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The SF3B1 inhibitor spliceostatin A (SSA) elicits apoptosis in Chronic Lymphocytic Leukemia cells through downregulation of Mcl-1

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Conflict of Interest
All authors declare no conflicts of interest.

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Running title: Spliceostatin induces apoptosis by inhibiting Mcl-1 expression

Keywords; CLL; SF3B1; spliceostatin A; Mcl-1; ABT-199, Meayamycin B

Abstract

The pro-survival Bcl-2 family member Mcl-1 is expressed in chronic lymphocytic leukemia (CLL), with high expression correlated with progressive disease. The spliceosome inhibitor spliceostatin A (SSA), is known to regulate Mcl-1 and so here we assessed the ability of SSA to elicit apoptosis in CLL. SSA induced apoptosis of CLL cells at low nanomolar concentrations in a dose- and time-dependent manner, but independently of *SF3B1* mutational status, *IGHV* status and CD38 or ZAP70 expression. However, normal B and T cells were less sensitive than CLL cells ($p=0.006$ and $p<0.001$, respectively). SSA altered the splicing of anti-apoptotic *MCL-1*$_L$ to *MCL-1*$_s$ in CLL cells coincident with induction of apoptosis. Overexpression studies in Ramos cells suggested Mcl-1 was important for SSA-induced killing since its expression inversely correlated with apoptosis ($p=0.001$). IL4 and CD40L, present in patient lymph nodes, are known to protect tumor cells from apoptosis and significantly inhibited SSA, ABT-263 and ABT-199 induced killing following administration to CLL cells ($p=0.008$). However, by combining SSA with the Bcl-2/Bcl-x_L antagonists ABT-263 or ABT-199, we were able to overcome this pro-survival effect. We conclude that SSA combined with Bcl-2/Bcl-x_L antagonists may have therapeutic utility for CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of neoplastic lymphocytes in peripheral blood, bone marrow, lymph nodes and spleen. It is incurable without allogeneic transplantation and exhibits a high degree of heterogeneity in clinical course. Progressive disease is characterised by unmutated immunoglobulin heavy variable (*IGHV*) genes, ZAP70 and CD38 expression.[1] Genetic markers including deletions of 17p, 11q and 13q, trisomy 12 and *TP53* mutations[2-4] have been known to impact prognosis for some time, however with the emergence of next generation sequencing, a plethora of new genetic alterations have been identified including *NOTCH1* (10-15%)[5-8] and *SF3B1* (10-17%)[8-10] mutations which identify patients with progressive disease.[8]

SF3B1 is one of the proteins that make up the spliceosome and regulates excision of introns from pre-mRNA[11]. The catalytic core is made up of 5 small nuclear protein complexes (snRNP), U1, U2, U4, U5 and U6. SF3B1 belongs to the U2 snRNP complex implicated in constitutive and alternative splicing within eukaryotic cells. Mutations in *SF3B1* are generally missense mutations, predicted to generate altered splicing function.[8] They elicit changes in the RNA splicing profile in CLL cells and can affect important genes such as *FOXP1, ATM* and *TNIP1* amongst others.[9, 12] Therefore targeting the spliceosome may be a novel therapeutic approach for the treatment of CLL. Several compounds are available for this purpose,[13-16] including meayamycin B[17, 18] and the structurally related spliceostatin A (SSA).[16] SSA is a synthetically modified analog of the natural product FR901464,[19] while meayamycin B is prepared by total synthesis.[20] SSA binds to SF3B1 and possibly other members of the splicing factor 3 complex and targets normal splicing in the cell, promoting alternative gene splicing, accumulation of unspliced mRNAs in the nucleus and a decrease in translation.[16] Numerous transcripts have been shown to be down-modulated by SSA-altered splicing in HeLa cells.[21] Furthermore, work in neuroblastoma cells has highlighted the importance of the spliceosome in regulating the expression of the pro-survival Bcl-2 family member Mcl-1.[22]

In CLL, Mcl-1 expression correlates with stage of disease, lymphocyte doubling time, *IGHV* mutation status, ZAP70 positivity and CD38 expression.[23] Furthermore, the Mcl-1 expression level and Mcl-1:Bax ratio correlate with resistance to fludarabine and time to first treatment.[23] Mcl-1 may also be expressed to a greater extent in CLL lymph nodes compared with blood, where resistance to apoptosis is known to occur.[24] The emergence of Bcl-2 inhibitors such as ABT-263 and ABT-199 are showing considerable promise for the treatment of CLL, however these drugs are unable to inhibit

the anti-apoptotic effects of Mcl-1 conferred by the microenvironment,[25] thus identifying a known mechanism of resistance to these BH3-mimetics.[26-29] Several pan-Bcl-2 family inhibitors such as obatoclax, sabutoclax and AT-101 have been developed with low micromolar potency against Mcl-1. Obatoclax (a BH-3 mimetic) has been investigated in a phase I clinical trial for advanced CLL, but its effect was limited.[30] Studies on AT-101 with rituximab showed some efficacy, resulting in partial remissions.[31, 32] Therefore, there is a need to develop alternative Mcl-1 inhibitors perhaps in combination with Bcl-2/Bcl-x_L inhibitors.

In this study we showed that the SF3B1 splicing inhibitor SSA induced robust apoptosis in CLL cells whilst normal lymphocytes were significantly more resistant. Apoptosis was induced independently of *SF3B1* mutation status. SSA-induced apoptosis correlated with altered *MCL-1* RNA splicing and downregulation in Mcl-1_L protein expression. IL4 and CD40L treatment conferred resistance to SSA-induced apoptosis; however, this resistance was reversed by combining SSA with the Bcl-2 family inhibitors ABT-199 or ABT-263. These data suggest that microenvironmental signals in the lymph node may make the CLL cells that reside there resistant to SSA. Therefore combining inhibitors of both Bcl-2/Bcl-x_L and Mcl-1 may be warranted for the treatment of CLL.

Methods

Patients

Diagnosis of CLL was according to the IWCLL-NCI 2008 criteria.[3] Fifty-nine samples were obtained from patients diagnosed with CLL at the Royal Bournemouth Hospital and Southampton General Hospital following ethical committee approvals (06/Q2202/30 and 228/02/t, respectively) under the declaration of Helsinki (Supplementary Table 1). Malignant cell isolation and the determination of their purity have been described previously.[33] Isolates typically contained >90% $CD19^+CD5^+$ cells. Normal B and T lymphocytes from peripheral blood were taken from healthy age-matched controls as previously described.[34] Mutational screening for *SF3B1* was carried out by Sanger sequencing as previously reported (Supplementary Table 2).[8]

Reagents and antibodies

Spliceostatin A was produced by Professor Yoshida (Riken Institute, Japan).[16] Meayamycin B was produced by the Koide group (University of Pittsburgh, USA).[20] ABT-199 and ABT-263 were purchased from Selleckchem (USA). Annexin V-FITC was produced in-house (Southampton CR-UK core protein production facility). Pan-caspase inhibitor Z-VAD.fmk (Z-VAD) was from Enzo Life Sciences (UK) and Q-VD-OPh was from Calbiochem (UK). Recombinant human IL4 and CD40L were

from R&D Systems (UK). Antibodies for flow cytometry and immunoblotting are detailed in Supplementary Table 3.

