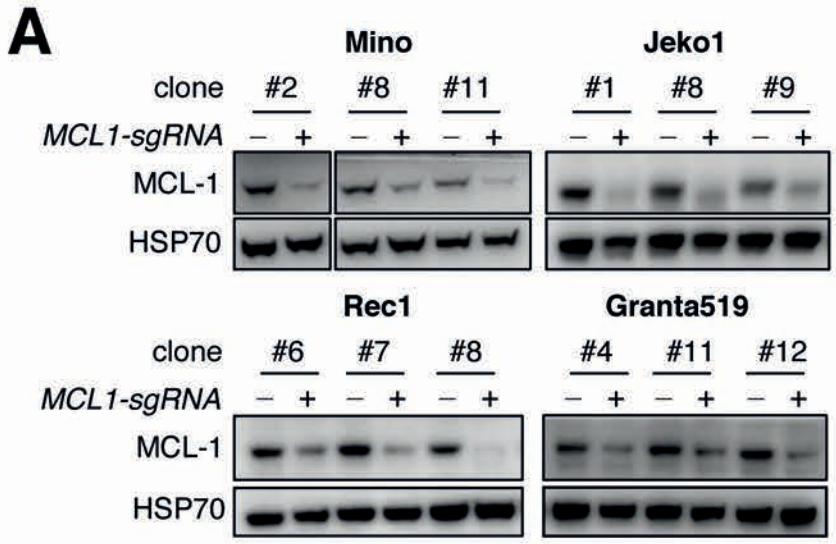
Patient	Status [#]	Blastoid variant	Cryo-preserved	$CD5^+CD19^+$ [%] [$] BM	$CD5^+CD19^+$ [%] [$] PB	LC50 [nM] S63845 unstimulated ^ BM	LC50 [nM] S63845 unstimulated ^ PB	LC50 [nM] S63845 CD40L + CK ^ BM	LC50 [nM] S63845 CD40L + CK ^ PB
4	D	+	+	81	73	19	35	>10000	8390
11	D	+	+	-	79	-	19	-	6757
20	D	-	+	72	67	201	70	5113	5077
30	D	-	+	-	56	-	229	-	2263
38	R	-	+	88	90	90	60	421	230
42	R	-	+	23	15	268	325	710	1125
42-2	RF*	-	+	33	28	349	280	3152	3428
44	D	-	+	-	65	-	207	-	544
231	RF	-	-	-	35	-	55	-	1429
250	D	-	-	40	6	56	37	>10000	>10000
257	D	-	-	10	6	679	150	>10000	>10000
265	D	-	-	8	12	443	468	1566	1165
299	D	-	+	-	32	-	117	-	8502
312	D	-	+	-	10	-	294	-	>10000

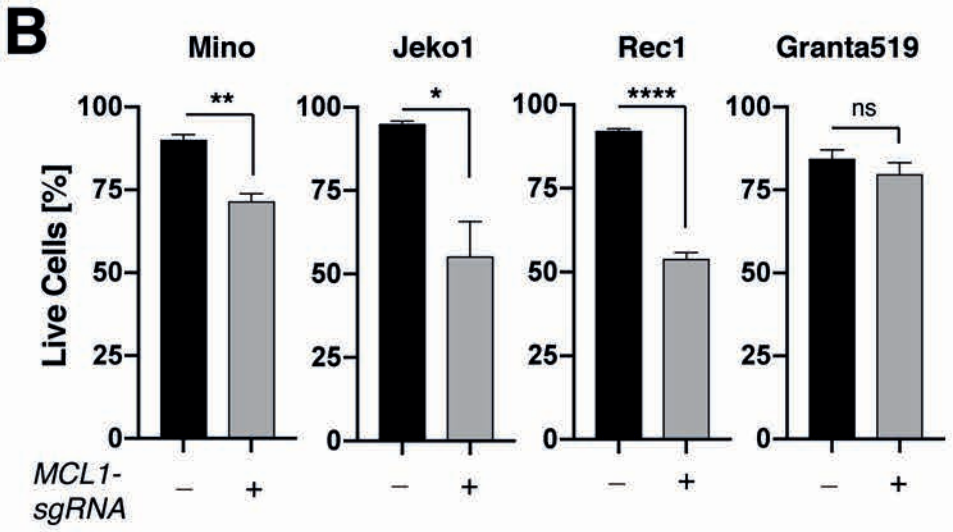
[#] D: at diagnosis; R: relapsed; RF: refractory; *: treated with BTK inhibitor for three months

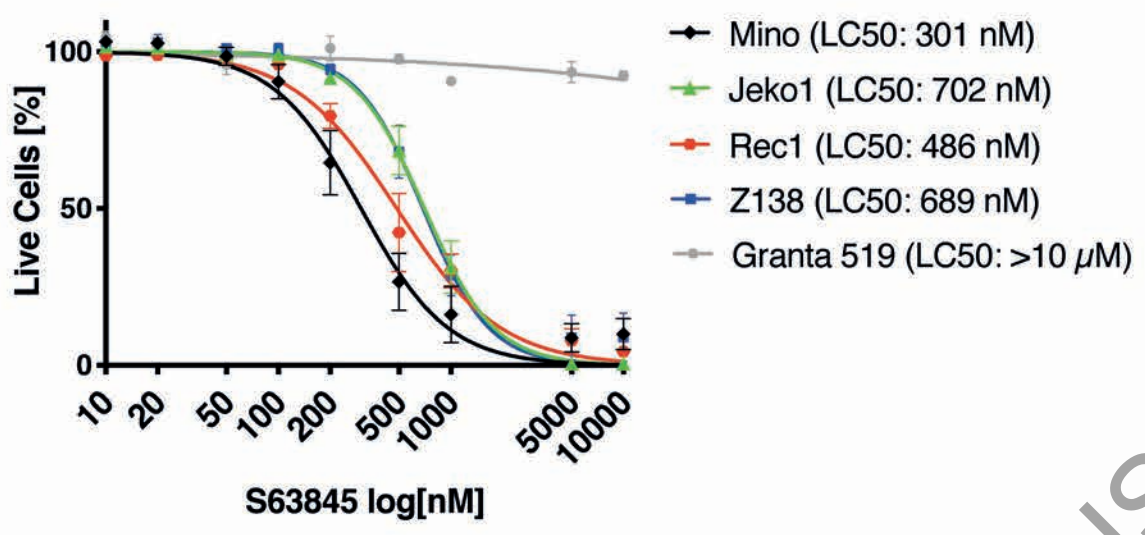
[$] Percentage of $CD5^+CD19^+$ cells determined by flow cytometry (see Fig. S4A).

