Published in final edited form as:

Apoptosis. 2014 January ; 19(1): 201–209. doi:10.1007/s10495-013-0910-y.

The putative BH3 mimetic S1 sensitizes leukemia to ABT-737 by increasing reactive oxygen species, inducing endoplasmic reticulum stress, and upregulating the BH3-only protein NOXA

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Abstract

S1 is a putative BH3 mimetic proposed to inhibit BCL2 and MCL1 based on cell-free assays. However, we previously demonstrated that it failed to inhibit BCL2 or induce apoptosis in chronic lymphocytic leukemia (CLL) cells, which are dependent on BCL2 for survival. In contrast, we show here that S1 rapidly increases reactive oxygen species, initiates endoplasmic reticulum stress, and upregulates the BH3-only protein NOXA. The BCL2 inhibitors, ABT-737, ABT-263, and ABT-199, have demonstrated pro-apoptotic efficacy in cell lines, while ABT-263 and ABT-199 have demonstrated efficacy in early clinical trials. Resistance to these inhibitors arises from the upregulation of anti-apoptotic factors, such as MCL1, BFL1, and BCLXL. This resistance can be induced by co-culturing CLL cells on a stromal cell line that mimics the microenvironment found in patients. Since NOXA can inhibit MCL1, BFL1, and BCLXL, we hypothesized that S1 may overcome resistance to ABT-737. Here we demonstrate that S1 induces NOXA-dependent sensitization to ABT-737 in a human promyelocytic leukemia cell line (NB4). Furthermore, S1 sensitized CLL cells to ABT-737 *ex vivo*, and overcame resistance to ABT-737 induced by co-culturing CLL cells with stroma.

Keywords

BCL2; MCL1; BCLXL; NOXA; reactive oxygen species; ATF3

Introduction

The evasion of apoptosis is an established hallmark of cancer, and is frequently mediated by the deregulation of BCL2 proteins [1]. The BCL2 family regulates the intrinsic apoptosis pathway at the mitochondrial membrane and can be divided into three classes: the pro-

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Conflict of Interest

All authors declare that they have no conflicts of interest

apoptotic activating proteins (BAX and BAK), pro-apoptotic BH3-only proteins, and anti-apoptotic proteins. The pro-apoptotic BAX and BAK oligomerize to form pores in the outer mitochondrial membrane [2]. This releases cytochrome c and initiates the caspase cascade, ultimately leading to the destruction of the cell. The anti-apoptotic proteins (such as BCL2, BCLXL, BFL1 and MCL1) bind the BH3 domain of BAX and BAK, thereby preventing oligomerization and apoptosis. The BH3-only proteins can bind to anti-apoptotic proteins, in turn releasing BAX and BAK and tipping the cells towards apoptosis [3]. These BH3-only proteins serve as sensors of cellular integrity and can be activated by a variety of stresses such as DNA damage (NOXA and PUMA), microtubule disruption (BIM), and nutrient deprivation (BAD) [4]. The upregulation of anti-apoptotic BCL2 proteins gives cancer cells a survival advantage, and is a frequent event in leukemias such as chronic lymphocytic leukemia (CLL).

A class of compounds termed BH3 mimetics have been developed to inhibit anti-apoptotic proteins by occupying the BH3 binding pocket, with the goal of selectively killing cancer cells. The BH3 mimetic, ABT-737, is a potent inhibitor of BCL2 and BCLXL, but not of other anti-apoptotic BCL2 family members. A related, orally bioavailable compound, navitoclax (ABT-263), has completed phase I clinical trials against chronic lymphocytic leukemia (CLL) [5] and small cell lung cancer [6]. Although this compound has demonstrated efficacy, resistance can occur when cancer cells rely on alternative BCL2 family members, such as MCL1 and BFL1 [7]. Therefore, additional compounds are needed which inhibit MCL1 and BFL1.

