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Combination of Pim kinase inhibitors and Bcl-2 antagonists in chronic lymphocytic leukemia cells

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Abstract

The Pim proteins are Ser/Thr kinases overexpressed in several hematological malignancies such as chronic lymphocytic leukemia (CLL) and some solid cancers like prostate cancer. Several small molecules have been developed to inhibit these kinases. In prostate cancer cell lines, the Pim kinase inhibitor SMI-4a and the Bcl-2 antagonist ABT-737 resulted in synergistic cytotoxicity. Akin to prostate cancer cells, CLL lymphocytes overexpress Pim and Bcl-2 proteins, we hypothesized that similar cytotoxic interaction should be observed in CLL. We evaluated the *in vitro* cytotoxic effect of 3 Pim kinase inhibitors (AZD1208, SGI-1776, and SMI-4a) combined with Bcl-2 antagonist (ABT-737 or ABT-199) in malignant CLL lymphocyte. Data indicated Pim kinase inhibitors in combination with ABT-737 or ABT-199 resulted mostly in additive cytotoxicity with a few synergistic responses; however, the extent of synergism was less robust than that observed previously in prostate cancer cell lines treated with SMI-4a and ABT-737.

Keywords

Pim kinase; CLL; ABT-199; SMI-4a; AZD1208; SGI-1776

INTRODUCTION

Pim (provirus integration site for Moloney murine leukemia virus) family proteins are serine/threonine kinases that have been shown to have oncogenic potential. These proteins coexist and cooperate with Myc. Three Pim kinases have been identified at the transcript level: Pim-1, Pim-2, and Pim-3. However, due to separate initiation sites for protein

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DECLARATION OF INTERESTS

All authors declare that they have no conflicts of interest

AUTHORS' CONTRIBUTIONS

V.G. designed the research. F.C.G and B.L. performed the experiments. M.J.K. and W.G.W. provided fresh CLL primary cells. V.G. and F.C.G. prepared the manuscript. All authors reviewed and agreed for the manuscript text.

translation, different size isoforms of Pim proteins have been identified. The Pim kinases have similar active sites and lack regulatory domains, and thus these kinases are constitutively active if expressed [1].

Increased expression of Pim mRNA transcripts has been detected in many hematological malignancies [2]. These include acute myelogenous leukemia [3], chronic lymphocytic leukemia (CLL) [4–6], lymphomas [7–9], and multiple myeloma [10, 11]. Additionally, the expression of Pim kinase proteins is much higher in CLL cells than in normal lymphocytes [6]. Similar observations have been made in mantle cell lymphoma, a B-cell malignancy [9].

Pim kinases have several substrates, which are involved in multiple signaling pathways that include the regulation of apoptosis through the phosphorylation of Bad [12–15], transcription control via the phosphorylation of Myc [16] and histone H3 at Ser10 [17], the regulation of protein translation through the phosphorylation of 4E-BP1 [18], and cell cycle regulation resulting from substrate activation [19–22]. These multifaceted actions of Pim kinases and their overexpression support the rationale for developing Pim kinase inhibitors as therapeutic agents for hematological malignancies [23].

CLL is also characterized by an overexpression of Bcl-2 family survival proteins. Among the 6 antiapoptotic proteins in this family, Bcl-2, Bcl-X_L, and Mcl-1 are abundant in circulating CLL lymphocytes [24, 25]. The inhibition of these proteins by genetic manipulation or by small-molecule inhibitors results in the induction of apoptosis through mitochondrial pathways [25].

