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HA14-1 potentiates apoptosis in B-cell cancer cells sensitive to a peptide disrupting IP_3 receptor / Bcl-2 complexes

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ABSTRACT Anti-apoptotic B-cell lymphoma 2 (Bcl-2) is commonly upregulated in hematological cancers, including B-cell chronic lymphocytic leukemia (B-CLL) and diffuse large B-cell lymphoma (DLBCL), thereby protecting neoplastic cells from oncogenic-stress-induced apoptosis. Bcl-2 executes its anti-apoptotic function at two different sites in the cell. At the mitochondria, Bcl-2 via its hydrophobic cleft interacts with pro-apoptotic Bcl-2 family members to inhibit apoptosis. At the endoplasmic reticulum (ER), Bcl-2 via its Bcl-2 homology (BH)4 domain, prevents excessive Ca^{2+} signals by interacting with the inositol 1,4,5-trisphosphate receptor (IP_3R), an intracellular Ca^{2+}-release channel. A peptide tool (BIRD-2) that targets the BH4 domain of Bcl-2 reverses Bcl-2's inhibitory action on IP_3Rs and can trigger pro-apoptotic Ca^{2+} signals in B-cell cancer cells. Here, we explored whether HA14-1, a Bcl-2 inhibitor that also inhibits sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA), could potentiate BIRD-2-induced cell death. We measured apoptosis in Annexin V/7-AAD stained cells using flow cytometry and intracellular Ca^{2+} signals in Fura2-AM-loaded cells using an automated fluorescent plate reader. HA14-1 potentiated BIRD-2-induced Ca^{2+} release from the ER and apoptosis in both BIRD-2-sensitive DLBCL cell lines (SU-DHL-4) and in primary B-CLL cells. BIRD-2-resistant DLBCL cells (OCI-LY-1) were already very sensitive to HA14-1. Yet, although BIRD-2 moderately increased Ca^{2+} levels in HA14-1-treated cells, apoptosis was not potentiated by BIRD-2 in these cells. These results further underpin the relevance of IP_3R-mediated Ca^{2+} signaling as a therapeutic target in the treatment of Bcl-2-dependent B-cell malignancies and the advantage of combination regimens with HA14-1 to enhance BIRD-2-induced cell death.

KEY WORDS: *cancer, apoptosis, Bcl-2, IP_3R, Ca^{2+}*

Introduction

Dysregulation of anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins and thus of the intrinsic apoptotic pathway is a common feature of several B-cell cancer types. Bcl-2 overexpression was observed in B-cell malignancies like the germinal center subtype of diffuse large B-cell lymphoma (DLBCL) and B-cell chronic lymphocytic leukemia (B-CLL). Bcl-2 up-regulation protects cancer cells against cell-death signaling that is induced due to the on-going oncogenic stress (Chipuk *et al.*, 2010, Letai, 2008). There is accumulating evidence that cancer cell survival depends on the presence of Bcl-2 and its action at the mitochondria and/or the endoplasmic

Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; B-CLL, B-cell chronic lymphocytic leukemia; Bcl-2, B-cell lymphoma 2; Bcl-Xl, B-cell lymphoma-extra large; BH, Bcl-2 homology; BIRD-2, Bcl-2-IP_3R disrupter-2; DLBCL, diffuse large B-cell lymphoma; ER, endoplasmic reticulum; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SPCA, secretory-pathway Ca^{2+}-ATPase.

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reticulum (ER), the main intracellular Ca^{2+} store (Akl *et al.*, 2014).

At the mitochondria, Bcl-2 neutralizes the executioner proteins Bax and Bak, the activator BH3-only proteins Bid and Bim, and sensitizer/de-repressor BH3-only proteins like Bad (Chipuk *et al.*, 2010; Letai, 2008). The point-of-no-return for apoptosis is Bax/Bak-mediated permeabilization of the mitochondrial outer membrane, which leads to cytochrome c release. Bak activation appears to be mediated by Bid, while Bax activation appears to be mediated by Bim (Sarosiek *et al.*, 2013). Through its hydrophobic cleft formed by the BH3, BH1 and BH2 domain, Bcl-2 binds to the BH3 domain of their pro-apoptotic counterparts, including BH3-only proteins and Bax/Bak. This effect of Bcl-2 prevents Bax/Bak activation and thus the subsequent mitochondrial outer membrane permeabilization (Chipuk and Green, 2008; Tait and Green, 2010). In many cancer cells, oncogenic stress results in the upregulation of BH3-only proteins like Bim thereby rendering these cells addicted to high Bcl-2 levels for neutralizing the pro-apoptotic proteins (Certo *et al.*, 2006; Del Gaizo Moore *et al.*; 2007, Letai, 2008). BH3-mimetic drugs targeting the hydrophobic cleft of Bcl-2 (like ABT-737 and ABT-199) cause Bim release and subsequent Bax/Bak activation (Del Gaizo Moore *et al.*, 2007; Del Gaizo Moore *et al.*, 2008; Deng *et al.*, 2007). Although therapeutic targeting of anti-apoptotic Bcl-2 proteins with BH3 mimetics is a promising new anticancer approach, BH3 mimetics like ABT-737 and other Bcl-2 antagonists like HA14-1, a small molecule that also bind the hydrophobic cleft of Bcl-2 (Wang *et al.*, 2000), drastically impair platelet survival and homeostasis (Akl *et al.*, 2013b, Kodama *et al.*, 2011, Vogler *et al.*, 2011). This can be explained by the fact that BH3 mimetics like ABT-737 target the hydrophobic cleft of both Bcl-2 and Bcl-Xl (Chonghaile and Letai, 2008), which is essential for the life span of platelets (Mason *et al.*, 2007). It has been proposed that the ABT-737-induced toxicity in platelets is due to the dysregulation of intracellular Ca^{2+} homeostasis (Vogler *et al.*, 2011), but this was contested by others (Harper and Poole, 2012; Schoenwaelder and Jackson, 2012). We have recently shown that Ca^{2+} dysregulation was not a primary cause for ABT-737-induced toxicity in platelets (Akl *et al.*, 2013b). In contrast, HA14-1-induced platelet toxicity was associated with cytosolic Ca^{2+} overload, due to its inhibitory effect on the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Akl *et al.*, 2013b).