Cell culture

CLL cells were cultured as previously described.[35] The Burkitt's lymphoma cell lines Ramos and Raji were obtained from ECACC. Cells were maintained in RPMI1640 supplemented with 10% FCS, 2mM glutamine, penicillin/streptomycin and 1mM sodium pyruvate (Life Technologies, UK).

Cell viability assay

Cell death was measured by flow cytometry of Annexin-V-FITC and propidium iodide (PI) labelled cells as previously described.[36] Viable cells were negative for both Annexin-V and PI. Fifty percent inhibitory concentration values (IC_{50}) were measured using CalcuSyn software (Biosoft, UK). The combination effect of SSA and ABT-199 or ABT-263 was calculated by comparing the expected and observed survival, as previously described for CLL.[37, 38]

RT-PCR

Total RNA was isolated with the RNeasy mini kit (Qiagen, UK) as described by the manufacturer. Total RNA was reverse-transcribed with M-MLV enzyme (Promega, UK). Conventional PCR was performed for *DNAJB1*, *RIOK3*, *MCL-1*, *BCL-X* and *UBIQUITIN* (Supplementary Table 3) using the GoTaq Green Master Mix (Promega, UK). Real time PCR was performed on a LightCycler 480 (Roche, UK) using Syto9 (Life technologies, UK). Each sample was analysed in triplicate with *UBIQUITIN* as a house-keeping control. The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was normalized to its non-treated matched sample.

Immunoblotting

Immunoblot analysis was performed as previously described.[36] Proteins were separated on 12% polyacrylamide gels (LifeTechnologies, UK). Blots were probed with primary antibodies (Supplementary Table 2) and secondary antibodies (Dako, UK). Blots were visualized with ChemiDoc-It Imaging System (UVP, UK) and densitometry performed with Image J.

Nucleofection

Nucleofection was performed with the Amaxa Cell Line Nucleofector Kit V (Lonza, UK), according to the manufacturer's instructions. Cells were nucleofected with the empty vector pcDNA3 (mock) or

pcDNA3-9E10-Mcl-1_L plasmid, and were selected in complete media with 1mg/ml geneticin (Life Technologies, UK). Clones were evaluated for Mcl-1_L overexpression after 14 days.

Statistical analysis

The normal distribution of the samples was tested by D'Agostino-Pearson test. Statistical differences between groups were evaluated by Student's t test or ANOVA when samples were normally distributed or by the Mann-Whitney U or Kruskal-Wallis test when samples were not. Correlation analysis was carried out using Pearson or Spearman test depending on the normality of the samples. Statistical analysis was performed using GraphPad Prism v6 (GraphPad Software Inc).

Results

SSA induces caspase-dependent apoptosis of CLL cells in a dose- and time-dependent manner

CLL samples (n=59) were treated with SSA (2.5-20nM) and cell death assessed by Annexin-V/PI staining and immunoblotting over 24h. SSA increased Annexin-V binding (Figure 1A) and cleaved caspase-3 and PARP (p89) (Figure 1B) within 24h at low nanomolar concentrations. CLL cell death was caspase-dependent since treatment with Z-VAD (Figure 1B-C) and QVD (Supplementary Figure 1A-B) significantly protected against SSA-induced cell death even at the highest concentrations (p=0.0027 and p=0.034 at 10nM and 20nM, respectively). Moreover SSA induced substantial apoptosis within 12-24h in a dose dependent manner (Figure 1D).

Sensitivity to SSA is independent of *SF3B1* mutational status

Due to the incidence of *SF3B1* mutations in CLL,[8, 10] we compared the effect of SSA on 13 patients carrying a mutation in this gene and 42 wild-type patients. The *SF3B1* mutations observed reflected the spectrum reported previously (Supplementary Table 2).[8] We confirmed that the splicing of *ATM*, *FOXP1* and *TNIP1* were altered in these samples as previously described[9, 12] (Supplementary Figure 2A-C). However, no significant difference between the mean SSA IC_{50} of wild-type (5.5nM) and *SF3B1* mutated samples (4.9nM; p=0.516) was observed (Figure 2A). In addition, SSA appears equally effective at inducing substantial apoptosis independently of *IGHV* mutational status, ZAP70 or CD38 expression, and genetic abnormalities [del(13q), Tri12, del(11q) and del(17p)] (Figure 2B-E, Supplementary Figure 3A). To determine whether *SF3B1* mutations conferred resistance to other drugs, we treated 8 samples with *SF3B1* mutations and 18 wild-type patients with chlorambucil (CHL). Samples with *SF3B1* mutations were significantly (p=0.0016) more resistant to CHL than wild-type patients (Supplementary Figure 3B-C).

Normal B and T cells are less sensitive to SSA

Normal B ($CD19^+$) and T ($CD3^+$) lymphocytes from control age-matched healthy donors (HD) were more resistant to SSA than CLL cells, with an IC_{50} of 12.1nM (p=0.003) and 61.7nM (p<0.001), respectively (Figure 2F). We also selected three CLL samples in which the proportion of $CD5^+CD19^+$ (B-CLL) to total lymphocytes was lower than 60% cells and analysed the effect of SSA in the CLL cells ($CD19^+CD5^+CD3^-$), B-cells ($CD19^+CD5^-CD3^-$) and T-cells ($CD3^+CD5^+CD19^-$). As above, T cells from CLL patients were significantly more resistant than CLL cells (~60nM; p=0.029). However, we only observed a trend for increased resistance of normal B cells to SSA (16.9nM) compared to CLL (6.67nM) in these patients (Figure 2G).

SSA induces changes in *MCL-1* splicing

To assess the effect of SSA on splicing within CLL cells, we studied the genes *DNAJB1* and *RIOK3* in which other splicing inhibitors have been shown to induce intron retention (larger RT-PCR products). SSA induced a dose-dependent increase in the unprocessed mRNA species compared to the mature mRNA (Figure 3A). Therefore, SSA was shown to decrease the capability of the CLL cells to correctly process pre-mRNAs. The siRNA-mediated knockdown of SF3B1 induces changes in the splicing of *MCL-1* and *BCL-X* in solid tumors,[39] so, we investigated the effect of SSA on *MCL-1* and *BCL-X* in CLL cells. SSA decreased *MCL-1*$_L$ and increased *MCL-1*$_s$ isoforms in a dose-dependent manner even in the presence of Z-VAD (Figure 3B), confirming that the accumulation of *MCL-1*$_s$ was not an indirect result of caspase cleavage and cell destruction. In contrast, *BCL-X* showed no altered splicing following SSA treatment (Figure 3C). SSA-induced altered splicing of *MCL-1* occurred in a time- and dose-dependent manner (Figure 3D-F), occurring as early as 6h after 5nM SSA administration and immediately prior to CLL apoptosis. Importantly, *MCL-1* splicing occurred to the same extent in both wild-type and *SF3B1* mutated samples (Supplementary Figure 4A-B). In addition, the changes in MCL-1 splicing were specific to SSA, as other drugs used to treat CLL did not affect this gene (Supplementary Figure 4C).