Because of the prevalence and survival role of Pim kinase and Bcl-2 family proteins, several small-molecule inhibitors of these proteins have been developed. Pim kinase inhibitors include SGI-1776 [6, 26], AZD1208 [27, 28], LGH447 [10], and SMI-4a [29, 30]. Several of these agents are being tested in humans, and both AZD1208 (ClinicalTrials.gov identifiers: NCT01588548) and LGH447 (ClinicalTrials.gov identifier: NCT01456689) are currently in clinical trials for hematological malignancies. Among Bcl-2 family antagonists, pan-Bcl-2 inhibitors AT-101, obatoclax, and sabutoclax have been tested preclinically [31–33] and clinically (ClinicalTrials.gov identifiers: NCT00286780 [AT-101] and NCT00438178 [obatoclax]) [34]. Furthermore, compounds that target only Bcl-2 (ABT-199) [35] or both Bcl-2 and Bcl-X_L (ABT-737) [36] are being tested clinically [37].

Similar to CLL studies, investigations in prostate cancer cell lines have established the overexpression of Pim kinases [38, 39] as well as Bcl-2 antiapoptotic family proteins [40, 41]. Furthermore, using prostate cancer cell lines and animal models, Song and Kraft [30] demonstrated that the combination of Pim kinase inhibitor SMI-4a and Bcl-2 antagonist ABT-737 resulted in synergistic cytotoxicity. Mechanistic investigations established that the synergy was due to a reduction in the level of Mcl-1 protein, which resulted from the inhibition of protein translation by SMI-4a. Additionally, there was a transcriptional increase in the proapoptotic protein Noxa.

Because Pim kinase upregulation and Bcl-2 family antiapoptotic protein overexpression are seen in both prostate cancer and CLL, we hypothesized that—as in the prostate cancer study—Pim kinase inhibitors would be synergistically cytotoxic with ABT-737 (a Bcl-2 and Bcl-

X_L antagonist) or ABT-199 (a Bcl-2 only inhibitor) in CLL lymphocytes. To test this hypothesis, we used 3 Pim kinase inhibitors (AZD1208, SGI-1776, or SMI-4a) in combination with ABT-737 or ABT-199 in primary malignant lymphocytes obtained from patients with CLL. Our studies demonstrated that these drug combinations resulted in additive or synergistic cytotoxicity in CLL; however, the extent of synergism was less robust than that previously reported in prostate cancer cell lines.

MATERIAL AND METHODS

Drugs and chemicals

SGI-1776 was obtained from SuperGen (Dublin, CA), AZD1208 was obtained from AstraZeneca Pharmaceuticals (Wilmington, DE), ABT-199 was purchased from XcessBio (San Diego, CA), and SMI-4a as well as ABT-737 (known in the clinic as its clinical derivative ABT-263) were gifts from Dr. Andrew Kraft (Medical University of South Carolina, Charleston, SC). All these inhibitors were dissolved in DMSO and stored at -20°C. All experiments, including a vehicle control, were conducted using 0.1% DMSO.

Due to the translational nature of our study, achievable clinical drug plasma levels derived from *in vitro* drug concentration evaluations are necessary. In the present study, drug concentrations were selected based on Phase I studies when available or from previously published studies. The concentration for AZD1208 was based on a personal communication made by the proprietary company (AstraZeneca) after conducting a human study analyzing for the pharmacodynamics and pharmacokinetics of the drug. SGI-1776 concentration was selected based on the work by Chen et al. of this drug in chronic lymphocytic leukemia [6]. Similarly SMI-4a concentration was based on the published work of Song et al. evaluating this drug in a prostate cancer mouse model [30]. ABT-737 (clinically used as ABT-263) concentration was based on the 1–4 µM maximal plasma concentrations of ABT-263 (navitoclax) achieved in clinical trials with a 110–250 mg daily dosing schedule [37] and also on its well published nanomolar *in vitro* IC₅₀ [42]. The same concentration selected for ABT-737 was used for ABT-199 (venetoclax) due to their structural similarity, mechanism of action, and IC₅₀ for CLL cells [42].

Patient samples

All investigations were carried out in freshly isolated primary CLL lymphocytes obtained from patients with CLL (n = 22). All patients gave written informed consent to participate in this laboratory protocol, which was approved by the institutional review board of The University of Texas MD Anderson Cancer Center.

