Inhibiting anti-apoptotic Bcl-2 in diffuse large B-cell lymphomas to provoke pro-apoptotic Ca^{2+} signaling

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ABSTRACT

Some diffuse large B-cell cancers are sensitized to programmed cell death at the endoplasmic reticulum by upregulation of the IP_3R2, an inositol 1,4,5-trisphosphate-dependent Ca^{2+}-release channel. These cancers express the anti-apoptotic protein Bcl-2 in abundance to prevent pro-apoptotic IP_3R2-mediated Ca^{2+} signaling. BIRD-2 (Bcl-2/IP_3R Disrupter-2) is a newly developed peptide that disrupts the Bcl-2/IP_3R complex, provoking toxic Ca^{2+} signaling. However, the exact working mechanism of BIRD-2 is unknown. We report a possible role for the mitochondria as downstream effectors of BIRD-2-induced cell death and validated a calcein-based staining to assess opening of the mitochondrial permeability transition pore in response to BIRD-2.

Keywords
Apoptosis, DLBCL, Ca^{2+} signaling, mPTP opening

INTRODUCTION

Apoptosis is a form of programmed cell death that plays a crucial role in development and homeostasis (1). It is executed by the activation of cysteine-containing aspartate specific proteases or caspases. This results in DNA fragmentation, cell shrinkage and non-inflammatory cell death (1).

Apoptosis is a mitochondrial process that is tightly regulated by the B-cell lymphoma-2 (Bcl-2)-protein family. At the mitochondria, anti-apoptotic Bcl-2 antagonizes pro-apoptotic B-cell homology (BH) 3-only members of the Bcl-2-protein family like Bim and pro-apoptotic executioners Bax and Bak via its BH1, -2 and -3 domains. This prevents cytochrome *c* release from the mitochondria, which causes initiation of apoptosis (2).

Bcl-2 expression is increased in several cancers like chronic lymphocytic leukemia (CLL) and diffuse large B-cell lymphomas (DLBCL) (3). These cancers depend on this mechanism to prevent apoptosis via the pro-apoptotic BH3-only proteins like Bim that show increased expression levels due to oncogenic stress; a state that was aptly coined "primed-to-death" by Letai, et al (2).

In addition to this, Bcl-2 was found to act at another cellular compartment: the endoplasmic reticulum (ER), which functions as the main intracellular Ca^{2+}-storage compartment (4). Large differences exist between the $[Ca^{2+}]_{cytosol}$ (~100 nM) and $[Ca^{2+}]_{ER}$ (~500 µM). This allows the cell to use Ca^{2+} fluxes as a signal that can be decoded in various subcellular compartments. One of the channels responsible for the release of Ca^{2+} from the ER is the inositol (1,4,5)-trisphosphate (IP_3) receptor (IP_3R), which opens in response to IP_3, a second messenger (5). IP_3Rs are encoded by 3 genes, yielding the expression of 3 isoforms (6).

A subpopulation of the IP_3R can be found in domains that link the ER and the mitochondria: mitochondria-associated membranes (MAMs). This allows for efficient Ca^{2+} signaling between the ER and the mitochondrial matrix. The different transport proteins involved in this ER-mitochondrial Ca^{2+} transport are displayed in Figure 1 (5).

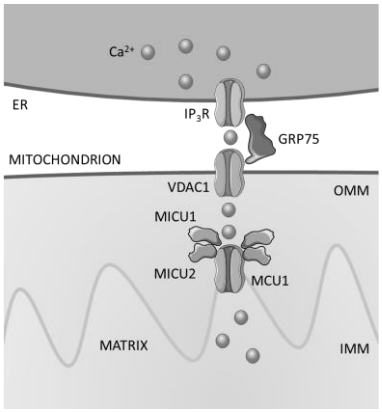

Figure 1: Schematic representation of the proteins involved in ER-mitochondrial Ca^{2+} transport. The IP_3R, a tetrameric, intracellular Ca^{2+} -release channel in the ER membrane is connected to the Ca^{2+}-permeable voltage dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane (OMM) via the chaperone glucose-regulated protein 75 (GRP75). Ca^{2+} readily passes the OMM via VDAC1. Subsequently, the mitochondrial Ca^{2+} uniporter (MCU) in the inner mitochondrial membrane (IMM) allows Ca^{2+} to pass to the mitochondrial matrix. MCU activity is regulated by the mitochondrial Ca^{2+} uptake (MICU) 1 and MICU2 (5,7)**.**

Bcl-2-dependent cancers can be "primed-to-death" at the ER. This "priming" is due to elevated expression levels of a specific IP_3R isoform: IP_3R2 (8). This isoform is most sensitive to IP_3 and the upregulation of this receptor is believed to cause toxic Ca^{2+} signaling that leads to apoptosis. However, pro-apoptotic Ca^{2+} signaling is

prevented by Bcl-2 forming a complex with the IP_3R2 via its BH4 domain (8).

