Regulation of the ryanodine receptor by anti-apoptotic Bcl-2 is independent of its BH3-domain-binding properties

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Abstract

The regulation of intracellular Ca^{2+} signaling is an important aspect of how anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins regulate cell death and cell survival. At the endoplasmic reticulum (ER) the Bcl-2 homology (BH) 4 domain of Bcl-2 is known to bind to and inhibit both inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs). Besides this, drugs that target the hydrophobic cleft of Bcl-2 have been reported to deplete ER Ca^{2+} stores in an IP_3R- and RyR-dependent way. This suggests that the hydrophobic cleft of Bcl-2 may also be involved in regulating these ER-located Ca^{2+}-release channels. However, the contribution of the hydrophobic cleft on the binding and regulatory properties of Bcl-2 to either IP_3Rs or RyRs has until now not been studied. Here, the importance of the hydrophobic cleft of Bcl-2 in binding to and inhibiting the RyR was assessed by using a genetic approach based on site-directed mutagenesis of Bcl-2's hydrophobic cleft and a pharmacological approach based on the selective Bcl-2 hydrophobic cleft inhibitor, ABT-199. Both binding assays and single-cell Ca^{2+} measurements indicated that RyR binding and the inhibition of RyR-mediated Ca^{2+} release by Bcl-2 is independent of its hydrophobic cleft.

Keywords: B-cell lymphoma 2, ryanodine receptors, hydrophobic cleft, BH3 mimetic, ABT-199, P2A

Abbreviations: Bcl-2-associated death promoter (Bad), Bcl-2 homologous antagonist killer (Bak), Bcl-2-associated X protein (Bax), B-cell lymphoma 2- (Bcl-2), Bcl-2 homology (BH), endoplasmic reticulum (ER), erythropoietin (Epo), inositol 1,4,5-trisphosphate receptor (IP_3R), mammalian protein-protein interaction trap (MAPPIT), ryanodine receptor (RyR), sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)

Highlights

- **Bcl-2's hydrophobic cleft is not involved in binding to and regulating RyRs**
- **The BH3 mimetic ABT-199 does not alter Bcl-2's ability to regulate RyRs**
- **Bcl-2-P2A-mCherry can efficiently be used in single-cell Ca^{2+} measurements**

Introduction

The modulation of intracellular Ca^{2+} signalling is an important aspect of how proteins of the B-cell lymphoma 2 (Bcl-2)-family regulate cell death and cell survival [1, 2, 3, 4]. The three C-terminally located Bcl-2 homology (BH) domains (BH1, BH2 and BH3) of anti-apoptotic Bcl-2 family members (such as Bcl-2 and Bcl-X_L) form a hydrophobic cleft, which is used to bind to the BH3 domains present in the pro-apoptotic Bcl-2-family members, thereby inhibiting their function [5]. The N-terminally located BH4 domain also critically determines its anti-apoptotic function in at least two ways: (i) by binding to and inhibiting the pro-apoptotic, multi-domain protein Bcl-2-associated X protein (Bax), which upon activation oligomerizes and permeabilizes mitochondrial outer membranes [6], and (ii) by binding to and inhibiting the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), an endoplasmic reticulum (ER)-located Ca^{2+}-release channel, thereby preventing mitochondrial Ca^{2+} overload [7].

We recently showed that, similarly to IP_3Rs, anti-apoptotic Bcl-2 via its BH4 domain also targets another important ER-located family of Ca^{2+}-release channels: the ryanodine receptors (RyRs) [8]. The BH4 domain of Bcl-2 was sufficient to interact with and suppress RyR-mediated Ca^{2+} release. For Bcl-X_L the situation was different. In contrast to the IP_3R, where the BH4 domain of Bcl-X_L does not bind to and inhibit the channel [9], the BH4 domain of Bcl-X_L is able to bind to and inhibit RyRs [10]. However, in the full-length Bcl-X_L Lys87 located in the BH3 domain, which is part of the hydrophobic cleft, also plays a role in targeting Bcl-X_L to the RyR. This suggests that the hydrophobic cleft of the anti-apoptotic Bcl-2 proteins may also play a role in targeting them to the RyR. In addition to this, drugs that target the hydrophobic cleft of Bcl-2 and thus compete with pro-apoptotic Bcl-2 family members have been reported to deplete the ER Ca^{2+} stores in pancreatic acinar cells [11]. As these cells express both IP_3Rs and RyRs [12], it was suggested that dissociation of the pro-apoptotic Bcl-2 family members from the anti-apoptotic family members by these Bcl-2 antagonists may increase the sensitivity of the IP_3R and RyR to Ca^{2+}-induced Ca^{2+} release [11]. However, it was not investigated whether these compounds effectively altered the binding of Bcl-2 to either of these ER-located Ca^{2+}-release channels. Furthermore, the Bcl-2 antagonists used in this study are not selective

71 Bcl-2 inhibitors, thereby affecting other proteins. For instance, the Bcl-2 antagonist HA14-1 also
72 inhibits the activity of the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA), thereby causing ER
73 Ca^{2+}-store depletion [13, 14].

74 Therefore, we here assessed the involvement of the hydrophobic cleft of Bcl-2 in binding to and
75 inhibiting the RyR by using both site-directed mutagenesis and a novel specific Bcl-2 inhibitor, ABT-
76 199. This analysis shows that RyR binding and inhibition by Bcl-2 is independent of its hydrophobic
77 cleft properties.

Materials and methods

Chemicals, antibodies and peptides

Unless otherwise mentioned all chemicals were obtained from Sigma-Aldrich. The antibodies used in this study include: HRP conjugated anti-FLAG M2 antibody (Sigma-Aldrich), mouse monoclonal anti-RyR antibody 34C (Thermo Scientific, Rockford, IL, USA, or Developmental Studies Hybridoma Bank, University of Iowa, USA) and rabbit polyclonal anti-Bax (Santa Cruz). The peptide used for elution of the FLAG pull-downs was MDYKDHDGDYKDHDIDYKDDDDK (Life Tein).