At the ER, Bcl-2 acts on the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), thereby limiting Ca^{2+} flux through the channel (Chen *et al.*, 2004; Rong *et al.*, 2008). Via its BH4 domain, Bcl-2 proteins target a highly conserved region in the modulatory domain of the IP_3R (Distelhorst and Bootman, 2011; Monaco *et al.*, 2012; Rong *et al.*, 2009). The site is conserved in all three IP_3R isoforms (Monaco *et al.*, 2012) and also in the related family of intracellular Ca^{2+}-release channels, the ryanodine receptors (Vervliet *et al.*, 2014). Bcl-2 binding to IP_3Rs dampens the pro-apoptotic Ca^{2+} flux from the ER into the mitochondria (Rong *et al.*, 2009). A cell-permeable peptide, corresponding to the Bcl-2-binding site located in the modulatory domain of IP_3R1, was developed and able to disrupt IP_3R/Bcl-2 complexes in a variety of cell models, including B-cell cancer cells (Zhong *et al.,* 2011; Akl *et al.*, 2013a). The stabilized version of this peptide (with a mutated aspartate cleavage site) and previously referred to as TAT-$IDP^{DD/AA}$ (Zhong *et al.*, 2011) or TAT-IDP^{S} (Akl *et al.*, 2013a; Akl *et al.*, 2014), will for convenience be referred to as BIRD-2 (Bcl-2-IP_3R Disrupter-2) to distinguish it from unrelated IP_3R sequence-derived peptides now in use in our laboratories. BIRD-2 triggered IP_3R-mediated Ca^{2+} signaling and subsequent apoptotic cell death in primary CLL cells (Zhong *et al.*, 2011) and in DLBCL cell lines (Akl *et al.*, 2013a), while normal peripheral mononuclear blood cells were resistant. DLBCL cancer cells displayed a large heterogeneity in their apoptotic responses to BIRD-2 with the SU-DHL-4 cells being most sensitive and the OCI-LY-1 cells being most resistant, a behavior linked to differences

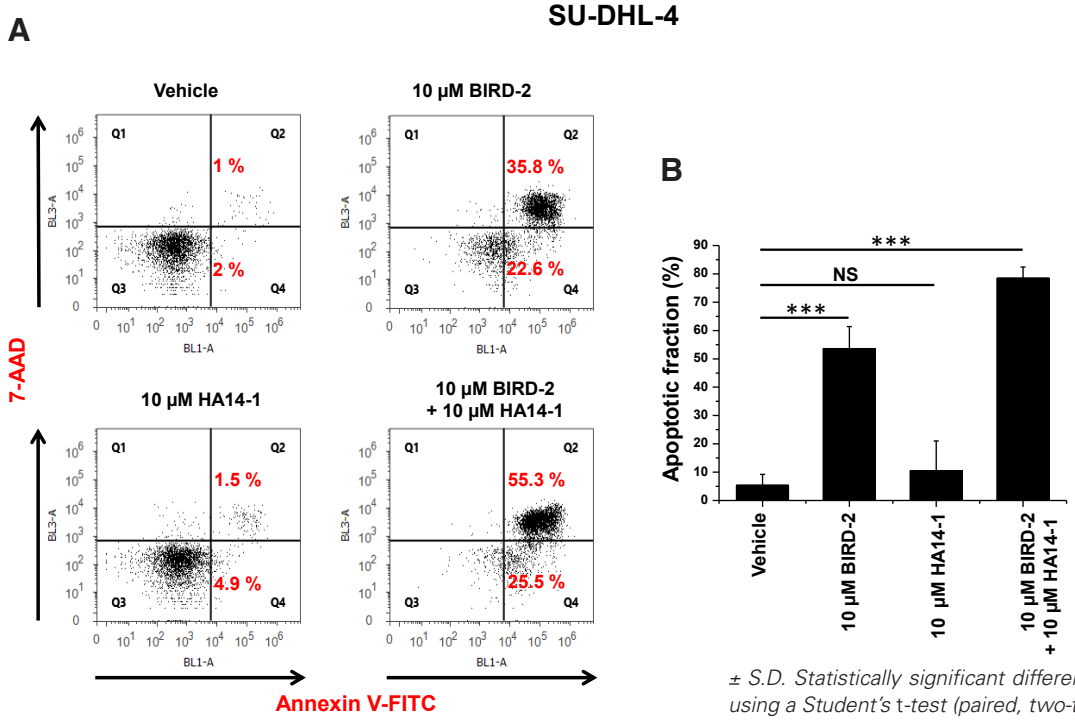

Fig. 1. BIRD-2 and HA14-1 have additive effects on apoptosis in SU-DHL-4 cells. (A) *Dot plots from a flow-cytometric analysis of apoptosis induced in SU-DHL-4 cells by a 24 hours treatment without or with 10 µM BIRD-2 and/or 10 µM HA14-1. The dot plots are representative for three independent experiments. The early apoptotic population is identified as the Annexin V-FITC-positive / 7AAD-negative fraction (Q4) and the secondary necrotic fraction (Q2) is identified as the AnnexinV-FITC-positive/7-AAD-positive population.* **(B)** *A quantitative analysis of the apoptotic fraction (Annexin V-FITC-positive fraction, i.e. Q2 + Q4 of each condition) for the three independent experiments is shown in the panel B. Data were calculated and shown as average ± S.D. Statistically significant differences are labeled with: *** p<0.001 using a Student's* t*-test (paired, two-tailed); NS, not significant.*