Many putative BH3 mimetics have been characterized based on experiments in cell free systems. We previously investigated 7 of these compounds for their ability to act as BH3 mimetics in intact cells [8]. These mimetics included ABT-737, gossypol, apogossypol (a chemical derivative of gossypol), HA14-1, 2-methoxy-antimycin A₃, obatoclax (GX15-070) and S1. We found that ABT-737 was the only compound that functioned as a true BH3-mimetic, in agreement with prior findings [9]. However, it was noted that all the other compounds increased the expression of the BH3-only protein NOXA at both the transcript and protein level [8]. We also demonstrated that these compounds could enhance apoptosis induced by ABT-737, presumably because NOXA is a potent inhibitor of MCL1 and BFL1. S1 was selected for further characterization, due to its robust NOXA induction, minimal toxicity as a single agent, and unique signaling properties (as will be described here). This compound was originally described as an inhibitor of BCL2 and MCL1 [10], which induces apoptosis in multiple cancer cell lines and a mouse xenograft model [11]. However, we report here that it fails to induce apoptosis in CLL cells, which are well known to rely on BCL2 for survival. Instead, we observed that S1 is a potent inducer of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and NOXA, and can enhance the efficacy of ABT-737 in leukemia cell lines and isolated patient samples.

Materials and Methods

Cell Culture and Reagents

NB4 cells were used as previously described [12]. Blood from patients with CLL, or healthy individuals was obtained from consenting donors at the Norris Cotton Cancer Center (Lebanon, NH). Lymphocytes were purified using Ficoll-Paque PLUS as previously described [13] and immediately incubated with the experimental compounds. NB4 and CLL cells were cultured in RPMI 1640 media supplemented with 10% (v/v) inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. For CLL and stroma co-culture experiments, CLL cells were plated on a confluent monolayer of CD154⁺ stroma cells [14] for 24 h, and then treated with compounds for 6 h. ABT-737 was obtained from Abbott Laboratories (Abbott Park, IL), S1 was synthesized according to a previously

published method [10], N-acetylcysteine (NAC), Trolox, and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO). Additional putative BH3 mimetics were obtained as previously described [8]. All compounds were dissolved in dimethyl sulfoxide (DMSO) except NAC and sodium azide, which were dissolved in water.

Immunoblotting

For protein analysis, 1×10^6 cells were pelleted by centrifugation, washed once with phosphate buffered saline (PBS), lysed with 100 μ L of urea lysis buffer, and boiled for 5 min. Protein expression was analyzed by standard SDS-PAGE and western blotting as described previously [12]. Antibodies were obtained from the following sources: rabbit anti-PARP (46D11), rabbit anti-phospho-eIF2 α (D9G8), and rabbit anti-PERK (D11A8) (Cell Signaling Technology, Danvers, MA); rabbit anti-ATF4 (H-290), rabbit anti-ATF-3 (C-19), rabbit anti-eIF2 α (FL-315) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-NOXA (OP180) (Calbiochem, Billerica, MA); actin-HRP conjugated antibody (AC-15) (Sigma Aldrich). Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were obtained from BioRad (Hercules, CA).

Cell transfection and RNA-knockdown

The Amaxa Cell Line Nucleofector Kit V (Lonza) was used according to the manufacturer's protocol. Briefly, 2×10^6 NB4 cells were transfected with 3 μ g of siRNA (in 100 μ L volume) using program X-001 and then incubated in RPMI 1640 (1.6 mL final volume) for 48 h prior to drug treatment. The siRNA were obtained from Ambion: ATF4 [s1702], (GCCUAGGUCUCUAGAUGAtt); ATF3 [s1699], (GCAAAGUGCCGAAACAAGAtt); NOXA (PMAIP1) [s10709] (AGUCGAGUGUGCUACUCAAtt).