Plasmids and constructs

The used restriction enzymes were obtained from New England BioLabs. 3XFLAG-tagged Bcl-2 (3XFLAG-Bcl-2) was obtained as described previously [9]. PCR site-directed mutagenesis using the 3XFLAG-Bcl-2 construct as template (primers forward: 5'ATCCCCATGGCAGCAGTAGATCAAGCGCTGAGGGAGGCA3', and reverse: 5'TGCCTCCCTCAGCGCTTGATCTACTGCTGCCATGGGGAT3') was performed to obtain the 3XFLAG-Bcl-2 G145A R146A (3XFLAG-Bcl-$2^{GR/AA}$) double mutant. The pCMV24 vector encoding the P2A-mCherry or 3XFLAG-Bcl-2-P2A-mCherry were obtained as follows. A P2A-encoding DNA sequence (corresponding to amino acid sequence GSGATNFSLLKQAGDVEENPGP) [15] was obtained as a duplex with overhanging ends corresponding to cleaved EcoRI and BglII restriction sites forward: 5'AATTCGGATCCGGAGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGA AAACCCTGGTCCTA3', and reverse 5'GATCTAGGACCAGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGT GGCTCCGGATCCG3'. The duplex was ligated into the empty pCMV24 vector utilizing EcoRI and BglII as restriction sites. After removing the BglII site from the mCherry construct via site-directed mutagenesis (primers forward: 5'GGACGAGCTGTACAAGAGGTCTCGAATCACAAGTTTG3', and reverse: 5' CAAACTTGTGATTCGAGACCTCTTGTACAGCTCGTCC3'), mCherry was cloned

at the 3' side of the P2A sequence (primers forward: 5'GCGGCGAGATCTGTGAGCAAGGGCGAGGAGGAC3', and reverse: 5'GCGGCGGTCGACTTACGTTTCTCGTTCAGC3') utilizing the BglII and SalI restriction enzymes. At the 5' side of the P2A sequence, Bcl-2 was cloned (primers forward: 5'GCGGCGGCGGCCGCAGCGCACGCTGGGAGAAC3', and reverse: 5'GCGGCGGAATTCCTTGTGGCCCAGATAGGCAC3') utilizing the NotI and EcoRI restriction enzymes and the 3XFLAG-Bcl-2 plasmid as template. One nucleotide was added just before the multiple cloning site in order to put the pCMV24-P2A-mCherry construct in the correct open reading frame (primers forward: 5'GGATGACGATGAGCAAGCTTGCGGC3', and reverse: 5'GCCGCAAGCTTGCTCATCGTCATCC3').

Cell culture and transfections

All media used in this study were obtained from Life Technologies. HEK293T cells, and HEK293 cells stably overexpressing RyR3 (HEK RyR3) were cultured as previously [10].

One day after seeding, the 3XFLAG-Bcl-2 or 3XFLAG-Bcl-$2^{GR/AA}$ construct were introduced into HEK RyR3 cells utilizing the X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. For single-cell Ca^{2+} measurements two different methods for introducing mCherry, as selection marker for transfected cells, into the cells were used. First, 48 hours after seeding the pCMV24 vector (negative control), 3XFLAG-Bcl-2 or the 3XFLAG-Bcl-$2^{GR/AA}$ construct were transfected together with a pcDNA 3.1(-) mCherry expressing vector as a selection marker at a 3:1 ratio as previously described [10]. Second, 48 hours after seeding, 0.25 µg of a pCMV24 vector encoding the P2A-mCherry or a pCMV24 vector encoding 3XFLAG-Bcl-2-P2A-mCherry was introduced into the HEK RyR3 cells utilizing the X-tremeGENE HP DNA transfection reagent. All single-cell Ca^{2+} measurements were performed two days after the transfection.

Co-immunoprecipitations, FLAG pull-downs and immunoblots

Co-immunoprecipitation experiments were performed utilizing a co-immunoprecipitation kit (Thermo Scientific) in HEK RyR3 cells as described previously [10]. The negative control IgG was obtained from Thermo Scientific. Cell lysis and wash steps were performed with a CHAPS-based buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% CHAPS and protease inhibitor tablets (Roche)). When indicated, 3 µM of ABT-199 (Active Biochem) was added during the overnight incubation. In the control co-immunoprecipitations an equal amount of the vehicle (DMSO) was included. For FLAG pull-downs, 300 µg of HEK RyR3 cells transfected with the empty vector, 3XFLAG-Bcl-2 or 3XFLAG-Bcl-$2^{GR/AA}$ were lysed (10 mM HEPES pH 7.5, 0.25 % Nonidet P-40, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM EGTA and protease inhibitor tablets) and incubated for 2 hours at 4°C with 30 µl of anti-DYKDDDDK (L5)–tag agarose (Biolegend). After extensive washing (50 mM Tris pH 8, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor tablets) elution was performed by incubating the resin for 30 min at 15°C with FLAG elution peptide (250 µg/ml 50 mM Tris-HCl pH 7.4, 150 mM NaCl). After the elution, the eluate was collected and 4X LDS (Life Technologies) supplemented with 1/200 β-mercaptoethanol. Samples were boiled (95°C) for 5 min just before SDS-PAGE on either NuPAGE 4-12% Bis-Tris gels or NuPAGE 3-8% Tris-Acetate gels (both from Life Technologies) when RyRs were visualized. gels. Immunoblot analysis was performed as previously [10].