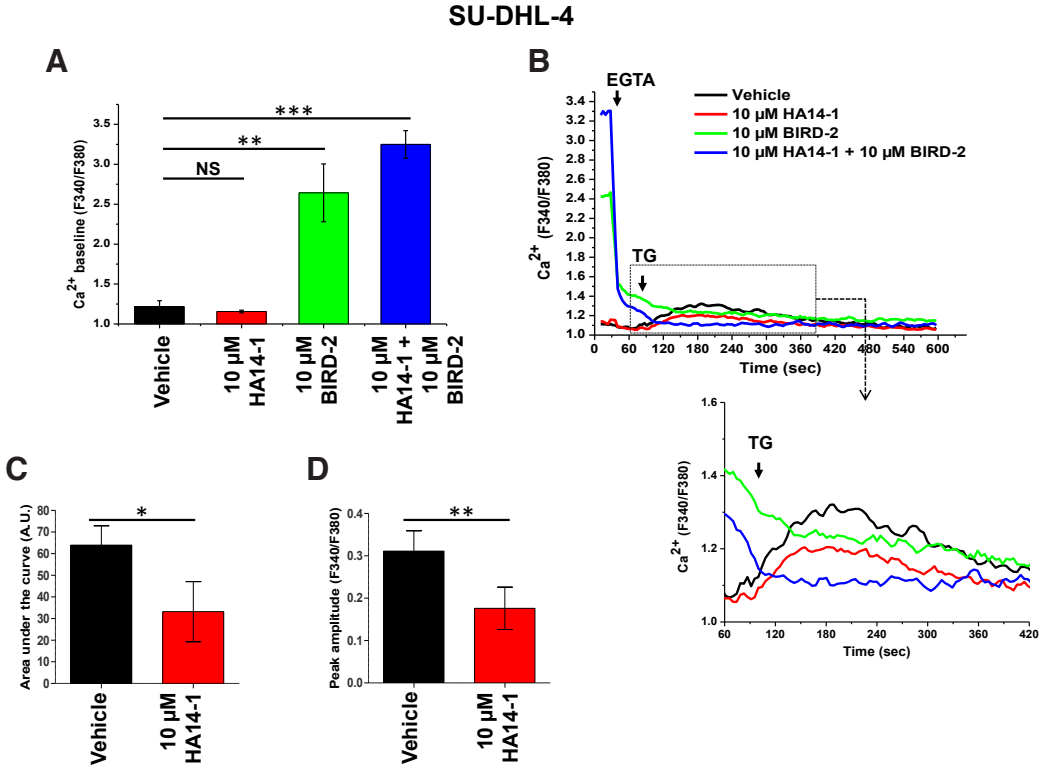

Fig. 2. Effect of BIRD-2 and HA14-1 on cytosolic Ca^{2+} levels in SU-DHL-4 cells. (A) *Averaged basal Ca^{2+} levels in SU-DHL-4 cells pretreated without (black bar) or with 10 μM HA14-1 (red bar), 10 μM BIRD-2 (green bar) or 10 μM HA14-1 + 10 μM BIRD-2 (blue bar) for 30 min. Values represent the mean ± S.E.M. of at least 3 independent experiments. Statistically significant differences are labeled with: *** p<0.001 using a Student's* t*-test (paired, one-tailed), ** p<0.01, NS = not significant.* **(B)** *Analysis of the thapsigargin (TG)-induced Ca^{2+} responses in SU-DHL-4 cells pretreated without (black line) or with 10 μM HA14-1 (red line), 10 μM BIRD-2 (green line) or 10 μM HA14-1 + 10 μM BIRD-2 (blue line) for 30 min. Thapsigargin concentration was 10 μM. The curves are representative for three independent experiments. For clarity's purpose, a magnification of the thapsigargin-releasable Ca^{2+} traces is provided. Quantification of the thapsigargin-releasable Ca^{2+} is provided as area under the curve* **(C)** *and as peak amplitude* **(D)**. *** p<0.01 using a Student's* t*-test (paired, one-tailed), * p<0.05.*

in IP_3R2 expression levels (Akl *et al.*, 2013a).

In this study, we explored the possibility to use HA14-1, which inhibits Bcl-2 by targeting its hydrophobic cleft (Wang *et al.*, 2000) and dysregulates Ca^{2+} homeostasis by inhibiting SERCA (Akl *et al.*, 2013b), to enhance the sensitivity of B-cell cancer cells to BIRD-2. Our data indicate that HA14-1 cannot only potentiate BIRD-2-induced Ca^{2+} signaling but also BIRD-2-induced cell death in a BIRD-2-sensitive DLBCL cell line (SU-DHL-4 cells) as well as in primary B-CLL cells isolated from patients. Taken together, these results further underpin the critical role of IP_3R-mediated Ca^{2+} signaling in causing cell death in B-cell cancer cells upon BH4-domain antagonism.

Results

HA14-1 enhances BIRD-2-induced apoptosis in BIRD-2-sensitive DLBCL cells

Recently, we have shown that SU-DHL-4 cells were very sensitive to BIRD-2-induced cell death (Akl *et al.*, 2013a). Treating SU-DHL-4 cells for 24 hours with 10 μM BIRD-2 caused apoptotic cell death in about 50% of the cells (Fig. 1A and B). We wondered whether we could boost BIRD-2-induced apoptosis using HA14-1. In contrast to BIRD-2, we found that 10 μM HA14-1 applied for 24 hours did not provoke cell death in SU-DHL-4 cells. Yet, combining HA14-1 with BIRD-2 potentiated cell death in SU-DHL-4 cells, causing cell death in ~80% of the cells. These data demonstrate a potentiating action of HA14-1 on BIRD-2-induced cell death in this DLBCL cell line.