Detection of reactive oxygen species

Reactive oxygen species (ROS) were detected using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Cells were incubated with 10 μ g/mL H₂DCFDA for 1 h and then washed once with PBS prior to drug treatment. Changes in ROS were assessed by monitoring FL-1 intensity using a FACScan flow cytometer (Becton Dickinson).

Chromatin condensation

One of the hallmarks of apoptosis is condensation of nuclear chromatin, and this is particularly evident in leukemia cells. To quantify apoptosis, cells were incubated with 2 μ g/mL Hoechst 33342 for 10 min at 37° C. Cells were analyzed by microscopy, and apoptosis was calculated as the percentage of cells with condensed chromatin. Error bars represent one standard error of the mean (SEM).

XBP1 analysis

mRNA was collected using RNeasy Plus mini kit (Qiagen), cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) and PCR was performed using DNA Taq polymerase (Invitrogen) according to the manufacturer's protocols. The following primers were obtained from Integrated DNA Technologies (Coralville, Iowa): XBP1 forward primer (5'- GTT GAG AAC CAG GAG TTA AGA CAG- 3'), XBP1 reverse primer (5'- CAG AGG GTA TCT CAA GAC TAG G- 3'). The PCR products were resolved using agarose electrophoresis and detected with ethidium bromide under UV. The larger PCR fragment represents the unprocessed form of XBP1, and the smaller fragment is processed XBP1 indicative of ER stress.

Results

S1 sensitization to ABT-737 is dependent on induced NOXA

We previously reported that S1 induces NOXA and sensitizes the acute promyelocytic leukemia line NB4 to ABT-737 at concentrations that induced NOXA [8]; those experiments were extended here (Fig. 1a, b). A 6-h incubation of NB4 cells with 20 μ M S1 induced NOXA but failed to induce apoptosis as assessed by caspase-mediated cleavage of poly (ADP-ribose) polymerase (PARP). ABT-737 alone also failed to induce apoptosis. However, 20 μ M S1 significantly increased apoptosis when combined with ABT-737. The dramatic increase in PARP cleavage observed between 3-10 nM ABT-737 (when combined with 20 μ M S1) likely represents a critical threshold for inhibiting anti-apoptotic proteins. As sensitization to ABT-737 correlated strongly with NOXA induction, we assessed the NOXA dependence using siRNA. Indeed, preventing NOXA induction with siRNA completely protected from apoptosis induced by this combination as assessed by PARP cleavage and chromatin condensation (Fig. 1c, d). Compared to Fig. 1b, there is a slight increase in sensitivity to ABT-737 and S1 as single agents, but this is likely due to the transfection process. The slight decrease in NOXA protein observed when NB4 cells are undergoing apoptosis is prevented by the caspase inhibitor (Fig. 1b). Treatment with ABT-737 increased MCL1 protein (Fig. 1b), which is likely due to the BIM displaced from BCL2 now binding to MCL1, as BIM is reported to stabilize MCL1 protein [15]. In addition, we previously observed that S1 increases MCL1 protein, but not mRNA [8], and we hypothesized that the increased MCL1 protein was due to stabilization of the MCL1:NOXA complex. In support of this, siRNA against NOXA completely prevented the induction of MCL1 protein by S1. Finally, the loss of MCL1 in cells undergoing apoptosis when treated with S1 and ABT-737 is prevented by pre-treatment with a caspase inhibitor (Fig. 1b). Given the potent effects of S1 and the potential impact of this novel NOXA induction, we set out to characterize the signaling events linking S1 to NOXA.