A recently developed cell-permeable peptide, Bcl-2/IP_3R Disrupter-2 (BIRD-2), targets the BH4 domain of Bcl-2 and disrupts the formation of IP_3R/Bcl-2 complexes, thereby boosting pro-apoptotic Ca^{2+}-signaling events (e.g. in Jurkat cells exposed to strong T-cell receptor activation). The apoptotic properties of BIRD-2 have also been exploited to kill primary CLL cells. Interestingly, naïve Jurkat cells or peripheral mononuclear blood cells derived from healthy donors were resistant to BIRD-2 exposure (9).

However, the exact working mechanism of BIRD-2 remains elusive. Ca^{2+} overload in the mitochondria has been shown to trigger the opening of the mitochondrial permeability transition pore (mPTP), a pore that permeabilizes the IMM (10). This, in turn, leads to osmotic swelling of the mitochondria, release of pro-apoptotic factors like cytochrome *c* and cell death (11). In this study, evidence is strengthened that different DLBCL cell lines display different sensitivity towards BIRD-2-induced apoptosis. Furthermore, we suggest the involvement of the mitochondria as downstream effectors of BIRD-2 and have validated a calcein-AM/$CoCl_2$ staining to investigate the opening of the mPTP.

MATERIALS AND METHODS

Cell culture

Human DLBCL cell lines (SU-DHL-4 and OCI-LY-1) were acquired from Dr. A. Letai from the Dana-Farber Cancer Institute in Boston, USA. Culture conditions were described previously (8).

Cell lysis and western blotting

Cell lysis and western blot analysis were performed as previously described (12). Instead of MES/SDS running buffer, MOPS running buffer was used. Primary antibodies that were used can be found in Table 1.

Table 1: Primary antibodies for western blotting.

Antibody	Company	Catalogue number
VDAC1	Cell signaling technology	#4866
GRP75	Abcam	#ab82591
IP_3R2	Produced in own lab	/
MCU1	Sigma-Aldrich	#HPA016480
β-actin	Sigma-Aldrich	#A5441

Caspase-3 activity assay

Cells (500 000/mL) were loaded with 10 µM of BIRD-2 and TAT-control (TAT-CTRL). Staurosporine (STS; 2 µM) was used as a positive control. After a 3 h treatment, cells were washed and resuspended in Dulbecco's phosphate buffered saline (DPBS) with 5 µM of NucView™ 488 Caspase-3 substrate (Gentaur, Kampenhout, Belgium). After 30 min incubation, fluorescence was assessed by fluorescence microscopy (inverted Zeiss Axio Observer Z1 (Zeiss, Jena, Germany)).

Cytosolic Ca^{2+} imaging via fluorescence microscopy

Cells (500 000/mL) were treated with Fura2-acetoxymethyl (AM) ester as described previously (8). Live imaging was performed via an inverted Zeiss Axio Observer Z1 (Zeiss, Jena, Germany) fluorescence microscope equipped with a 40× oil immersion objective and AxioCam HSm camera (Zeiss, Jena, Germany).

Calcein-AM/$CoCl_2$ staining

Cells (500 000/mL) were washed with Hank's balanced salt solution (HBSS). Then the cells were resuspended in HBSS (supplemented with Ca^{2+} and Mg^{2+}) with 20 nM calcein-AM with or without $CoCl_2$ (100 µM) at a density of 1 000 000/mL. After 15 min of incubation at 37 °C in the dark, fluorescence was assessed by confocal microscopy (Zeiss LSM510 confocal microscope, 63× lens, Jena, Germany) in presence or absence of ionomycin. Reagents for this assay were taken from the Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit (Life Technologies, Ghent, Belgium).

Reagents

BIRD2:RKKRRQRRRGGNVYTEIKCNSLLPLAAIVR-V and TAT-CTRL: RKKRRQRRRGGSI-ELDDPRPR were purchased from LifeTein (South Plainfield, New Jersey, USA) (purity > 85%). Fura2-AM (Biotium, Kampenhout, Belgium), Staurosporine (LC Laboratories, Kampenhout, Belgium).