Next, we examined whether the impact of these compounds on SU-DHL-4 viability was related to perturbations of the cytosolic Ca^{2+} homeostasis. Consistent with our previous data (Akl *et al.*, 2013a), BIRD-2 caused a considerable rise in the basal cytosolic Ca^{2+} levels (Fig. 2A). In contrast, HA14-1 did not affect the basal cytosolic Ca^{2+} levels in SU-DHL-4 cells. These Ca^{2+} data correlate with the cell death data obtained with both compounds separately (Fig. 1A and B). Importantly, combining HA14-1 with BIRD-2 caused a rise in the basal cytosolic Ca^{2+} levels that was significantly higher than the rise in cytosolic Ca^{2+} levels obtained upon the exposure of SU-DHL-4 to BIRD-2 alone. This indicates that there is a synergistic effect between HA14-1 and BIRD-2 at the level of cytosolic Ca^{2+} levels. To assess whether the increased cytosolic Ca^{2+} levels were due to Ca^{2+} released from the ER Ca^{2+} stores, we quantified the thapsigargin-releasable Ca^{2+} after the different treatments (Fig. 2B). We used 10 μM of thapsigargin, a concentration that is high enough to inhibit all SERCA isoforms and that has previously been used to discriminate between the ER Ca^{2+} stores and the Ca^{2+} stores of the Golgi compartment (Missiaen *et al.*, 2002). Treatment of the cells with HA14-1 (10 μM) decreased both the area under the curve (Fig. 2C) and the peak amplitude (Fig. 2D) of the thapsigargin-releasable Ca^{2+} by respectively 50% and 45%. Prior treatment with either BIRD-2 (10 μM) or a combination of BIRD-2 (10 μM) and HA14-1 (10 μM) completely depleted the ER Ca^{2+} stores. Hence, in these conditions, thapsigargin did not trigger a Ca^{2+} rise in the cytosol (Fig. 2B). These results indicate that the increase in cytosolic Ca^{2+} levels by these treatments was at least partially due to ER Ca^{2+}-store depletion. Identical results were obtained using ionomycin to release Ca^{2+} from intracellular stores, showing again that intracellular Ca^{2+} stores were most de-

pleted by treatment with the combination of BIRD-2 and HA14-1 (Supplemental Fig. 1A).

BIRD-2 does not increase the total number of apoptotic cells in BIRD-2-resistant DLBCL cells exposed to HA14-1

In contrast, the BIRD-2-resistant DLBCL cell line OCI-LY-1 is already very sensitive to 10 μM HA14-1 (about 30% of apoptotic cells in HA14-1-treated OCI-LY-1 cells, compared to about 6% in SU-DHL-4 cells, Fig. 3A). We investigated whether a combined treatment with BIRD-2, at a concentration which was able to kill SU-DHL-4 cells but was not able to kill OCI-LY-1 cells by itself, could potentiate HA14-1-induced apoptosis in OCI-LY-1 cells. However, the total number of apoptotic OCI-LY-1 cells induced by 10 μM HA14-1 was not increased by the addition of 10 μM BIRD-2. Of note, BIRD-2 seems to increase the number of 7-AAD-positive cells within the Annexin V-FITC-positive fraction, suggesting that apoptosis in cells sensitive to HA14-1 is expedited by BIRD-2, but that the total number of OCI-LY-1 cells sensitive to HA14-1 is not increased by BIRD-2. Consistent with these findings, we found that the combined treatment of HA14-1 and BIRD-2 increased the basal cytosolic Ca^{2+} levels more than treatment with either compound alone (Fig. 3B). Moreover, the cytosolic Ca^{2+} levels reached by the combination of HA14-1 and BIRD-2 were still much lower than those observed in SU-DHL-4 cells (Fig. 3B and C). We again quantified the thapsigargin-releasable Ca^{2+}. Both the area under the curve (Fig. 3D) and the peak amplitude (Fig. 3E) were reduced after prior treatment with HA14-1 (10 μM) though in contrast with the experiments performed on the SU-DHL-4 cells, treatment with BIRD-2 did not significantly decrease the Ca^{2+} content of the thapsigargin-sensitive Ca^{2+} stores. Identical results were obtained using ionomycin to induce Ca^{2+} release from the intracellular stores (Supplemental Fig. 1B). The lack of BIRD-2's ability to sensitize HA14-1-induced apoptosis in OCI-LY-1 cells was further scrutinized by using "sub-lethal" concentrations of HA14-1 in the OCI-LY-1 cells (Supplemental Fig. 2). For these experiments, 5 μM HA14-1 was used. This concentration had no