Isolation of lymphocytes

Whole blood was collected in heparinized tubes, diluted with PBS, and layered onto Ficoll-Hypaque (specific gravity, 1.086; Life Technologies, Grand Island, NY) for cell separation as previously described [6]. The isolated lymphocytes were resuspended in RPMI 1640 media supplemented with 10% human AB serum (Cambrex Biosciences, East Rutherford, NJ) in the presence of 5% CO₂ at 37°C. The cell number and mean cell volume were determined using a Coulter channelyzer (Coulter Electronics, Hialeah, FL). The

lymphocytes were suspended at a concentration of 1×10^7 cells/mL for all experiments and were used fresh.

Apoptosis assay

CLL lymphocyte cells were left untreated or treated with DMSO alone; 0.5 nM ABT-199 or 1 nM ABT-737; 3 or 10 μ M AZD1208, SMI-4a, or SGI-1776; or a combination of a Bcl-2 antagonist and a Pim kinase inhibitor at the indicated concentrations. Cells were washed, resuspended in 200 μ L of Annexin binding buffer (Roche, Indianapolis, IN), mixed with 5 μ L of Annexin V solution (BD Pharmingen, San Diego, CA) plus 5 μ L of propidium iodide (PI; Sigma-Aldrich, St. Louis, MO), and incubated for 15 min in the dark at room temperature. At least 1×10^4 cells were measured per sample using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA).

Immunoblot analysis

CLL cells were left untreated, treated with single agents, or treated with a combination of a Pim kinase inhibitor plus ABT-737 or ABT-199 as described above. The cell pellets were washed with ice-cold PBS and lysed at 4°C in radioimmunoprecipitation assay buffer supplemented with 1 mini Complete™ Protease Inhibitor (Roche) tablet per 10 mL of buffer. The lysate protein content was measured using a DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Aliquots (30–50 μ g) of total protein were loaded onto 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Osmonics Labstore, Minnetonka, MN) as previously described [11]. The membranes were blocked at room temperature for 1 h in Odyssey blocking buffer (LI-COR Inc., Lincoln, NE) and then incubated overnight at 4°C with the following primary antibodies: Bcl-2 (Dako, Carpinteria, CA), Mcl-1, Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-4E-BP1 (Thr 37/46), total 4E-BP1, phospho-p70S6K (Thr 389), or GAPDH (Cell Signaling Technology, Danvers, MA), and PARP (BD Pharmingen). After washing, the membranes were incubated with infrared-labeled secondary antibodies (LI-COR, Lincoln, NE) for 1 h and visualized using a LI-COR Odyssey Infrared Imager.

Fractional product of Webb to determine synergistic, additive, or antagonistic cytotoxicity

This method was used to determine whether the induced cell death by the combinations was additive, synergistic or antagonistic. This calculation is appropriate in our setting since our combination strategy involves nonexclusive drugs (agents with independent mechanisms of action). Total cell death was determined using the flow cytometer as described above. The endogenous cell death observed in controls was subtracted from all the conditions, and the expected percentage of cells surviving after treatment with the combination was calculated using fractional two-drug combinational analysis. To compare the expected and observed (Annexin V/PI staining) levels of cell death for the combination treatments, we first calculated the expected level of cell survival for each combination. This was done by multiplying the percentage of cells surviving Pim kinase inhibitor treatment (100% - X% Annexin V/PI staining) by the percentage of cells surviving ABT-737 treatment (100% - X% Annexin V/PI staining) divided by 100. The resulting number (survival in combination) was then subtracted from 100 (to obtain cell death). When the observed cell death was 20% lower than the expected cell death, the interaction was considered antagonistic; when the

observed cell death was 20% higher than the expected cell death, the interaction was considered synergistic. When the observed cell death was > 0% but < 20% higher than the expected cell death, the interaction was considered additive.