NOXA induction by S1 is dependent on ER stress signaling involving ATF4 and ATF3 transcriptional activity

We previously reported that six putative BH3 mimetics induce the integrated stress pathway, including phosphorylation of eIF2 α , ATF4 protein induction, ATF3 mRNA and protein induction in sequence [8]. As the integrated stress response can be induced by ER stress, we probed for an additional marker of ER stress, IRE1 activity. IRE1 is an endonuclease which, when activated, splices XBP1 mRNA from the inactive (long) to the active isoform (short) [16]. Using a PCR assay, only S1 was found to induce XBP1 splicing (Fig. 2a), suggesting that S1, but none of the other putative BH3 mimetics, induces ER stress. PERK is one of four eIF2 α kinases (the others being PKR, GCN2, and HRI), although PERK is the only one exclusively linked to ER stress. Therefore, we determined whether PERK was activated following incubation with S1. Phosphorylation of PERK occurred rapidly following S1 treatment in NB4 cells as indicated by a shift in the PERK band analyzed via SDS-PAGE (Fig. 2b). This phosphorylation of PERK is a recognized autophosphorylation event, coincides with eIF2 α phosphorylation, and precedes ATF4, ATF3 and NOXA induction following S1 treatment. The transcription factors ATF4 and ATF3 have been shown to induce NOXA transcription by acting at the CRE site in the NOXA promoter and both ATF4 and ATF3 need to be silenced to completely prevent the induction of NOXA [17]. Therefore, we co-transfected NB4 cells with siATF4 and siATF3 to test if NOXA induction by S1 is dependent on ATF4/ATF3. Bortezomib was used as a positive control, as this drug induces ATF4/ATF3-dependent NOXA induction [17]. This combination of siRNA completely prevented the induction of ATF4, ATF3, and NOXA by both S1 and bortezomib (Fig. 2c) strongly supporting the hypothesis that NOXA induction by S1 is mediated by these transcription factors. In addition, the siATF4 and siATF3 co-transfection protected

from apoptosis induced by the combination of S1 and ABT-737 (Fig. 2d). Taken together, these data show that S1 activates PERK, which in turn increases eIF2 α phosphorylation, ATF4, ATF3, and NOXA in sequence.

S1 rapidly increases reactive oxygen species that are required for NOXA induction and sensitization to ABT-737

We previously analyzed the spectrum of genes induced by S1 [8]. On reanalysis, we noted that some of these genes, such as OSGIN and GCLM, are involved in cellular response to ROS. Previous studies have also shown that increases in ROS can induce ER stress [18]. Therefore, we investigated whether S1 induces ROS, which in turn leads to ER stress and NOXA induction. Using H₂DCFDA (an intracellular ROS probe), we found S1 increased ROS in NB4 cells as early as 15 min (Fig. 3a), and the increase was approximately 5-fold greater than the H₂O₂ positive control. In addition, none of the other putative BH3 mimetics induced ROS at concentrations which induce NOXA, further showing that S1 is unique among these compounds (Fig. 3b). Although it appeared that GX15-070 slightly increased ROS, this is entirely due to auto-fluorescence of GX15-070 at the wavelengths used in the ROS detection assay (Suppl. Fig. 1C). Taken together, ROS levels are increased before the induction of the ER stress signaling pathway, supporting the hypothesis that ROS generated by S1 activates this pathway. To further implicate ROS as important in NOXA induction by S1, we used N-acetylcysteine (NAC) an anti-oxidant which can decrease ROS directly through its thiol moiety, or indirectly by increasing glutathione levels [19]. In NB4 cells treated for 1 h with 0.1 - 10 mM NAC, there was a concentration-dependent decrease in ROS levels after a 1 h S1 treatment (Fig. 3c). Importantly, this coincided with a decrease in PERK phosphorylation, p-eIF2 α , ATF4, ATF3, and NOXA after a 6 h treatment with S1 (Fig. 3d). Lastly, we found that pre-treatment of NB4 cells with NAC decreased apoptosis induced by the S1 and ABT-737 combination (Fig. 3e). In addition, the singlet-oxygen scavenger, sodium azide, significantly attenuated ROS formation and NOXA induction following S1 treatment (Suppl. Fig. 1A, B). Interestingly, Trolox (a peroxyl radical scavenger) failed to suppress ROS or NOXA induction following S1 treatment. Many additional anti-oxidants, such as ascorbic acid and Tiron, were tested in conjunction with S1 treatment, but only NAC and sodium azide prevented the induction of ROS and NOXA (data not shown). Taken together, these data support the hypothesis that S1 rapidly increases ROS, of which singlet-oxygen is likely the dominant species, which in turn leads to ER stress, NOXA induction and sensitization to ABT-737.