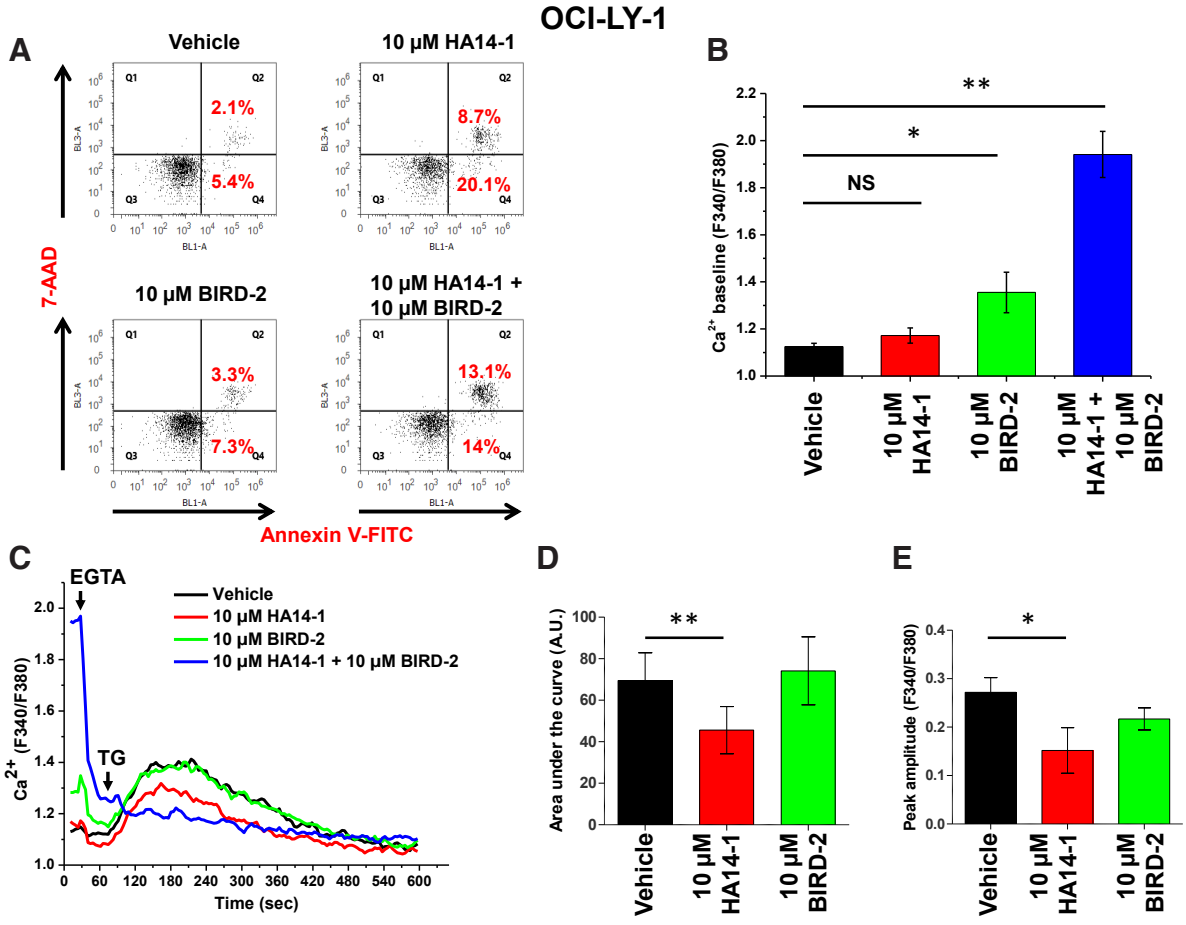

Fig. 3. Effect of BIRD-2 and HA14-1 on apoptosis and cytosolic Ca^{2+} levels in OCI-LY-1 cells. (A) *Dot plots from a flow-cytometric analysis of apoptosis induced in OCI-LY-1 cells by a 24 hours treatment without or with 10 μM HA14-1 and/or 10 μM BIRD-2. The dot plots are representative for three independent experiments.* **(B)** *Averaged basal Ca^{2+} levels in OCI-LY-1 cells pretreated without (black bar) or with 10 μM HA14-1 (red bar), 10 μM BIRD-2 (green bar) or 10 μM HA14-1 + 10 μM BIRD-2 (blue bar) for 30 min. Values represent the mean ± S.E.M. of at least 3 independent experiments. Statistically significant differences are labeled with: ** p<0.01 using a Student's* t*-test (paired, one-tailed), * p<0.05, NS = not significant.* **(C)** *Analysis of the thapsigargin (TG)-induced Ca^{2+} responses in OCI-LY-1 cells pretreated without (black line) or with 10 μM HA14-1 (red line), 10 μM BIRD-2 (green line) or 10 μM HA14-1 + 10 μM BIRD-2 (blue line) for 30 min. Thapsigargin concentration was 10 μM. The curves are representative for three independent experiments. Quantification of the thapsigargin-releasable Ca^{2+} is provided as area under the curve* **(D)** *and as peak amplitude* **(E)**. *** p<0.01 using a Student's* t*-test (paired, one-tailed), * p<0.05.*

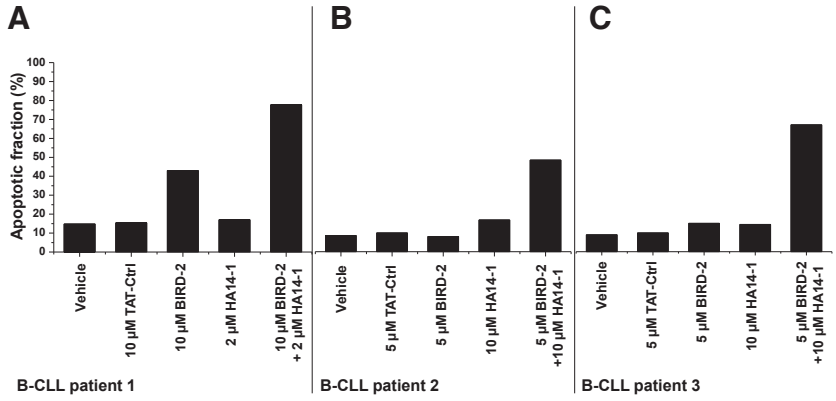

Fig. 4. BIRD-2 and HA14-1 have synergistic effects on apoptosis in primary B-CLL cells. *The apoptotic population was identified as the Annexin V-FITC-positive fraction in each of the indicated conditions.* **(A)** *In cells from the B-CLL patient 1 the combination of 2 μM HA14-1 and 10 μM BIRD-2 increased the number of Annexin V-FITC-positive B-CLL cells in comparison to B-CLL cells separately treated with either 2 μM HA14-1 or 10 μM BIRD-2. In cells from* **(B)** *patient 2 and* **(C)** *patient 3 a synergistic effect was observed when using 10 μM HA14-1 +5 μM BIRD-2 in comparison to 10 μM HA14-1 or 5 μM BIRD-2 alone. In none of the cells the control peptide TAT-Ctrl demonstrated any effect.*

effect on the viability of OCI-LY-1 cells. Combining 10 μM BIRD-2 with 5 μM HA14-1 did however also not trigger any increase in cell death in OCI-LY-1 cells.