Altered *MCL-1* splicing reduces Mcl-1$_L$ protein expression

Next, we evaluated what impact altered splicing of *MCL-1* mRNA had on protein expression and whether SSA regulated expression of other Bcl-2 family members. SSA caused a marked reduction in Mcl-1_L protein expression, which was equivalent in the presence of Z-VAD, indicating this decrease was not due to proteolytic cleavage after caspase activation. In contrast, Bcl-2 and Bcl-x_L showed no consistent change in expression (Figure 4A). The pro-apoptotic BH3-only protein Puma decreased alongside Mcl-1_L whereas Noxa increased in 5/7 samples. As such, Mcl-1_L reduction was the only

consistent Bcl-2 family protein change observed and coincided with apoptosis, as measured by PARP cleavage (Figure 4A). A summary of the effect of SSA on Mcl-1_L protein expression is presented in Figure 4B.The decrease in Mcl-1_L protein started at 6h with 10nM SSA, coincident with the decrease in mRNA levels (Figure 4C). To indicate whether the reduction in Mcl-1_L induced by SSA may be due to inhibition of SF3B1 we used a second inhibitor meayamycin B and showed similar results (Figure 4D). Finally, we correlated Mcl-1_L and Bcl-x_L expression at a basal level with sensitivity to SSA. Mcl-1_L but not Bcl-x_L expression inversely correlated with increased IC_{50} values (p=0.045; Supplementary Figure 5A-C).

Overexpression of Mcl-1_L protects Ramos cells from SSA-induced apoptosis

To confirm that Mcl-1_L protein reduction was responsible for the SSA-induced apoptosis, we attempted to overexpress Mcl-1_L directly. These studies are difficult to perform in CLL cells due to low transfection efficiency and/or high background apoptosis following nucleofection. Therefore, we undertook these studies in Ramos B cells in which we could more readily manipulate Mcl-1 protein expression. We first showed that SSA was able to induce apoptosis of Ramos cells with a comparable IC_{50} to that of CLL cells (4.68nM) (Figure 5A-B), in a dose-, time- and caspase-dependent manner (Figures 5B-C). We also showed that SSA downregulated Mcl-1_L expression in Ramos cells, akin to that in CLL (Figure 5A). We then nucleofected Ramos cells with plasmids containing a Myc-tagged Mcl-1_L protein or the empty plasmid (mock) and selected three stable clones which overexpressed Myc-tagged Mcl-1_L that is unable to be alternatively spliced by SSA (Figure 5D). Importantly, these clones were selected to reflect levels of ectopic Mcl-1_L that could be considered physiological. Ramos cells which overexpressed Mcl-1_L still underwent apoptosis but were significantly less sensitive to SSA induced killing at the concentrations investigated (Figure 5E-F). In contrast to CLL, SSA also strongly reduced Bcl-x_L expression in Ramos cells, potentially explaining why they still undergo some apoptosis in the presence of sustained Mcl-1_L. To explore whether Bcl-x_L levels could help explain differential sensitivity to SSA, a second B cell-line, Raji, was examined. In Raji cells, SSA again reduced Mcl-1_L expression in a dose dependent manner but was unable to promote extremely high levels of apoptosis even at high doses (20-80nM). However, in contrast to Ramos cells Bcl-x_L levels in Raji cells remained high in the presence of SSA, (Supplementary Figure 6), supporting the role of Bcl-x_L in inhibiting SSA toxicity.

SSA-induced apoptosis is reduced in the presence of microenvironment support

Microenvironment signals are known to confer drug resistance to CLL cells and influence Mcl-1_L expression.[36, 40-42] In order to analyse the potential effect of these signals on SSA-induced apoptosis,

we tried to mimic lymph node conditions *in vitro*. T cells are abundant in CLL lymph nodes,[43] therefore we treated CLL cells with CD40L plus IL4, mimicking T cell help. This combination caused the expected increase in phosphorylated STAT6, an indicator of IL4 signalling, phosphorylated IκBα, an indicator of NFκB activation and accumulation of the NFκB downstream targets Mcl-1 and Bcl-x_L (Figure 6A). IL4/CD40L treatment doubled the expression of Mcl-1_L, whilst Bcl-x_L increased further, up to ~3.5-fold compared to the control. This increase in Mcl-1_L and Bcl-x_L significantly correlated with resistance to SSA (p=0.008) (Figure 6B). However SSA was still able to promote *MCL-1* splicing (Figure 6C). These data again suggest that increased Bcl-x_L expression by IL4/CD40L is associated with resistance to SSA.

SSA in combination with ABT-199 or ABT-263 synergises to overcome microenvironment signals

To confirm that the reduced sensitivity to SSA was a result of increased Bcl-x_L, we next evaluated SSA in combination with the BH3-mimetic ABT-263, which inhibits Bcl-2, Bcl-x_L and Bcl-w but not Mcl-1, or ABT-199 which preferentially targets Bcl-2. These compounds alone or in combination were evaluated in CLL cells treated with (Figure 7) or without IL4/CD40L (Supplementary Figure 7). SSA, but not ABT-199 or ABT-263, induced a dose-dependent reduction of Mcl-1_L whilst Bcl-2 and Bcl-x_L expression did not significantly change (Figure 7A. Supplementary Figure 7A). In the absence of IL4/CD40L, all compounds induced efficient dose-dependent apoptosis (Supplementary Figure 7A-B). However, any combination effects were difficult to discern due to the substantial amount of apoptosis with the compounds alone (Supplementary Figure 7B). In contrast, following IL4/CD40L stimulation, CLL cells underwent significantly less SSA- and ABT-199-induced cell death, whilst becoming completely resistant to ABT-263 (Figure 7B). Nevertheless, the combination of SSA with ABT-263 or ABT-199 significantly augmented CLL apoptosis (Figure 7A-B). Using the fractional 2-drug analysis method previously described for CLL,[38, 44] we evaluated the synergistic interaction between SSA and ABT compounds. Values above the diagonal line represent additive interaction whilst those under the line are synergistic. Under basal conditions, the drug combinations presented additive or synergistic effects in most of the patients (Supplementary Figure 7C-D). In the presence of IL4/CD40L, a strong synergistic relationship was observed following treatment with SSA and ABT-199 (Figure 7C) or ABT-263 (Figure 7D). It was noteworthy that the combination of SSA and ABT-199 or ABT263 also presented marked synergistic effects on 17p deleted samples after IL4/CD40L stimulation (Supplementary Figure 8). These data suggest that SSA in combination with Bcl-2/Bcl-x_L inhibitors may be a useful strategy for targeting cells receiving microenvironmental signals within the protective lymph node niche.