S1 enhances apoptosis induced by ABT-737 and overcomes stroma-mediated resistance in CLL cells *ex vivo*

While we have shown S1 can sensitize NB4 cells to ABT-737 (Fig. 1), we were interested in translating our findings to a more clinically-relevant model. Lymphocytes were obtained from patients with CLL, and incubated with S1 and ABT-737 alone or in combination *ex vivo*. Using chromatin condensation and PARP cleavage as markers of apoptosis, we found that CLL cells were very sensitive to ABT-737 as a single agent (Fig. 4a, b), consistent with previous reports [20]. In contrast, S1 as a single agent failed to induce apoptosis in CLL cells, which directly contradicts the proposed function of S1 as a BH3 mimetic that targets BCL2. However, co-treatment of S1 with ABT-737 further enhanced the apoptosis observed in CLL cells *ex vivo*. Interaction between CLL cells and surrounding stromal cells increases the expression of additional BCL2 family members (such as MCL1 and BFL1) and promotes resistance to BCL2 inhibitors [21]. This resistance can be modeled *ex vivo* by co-culturing CLL cells on a stromal monolayer. Using a CD154-expressing stromal cell line, we observed about 100-fold resistance to ABT-737 (Fig. 4c). Importantly, we found that the addition of S1 partially resensitized CLL cells to ABT-737 in this co-culture model. Lastly, we tested if this combination was toxic to normal lymphocytes. Importantly, we found that

normal lymphocytes were significantly resistant to the combination of S1 and ABT-737 (Fig. 4d). These data suggest that the combination of ABT-737 with S1 would preferentially kill CLL cells compared to normal lymphocytes.

Discussion

ABT-737 is a potent inhibitor of BCL2 and BCLXL, has demonstrated efficacy in a variety of cancer models, and the related compound navitoclax has yielded promising results in clinical trials. However, these drugs fail to inhibit additional antiapoptotic proteins, such as MCL1 and BFL1, making reliance on these proteins a common mechanism of resistance. Therefore, finding means to inhibit MCL1 and BFL1, either directly or indirectly, is an unmet clinical need. Many of the compounds reported to inhibit MCL1 directly (e.g. gossypol, obatoclax) have been shown to kill cells independent of BAX and BAK [22]. Other promising leads for MCL1 inhibitors are a “stapled-peptide” based on the BH3 domain of MCL1,[23] or the recently identified maritoclax [24].

Although MCL1 is a recognized resistance factor for ABT-737 and navitoclax [25], it is becoming more appreciated that BFL1 can also protect from the BCL2 inhibitors [7]. Keeping this in mind, it is likely that, even if a specific inhibitor of MCL1 is discovered, resistance will still occur due to protection by other anti-apoptotic proteins. Several strategies could be employed to prevent this. First, compounds could be synthesized which inhibit multiple antiapoptotic proteins (just as ABT-737 inhibits BCL2 and BCLXL). For instance, an inhibitor of both MCL1 and BFL1 would be more likely to overcome resistance and kill cancer cells. However, inhibition of multiple anti-apoptotic proteins would be more likely to increase toxicity. A second approach could utilize individual inhibitors of MCL1 or BFL1 which could be added in combination with ABT-737/navitoclax as needed. Another approach for overcoming resistance would be to target MCL1 and BFL1 indirectly, by either decreasing their expression [13] or upregulating a BH3-only protein, such as NOXA, which inhibits both [26]. Given the robust NOXA induction seen upon S1 treatment, and its low toxicity as a single agent, we were very interested to see if this compound would sensitize to ABT-737. Indeed, we found S1 lowered the ABT-737 concentration required to induce apoptosis, both in a leukemia cell line (NB4) and CLL cells *ex vivo*. We anticipate that S1 would similarly lower the concentration of navitoclax required to kill circulating CLL cells in patients. Lowering the effective concentration of navitoclax is of clinical significance because it may help prevent or reduce the severity of the dose-limiting thrombocytopenia observed in patients (due the dependence of platelets on BCLXL) [5]. Importantly, S1 resensitizes CLL cells to ABT-737 following co-culture on CD154⁺ stroma cells, which suggests that this combination may also kill the CLL cells residing in lymph nodes or other protective niches. If this is true, this combination may provide more effective therapy for CLL patients.