HA14-1 enhances BIRD-2-induced apoptosis in B-CLL primary patient cells

We aimed to investigate whether the potentiating effect of HA14-1 on BIRD-2-induced cell death could also be observed in primary B-CLL cells obtained from the peripheral blood of patients (Fig. 4). We used concentrations of HA14-1 that in a single treatment are not able to cause cell death in the B-CLL cells. In B-CLL patient 1, 2 μM HA14-1 was used, which was unable to induce cell death by itself, while 10 μM BIRD-2 triggered cell death in about 50% of the cells (Fig. 4A). Combining 2 μM HA14-1 with 10 μM BIRD-2 synergistically caused cell death in about 80% of the cells.

Finally, we wondered whether HA14-1 could potentiate cell death induced by "sub-lethal" concentrations of BIRD-2. For these experiments, 10 μM HA14-1 was used. These concentrations had only a limited effect on the viability of B-CLL cells (B-CLL patient 2 and B-CLL patient 3; Fig. 4B and 4C, resp.). Moreover, a single treatment of 5 μM BIRD-2 was ineffective in causing cell death in these B-CLL samples. Yet, combining 10 μM HA14-1 with 5 μM BIRD-2 triggered cell death in about ~50% of the cells in B-CLL patient 2 (Fig. 4B) and in about ~70% of the cells in B-CLL patient 3 (Fig. 4C). These data show that "sub-lethal" concentrations of HA14-1 and "sub-lethal" concentrations of BIRD-2 act synergistically to provoke apoptosis in primary B-CLL cells. Collectively, these data indicate that a synergism between BIRD-2 and HA14-1 can trigger increased cell death through Ca^{2+} signaling in various B-cell cancer cells, including B-CLL and DLBCL.

Discussion

The major finding of this study is that combining a Bcl-2 inhibitor that antagonizes Bcl-2's functions at the ER by targeting the BH4 domain (BIRD-2) and a Bcl-2 inhibitor that antagonizes Bcl-2's functions at the mitochondria by targeting the hydrophobic cleft and impacts Ca^{2+} signaling by inhibiting SERCA (HA14-1) acts synergistically to cause cell death in a DLBCL cell line and in a set of primary B-CLL cells from patients. In particular, BIRD-2-induced cell death in BIRD-2-sensitive cancer cells was boosted by HA14-1, which not only inhibited Bcl-2 at both the ER and the mitochondria but also increased "toxic" Ca^{2+}-signaling events in these cancer cells. These data also further underpin the important role of "toxic" Ca^{2+} rises in the cytosol for provoking apoptosis in B-cell cancer cells, in particular cells already sensitive to BH4-domain antagonists. Hence, this study shows that boosting Ca^{2+} signaling may be a promising strategy to target B-cell cancers. This further underscores the importance and potential of Ca^{2+} signaling for anti-cancer strategies, as has also recently been demonstrated *in vivo* in solid tumors exposed to photodynamic therapy (Giorgi *et al.*, 2014).

BIRD-2 has previously been shown to disrupt IP_3R/Bcl-2-protein complexes by targeting the BH4 domain of Bcl-2 (Monaco *et al.*, 2012; Rong *et al.*, 2008; Rong *et al.*, 2009). While this peptide by itself is not cytotoxic in non-malignant T cells (like Jurkat cells), it does boost pro-apoptotic Ca^{2+} signaling in these cells in response to excessive T-cell-receptor stimulation (Rong *et al.*, 2008). In B-cell leukemic/lymphoma cells, but not in normal mononuclear peripheral blood cells, BIRD-2 was toxic by itself via a mechanism that caused spontaneous, excessive Ca^{2+} oscillations and subsequent apoptotic cell death (Akl *et al.*, 2013a; Zhong *et al.*, 2011). In DLBCL, a heterogeneous response to BIRD-2 has been observed. The sensitivity of DLBCL to BIRD-2 could be linked to the expression level of the IP_3R2, the IP_3R isoform with the highest sensitivity to IP_3 (Akl *et al.*, 2013a; Ivanova *et al.*, 2014; Vervloessem *et al.*, 2015). Given the important role of Ca^{2+} signaling in BIRD-2-induced cell death, we opted to study whether this could be enhanced by a Bcl-2-inhibiting tool that also impacts Ca^{2+} signaling by partially inhibiting SERCA Ca^{2+}-pump activity, i.e. HA14-1 (Akl *et al.*, 2013b). This inhibitory effect of HA14-1 on SERCA might account for its ability to kill cells in a caspase-independent manner (Vogler *et al.*, 2009). Also, a stabilized version of this compound (sHA14-1) has been reported to display a dual action, including inhibition of Bcl-2 at the mitochondrial level and inhibition of SERCA by impacting its Ca^{2+}-ATPase activity (Hermanson *et al.*, 2009). In this study, it was proposed that the dual action of sHA14-1 was needed to cause cell death, since thapsigargin, an irreversible SERCA inhibitor without reported impact on anti-apoptotic Bcl-2 proteins, failed to induce mitochondrial depolarization and induction of the apoptosis cascade. In an independent study (Akl *et al.*, 2013b), the SERCA-inhibitory properties of HA14-1 were confirmed using HeLa cells. Inhibition of ER Ca^{2+} uptake by HA14-1 already occurred in those cells at concentrations as low as 3 μM. In addition to its inhibitory action on SERCA, concentrations of HA14-1 higher than 10 μM were found to negatively impact IP_3R-mediated Ca^{2+} release. Hence, the potentiating effect of HA14-1 on BIRD-2-induced cell death might be limited to sub-IP_3R-inhibitory concentrations of HA14-1,