Statistical analysis

Graphs were done using GraphPad Prism (GraphPad Software version 5, San Diego, CA). This same software was used to calculate statistical significance by t test analysis.

RESULTS

Simultaneous and sequence-dependent combination of AZD1208 with ABT-737

Five patient samples were treated with 1 nM ABT-737 alone, 3 or 10 μ M AZD1208 alone, or 1 nM ABT-737 with either dose of AZD1208 in simultaneous combination (Figure A). As mentioned in materials and methods, these concentrations were selected based on plasma levels of these drugs achieved during clinical trials or their use in preclinical studies [37]. In parallel, cultures were treated with AZD1208 first followed by ABT-737 8 h later (Figure 1B) or with ABT-737 first followed by AZD1208 8 h later for a total incubation period of 24 h (Figure 1C). Simultaneous treatment with both drugs resulted in an antagonistic interaction in CLL patients 878, 967, 322, and 741; however, this same combination resulted in an additive or synergistic effect (20% increase from observed value) in CLL patient 247 (Figure 1A). AZD1208 treatment followed by ABT-737 resulted in antagonistic cytotoxicity in samples from all CLL patients (Figure 1B). Similarly, ABT-737 followed by AZD1208 did not result in additive or synergistic cell death in any of the 5 CLL patients (Figure 1C).

Effect of Pim kinase inhibitors and ABT-737 in simultaneous incubation

Because the highest additive or synergistic interactions was observed in 1 CLL sample (patient 247) when incubated with AZD1208 and ABT-737 simultaneously, we evaluated this combination in 17 additional samples obtained from patients with CLL (Table I). Treatment with AZD1208 alone resulted in a dose-dependent cell death: at 3 μ M, cell death was between 0% and 10%, and at 10 μ M, cell death was between 0% and 29%. Synergistic cytotoxicity was observed after simultaneous incubation with ABT-737 and 3 μ M AZD1208 in 4 CLL patient samples and 10 μ M AZD1208 in 5 samples.

To expand our observation and to replicate previous investigations of prostate cancer cell lines, we also evaluated simultaneous treatment of ABT-737 with the other two Pim kinase inhibitors, SGI-1776 and SMI-4a. Eleven samples were simultaneously treated with SGI-1776 and ABT-737 (Table II). Of these, 4 and 4 showed synergistic cytotoxicity at 3 and 10 μ M SGI-1776, respectively. As shown in Table III, 11 samples were simultaneously treated with ABT-737 and SMI-4a. Out of these samples, 3 demonstrated synergistic cytotoxicity at 3 μ M SMI-4a, and 3 demonstrated synergistic cytotoxicity at 10 μ M SMI-4a.

Effect of other Pim kinase inhibitors with ABT-737 or ABT-199

To further expand our observations, we did similar cell death assays with ABT-737 or ABT-199 in combination with each of the 3 Pim kinase inhibitors (AZD1208, SMI4a, and SGI-1776) (Figure 2). Furthermore, to compare data among the various treatment

combinations, we studied all these combinations in CLL cells obtained from the same patient. In total, samples from 5 patients with CLL disease were evaluated. Data presented in Figure 2 show that in general, treatment of CLL cells with each of the 3 Pim kinase inhibitors alone resulted in low (AZD1208, 0%–29%; SGI-1776 0%–46%; SMI-4a, 0%–28%) Annexin V/PI positivity. Among these 3 inhibitors, SGI-1776 resulted in the highest percentage of cell death. Among samples treated with Bcl-2 antagonists alone, 3 of 5 samples showed a higher percentage of cell death with ABT-199, 1 with ABT-737, and 1 showed similar percentages of cell death between the 2 drugs. Combination treatment with ABT-199 and Pim kinase inhibitors resulted in synergistic cytotoxicity in 2 patient samples. Similarly, treatment with ABT-737 and Pim kinase inhibitors had 2 samples with synergistic cytotoxicity.

