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_{3}R), 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_{3}Rs 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_{3}R-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_{3}R, 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...
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 Bim, while Bax activation appears to be mediated by Bim (Sarasiek 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 Ca²⁺ homeostasis (Vogler et al., 2011), but this was contested by others (Harper and Poole, 2012; Schoenwaelder and Jackson, 2012). We have recently shown that Ca²⁺ 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 Ca²⁺ overload, due to its inhibitory effect on the sarco-/endoplasmic reticulum Ca²⁺ ATPase (SERCA) (Akl et al., 2013b).

At the ER, Bcl-2 acts on the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃-R), thereby limiting Ca²⁺ 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 IP₃-R (Distelhorst and Bootman, 2011; Monaco et al., 2012; Rong et al., 2009). The site is conserved in all three IP₃-R isoforms (Monaco et al., 2012) and also in the related family of intracellular Ca²⁺ release channels, the ryanodine receptors (Vervliet et al., 2014). Bcl-2 binding to IP₃-Rs dampens the pro-apoptotic Ca²⁺ 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 IP₃-R1, was developed and able to disrupt IP₃-R/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-IDP³/AA (Zhong et al., 2011) or TAT-IDP³ (Akl et al., 2013a; Akl et al., 2014), will for convenience be referred to as BIRD-2 (Bcl-2-IP₃ disruptor-2) to distinguish it from unrelated IP₃-R sequence-derived peptides now in use in our laboratories. BIRD-2 triggered IP₃-R-mediated Ca²⁺ 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

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**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 10µM 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 defined as the Annexin V-FITC-positive / 7AAD-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.
in IP3R2 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 Ca2+ 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 Ca2+ 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 IP3R-mediated Ca2+ 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 Ca2+ homeostasis. Consistent with our previous data (Akl et al., 2013a), BIRD-2 caused a considerable rise in the basal cytosolic Ca2+ levels (Fig. 2A). In contrast, HA14-1 did not affect the basal cytosolic Ca2+ levels in SU-DHL-4 cells. These Ca2+ 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 Ca2+ levels that was significantly higher than the rise in cytosolic Ca2+ 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 Ca2+ levels. To assess whether the increased cytosolic Ca2+ levels were due to Ca2+ released from the ER Ca2+ stores, we quantified the thapsigargin-releasable Ca2+ as provided by these treatments was...
completed 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...
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\(_R\)-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, R2, the IP, R isoform with the highest sensitivity to IP\(_R\) (Akl et al., 2013a; Ivanova et al., 2014; Vervoessel 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, R-mediated Ca\(^{2+}\) release. Hence, the potentiating effect of HA14-1 on BIRD-2-induced cell death might be limited to sub-IP, R-inhibitory concentrations of HA14-1,

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**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.
on the basal cytosolic Ca\textsuperscript{2+}-elevating properties. Indeed, HA14-1 did not have a major impact on the number of dying cells. We also noticed 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\textsuperscript{2+} to the mitochondria and the Ca\textsuperscript{2+}-elevating properties of HA14-1 potentiate 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\textsuperscript{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\textsuperscript{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 (In Vitrogen, Mellebeke, 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 in accordance with 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\textsubscript{2}.

Reagents were as follows: ethylene glycol tetraacetic acid (EGTA) (Acros Organics, Geel, Belgium, 409910260), thapsigargin (Enzo Life Sciences, Farmingdale, NY, USA, ALX-350-004-M010), Fura-2 AM (Biolum, Kampenhout, Belgium, 50033), Annexin V-Fluorescein Isothiocyanate (FITC) (Becton Dickinson, Franklin Lakes, NJ, USA, 556419), 7-Aminoactinomycin D (7-AAD) (Becton Dickinson, 556815), HA14-1 (Sigma, H8787-5MG) and PE-Cy™5 Mouse Anti-Human CD19 antibody (Eurogentec, 54775).

The peptides BIRD-2 (RKKRRQRRRGGNVYTEIKCNSLPLAAIVRV) and the schematic representation 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\textsuperscript{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\textsuperscript{2+}-signaling events triggered by BIRD-2.
Apoptosis assay

DLBCL and B-CLL primary cells were treated as indicated at 5×10^5 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 5×10^4 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 alternating 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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