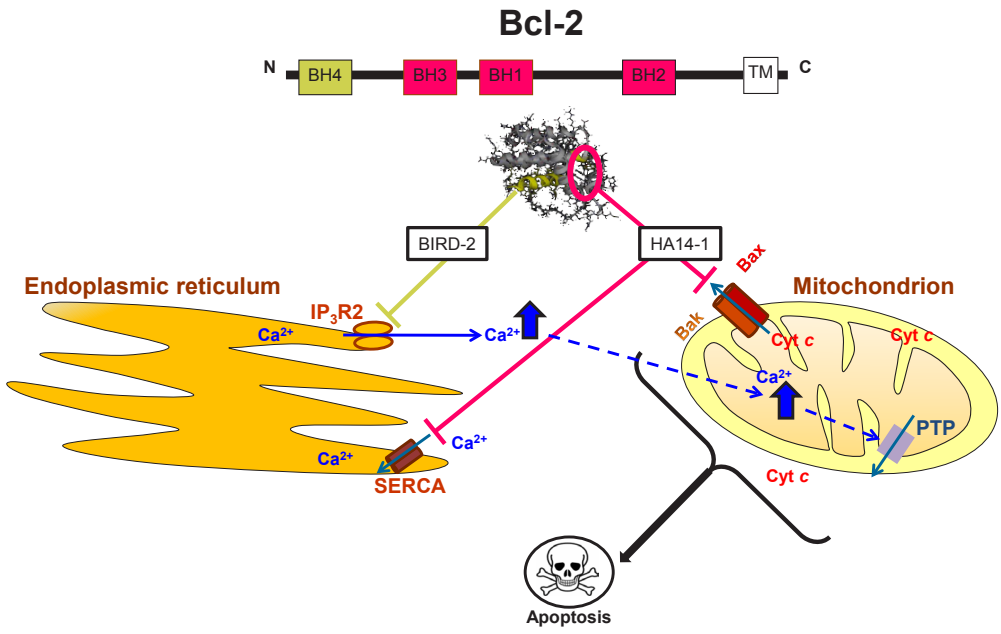

Fig. 5. Targeting anti-apoptotic Bcl-2 at both the mitochondria and the endoplasmic reticulum of cancer B-cells can lead to apoptosis in a Ca^{2+}-dependent manner. *Bcl-2 protects cancer B-cells from apoptosis at two different intracellular locations. At the mitochondria, Bcl-2 binds Bax/Bak via its hydrophobic cleft composed of the BH3, BH1 and BH2 domains (fuchsia boxes), preventing their oligomerization and inhibiting Bax/Bak-pore formation. This can be counteracted by small molecules, like HA14-1, that target the hydrophobic groove of Bcl-2. At the ER, Bcl-2 interacts with the IP_3R via its N-terminal BH4 domain (yellow box), thereby inhibiting its Ca^{2+}-flux properties. The IP_3R mimetics (like BIRD-2) can relieve the inhibition of IP_3Rs by Bcl-2, leading to increased Ca^{2+} signaling likely triggered by the constitutive IP_3 production downstream of the B-cell receptor in these cancer cells. We also speculate that Ca^{2+} released from the ER is transferred into the mitochondria, likely causing opening of the permeability transition pore (PTP) and subsequent cytochrome c (Cyt c) release. In addition to its capacity to bind the hydrophobic cleft of Bcl-2, HA14-1 also inhibits SERCA activity. This may lead to increased Ca^{2+} levels in the cytosol. Therefore, in anti-cancer therapy HA14-1 can be used in a combined regimen with BIRD-2, in order to also target Bcl-2 at the mitochondria and to aggravate the "toxic" Ca^{2+}-signaling events triggered by BIRD-2.*

thus 10 μM and lower. Interestingly, the synergy between HA14-1 and BIRD-2 observed in BIRD-2-sensitive cancer cells did not occur in BIRD-2-resistant cells. The rise in cytosolic Ca^{2+} levels in OCI-LY-1 cells treated with the combination of HA14-1 and BIRD-2 (Fig. 3B) was lower than the rises in cytosolic Ca^{2+} levels obtained upon the exposure of SU-DHL-4 to BIRD-2 alone or to BIRD-2 together with HA14-1 (Fig. 2A). Consistent with these findings, the total number of apoptotic OCI-LY-1 cells was very similar when BIRD-2 was combined with HA14-1 or when HA14-1 was applied by itself. However, we did notice that the BIRD-2/HA14-1 combination might accelerate apoptosis in the cells already sensitive to HA14-1, thereby shifting apoptosis to more secondary necrosis without increasing the total number of dying cells. We also noticed that the SU-DHL-4 cells that are more sensitive to BIRD-2 were more resistant to HA14-1 and oppositely, that the OCI-LY-1 cells that are resistant to BIRD-2 were more sensitive to HA14-1. This suggests that cancer cells might display a dual addiction to Bcl-2 either at the ER or the mitochondria (Fig. 5) (Akl *et al.*, 2014), although this ought to be further scrutinized in more cell lines and using Bcl-2 inhibitors that do not impact SERCA, like ABT-199 (Souers *et al.*, 2013). We hypothesize that in SU-DHL4 and primary B-CLL cells Bcl-2 is needed at the ER to prevent the excessive transfer of Ca^{2+} to the mitochondria and the Ca^{2+}-elevating properties of HA14-1 potentiates the apoptotic effect of BIRD-2. However, in OCI-LY-1 cells, Bcl-2 is only needed at the mitochondria to prevent excessive Bim and Bid activity, and the Bcl-2 antagonist HA14-1 induces apoptosis. The Ca^{2+}-elevating properties of neither BIRD-2 alone nor HA14-1 combined with BIRD-2 further potentiated the level of apoptosis. In these cells, we speculate that HA14-1-induced cell death is due to its Bcl-2's inhibitory properties rather than its SERCA inhibitory properties. Indeed, HA14-1 did not have a major impact on the basal cytosolic Ca^{2+} levels in OCI-LY-1 cells.