Effects of Pim kinase inhibitors on Pim kinase targets of translation

To elucidate their mechanism of action, we evaluated the effect of all 3 Pim kinase inhibitors on proteins involved in translation. These proteins were selected because they were previously found to be affected by Pim kinase inhibitors in prostate cancer cell lines [30]. CLL cells were treated with vehicle DMSO alone or with 3 or 10 μ M AZD1208, SMI-4a, or SGI-1776 for 24 h, after which they were harvested and compared with untreated CLL cells (Figure 3). There was no change in phospho-p70S6K protein from treatment with SMI-4a or SGI-1776 (Figure 3). However, a decrease in phospho-p70S6K protein was observed at 10 μ M AZD1208 in one patient sample (CLL350) (Figure 3B). None of the Pim kinase inhibitors affected the levels of total and phosphorylated 4E-BP1 except AZD1208, which resulted in a decrease in phospho 4E-BP1 (Figure 3B).

Effects of Pim kinase inhibitors on survival proteins

We also evaluated the impact of Pim kinase inhibitors on the Bcl-2 family antiapoptotic proteins because Mcl-1 was down-regulated after treatment with Pim kinase inhibitors in prostate cancer cell lines [30]. Treatment with each of the 3 Pim kinase inhibitors elicited an increase in PARP cleavage (Figure 4). However, no significant change in phospho-Akt was observed. Similarly, the Bcl-2 family proteins Bcl-2, Bcl-X_L, and Mcl-1 remained unchanged after treatment with AZD1208, SMI-4a, or SGI-1776 (Figure 4).

DISCUSSION

Our results show that *in vitro* combined treatment with a Bcl-2 antagonist (ABT-737 or ABT-199) and a Pim kinase inhibitor (AZD1208, SGI-1776, or SMI-4a) can cause additive cytotoxicity in primary CLL lymphocytes with some synergistic responses; however, the extent of synergism was less than that previously observed in prostate cancer cell lines [30].

Initially, we focused our investigations on AZD1208 and ABT-737. There were several reasons to select AZD1208. First, this agent is a pan-Pim kinase inhibitor that impacts all 3 Pim kinases [27, 28]. In contrast, the other 2 inhibitors have a limited effect on Pim-2. Second, compared to SMI-4a and SGI-1776, AZD1208 is a potent inhibitor with IC₅₀ values in the low nanomolar range [28]. Third, neither SMI-4a nor SGI-1776 is a clinical candidate. Fourth, and in contrast to SMI-4a and SGI-1776, AZD1208 was in clinic for patients with

acute myelogenous leukemia and another clinical trial investigations (ClinicalTrials.gov Identifier: NCT01588548) for patients with lymphoma was just completed; hence, it would be feasible to also test this compound in other leukemias such as CLL.

Among the 3 Pim kinase inhibitors that were tested, SGI-1776 showed the highest overall level of cytotoxicity. This cannot be explained by the IC₅₀ values for these inhibitors on the 3 Pim kinases, as these values predict that, based on IC₅₀ values, the most potent inhibitor would be AZD1208 [28]. Furthermore, this drug inhibits all 3 Pim kinases which is in contrast to the other 2 inhibitors which have limited effect on Pim 2. However, it should be noted that the IC₅₀ studies were conducted separately and different conditions were used. In addition, studies conducted in cells such as CLL (in contrast to cell extracts), the binding of these drugs to serum may also affect their activity.