Discussion

Solid and haematological malignancies commonly overexpress anti-apoptotic Bcl-2 family members, allowing them to evade apoptosis.[45, 46] Whilst compounds have been developed to mimic BH3 proteins, many of these target Bcl-2, Bcl-x_L and Bcl-w with little to no effect on Mcl-1.[45, 47] It is known that targeting all pro-survival members is critical for efficient apoptosis[48] and CLL is known to have elevated levels of Bcl-2, Bcl-x_L and Mcl-1 in lymph nodes.[24] Furthermore, Mcl-1 expression is known to correlate with progressive disease, resistance to chemotherapy and time to first treatment[23], therefore overcoming Mcl-1 is likely to be critical for effectively treating CLL.

In this study, SSA, an analogue of FR901464 that inhibits the SF3B1 protein, induced altered splicing and down-regulation of Mcl-1_L, which was concordant with caspase-dependent apoptosis of CLL cells at low nanomolar concentrations. In agreement with the key role of Mcl-1 in regulating CLL survival, we saw an inverse correlation between Mcl-1_L expression and sensitivity to SSA in CLL samples. Interestingly, 2- and 14-fold higher concentrations of SSA were required to kill normal B and T cells, respectively. This sensitivity profile indicates that B cells and particularly tumor B cells are more sensitive to spliceosome interference than T cells. The molecular basis behind this is unclear but since Mcl-1 and Bcl-x_L are essential for T cell survival,[49] resistance may relate to Bcl-x_L overexpression or differential splicing by SSA in B and T cells.

SSA-induced killing of CLL cells was independent of the mutational status of *SF3B1 and IGHV*, as well as, CD38 and ZAP70 expression. *SF3B1* mutations have been shown to be present at sub-clonal levels,[50] however all our *SF3B1* mutant samples exhibited deregulated splicing supporting the notion that the SF3B1 mutant protein in these cases has a functional impact. The equal sensitivity of CLL samples to SSA indicates a common mechanism of cell death irrespective of the size of the SF3B1 mutated clone within the population. Therefore further correlations between SSA sensitivity and *SF3B1* mutant sub-clonal levels from deep-sequencing data will be required to fully elucidate this relationship and these studies are ongoing. Furthermore, we showed that *SF3B1* mutated samples have a significantly greater IC_{50} for chlorambucil than WT samples. These data suggest that *SF3B1* mutations may confer reduced sensitivity to drug induced apoptosis supporting previous reports with fludarabine.[51]

The binding of SSA to SF3B1 inhibits pre-RNA splicing[16] but also induces changes in alternative splicing.[52] We have demonstrated in CLL cells that SSA promotes alternative-splicing of *MCL-1*. Laetsch *et al*. observed similar results in neuroblastoma cell lines.[22] A reduction of SF3B1 protein by

siRNA in HeLa cells resulted in a similar increase in the *MCL-1$_S$:MCL-1$_L$* ratio[39], however in neuroblastoma cell lines SF3B1 knockdown increased the production of *MCL-1$_L$* modestly and *MCL-1$_S$* markedly.[22] Together these data hint to a complex regulation of *MCL-1* which likely varies in a cell and context dependent manner. In resting CLL cells, *MCL-1$_L$* is the main isoform produced by alternative splicing. After SSA treatment, RT-PCR analysis indicated a preferential alternative splicing towards the *MCL-1$_S$* isoform which resulted in a downregulation of the anti-apoptotic Mcl-1$_L$ protein. Previous studies have indicated that Mcl-1$_S$ might have pro- rather than anti-apoptotic properties.[53] We saw no evidence for the generation of mature Mcl-1$_S$ protein before or after SSA treatment in CLL or Ramos cells even in the presence of Z-VAD, suggesting that the Mcl-1$_s$ form is not stable and/or biologically functional in these cells. Supporting our hypothesis that Mcl-1$_L$ reduction is the primary reason for the apoptosis induced by SSA, we saw tight correlation between the reduction in *MCL-1$_L$* transcripts, protein expression and apoptosis. Furthermore, overexpression of unspliceable Mcl-1$_L$ in Ramos cells partially protected from SSA-induced apoptosis. This incomplete protection may be due to the fact that SSA also downregulated Bcl-x$_L$, in Ramos cells (but not in CLL or Raji cells). Bcl-x$_L$ is not highly expressed in CLL cells in the blood, but is significantly expressed at higher levels in the lymph nodes.[24] In addition, our current observations using IL4/CD40L to mimic this microenvironment suggest that SSA alone may be less effective at treating CLL cells in the lymph node niche. These data are supported by results with Raji cells, which retained Bcl-x$_L$ expression and were relatively insensitive to SSA-induced apoptosis, although Mcl-1 was spliced and down-regulated. Another FR901464 analogue, meayamycin B, induced the same reduction on Mcl-1$_L$ expression (this study) and non-small cell lung cancer and head and neck cancer cell lines.[18, 54] Although, differential and off-target effects cannot be ruled out completely, the similar responses with two structurally different compounds suggests that cell death is regulated by altered *MCL-1* splicing.

In our study, IL4/CD40L stimulation increased the expression of Bcl-x$_L$ and Mcl-1$_L$ and reduced the sensitivity of CLL cells to SSA. One explanation for this is that the induction of anti-apoptotic proteins was higher in IL4/CD40L-stimulated cells, indeed the levels of Bcl-x$_L$ appeared higher with IL4/CD40L treatment compared to the control. Several Bcl-2 inhibitors have been developed in the last 20 years, including the series of BH3-mimetics pioneered by Abbot.[55] ABT-737, ABT-263 and ABT-199 reflect the best developed exponents of this approach and have shown promising results as single agents or in combination with other cytotoxic agents.[56] The clinical trial of ABT-263 in CLL demonstrated rapid and profound reduction of CLL cells in the blood, whilst bone marrow biopsies showed evidence of residual CLL cells.[26] In that study, resistance to ABT-263 correlated with higher Mcl-1 expression

levels.[26] However, another study demonstrated that the resistance of CLL cells to ABT-737 was due to Bcl-2A1 and not Mcl-1 expression.[29, 57]

Under IL4/CD40L stimulation, ABT-199 or ABT-263 alone failed to induce significant apoptosis in CLL cells, whilst its combination with SSA produced a dramatic reduction in cell viability. Combinations of the BCR kinase inhibitors (ibrutinib or idelalisib) with Bcl-2 family inhibitors (ABT-199) have been proposed, since ibrutinib and idelalisib cause an efflux of CLL cells out of the lymph nodes into the blood where they can be readily killed by ABT-199. However, patients treated with BCR kinase inhibitors alone still have residual disease in their nodes. We therefore propose that a combination of ABT-199 with SSA may be a more successful approach for targeting residual disease in the lymph nodes in order to provide complete and durable responses for this currently incurable disease. Whilst costs preclude our investigation of SSA and ABT-199 or ABT-263 either alone or in combination *in vivo*, previous studies have been performed with meayamycin B and FR901464 alone and shown some antitumor effect *in vivo*, further suggesting their promise for use as effective new therapies.[59, 60]

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Author contributions: ML, MY, MSC, JCS and AJS designed and analysed experiments and wrote the manuscript. ML, SJB, RD, MJJR and MDB performed experimental work. MY and KK synthesized SSA and meayamycin B, respectively. FF, RW, AD and DO obtained patient consent and analysed clinical data. DO, MY, KK and GP provided critical review.