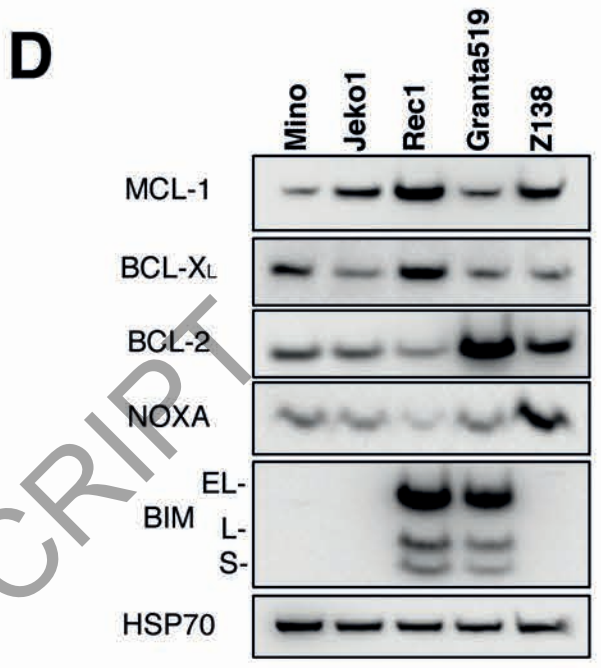
^ LC50 values for $CD5^+CD19^+$ cells were determined by treating unstimulated or