There are 2 fundamental similarities between CLL and prostate cancer, in which these drug combinations were previously studied. First, both these malignancies show a prevalence of increased Pim kinase expression, suggesting that Pim inhibitors may be a treatment modality for both these cancers. Furthermore, the inhibition of Pim kinases by a variety of inhibitors showed “*in vitro*” efficacy against both these neoplasms [6, 30]. Second, both CLL lymphocytes and prostate cancer cells have an overabundance of Bcl-2 family survival proteins (Bcl-2, Bcl-X_L, and Mcl-1). Interestingly, studies using transgenic mice established a cooperation between Bcl-2 and Pim-1 in lymphomagenesis [43]. These similarities directed us to obtain similar results when combining Pim kinase inhibitors and Bcl-2 antagonists ABT-737 and ABT-199. However, there are differences between these neoplasms. First, CLL cells are replicationally quiescent while prostate cancer cells are highly proliferative. The mature and differentiated nature of CLL cells will limit the effects of Pim kinase inhibitors on cell cycle–related proteins, as these inhibitors have several targets in the cell cycle process, cell cycle–related proteins include p27, p21, cdc251 and cdc25c [19–22]. Second, as observed in Figure 3, protein substrates of Pim kinases that were affected by SMI-4a in prostate cancer cell lines [30] were unaffected in CLL cells. With 16 h of treatment with SMI-4a, 4E-BP1 was dramatically decreased in the prostate cancer study. Third and consistent with 4E-BP1 data, Mcl-1 protein levels also declined with SMI-4a in prostate cancer cell lines. The decrease was apparent as early as 4 h into treatment. However, in our study using CLL cells, Mcl-1 protein levels were unchanged after treatment with AZD1208, SMI-4a, or SGI-1776. Collectively, these context- or cell type–specific differences resulted in data that were unlike those observed in prostate cancer cell lines.

Even though the dramatic synergistic interaction observed in prostate cancer cell lines treated with a Bcl-2 antagonist and a Pim kinase inhibitor were not reproduced in CLL lymphocytes, this strategy warrants further evaluation in an *in vitro* setting as well as animal and clinical studies. This assertion is based on our finding that among the 34 combination experiments CLL samples treated with the combination of 3 μ M or 10 μ M AZD1208 and 1 nM ABT-737, only 5 showed antagonistic interactions (Table I). Similarly, among the 22 samples treated with the combination of SGI-1776 and ABT-737, 3 showed antagonistic interactions (Table II). Furthermore, only 4 of 22 samples treated with 3 μ M or 10 μ M SMI-4a and ABT-737 displayed antagonistic cytotoxicity (Table III). Collectively, these data suggest that in most cases, Bcl-2 antagonist and Pim kinase inhibitors resulted in additive or

synergistic interactions. Because AZD1208 and ABT-737 as well as ABT-199, are being evaluated for patients with hematological malignancies, these drugs provide an opportunity to test a combination strategy using a Bcl-2 antagonist and a Pim kinase inhibitor for a clinical trial in CLL patients [37].

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Figure 1.

Cytotoxic effect of AZD1208 and ABT-737 alone and in combination on CLL lymphocytes. CLL cells were isolated from 5 patients (rows) and treated for 24 h with 3 or 10 µM AZD1208, 1 nM ABT-737, or the 2 drugs combined (Column A). Parallel cultures were incubated with AZD1208 for 8 h, and then ABT-737 was added; incubation and then continued for an additional 16 h (Column B). In a third group of cultures, cells were treated first with ABT-737 followed by AZD1208 (Column C). After 24 h of incubation, cells were harvested, stained with Annexin V/PI, and analyzed using the flow cytometer. For controls, untreated or DMSO cells were kept for 24 h and then stained with same procedure. Endogenous cell death in the control samples was subtracted from all treated conditions.

Figure 2.

Cytotoxic effect of Pim kinase inhibitors and ABT-199 or ABT-737 alone and in combination on CLL lymphocytes. CLL cells were isolated from 5 patients (rows) and were treated for 24 h with 3 or 10 μ M AZD1208, SMI-4a, or SGI-1776; 0.5 nM ABT-199 or 1 nM ABT-737; or a combination of a Bcl-2 antagonist with a Pim kinase inhibitor. After 24 h of incubation, cells were harvested, stained with Annexin V/PI, and analyzed using the flow cytometer. For controls, untreated or DMSO cells were kept for 24 h and then stained with same procedure. Cell death in the control samples was considered as endogenous apoptosis, which varied between 7% and 42%, and was subtracted from each sample's value.