To conclude, we demonstrated that BIRD-2-induced cell death in DLBCL cell models and B-CLL can be boosted by a "sub-lethal" concentration of HA14-1, a compound counteracting both mitochondrial functions of Bcl-2 and impairing SERCA activity. These data indicate the potential of combination regimens of Bcl-2 inhibitors with different properties to enhance BIRD-2-induced cell death in B-cell cancer cells.

Materials and Methods

Cells, reagents and peptides

The DLBCL cell lines SU-DHL-4 and OCI-LY-1 were cultured in suspension in RPMI-1640 and Iscove modified Dulbecco media (Invitrogen, Merelbeke, Belgium) respectively. Primary lymphocytes were separated using a Ficoll Hypaque density gradient from the peripheral blood of adult patients with B-CLL, and suspended in RPMI-1640 medium. The resulting samples contained more than 80% B cells, as determined by CD19 surface staining and fluorescence-activated cell sorting analysis. The latter blood samples were obtained with the agreement of the UZ Leuven Ethical Committee (Belgian Number: B32220071536) according to the principles established by the International Conference on Harmonization Guidelines on Good Clinical Practice. An informed consent form was obtained from all B-CLL patients. All media were supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (100x GlutaMAX, Gibco/Invitrogen, 35050) and penicillin and streptomycin (100x Pen/Strep, Gibco/Invitrogen, 15070-063) and cells were cultured at 37°C and in the presence of 5% CO_2.

Reagents were as follows: ethylene glycol tetraacetic acid (EGTA) (Acros Organics, Geel, Belgium, 409910250), thapsigargin (Enzo Life Sciences, Farmingdale, NY, USA, ALX-350-004-M010), Fura-2 AM (Biotium, Kampenhout, Belgium, 50033), Annexin V-Fluorescein isothiocyanate (FITC) (Becton Dickinson, Franklin Lakes, NJ, USA, 556419), 7-Aminoactinomycin D (7-AAD) (Becton Dickinson, 555815), HA14-1 (Sigma, H8787-5MG) and PE-Cy™5 Mouse Anti-Human CD19 antibody (Eurogentec, 54775).

The peptides BIRD-2 (RKKRRQRRRGGNVYTEIKCNSLLPLAAIVRV)

and TAT-Ctrl (RKKRRQRRRGGSIELDDPRPR) were purchased from LifeTein (South Plainfield, New Jersey, USA) (purity> 85%).

Apoptosis assay

DLBCL and B-CLL primary cells were treated as indicated at $5x10^5$ cells/ml, pelleted by centrifugation, and incubated with Annexin V-FITC and 7-AAD. Cell suspensions were analyzed with a FACSCanto (Becton Dickinson) or Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems). Cell death by apoptosis was scored by quantifying the population of Annexin V-FITC-positive cells. Flow-cytometric data were plotted and analyzed using BD FACS Diva Software (Becton Dickinson) or Attune version 2.1.0 (Applied Biosystems).

Fluorescence Ca^{2+} measurements in intact cells

For the Ca^{2+} measurements in intact cells, DLBCL cells were seeded in poly-L-lysine-coated 96-well plates (Greiner) at a density of 5×10^5 cells/ml. The cells were loaded for 30 min with 1 μM Fura-2 AM at 25°C in modified Krebs solution, followed by a 30-min de-esterification step in the absence of Fura- 2 AM. Fluorescence was monitored on a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) by alternately exciting the Ca^{2+} indicator at 340 and 380 nm and collecting emitted fluorescence at 510 nm, as described previously (Decuypere *et al*., 2011). EGTA (final concentration 3 mM), and thapsigargin (10 μM) or ionomycin (10 μM) were added as indicated. All data were obtained in triplicate and are plotted as F340/F380. At least, three independent experiments were always performed.

Statistical analysis

Results are expressed as average ± S.D. or S.E.M. as indicated. The number of independent experiments is always indicated. Significance was determined using a one- or two-tailed paired Student's t-test as appropriate. Differences were considered significant at $P < 0.05$.

Conflict of interest

The authors declare no conflict of interests.

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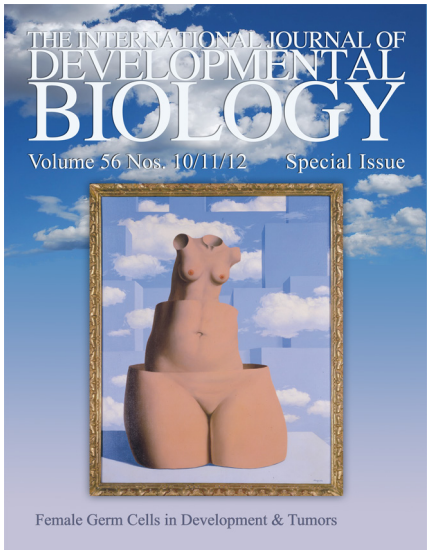

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