Mammalian protein-protein interaction trap (MAPPIT)

Bcl-2-associated death promoter (Bad), and Bcl-2 were cloned in the pSEL+2L bait vector, TRIP13 and Bcl-2 lacking its transmembrane domain were cloned in the pMG1 or pMG2 prey vector, respectively [16]. Bad and TRIP13 entry clones are from the ORFeome8.1 collection received from the Vidal lab (CCSB, Boston, USA), and were cloned as bait or prey, respectively using the Gateway recombination technology as described by the manufacturer (Life Technologies). Using the pCAGGS-Bcl-2 plasmid, obtained from BCCM/LMBP (Gent, Belgium) as template, Bcl-2 was amplified by PCR using the following primers, forward: 5'GCGGAATTCATGGCGCACGCTGGGAGA3', and

reverse: 5‘CGCGCGGCCGCTCACTTGTGGCCCAGATAGG3’, and was cloned in the pMG2 prey vector using the restriction enzymes EcoRI and NotI. Bcl-2 was cloned in the bait vector, by EcoRI digestion of the above described Bcl-2 prey vector, made blunt by nucleotide fill-in, and followed by a NotI restriction digest, and SalI digestion of the pSEL+2L bait vector, made blunt by nucleotide fill-in and subsequent NotI digestion. The Bcl2 prey lacking its transmembrane domain was obtained by PCR amplification on the Bcl-2 prey construct using the same forward primer as described above and the following reverse primer: 5’CGCGCGGCCGCTCAGGAGAAATCAAACAGAGGCC3’, and was cloned in the pMG2 prey vector using the restriction enzymes EcoRI and NotI.

MAPPIT experiments were performed as previously [10], with minor changes. Briefly, HEK293T cells were seeded in 96-well plates. Six wells per condition were transfected with the different combinations of bait, prey and reporter plasmid (rPAP1-luci) using the calcium phosphate method. The next day, a serial dilution of the ABT-199 compound was added and 4 hours later half of the wells were stimulated with 5 ng/ml erythropoietin (Epo) while the other half were left untreated. One day later the cells were lysed and after the addition of substrate the luciferase activity was determined using a luminometer. The fold induction was obtained by dividing the average value of the stimulated cells by the average value of the non-stimulated cells.

Single-cell Ca^{2+} measurements and fluorescence microscopy

Single-cell Ca^{2+} measurements were performed as previously [8]. mCherry was used as selection marker for the transfected cells. When used, ABT-199 or an equal volume of the vehicle (DMSO) was included during the loading and de-esterification (1 hour in total) of the Fura2-AM in the HEK RyR3 cells. A Zeiss Axio Observer Z1 Inverted Microscope equipped with a 20x air objective and a high-speed digital camera (Axiocam Hsm, Zeiss) was used for imaging mCherry expression.

Statistical analysis

Repeated measure ANOVA tests were performed. * indicates significantly different results ($p<0.05$). Exact p-values are indicated in the figure legends. NS indicates non-significant.

Results and discussion

The hydrophobic cleft of Bcl-2 has been extensively characterized with respect to its binding to BH3 domains of the pro-apoptotic Bcl-2 family members. Mutations disrupting the hydrophobic cleft of Bcl-2 and thereby abolishing binding of Bcl-2 to Bax and Bcl-2 homologous antagonist killer (Bak) have been described [17]. Using a Bcl-2 hydrophobic cleft double mutant (G145A and R146A (Bcl-$2^{GR/AA}$)), we aimed to elucidate whether or not this region is involved in regulating RyRs.

We first validated that the Bcl-$2^{GR/AA}$ mutant was unable to bind the pro-apoptotic protein Bax by performing FLAG-pull-down assays in HEK RyR3 cells transiently overexpressing the empty vector (pCMV24), 3XFLAG-Bcl-2 or the 3XFLAG-Bcl-$2^{GR/AA}$ mutant (Fig. 1A). The 3XFLAG-tagged constructs were pulled down (Fig 1A, top) after which the presence of endogenous Bax was assessed. Bax specifically co-immunoprecipitated with 3XFLAG-Bcl-2, while it was absent in empty vector control samples. Furthermore, in contrast to wild-type 3XFLAG-Bcl-2 the 3XFLAG-Bcl-$2^{GR/AA}$ mutant could not pull-down endogenous Bax (Fig. 1A, bottom), indicating that these mutations disrupt the hydrophobic cleft as reported. Next, co-immunoprecipitation experiments were set up in order to verify whether the hydrophobic cleft of Bcl-2 is involved in binding to RyR3. RyR3 was immunoprecipitated from HEK RyR3 cells (Fig. 1B, top) transiently overexpressing 3XFLAG-Bcl-2 or 3XFLAG-Bcl-$2^{GR/AA}$. Strikingly, mutating the hydrophobic cleft of Bcl-2 resulted in an increased binding to RyR3 (Fig. 1B, bottom). This might indicate that more of 3XFLAG-Bcl-$2^{GR/AA}$ may be available to bind to RyR3 due to its impaired recruitment to pro-apoptotic Bcl-2-family members.

Finally, Fura2-AM single-cell Ca^{2+} measurements were set up to assess whether disruption of Bcl-2's hydrophobic cleft affected its ability to inhibit RyR3-mediated Ca^{2+} release. In these experiments, the empty vector (pCMV24), 3XFLAG-Bcl-2 or the 3XFLAG-Bcl-$2^{GR/AA}$ mutant were transfected in HEK RyR3 cells at a 3:1 ratio with mCherry. Only mCherry-positive cells were included in these measurements. RyR-mediated Ca^{2+} release was evoked with caffeine (1.5 mM) after chelating extracellular Ca^{2+} with BAPTA (3 mM). An average Ca^{2+} trace of a typical experiment is shown in Fig. 2A whereas the summary of all performed Ca^{2+} measurements is presented in Fig. 2B. From this it was clear that, Bcl-2 mutations that disrupt the hydrophobic cleft did not affect the ability of Bcl-2 to inhibit RyR3. This suggests that the hydrophobic cleft of Bcl-2 is not involved in targeting RyR3.

To further substantiate our findings, an independent pharmacological approach was performed, utilizing a BH3-mimetic drug which occupies the hydrophobic cleft of Bcl-2. In recent years, several advances have been made in generating highly specific BH3-mimetic drugs [18]. One of the most recent compounds, ABT-199, was designed to have a high specificity for Bcl-2 over Bcl-X_L thereby limiting side effects generated by unwanted Bcl-X_L inhibition [18]. In this study we used this ABT-199 compound in order to independently assess the importance of the hydrophobic cleft of Bcl-2 for RyR interaction and regulation by Bcl-2.