CD40L plus CK-stimulated cells with increasing S63845 (50, 100, 200, 500, 1000, 5000 and 10000 nM) for 24 h followed by Annexin V staining and flow cytometry.

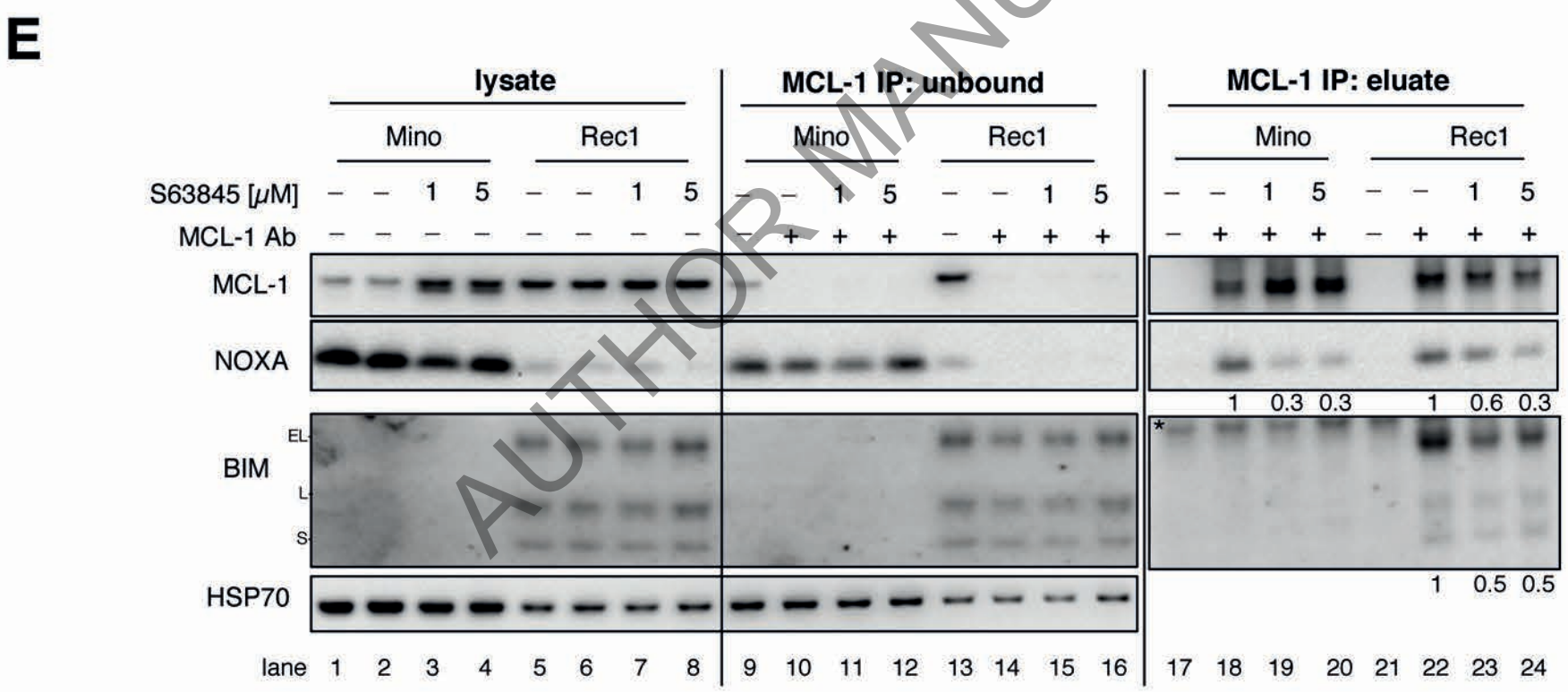
Abbreviations: BM: bone marrow-derived; PB: peripheral blood-derived

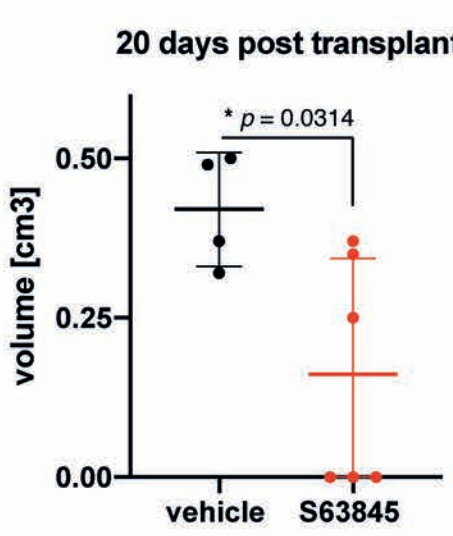


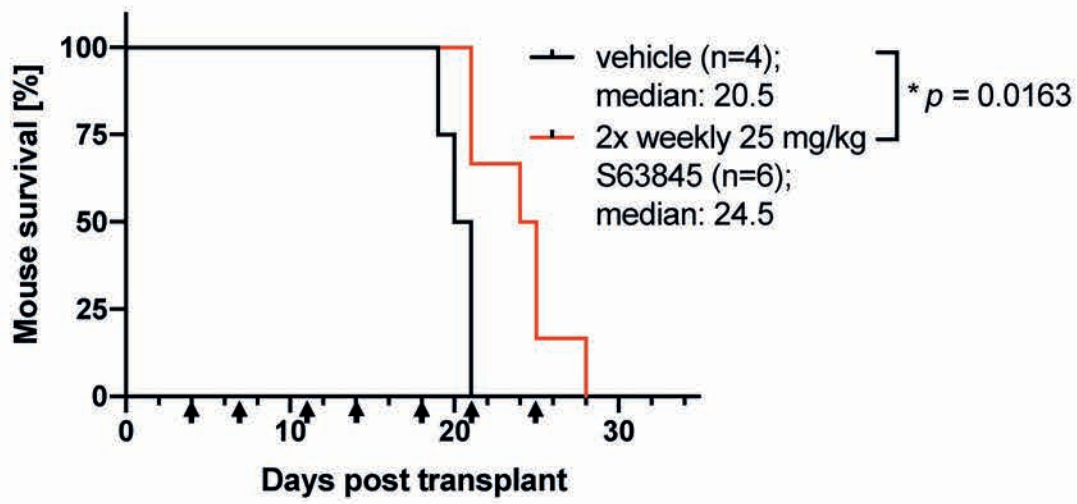