RESULTS

As previously indicated, Bcl-2-dependent cancers display distinct mechanisms for the evasion of apoptosis (8,9). Therefore, we compared BIRD-2-induced caspase-3 activity in two DLBCL cell lines: SU-DHL-4 and OCI-LY-1. Cells were treated with TAT-CTRL, as a negative control, BIRD-2 and STS, as a positive control. Figure 2 shows typical fluorescent images in SU-DHL-4 and OCI-LY-1 cells stained with the NucView™ 488 Caspase-3 substrate, which becomes fluorescent upon caspase-3 activaton. The bright field images in Figure 2 display the complete cell population.

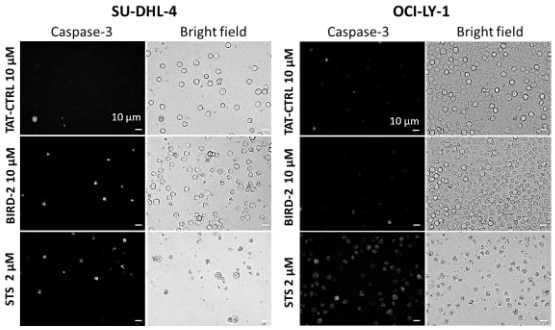

Figure 2: BIRD-2 induced caspase-3 activity in SU-DHL-4 but not in OCI-LY-1 cells. NucView™ 488 Caspase-3 substrate was used and fluorescence analysis was performed by fluorescence microscopy at the single-cell level. Bright field images show the complete cell population. In SU-DHL-4 cells, BIRD-2 or STS treatment led to an increase in number of caspase-3-positive cells, while in OCI-LY-1 cells, only STS exposure, but not BIRD-2 exposure, resulted in an increased number of caspase-3-positive cells.

These data indicate that in SU-DHL-4 cells caspase-3 activation occurs upon BIRD-2 or STS addition. Yet, in OCI-LY-1 cells, BIRD-2 did not trigger caspase-3 activaton. This was not due to a general block in apoptotic signaling in OCI-LY-1, since STS exposure could result in caspase-3 activation in these cells. TAT-CTRL did not trigger cell death in either cell line. These data support the concept that SU-DHL-4 cells are "addicted" to IP_3R/Bcl-2 complexes for their survival, while OCI-LY-1 cells are not.

To investigate if these differences could be seen at the level of Ca^{2+} signaling, single-cell Ca^{2+} live imaging experiments were performed. Cells were loaded with Fura2-AM, which is a ratiometric probe for intracellular Ca^{2+}. The ratio of emitted fluorescence at 340 and 380 nm is directly proportional to the cytosolic Ca^{2+} concentration. Figure 3 shows Fura2 fluorescence in SU-DHL-4 and OCI-LY-1 cells before and after 600 sec BIRD-2 treatment. BIRD-2 triggered an increase in $[Ca^{2+}]_{cytosol}$ in SU-DHL-4 cells, whereas in OCI-LY-1 cells no such event was witnessed.

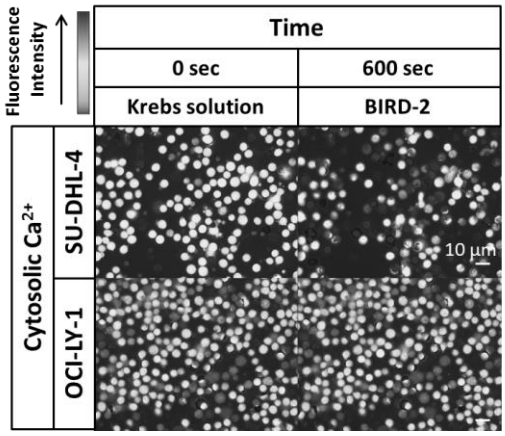

Figure 3: BIRD-2 increased cytosolic Ca^{2+} in SU-DHL-4 but not in OCI-LY-1 cells. Representative images by fluorescence microscopy of Fura2 fluorescence in SU-DHL-4 and OCI-LY-1 cells taken before BIRD-2 addition and 600 sec after BIRD-2 addition.