First, we assessed the efficiency of the compound in disrupting the binding of Bcl-2 to the pro-apoptotic Bcl-2 family members. For this we utilized an *in cellulo* protein-protein interaction assay, MAPPIT [10, 19]. In this technique, functional complementation of a chimeric cytokine receptor serves as a read out for the binding of two proteins. The first binding partner , Bad (bait) was fused to the membrane bound chimeric cytokine receptor consisting of the extracellular domain of the Epo receptor fused to the transmembrane and mutated cytosolic part of the leptin receptor. The second binding partner, Bcl-2 lacking its transmembrane domain (prey) was fused to a part of the glycoprotein 130 receptor which can trigger Epo-dependent STAT signaling when binding to the bait occurs. The latter is monitored via a luciferase reporter assay driven by a STAT-sensitive promoter. As expected, Bad could bind to Bcl-2 which could be inhibited by ABT-199 in a concentration-dependent way (EC50~20 nM) (Fig 3A). A positive control, consisting of Bcl-2 as bait and TRIP13 as prey, which triggers STAT signaling independently from binding to the bait, showed no decreased binding in the presence of ABT-199 indicating that the compound disrupts the Bad/Bcl-2 interaction specifically. Next, we wanted to assess the effect of ABT-199 on the binding of Bcl-2 to full size RyR3. For this, similar co-immunoprecipitations as in Fig. 1B were set up. In the next experiments 3 µM of ABT-199 was used as this concentration was more than sufficient to completely disrupt binding of Bad and Bcl-2 and we wanted to maximize the possible effects of the compound on the RyR/Bcl-2 interaction. RyR3 was immunoprecipitated from lysates from HEK RyR3 cells overexpressing 3XFLAG-Bcl-2 after overnight incubation with ABT-199 or the vehicle (DMSO) (Fig. 3B, top). Similar amounts of 3XFLAG-Bcl-2 could be co-immunoprecipitated under these two conditions (Fig. 3B, bottom) underpinning the data obtained with the Bcl-2 hydrophobic cleft mutant (Fig. 1B).

Next, we assessed the effect of the ABT-199 compound on the ability of Bcl-2 to inhibit RyR-mediated Ca^{2+} release. For this we optimized the use of a different plasmid combining the 3XFLAG-Bcl-2 construct with the mCherry selection marker in the same vector. The previously described virus-derived P2A sequence was used for this purpose [15]. This sequence, once translated, is known to cleave itself without any involvement of the host cell. The P2A sequence was introduced in the pCMV24 vector flanked on its N-terminus by 3XFLAG-Bcl-2 and at the C-terminus by mCherry (3XFLAG-Bcl-2-P2A-mCherry). A pCMV24 vector encoding P2A-mCherry was also made as control. Before proceeding we verified whether the P2A sequence produced two separate proteins. First, lysates from HEK RyR3 cells expressing either 3XFLAG-Bcl-2-P2A-mCherry or 3XFLAG-Bcl-2 were immunoblotted. These showed in both cells the presence of 3XFLAG-Bcl-2 (Fig. 4A). In the 3XFLAG-Bcl-2-P2A-mCherry expressing cells this migrated at a slightly higher molecular mass because part of the P2A sequence remains attached to the protein preceding the P2A sequence. As reported previously, a weaker second product was also recognized at a slightly lower molecular mass [15, 20]. Since this is also recognized by the anti-FLAG antibody, this is likely also 3XFLAG-tagged-Bcl-2 protein lacking the P2A sequence. This band migrates slightly lower than the 3XFLAG-Bcl-2 next to it because the Myc-tag, present at the C-terminal end of the multiple cloning site of the pCMV24 vector, is in this case attached to the mCherry protein and not to the 3XFLAG-Bcl-2. Importantly, no FLAG-reactive signal could be detected at higher molecular weights. Since the 3XFLAG tag allows for a very sensitive detection, this result indicates that the vast majority of the P2A-containing constructs was cleaved, resulting in the overexpression of two separate proteins. Next, the two transfection protocols for single-cell Ca^{2+} measurements utilized in present study were compared for their mCherry expression (Fig. 4B). Both methods resulted in a similar number of mCherry-positive cells, indicating that the P2A-containing constructs can be used as selection marker in the single-cell Ca^{2+} measurements. Fura2-AM single-cell Ca^{2+} measurements were performed in HEK RyR3 cells overexpressing either P2A-mCherry as a control or 3XFLAG-Bcl-2-P2A-mCherry. During the loading and de-esterification steps (1 hour in total), ABT-199 (3 µM) or the vehicle control (DMSO) was added to the cells. The Ca^{2+} measurements were similarly performed as above. Introducing 3XFLAG-Bcl-2 into these HEK RyR3 cells via the 3XFLAG-Bcl-2-P2A-mCherry

construct resulted in about 50% decrease in caffeine-induced Ca^{2+} release (Fig. 4 C, D). ABT-199 did not impact the ability of Bcl-2 to inhibit caffeine-induced RyR-mediated Ca^{2+} release. It should be noted that ABT-199 slightly and non-significantly reduced caffeine-induced Ca^{2+} release in control conditions, which may suggest a minor direct impact of these drugs on RyRs.

The data obtained with the ABT-199 compound support the results using the Bcl-2 hydrophobic cleft mutant and suggest that the ABT-199 BH3 mimetics do not alter Bcl-2's ability to inhibit RyRs thereby decreasing the risk of potential side effects of these drugs.

In conclusion, disrupting the binding between the hydrophobic cleft of Bcl-2 and the pro-apoptotic family members, does not alter Bcl-2-mediated regulation of the RyR.

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References

[1] S. Ahmad, A. Ahmad, E.S. Dremina, V.S. Sharov, et al., Bcl-2 suppresses sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase expression in cystic fibrosis airways: role in oxidant-mediated cell death, Am. J. Respir. Crit. Care Med. 179 (2009) 816-26.