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Supplementary information is available at Leukemia's website

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Figure Legends

Figure 1. SSA induces apoptosis in CLL cells in a dose- and time-dependent manner.

CLL cells were incubated with vehicle control or 2.5-20nM SSA for 24h. (A) Apoptosis was quantified by Annexin V/PI staining and flow cytometry. (B) CLL cells were treated as above but in the presence or absence of Z-VAD.fmk. Immunoblotting was performed for cleaved caspase-3 and its substrate PARP with Hsc70 as a loading control. A representative blot of 6 patients is shown. (C) CLL cells (n=6) were incubated with SSA in the presence or absence of Z-VAD.fmk. Apoptosis was measured by Annexin V/PI and the number of viable cells remaining normalized to untreated control samples. (D) CLL cells were incubated with (5, 10 or 20nM) or without SSA for 6, 12 or 24h. Cell viability was assessed by Annexin V/PI (n=5) and normalized to untreated samples. Bars represent mean ± SD. *: P<0.05; **: P<0.01; ***: P<0.001.

Figure 2. Sensitivity of CLL and normal B and T cells to SSA-induced apoptosis

Cell viability assays were used to generate IC_{50} values for CLL samples treated with SSA (2.5-40nM SSA) for 24h. The IC_{50} values were plotted for (A) *SF3B1* mutated (n=13) and wild-type samples (n=42), (B) mutated-*IGHV* (M-CLL; n=26) and unmutated-*IGHV* (U-CLL; n=29), (C) $ZAP70^+$ (>30%) and $ZAP70^-$ (<30%), (D) $CD38^+$ (>30%) and $CD38^-$ (<30%), (E) genetic abnormalities del(13q) (n=21), Tri12 (n=5), del(11q) (n=11) and del(17p) (n=8) and wild-type (n=10). (F) Cell viability assays were performed in healthy age-matched donors (n=5). Apoptosis was evaluated by Annexin V/PI staining following gating on the normal B ($CD19^+$) or T ($CD3^+$) lymphocyte populations. A representative cohort of CLL samples is shown for comparison. (G) CLL, B and T cells from individual CLL patients (n=3) were compared in the same assay after staining with CD19, CD5 and CD3 to identify CLL ($CD19^+CD5^+CD3^-$), B ($CD19^+CD5^-CD3^-$) or T cells ($CD19^-CD5^+CD3^+$). Lines represented mean ± SD. *: P<0.05; **: P<0.01; ***: P<0.001.

Figure 3. SSA treatment causes intron retention and modifies *MCL-1* RNA splicing in CLL cells.

(A) CLL samples were incubated with 2.5-20nM SSA for 24h and assessed for intron retention by PCR for *DNAJB1* and *RIOK3* in the presence (+RT) or absence (-RT) of reverse transcriptase (RT) (n=4). mRNA species with intron retention are highlighted by (»).*MCL-1* (B) and *BCL-X* (C) splice variants analysed by RT-PCR after SSA treatment in the presence or absence of Z-VAD.fmk (n=6). (D) The effect of SSA on *MCL-1* splicing was assessed in a dose- and time-dependent manner by RT-PCR (D) and real-time PCR (E-F). The relative expression of $MCL\text{-}1_L$ and $MCL\text{-}1_S$ was normalized to the control sample (0nM SSA) at each time point. All samples were assessed in triplicate and represent the mean ± SD. *UBIQUITIN* was used as house-keeping gene. *: P<0.05; **: P<0.01; ***: P<0.001.

Figure 4. SSA/Meayamycin B treatment reduces Mcl-1_L protein expression

Immunoblotting was performed on CLL cells treated in a (A, B) dose- (24h) and (C) time-dependent manner (10nM) with SSA as indicated and various pro- and anti-apoptotic proteins investigated. CLL cells in (A) were also treated in the presence or absence of Z-VAD.fmk. (B) Quantitation of Mcl-1_L protein expression normalised to Hsc70 in CLL samples treated with SSA (n=7); bars represent mean ± SD. (D) CLL cells were treated with Meayamycin B in a dose dependent manner and immunoblotting was performed for PARP and Mcl-1 . Hsc70 was used as a loading control. *: P<0.05; ***: P<0.001

Figure 5. Mcl-1_L overexpression in Ramos cell lines induces resistance to SSA.

(A) Ramos cells were treated with SSA (1.25-10nM) for 24h in the presence or absence of Z-VAD.fmk (25μM) and PARP cleavage, Mcl-1_L and Bcl-x_L expression was investigated by immunoblotting. Hsc70 was used as a loading control for immunoblotting. (B) Ramos cells were incubated as above and viability assessed with Annexin V/PI by flow cytometry, plotted as the mean ± SD of 4 independent experiments. (C) Ramos cells were exposed to 5nM SSA for 6, 12, 18 and 24h. Data represent the mean ± SD of 4 independent experiments. (D) Ramos cells were nucleofected with an empty vector (Mock) or a vector containing the sequence for Mcl-1_L tagged with myc (Mcl-1-tag myc). After selection, cellular extracts were subjected to immunoblot analysis for Mcl-1_L. (E) Annexin V/PI was

used to quantify cell viability following SSA treatment for 24h in Ramos cells overexpressing Mcl-1_L or vector control. Raw data from a representative experiment is shown.(F) Summary of 3 independent experiments where data is represented as mean ± SD. **: P<0.01; ***: P<0.001 .

Figure 6. The effect of microenvironment signals on SSA-induced apoptosis.
CLL cells were treated with IL4 (10ng/ml) and/or CD40L (300ng/ml either alone or in combination, in the presence or absence of SSA (5-20nM). (A) Immunoblotting for PARP, phosphorylated STAT6 (pSTAT6), phosphorylated IκBα (pIκBα), Mcl-1_L, Bcl-x_L, Bcl-2 and Hsc70, as a loading control, were performed. (B) Annexin V/PI analysis was quantified following 24h of SSA treatment and the percentage of viable cells determined. (C) PCR was performed to investigate whether *MCL-1* underwent alternative splicing following 5-10nM SSA treatment in the presence of IL4 and CD40L alone or in combination. Mean values are shown. *: P<0.05; **: P<0.01.