Another benefit of targeting MCL1 and BFL1 indirectly through NOXA induction is the potential for selectivity if the pathway(s) leading to NOXA are upregulated in cancer. In the case of S1, this is particularly relevant because it has been shown that cancer cells exhibit higher basal ROS compared to normal cells [27], and are more sensitive to increased ROS [28]. Indeed, a variety of compounds which directly increase ROS or inhibit ROS-scavenging machinery are in clinical trials [29]. It is therefore possible that many of these agents also upregulate NOXA, and sensitize to ABT-737 similar to S1. Taken together, we have identified a mechanism that may apply to other ROS-generating agents. While it has been reported that ABT-737 can increase ROS in resistant cell lines, this occurs at concentrations greater than required to inhibit BCL2 (1 μ M and above) and after a 24 hour incubation [30]. In addition, obatoclax was reported to increase ROS after a 24 hour

incubation [31], but we question these results due to the fact that obatoclax autofluoresces at the wavelength used in the ROS assay.

To the best of our knowledge, only one other group is studying S1 as an anti-cancer agent [10, 11, 32]. S1 was originally identified in a cytotoxicity screen, and was found to induce caspase-dependent apoptosis, in BCL2-overexpressing cells. Based on follow-up experiments in cell-free systems, it was concluded that S1 functions as a pan-BCL2 inhibitor. However, treatment with S1 does not induce apoptosis in CLL cells (Fig. 4) and does not disrupt the binding of BIM or BAD to BCL2, or NOXA to MCL1 [8], which suggests that S1 does not function as a pan-BCL2 inhibitor in cells. In addition, we found S1 does not induce apoptosis in freshly-isolated platelets (data not shown), which suggests that it does not inhibit BCLXL, as platelets require BCLXL for survival. Instead in cells, S1 functions mainly by inducing NOXA, which in turn inhibits MCL1 and sensitizes cells to apoptosis. Additionally, it was reported that S1 can induce autophagy [33] after a 12 h incubation, but this may also be a consequence of the rapid increase in ROS and induction of ER stress demonstrated here. Based on our result in cell-based systems, S1 should not be considered a BCL2 inhibitor, but instead re-purposed as a NOXA-inducing agent. If S1 were to enter clinical trial, these observations should be given serious consideration when selecting pharmacodynamics biomarkers.

In summary, S1 is a potent inducer of ROS which activates ER stress signaling, induces NOXA, and enhances apoptosis when combined with ABT-737. Although we have found that high concentrations of S1 can induce BAX/BAK-independent apoptosis at later time points, the concentrations and incubations used here are BAX/BAK dependent [8]. Taken together, the rapid increase in ROS and ER stress observed following S1 treatment call into question prior claims that S1 acts as a true BH3 mimetic. However, experiments with S1 in mice found it to be well tolerated and demonstrated anti-tumor activity at 0.3 mg/kg every other day [11]. This data, along with our results showing no apoptosis in normal lymphocytes, suggests that S1 may be well tolerated *in vivo*. Additional toxicity data would be required before S1 could enter clinical trials, but these results suggest that it might be particularly efficacious against CLL when combined with navitoclax. Based on this data, we hope to translate these findings with S1 or other ROS-inducing compounds to a clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by a Translational Research Grant from the Leukemia and Lymphoma Society and a Cancer Center Support Grant to the Norris Cotton Cancer Center (NIH CA23108). Support to A.V.D. was provided by a National Cancer Institute new faculty award (NIH CA023108-31S4) to the Norris Cotton Cancer Center.