[2] N. Arbel, V. Shoshan-Barmatz, Voltage-dependent anion channel 1-based peptides interact with Bcl-2 to prevent antiapoptotic activity, J. Biol. Chem. 285 (2010) 6053-62.

[3] P.E. Ferdek, J.V. Gerasimenko, S. Peng, A.V. Tepikin, et al., A novel role for Bcl-2 in regulation of cellular calcium extrusion, Curr. Biol. 22 (2012) 1241-6.

[4] Y.P. Rong, A.S. Aromolaran, G. Bultynck, F. Zhong, et al., Targeting Bcl-2-IP_3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals, Mol. Cell 31 (2008) 255-65.

[5] A.G. Letai, Diagnosing and exploiting cancer's addiction to blocks in apoptosis, Nat Rev Cancer 8 (2008) 121-32.

[6] L.A. Barclay, T.E. Wales, T.P. Garner, F. Wachter, et al., Inhibition of Pro-Apoptotic BAX by a Noncanonical Interaction Mechanism, Mol Cell 57 (2015) 873-86.

[7] Y.P. Rong, G. Bultynck, A.S. Aromolaran, F. Zhong, et al., The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP_3 receptor, Proc Natl Acad Sci U S A 106 (2009) 14397-402.

[8] T. Vervliet, E. Decrock, J. Molgo, V. Sorrentino, et al., Bcl-2 binds to and inhibits ryanodine receptors, J. Cell Sci. 127 (2014) 2782-92.

[9] G. Monaco, E. Decrock, H. Akl, R. Ponsaerts, et al., Selective regulation of IP_3-receptor-mediated Ca^{2+} signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-Xl, Cell Death Differ. 19 (2012) 295-309.

[10] T. Vervliet, I. Lemmens, E. Vandermarlieren, E. Decrock, et al., Ryanodine receptors are targeted by Bcl-X_L involving the BH4 domain and Lys87 from its BH3 domain, Sci. Rep. in press (2015).

[11] J. Gerasimenko, P. Ferdek, L. Fischer, A.S. Gukovskaya, et al., Inhibitors of Bcl-2 protein family deplete ER Ca^{2+} stores in pancreatic acinar cells, Pflugers Arch 460 (2010) 891-900.

[12] J.V. Gerasimenko, M. Sherwood, A.V. Tepikin, O.H. Petersen, et al., NAADP, cADPR and IP_3 all release Ca^{2+} from the endoplasmic reticulum and an acidic store in the secretory granule area, J Cell Sci 119 (2006) 226-38.

[13] H. Akl, I. Vandecaetsbeek, G. Monaco, A. Kauskot, et al., HA14-1, but not the BH3 mimetic ABT-737, causes Ca2+ dysregulation in platelets and human cell lines, Haematologica 98 (2013) e49-51.

[14] D. Hermanson, S.N. Addo, A.A. Bajer, J.S. Marchant, et al., Dual mechanisms of sHA 14-1 in inducing cell death through endoplasmic reticulum and mitochondria, Mol Pharmacol 76 (2009) 667-78.

[15] J.H. Kim, S.R. Lee, L.H. Li, H.J. Park, et al., High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice, PLoS One 6 (2011) e18556.
[16] I. Lemmens, S. Eyckerman, L. Zabeau, D. Catteeuw, et al., Heteromeric MAPPIT: a novel strategy to study modification-dependent protein-protein interactions in mammalian cells, Nucleic Acids Res 31 (2003) e75.
[17] G.U. Gurudutta, Y.K. Verma, V.K. Singh, P. Gupta, et al., Structural conservation of residues in BH1 and BH2 domains of Bcl-2 family proteins, FEBS Lett 579 (2005) 3503-7.
[18] A.J. Souers, J.D. Leverson, E.R. Boghaert, S.L. Ackler, et al., ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets, Nat Med 19 (2013) 202-8.
[19] S. Lievens, F. Peelman, K. De Bosscher, I. Lemmens, et al., MAPPIT: a protein interaction toolbox built on insights in cytokine receptor signaling, Cytokine Growth Factor Rev 22 (2011) 321-9.
[20] E. Provost, J. Rhee, S.D. Leach, Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos, Genesis 45 (2007) 625-9.

Figure legends

Figure 1: Mutational disruption of the hydrophobic cleft of Bcl-2 does not disrupt Bcl-2/RyR complexes.

(A) Immunoblot of FLAG pull-downs performed on lysates from HEK RyR3 cells transiently overexpressing the empty vector (pCMV24), 3XFLAG-Bcl-2 or the 3XFLAG-Bcl-$2^{GR/AA}$ mutant. The FLAG-tagged proteins were pulled down using agarose coupled to a FLAG-recognizing antibody. The empty vector (pCMV24) was used as negative control. Immunoblots were stained with an anti-FLAG-HRP-conjugated antibody (top) or an anti-Bax antibody (bottom). All experiments were performed independently at least three times. (B) Immunoblot of the performed co-immunoprecipitation experiments using HEK RyR3 lysates overexpressing 3XFLAG-Bcl-2 or the 3XFLAG-Bcl-$2^{GR/AA}$ mutant. RyR3 was immunoprecipitated using a RyR antibody recognizing all RyR isoforms (top). Co-immunoprecipitations with non-specific IgG antibodies were used for every condition as negative control. The presence of the co-immunoprecipitated FLAG-tagged proteins was assessed with an anti-FLAG HRP-conjugated antibody (bottom). The experiments were performed independently at least three times. For clarity reasons, a longer exposure of the input sample lanes obtained from the same gel/blot is shown.

Figure 2: Mutational disruption of the hydrophobic cleft of Bcl-2 does not alter Bcl-2-mediated inhibition of RyR-mediated Ca^{2+} release.