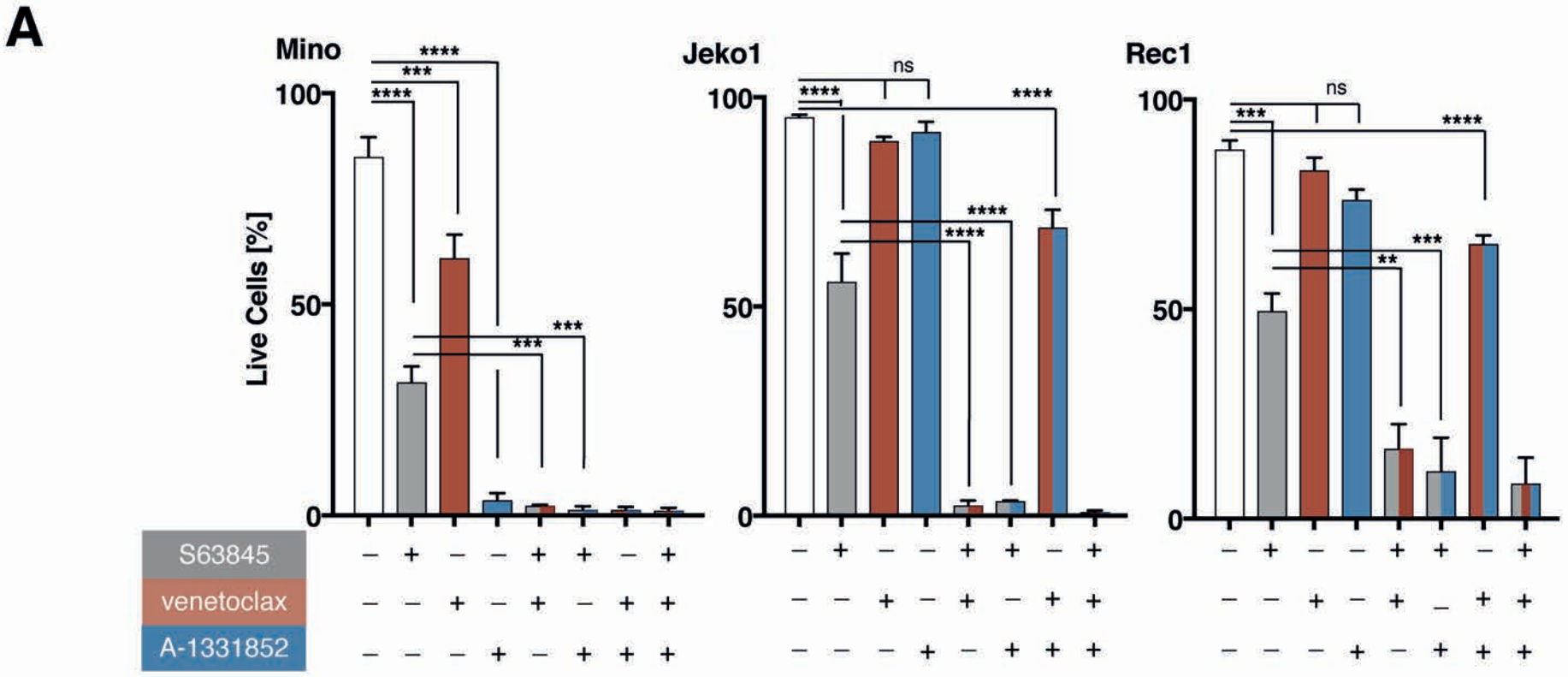


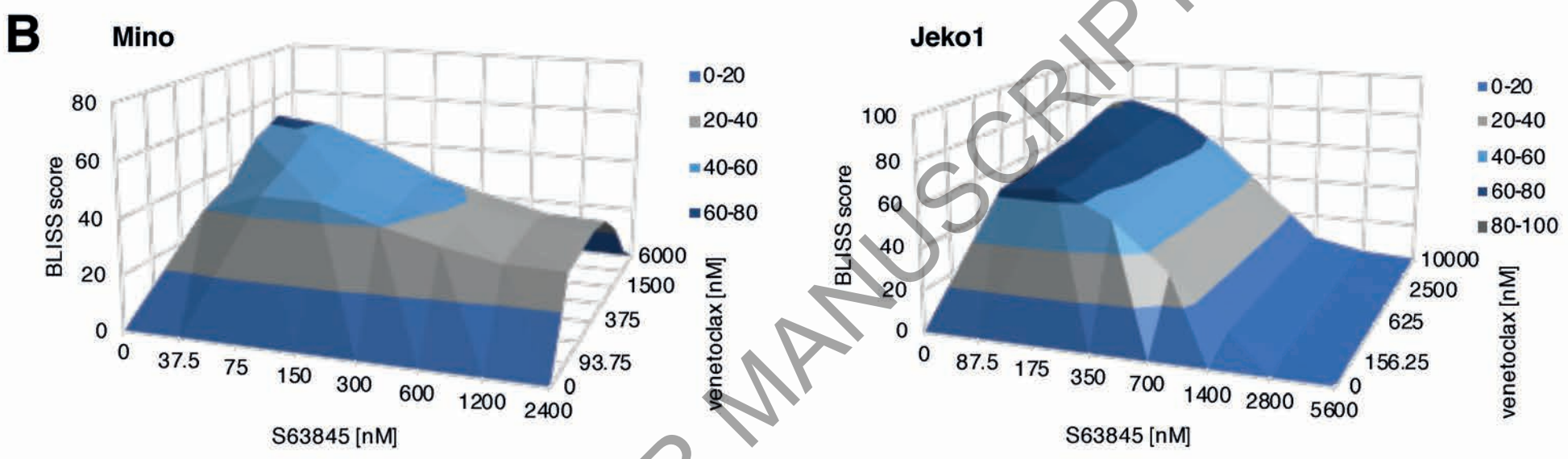












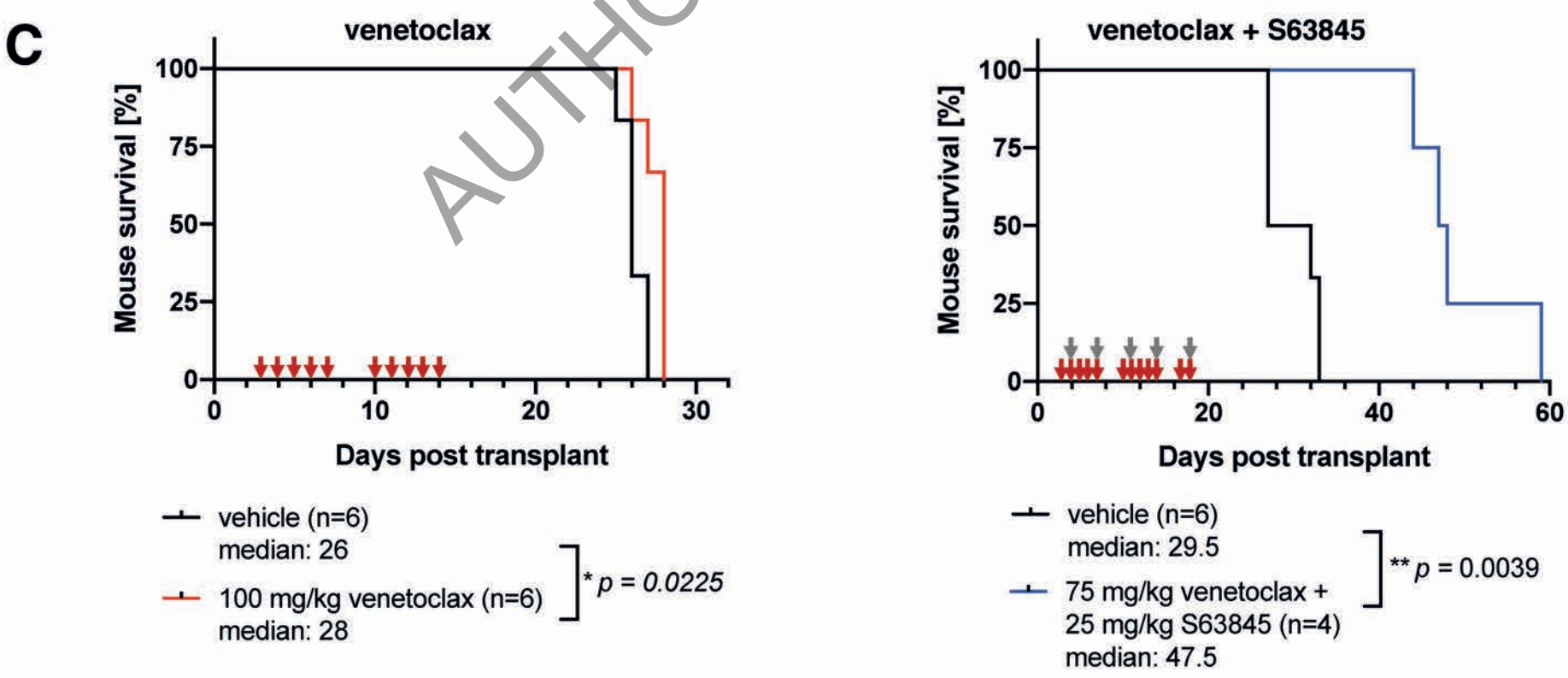