Figure 3.

Immunoblot analysis of potential target proteins in CLL cells treated with Pim kinase inhibitors. (A) CLL cells (patient numbers indicated on top of each figure) were treated with 0.1% vehicle DMSO alone, 3 or 10 μ M SMI-4a for 24 h, after which the cells were harvested and lysed. The protein levels of phospho-p70S6K (Thr 389), phospho-4E-BP1 (Thr 37/46), total 4E-BP1, and GAPDH (used as a loading control) were visualized by immunoblot analysis. (B) Similar immunoblot analyses were performed for CLL cells treated with 0.1% vehicle DMSO alone, 3 or 10 μ M AZD1208 or 3 μ M SGI-1776. Total cell death was determined by flow cytometry for each treated sample and is displayed at the bottom of each figure.

Figure 4.

Immunoblot analysis of antiapoptotic and proapoptotic proteins and cell death in CLL cells treated with Pim kinase inhibitors. (A) CLL cells were treated with 0.1% vehicle DMSO alone or 3 or 10 μ M of SMI-4a for 24 h, after which the cells were harvested and lysed. The protein levels of PARP, phosphor-Akt (Ser 473), total Akt, Mcl-1, Bcl-X_L, Bcl-2, and GAPDH (used as a loading control) were immunoblotted from the indicated CLL cell lysates. (B) Similar immunoblot analyses were performed for CLL cells treated with 0.1% vehicle DMSO alone, 3 or 10 μ M AZD1208, or 3 μ M SGI-1776. Total cell death was determined by flow cytometry for each treated sample and is displayed at the bottom of each figure.

Table 1
CLL cell death after treatment with ABT-737, AZD1208, and their combination

CLL cells were obtained from several patients and were treated with DMSO (vehicle), 1 nM ABT-737, 3 or 10 μ M AZD1208, or the combination of ABT737 plus AZD1208 for 24 h. Total cell death was determined by flow cytometry, and endogenous cell death observed in DMSO control was subtracted from the drug-treated conditions. The expected percentage of cells surviving after treatment with the combination was calculated using the fractional product of Webb and then subtracted from 100 to obtain the expected cell death. *P* values compare $\hat{r}$ expected versus $\hat{r}$ observed cell death for the 3 and 10 μ M combinations.

CLL patient	ABT-737 1 nM	AZD1208 3 μ M	AZD1208 10 μ M	% Cell Death			
				Combination 3 μ M Expected	Combination 3 μ M Observed	Combination 10 μ M Expected	Combination 10 μ M Observed
736	13	7	14	19	16	25	23
284	23	10	17	30	26	36	30
462	23	10	24	30	30	42	40
833	17	3	0	θ_{19}	4	θ_{17}	2
081	6	-1	-1	5	5	$\hat{r}_5$	17
878	16	9	14	θ_{24}	16	28	25
350	4	7	16	11	10	$\hat{r}_{19}$	34
967	38	-2	2	36	32	θ_{39}	29
322	37	-9	9	31	35	43	40
247	26	3	17	$\hat{r}_{29}$	40	39	45
741	32	7	29	θ_{36}	7	52	53
106	12	1	11	13	14	22	21
966	22	-7	-6	16	9	18	15

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CLL patient	ABT-737 1 nM	AZD1208 3µM	10µM	% Cell Death			
				Expected	Observed	Expected	Observed
403	10	2	8	7 ₁₂	21	7 ₁₇	25
203	7	6	26	7 ₁₂	17	7 ₃₀	37
087	33	6	16	37	30	44	36
247	9	5	8	7 ₁₃	23	7 ₁₇	33
				<i>p value = 0.007</i>		<i>p value= 0.003</i>	

7₁₂ Indicates synergistic action

7₁₃ Indicates antagonistic action

Based on heterogeneity among patient samples, a 20% increase or decrease from the expected value to the observed value was considered synergistic or antagonistic, respectively.