(A) Average calibrated trace (15-20 cells) of a typical single-cell Ca^{2+} measurement utilizing Fura-2AM as Ca^{2+} indicator. The empty vector (pCMV24), 3XFLAG Bcl-2 or 3XFLAG-Bcl$2^{GR/AA}$ was cotransfected with mCherry (3:1 ratio) to identify transfected cells. One minute after chelating extracellular Ca^{2+} with BAPTA (3 mM), caffeine was added (1.5 mM) as indicated by the arrows. (B) Summary of the performed single-cell Ca^{2+} measurement experiments. The data points indicate the average caffeine-induced Ca^{2+} release ($[Ca^{2+}]_{peak}$-$[Ca^{2+}]_{before\ caffeine}$) of all traces performed on the same day. All experiments were repeated three times independently and experiments performed on the same

day are indicated by the same symbol in the same color (N>80 cells/condition). The average ±S.E.M. is indicated in gray (p=0.0091).

Figure 3: The BH3 mimetic ABT-199 does not disrupt Bcl-2/RyR complexes.

(A) A representative example of the performed MAPPIT experiments. The binding is shown as fold induction value, calculated by dividing the average luciferase activity of Epo-stimulated cells by the average of non-stimulated cells. Binding of the positive control or of Bad to Bcl-2 is shown in the presence of increasing concentrations of ABT-199. (B) Immunoblot of the performed co-immunoprecipitation experiments in HEK RyR3 cells overexpressing 3XFLAG Bcl-2 as performed in Figure 1B (top). ABT-199 (3 µM) or an equal volume of DMSO was included during the overnight incubation step. Quantification of the performed experiments. Non-specific 3XFLAG-Bcl-2 binding to the corresponding negative control (IgG) was subtracted from the amount of 3XFLAG-Bcl-2 co-immunoprecipitated with RyR3 and expressed relative to the DMSO control. Values depict average ± SD. Experiments were repeated three times independently (bottom).

Figure 4: The BH3 mimetic ABT-199 does not alter Bcl-2-mediated inhibition of RyR-mediated Ca^{2+} release.

(A) Immunoblot stained with anti-FLAG HRP-conjugated antibody of HEK RyR3 lysates transiently transfected with 3XFLAG-Bcl-2 or 3XFLAG-Bcl-2-P2A-mCherry. The * indicates a byproduct of the P2A cleavage process also recognized by the anti-FLAG antibody. (B) Fluorescence and bright field overlay image, comparing the mCherry signal after transfection with 3XFLAG-Bcl-2 at 3:1 ratio with mCherry (top) or the 3XFLAG-Bcl-2-P2A-mCherry construct (bottom). (C) Average calibrated trace (15-20 cells) of single cell Ca^{2+} measurements, performed as in Figure 2, in HEK RyR3 cells transfected with the pCMV24 containing P2A-mCherry (control) or the 3XFLAG-Bcl-2-P2A-mCherry construct. ABT-199 (3 µM) or an equal volume of DMSO was added to the cells during the loading and de-esterification (one hour in total) of Fura2-AM. (D) Summary of the performed single-cell Ca^{2+} measurements. Experiments performed during the same day are indicated by the same symbol and color. The data points indicate average caffeine-induced Ca^{2+} release ($[Ca^{2+}]_{peak}$-$[Ca^{2+}]_{before}$

385 $_{caffeine}$) of all traces performed on the same day. All experiments were performed three times

386 independently (N>80 cells/condition). The average ±S.E.M. is indicated in gray (p=0.0015).

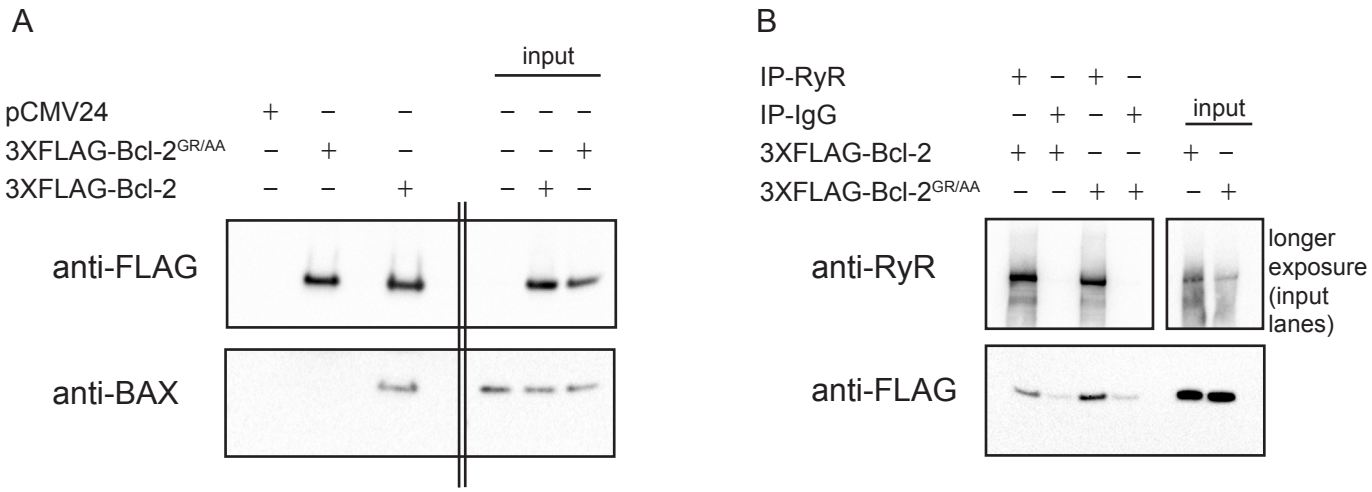
A
input
pCMV24 + − − − − −
3XFLAG-Bcl-2GR/AA − + − − − +
3XFLAG-Bcl-2 − − + − + −
anti-FLAG
anti-BAX
B
IP-RyR + − + −
IP-IgG − + − +
3XFLAG-Bcl-2 + + − − + −
3XFLAG-Bcl-2GR/AA − − + + − +
input
anti-RyR
longer exposure (input lanes)
anti-FLAG