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Figure 1.

S1 induces NOXA and sensitizes NB4 cells to ABT-737. **a** NB4 cells were incubated with 0–40 μ M S1 for 6 h; NOXA expression was analyzed by western blotting. **b** NB4 cells were incubated with 0–100 nM ABT-737 with or without S1 for 6 h. PARP cleavage was used as a marker of apoptosis. **c** NB4 cells were transfected with 3 μ g siRNA (control or NOXA) incubated for 48 h, and then incubated with S1, ABT-737 or both in combination for 6 h. PARP cleavage and NOXA were assessed by western blotting. **d** Cells from “c” were incubated as indicated for 6 h and scored for apoptosis. Survival is expressed as the percentage of cells which did not exhibit condensed chromatin.

Figure 2.

S1 induces NOXA through ER stress signaling. **a** NB4 cells were incubated as indicated for 6 h. RNA was purified and subjected to PCR to assess XBP1 processing. **b** NB4 cells were incubated with S1 for 0-6 h. Protein levels were analyzed by western blotting. The mobility shift in PERK is indicative of phosphorylation and activation. **c** NB4 cells were transfected with control siRNA or siATF4 plus siATF3 and incubated for 48 h. Transfected cells were then incubated with S1 or bortezomib for 6 h and probed for the indicated proteins. **d** NB4 cells transfected as in “C” were incubated with ABT-737, S1, or both for 6 h and scored for apoptosis.

Figure 3.

S1 increases reactive oxygen species, which is required for ER stress, NOXA induction, and sensitization to ABT-737. **a** NB4 cells were loaded with H_2DCFDA for 1 h, then incubated for 0-1 h with 0-20 μ M S1 and analyzed by flow cytometry. Increases in mean FL-1 signal reflect increased ROS. Values represent mean and 1 SEM ($n=3$). **b** NB4 cells were incubated with H_2DCFDA , treated as indicated for 1 h, and scored for increases in ROS. A 2-h incubation with hydrogen peroxide (H_2O_2) was used as a positive control. The increased signal seen with GX15-070 is due to autofluorescence (see supplemental Fig S1). **c** NB4 cells were incubated with H_2DCFDA plus 0-10 mM NAC for 1 h. Cells were then incubated with S1 for 1 h and analyzed for changes in ROS. Error bars represent 1 SEM ($n=3$). **d** NB4 cells were incubated with 0-10 mM NAC for 1 h, and then incubated with S1 for 6 h. Proteins were probed as indicated. **e** NB4 cells were incubated for 1 h with 0-10 mM NAC

and then incubated with ABT-737, S1, or both for 6 h. PARP cleavage was used as a marker for apoptosis.

Figure 4.

S1 sensitizes CLL cells, but not normal lymphocytes, to ABT-737. **a** CLL cells were incubated with S1 and ABT-737 as indicated. Cells were then incubated with Hoechst 33342 and scored for condensed chromatin. Survival is calculated as the percentage of cells which did not exhibit condensed chromatin. Values represent the mean and 1 SEM (n=3). **b** Cells treated as in “a” were assessed for PARP cleavage. **c** CLL cells were isolated and co-cultured for 24 h on CD154⁺ stroma cells, and then treated as indicated and scored for apoptosis. Values represent the mean and 1 SEM (n=3). **d** Lymphocytes were isolated from healthy donors, treated as indicated, and scored for apoptosis. Values represent the mean and 1 SEM (n=2).