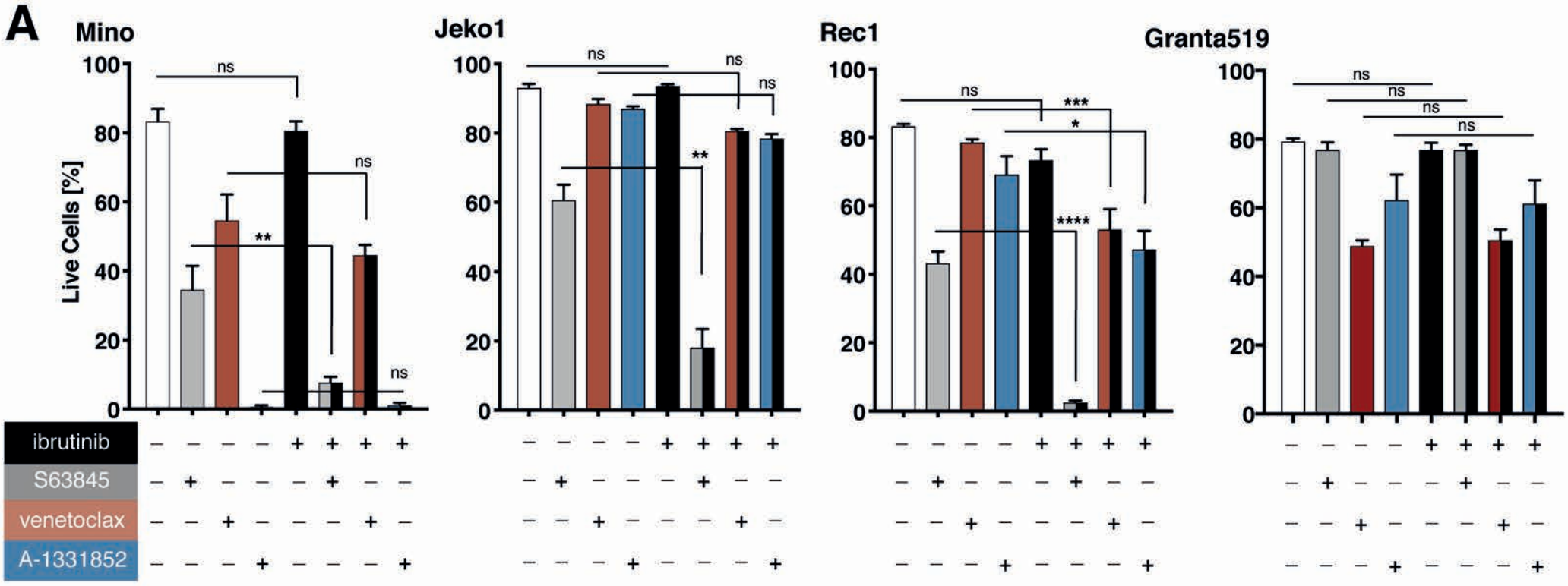
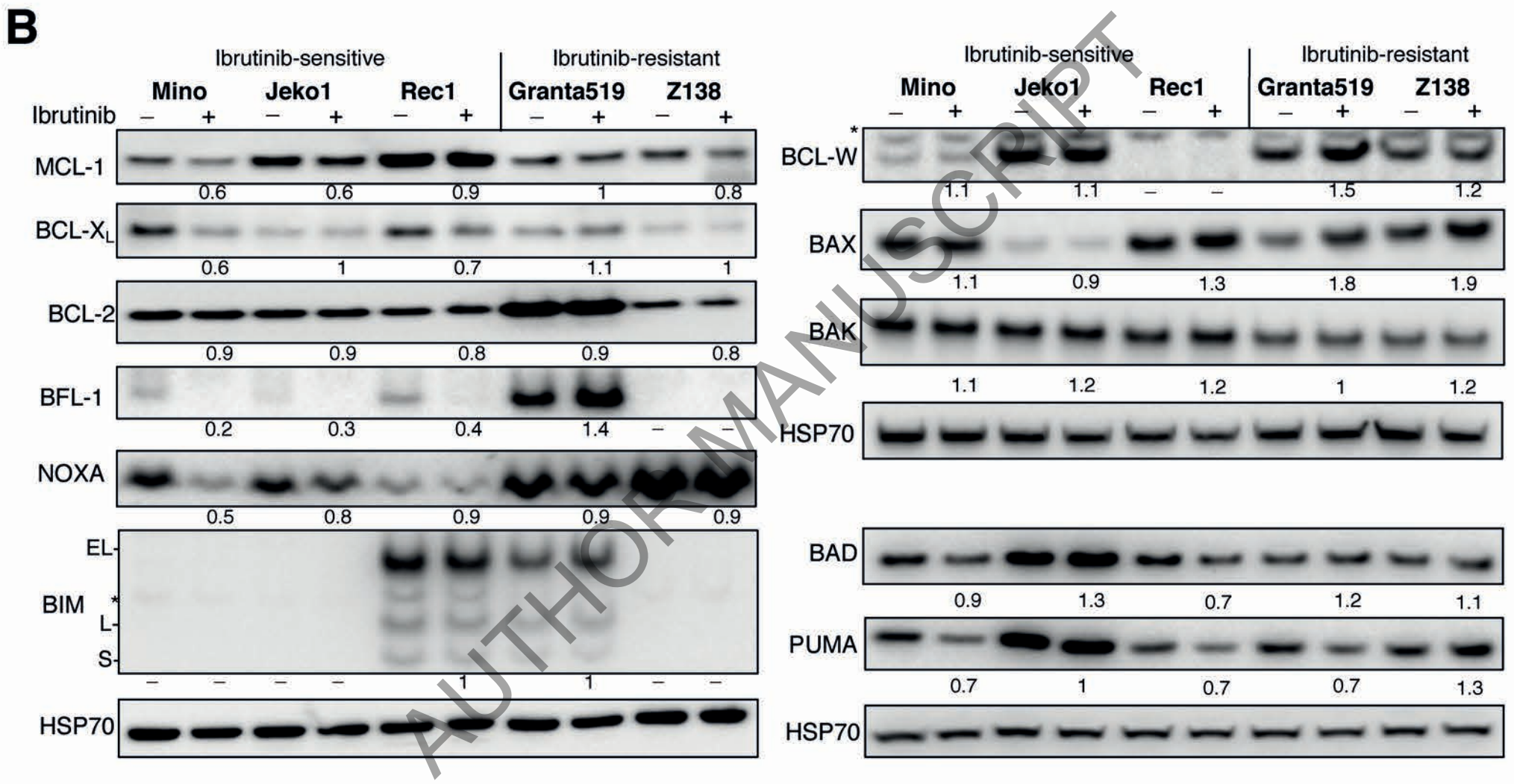


Figure 3





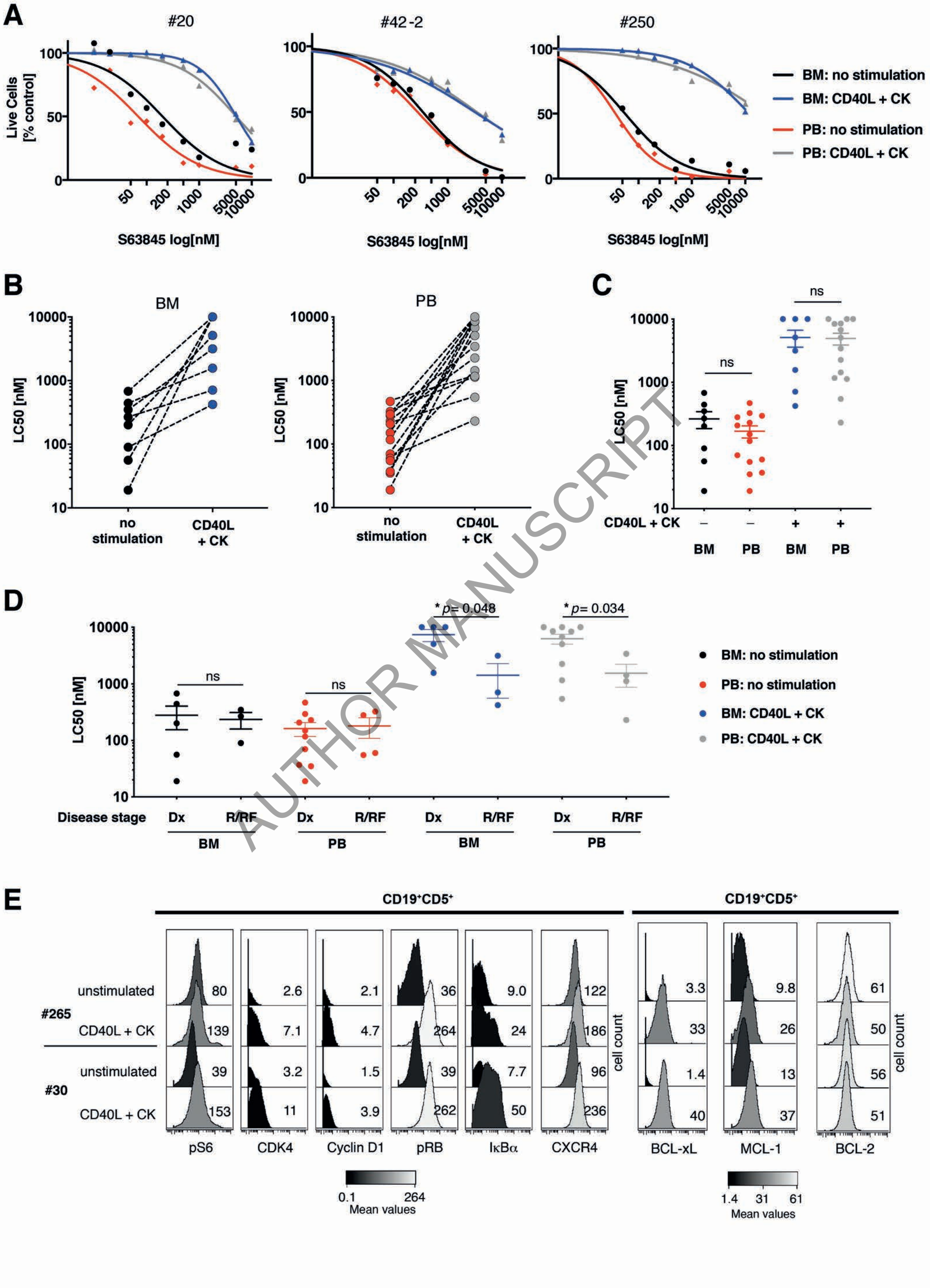