Figure 1

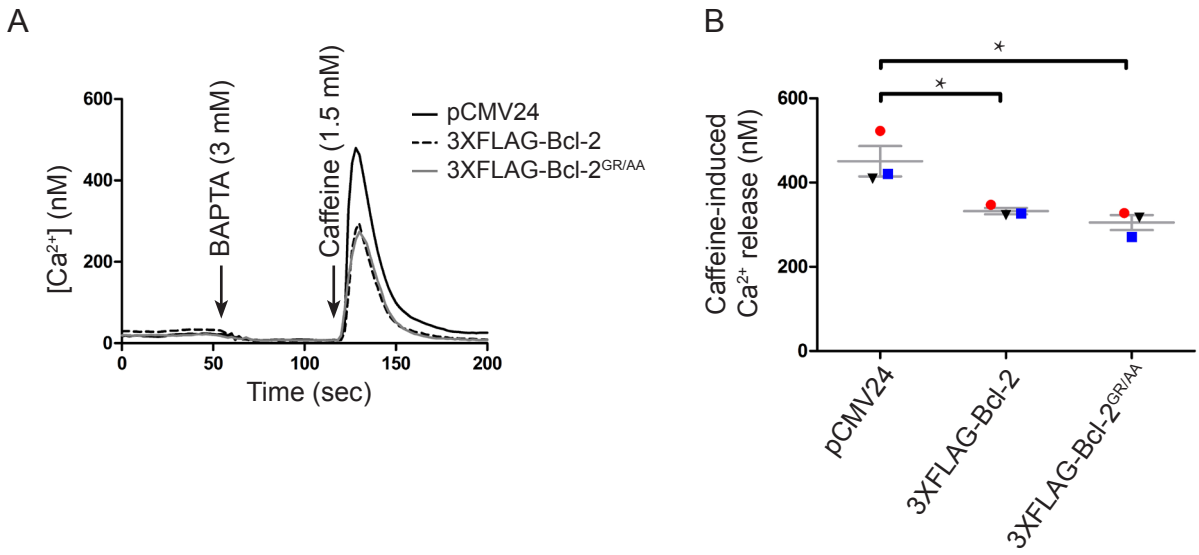
A
B
[Ca2+] (nM)
600
400
200
0
0
50
100
150
200
Time (sec)
BAPTA (3 mM)
Caffeine (1.5 mM)
pCMV24
3XFLAG-Bcl-2
3XFLAG-Bcl-2GR/AA
Caffeine-induced Ca2+ release (nM)
*
*
pCMV24
3XFLAG-Bcl-2
3XFLAG-Bcl-2GR/AA

Figure 2

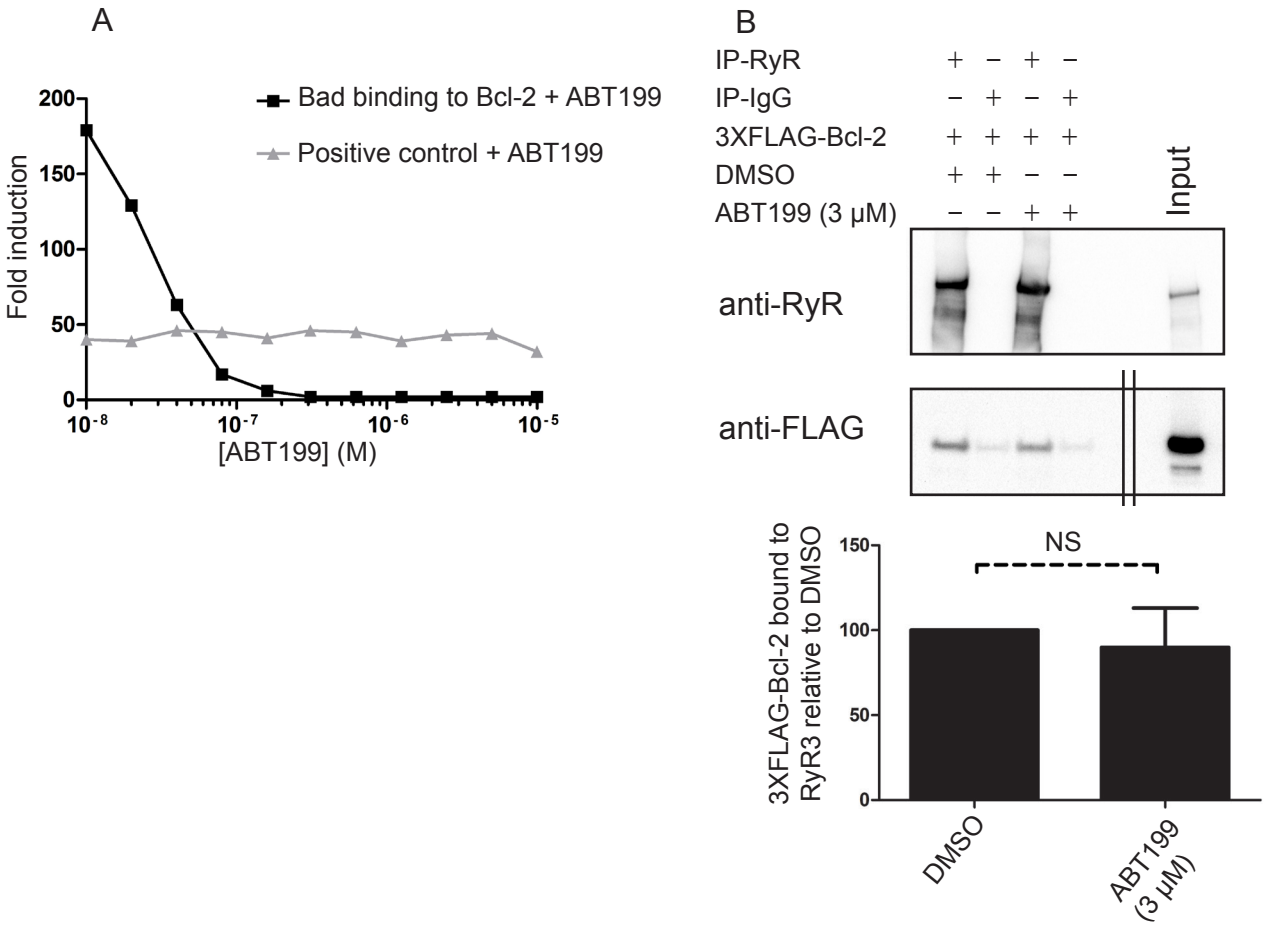
A
Bad binding to Bcl-2 + ABT199
Positive control + ABT199
Fold induction
200
150
100
50
0
10-8
10-7
10-6
10-5
[ABT199] (M)
B
IP-RyR + − + −
IP-IgG − + − +
3XFLAG-Bcl-2 + + + +
DMSO + + − −
ABT199 (3 μM) − − + +
Input
anti-RyR
anti-FLAG
3XFLAG-Bcl-2 bound to RyR3 relative to DMSO
NS
150
100
50
0
DMSO
ABT199 (3 μM)