Figure 7. SSA in combination with the Bcl-2 family inhibitors ABT-199 or ABT-263 augments apoptosis in CLL cells following IL4/CD40L treatment
CLL cells were treated with IL4 and CD40L for 6h. The cells were subsequently treated with SSA, ABT-199, ABT-263 or their combinations at a 1:1 ratio for 24h. (A) Immunoblotting was performed and analysed for the expression of PARP, Mcl-1_L, Bcl-x_L, Bcl-2 and Hsc70 as a loading control. (B) Annexin V/PI analysis was performed and the percentage of viable cells calculated. Data represent mean ± SD of seven independent experiments. (C) Synergistic interactions were evaluated at a 1:1 ratio between SSA and ABT-199 as indicated. (D) Synergistic interactions were evaluated at a 1:1 ratio between SSA and ABT-263. Points below the diagonal line represent synergistic interactions, above the line are additive. **: P<0.01; ***: P<0.001.

Figure 1

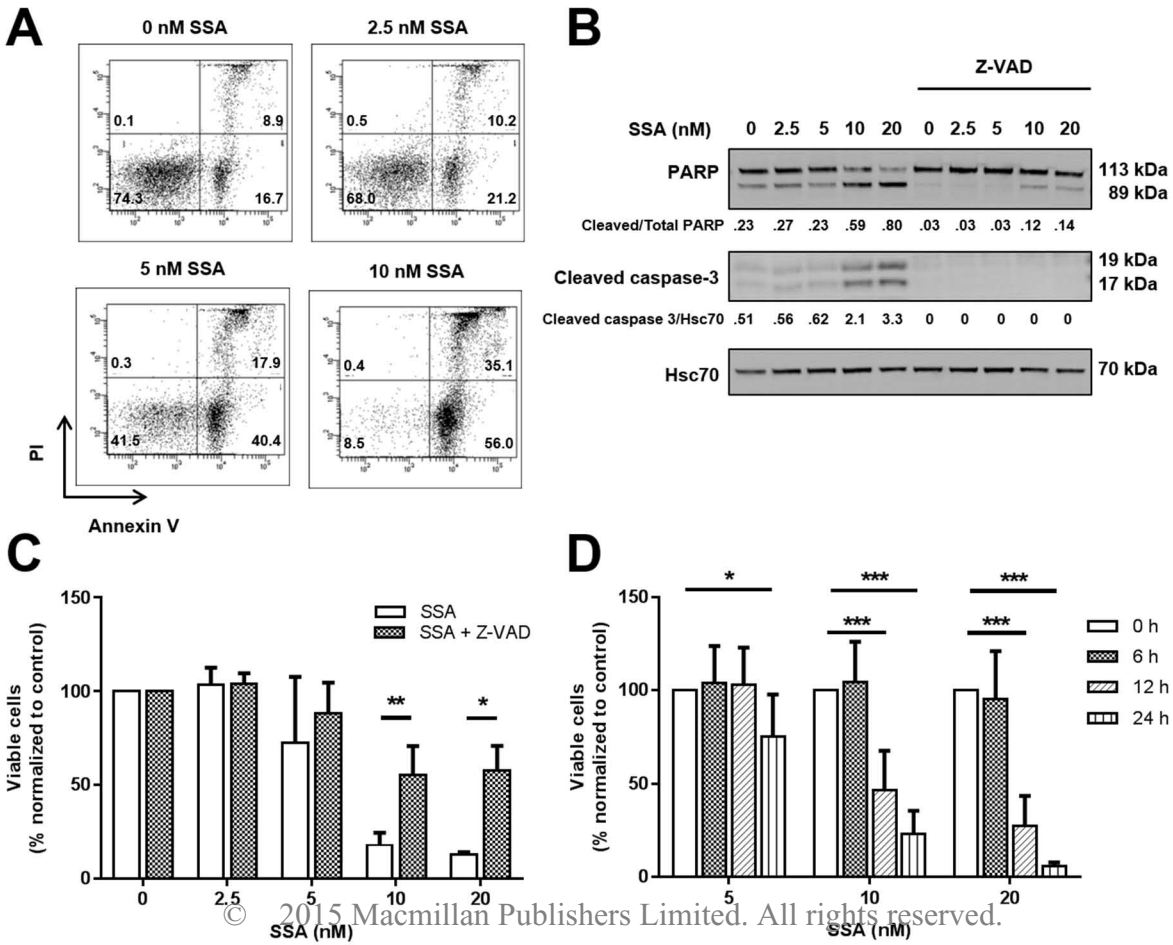

Figure 2

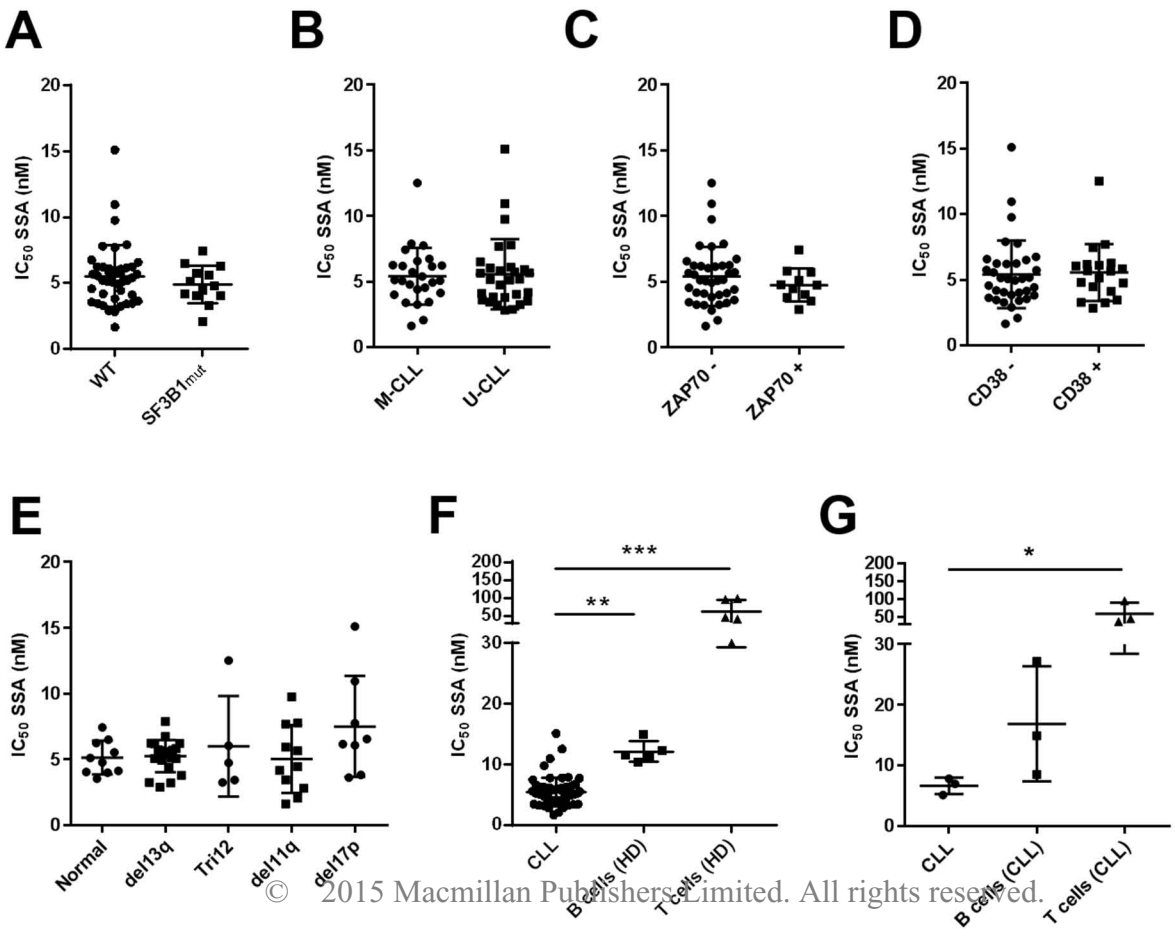

Figure 3

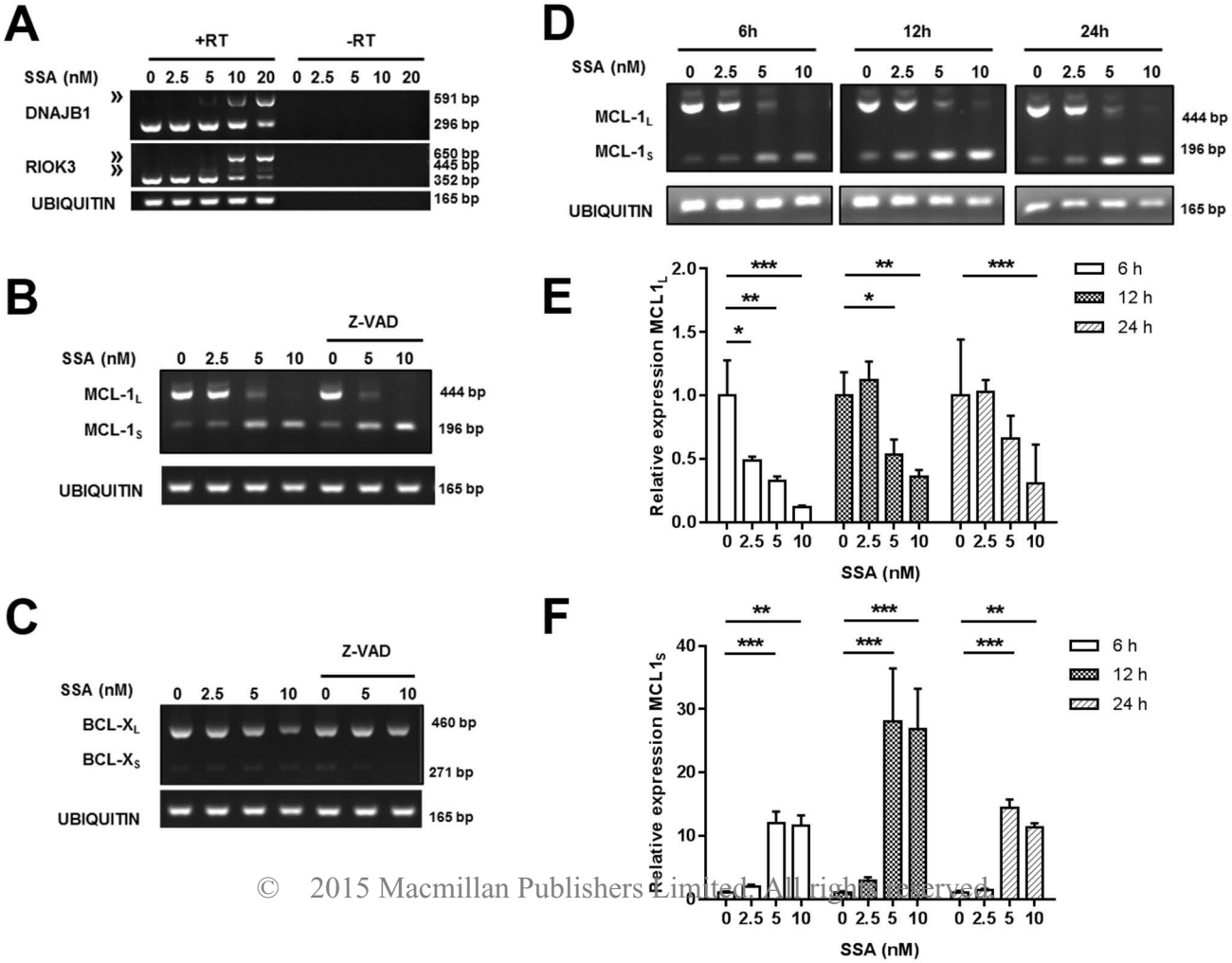

Figure 4

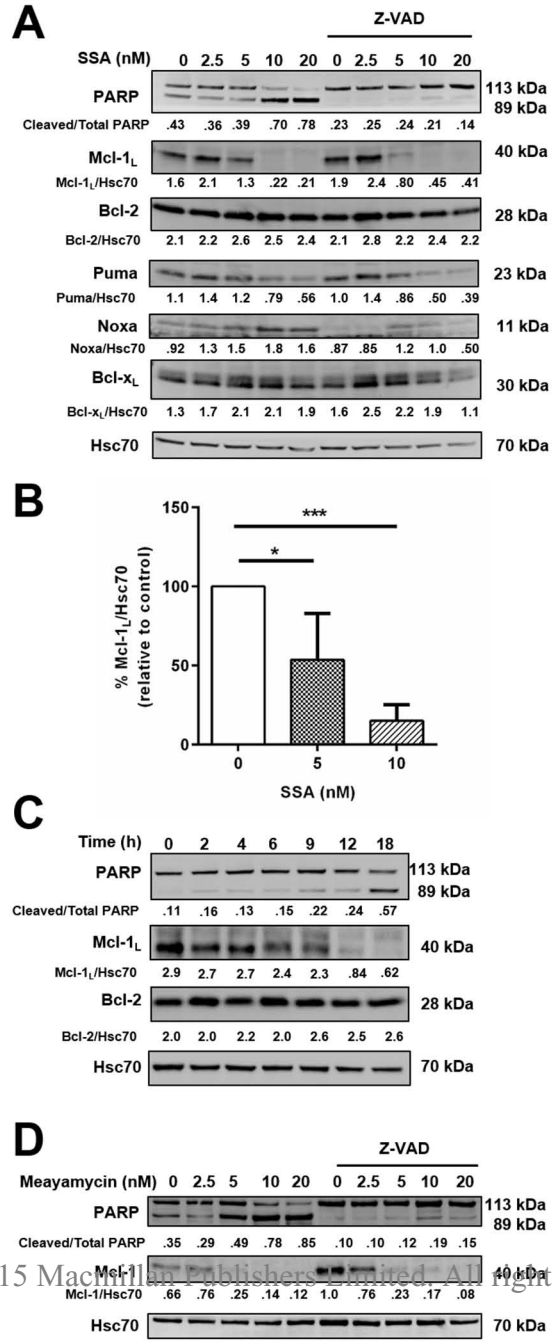

Figure 5

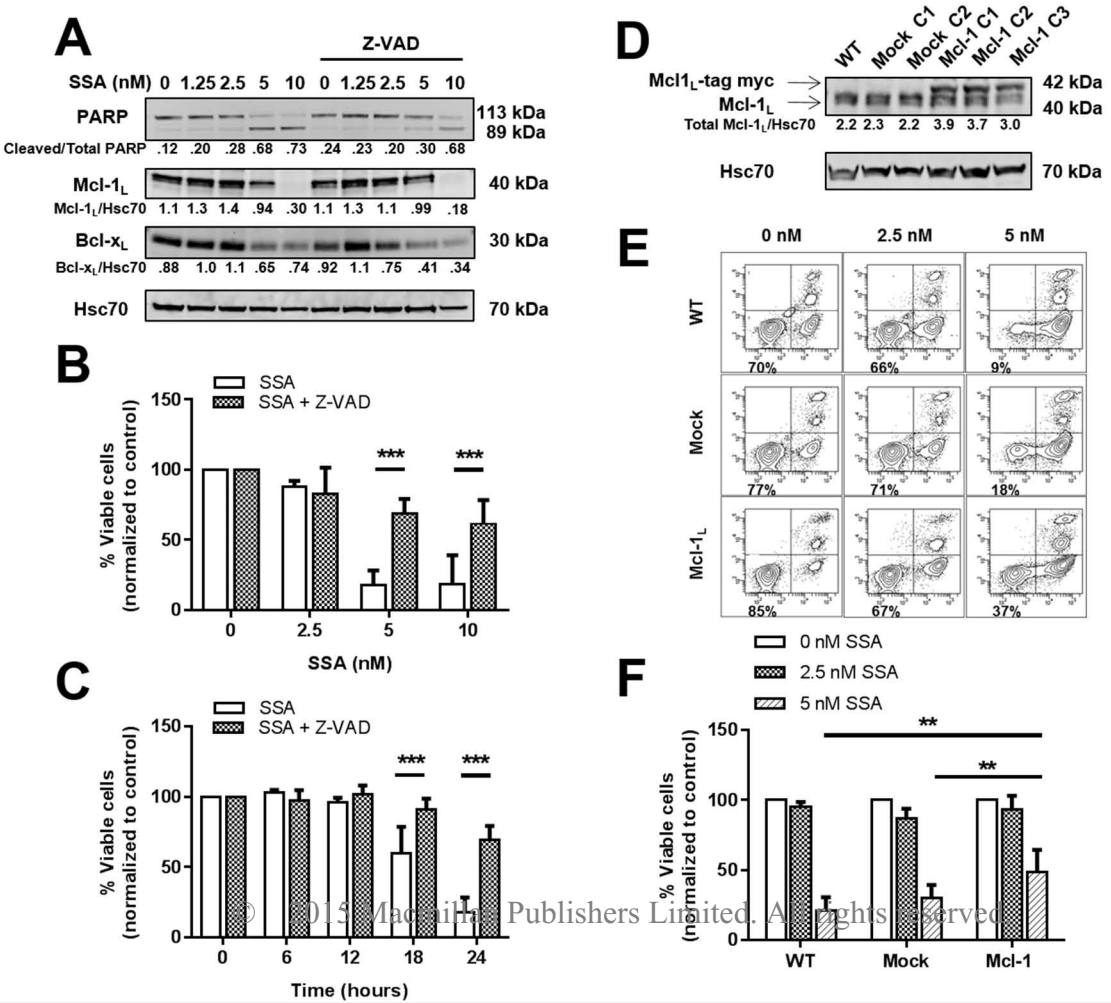

Figure 6

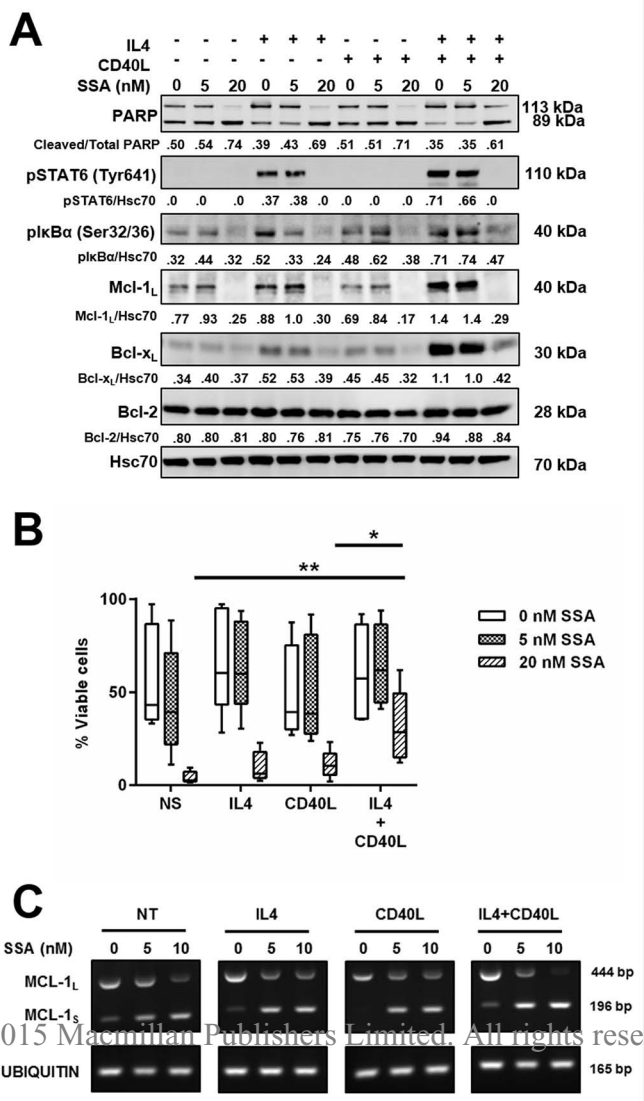

Figure 7