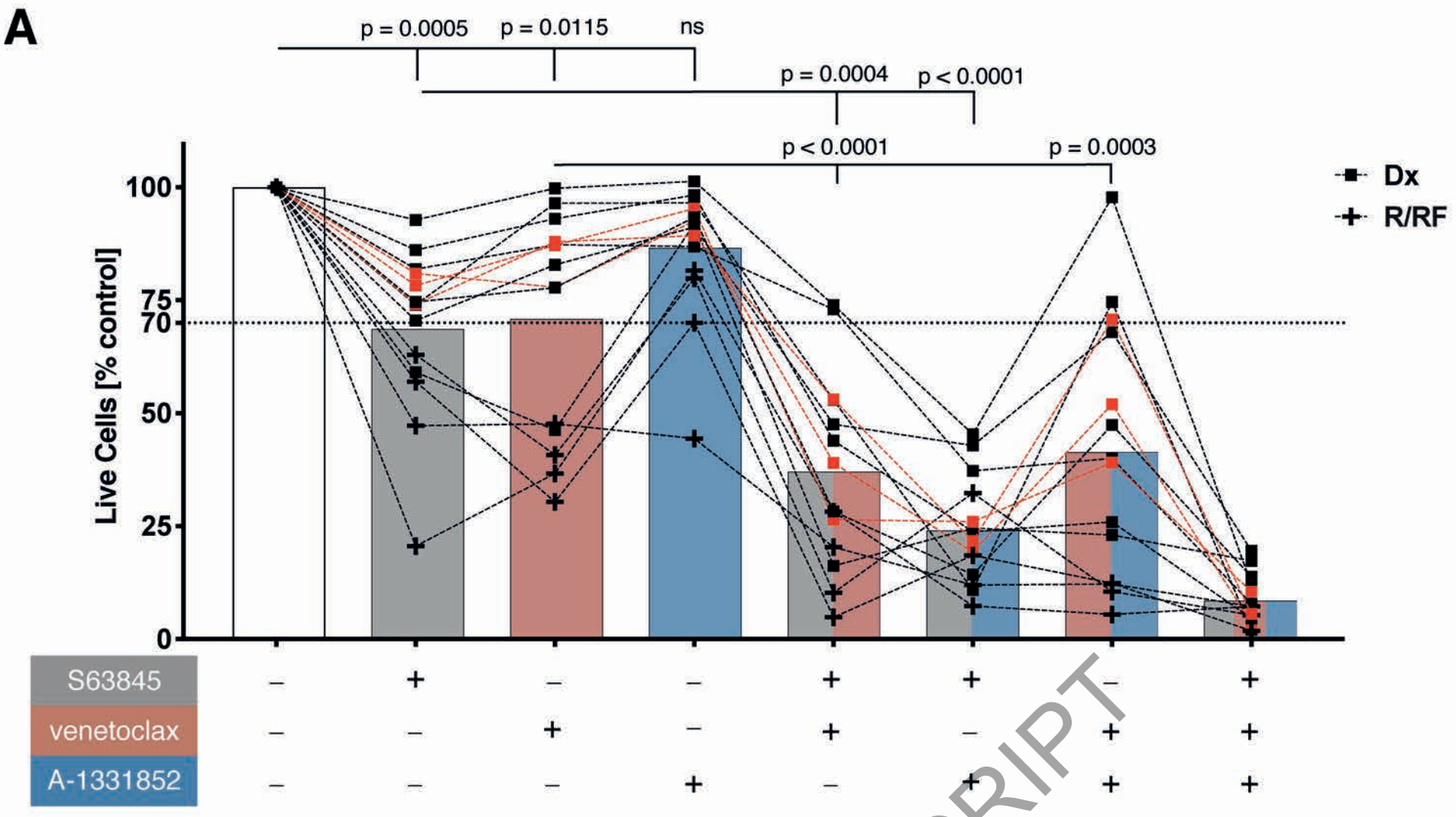


Figure 5



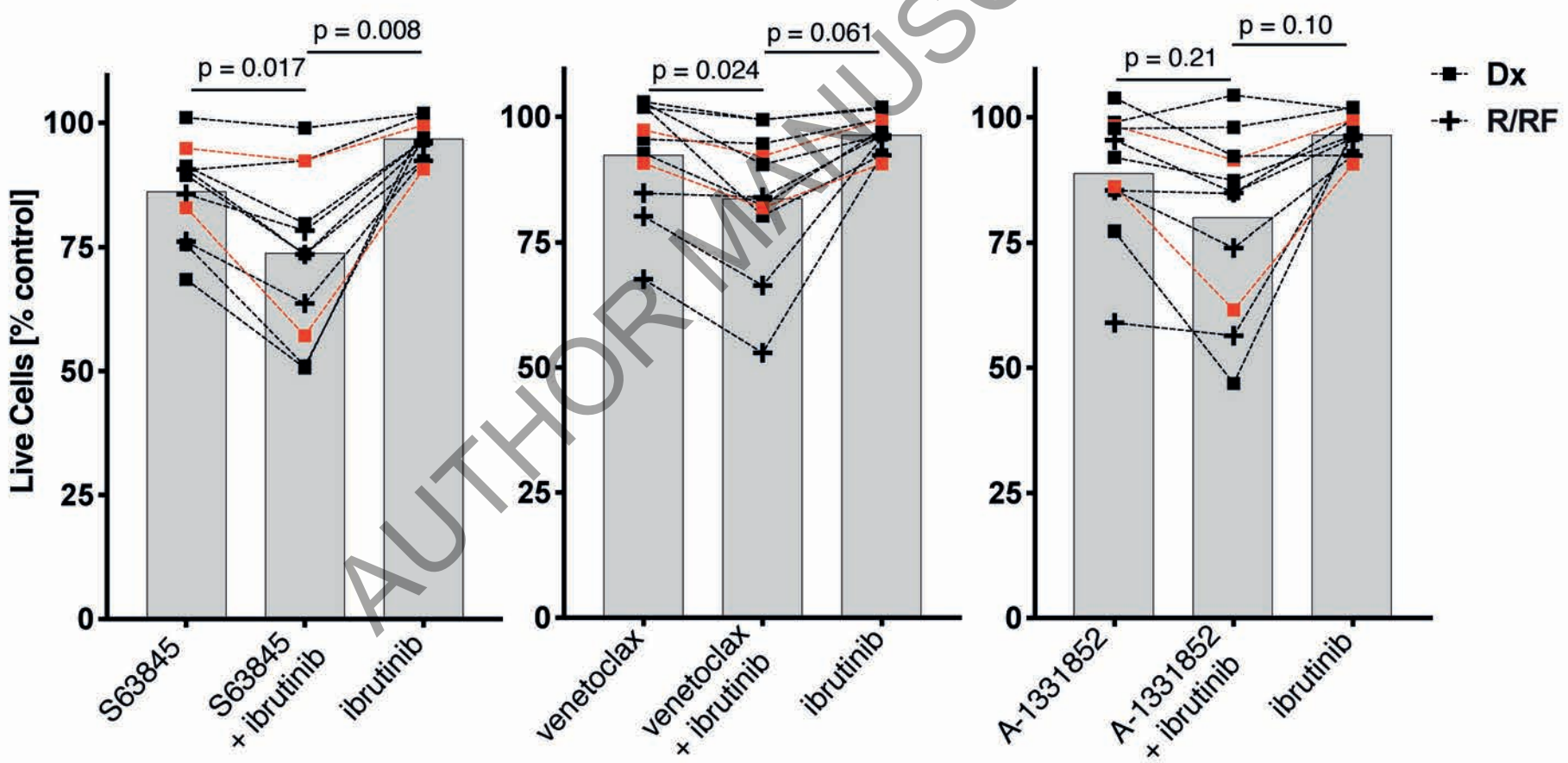


Figure 6