Figure 3

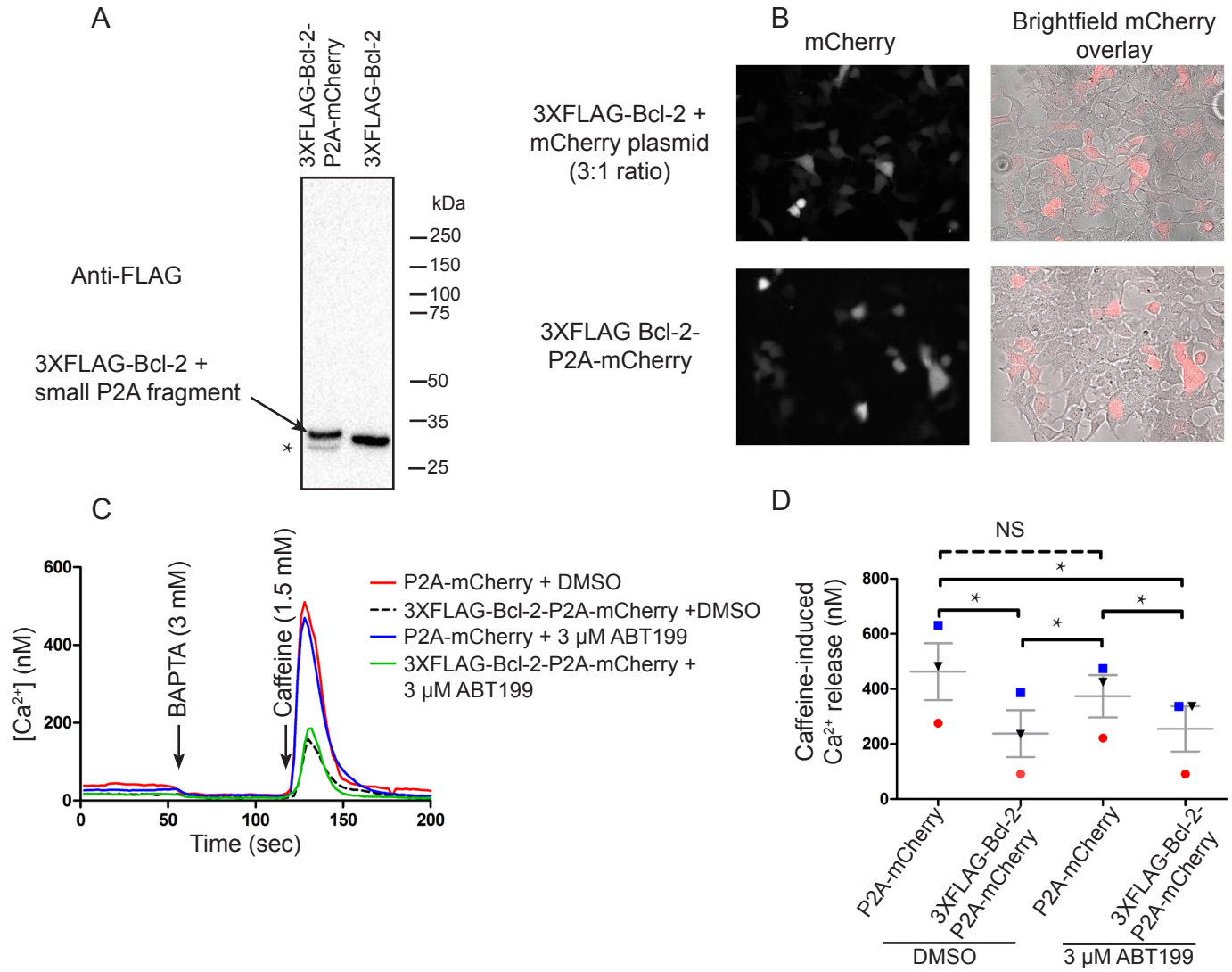
A
3XFLAG-Bcl-2-P2A-mCherry
3XFLAG-Bcl-2
kDa
250
150
100
75
50
35
25
Anti-FLAG
3XFLAG-Bcl-2 + small P2A fragment
*
B
mCherry
Brightfield mCherry overlay
3XFLAG-Bcl-2 + mCherry plasmid (3:1 ratio)
3XFLAG Bcl-2-P2A-mCherry
C
[Ca2+] (nM)
600
400
200
0
BAPTA (3 mM)
Caffeine (1.5 mM)
0
50
100
150
200
Time (sec)
P2A-mCherry + DMSO
3XFLAG-Bcl-2-P2A-mCherry +DMSO
P2A-mCherry + 3 μM ABT199
3XFLAG-Bcl-2-P2A-mCherry + 3 μM ABT199
D
Caffeine-induced Ca2+ release (nM)
800
600
400
200
0
NS
*
*
*
*
P2A-mCherry
3XFLAG-Bcl-2-P2A-mCherry
P2A-mCherry
3XFLAG-Bcl-2-P2A-mCherry
DMSO
3 μM ABT199

Figure 4