Table II
CLL cell death after treatment with ABT-737, SGI-1776, and their combination

CLL cells were obtained from several patients and were treated with DMSO (vehicle), 1 nM ABT-737, 3 or 10 μ M AZD1208, or the combination of ABT737 plus AZD1208 for 24 h. Total cell death was determined by flow cytometry, and endogenous cell death observed in DMSO control was subtracted from the drug-treated conditions. The expected percentage of cells surviving after treatment with the combination was calculated using the fractional product of Webb and then subtracted from 100 to obtain the expected cell death. *P* values compare $\hat{r}$ expected versus $\hat{r}$ observed cell death for the 3 and 10 μ M combinations.

CLL patient	ABT-737 1 nM	SGI-1776 3 μ M	10 μ M	% Cell Death			
				Expected	Observed	Expected	Observed
462	18	6	1	23	18	18	20
967	38	-1	28	37	35	55	44
741	19	1	46	$\hat{r}_{20}$	42	56	48
527	6	4	41	$\hat{r}_9$	23	$\hat{r}_{45}$	55
214	12	11	22	21	24	31	24
106	13	-5	-4	8	7	9	7
966	22	-8	1	16	14	θ_{23}	12
403	10	3	37	$\hat{r}_{13}$	32	θ_{44}	32
203	7	13	25	19	24	$\hat{r}_{30}$	39
087	33	9	28	39	37	θ_{52}	36
247	9	-2	17	$\hat{r}_7$	14	24	25
				<i>p</i> value = 0.02		<i>p</i> value = 0.03	

$\hat{r}$ Indicates synergistic action

θ Indicates antagonistic action

Based on heterogeneity among patient samples, a 20% increase or decrease from the expected value to the observed value was considered synergistic or antagonistic, respectively.

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Table III
CLL cell death after treatment with ABT-737, SMI-4a, and their combination

CLL cells were obtained from several patients and were treated with DMSO (vehicle), 1 nM ABT-737, 3 or 10 μ M AZD1208, or the combination of ABT737 plus AZD1208 for 24 h. Total cell death was determined by flow cytometry, and endogenous cell death observed in DMSO control was subtracted from the drug-treated conditions. The expected percentage of cells surviving after treatment with the combination was calculated using the fractional product of Webb and then subtracted from 100 to obtain the expected cell death. *P* values compare $\hat{r}$ expected versus $\hat{r}$ observed cell death for the 3 and 10 μ M combinations.

CLL patient	ABT-737 1 nM	% Cell Death					
		SMI4a		Combination 3 μ M		Combination 10 μ M	
		3 μ M	10 μ M	Expected	Observed	Expected	Observed
89	0	0	1	-1	1	1	0
527	10	-1	2	θ_9	5	θ_{12}	5
637	1	0	0	1	1	1	2
778	14	2	4	$\hat{r}_{16}$	22	$\hat{r}_{17}$	22
214	8	-1	1	8	7	θ_9	3
106	14	-1	5	$\hat{r}_{13}$	17	18	20
966	22	2	6	24	26	27	29
403	10	-1	-2	9	8	$\hat{r}_9$	16
203	7	0	1	7	9	7	9
087	33	-6	28	29	25	θ_{52}	31
247	9	2	6	$\hat{r}_{11}$	23	$\hat{r}_{15}$	27
				<i>p</i> value = 0.09		<i>p</i> value = 0.06	

$\hat{r}$ Indicates synergistic action

θ Indicates antagonistic action

Based on heterogeneity among patient samples, a 20% increase or decrease from the expected value to the observed value was considered synergistic or antagonistic, respectively.

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