Considering the importance of Ca^{2+} signaling for the working mechanism of BIRD-2 and the known role for mitochondria in apoptosis and as targets of Ca^{2+} signaling (5,8), the expression levels of proteins that are involved in Ca^{2+} transport to the mitochondrial matrix were assessed in SU-DHL-4 and OCI-LY-1 cells to detect potential differences. In Figure 4, a typical western blot of IP_3R2, GRP75, VDAC1 and MCU1 is represented. β-actin was used as a loading control. The IP_3R2 seemed to display higher expression levels in SU-DHL-4 than in OCI-LY-1 cells. Furthermore, MCU1 expression appeared to be higher in OCI-LY-1 cells compared to SU-DHL-4 cells, while VDAC1 levels appeared to be higher in SU-DHL-4 compared to OCI-LY-1 cells. The expression levels of GRP75 seemed to be very similar between SU-DHL-4 and OCI-LY-1 cells. The high difference in IP_3R2 expression levels, underpin previous observations of Akl, et al (8). Further quantitative analysis of the expression levels of these proteins in independent experiments ought to be performed to allow statistical analysis. In addition, the expression levels of MCU1 regulators ought to be assessed. Also, the functional implications of altered VDAC1/MCU1 expressions between SU-DHL-4 and OCI-LY-1 cells must be investigated. In any case, VDAC1 has an important role in ER-mitochondrial Ca^{2+} transport and apoptosis, and thus, these findings might point to the mitochondria as effectors of BIRD-2-induced Ca^{2+} signaling (13,14).

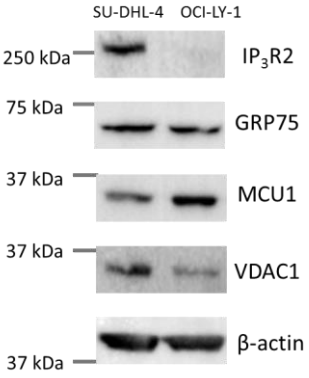

Figure 4: A typical immunoblot showing the protein-expression levels of IP_3R2, GRP75, MCU1 and VDAC1. β-actin was used a loading control. All samples were obtained from the same SDS-PAGE gel.

Since Ca^{2+} overload in the mitochondrial matrix is known to induce opening of the mPTP, which leads to apoptosis, a calcein-AM/$CoCl_2$ staining was validated to assess mPTP opening (10). Calcein-AM is a fluorescent probe that easily penetrates the cell membrane and the membranes of intracellular organelles. In this way it accumulates in the mitochondria. After introduction to the cell, esterases hydrolyse the acetoxymethyl ester, which traps calcein for example in the mitochondrial matrix, since it is too polar to cross the membrane again. To obtain the mitochondria-specific calcein signal, $CoCl_2$ is added to the cells. $CoCl_2$ quenches calcein fluorescence, but cannot reach the mitochondrial matrix due to the selective permeability of the IMM. Hence, in healthy cells, the fluorescent signal of cytosolic calcein is quenched by $CoCl_2$, yielding only the fluorescent signal of the calcein trapped inside the mitochondria. However, when a cytotoxic stimulus that opens the mPTP is added, $CoCl_2$ readily enters the mitochondrial matrix and quenches the mitochondrial calcein fluorescence.

We validated a method to stain SU-DHL-4 cells with calcein-AM/$CoCl_2$ via confocal microscopy.

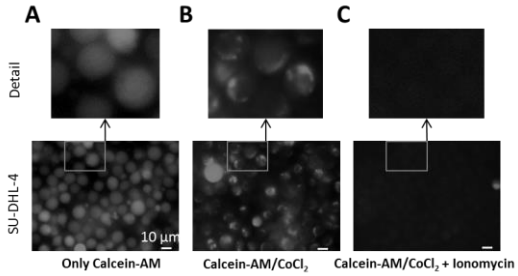

Figure 5: Validation of calcein-AM/$CoCl_2$ staining in SU-DHL-4 cells by confocal microscopy. (A) Calcein fluorescence before $CoCl_2$ quenching. (B) Calcein fluorescence was rendered mitochondria-specific by the addition of $CoCl_2$. (C) Addition of ionomycin permeabilizes the IMM to $CoCl_2$, followed by quenching of the mitochondrial calcein fluorescence.

Figure 5.A shows calcein fluorescence without $CoCl_2$ quenching. $CoCl_2$ renders the calcein fluorescence mitochondria-specific (Figure 5.B). As a proof of concept, ionomycin, a Ca^{2+} ionophore, previously established to open the mPTP, was added (15). Figure 5.C shows that the addition of ionomycin abolishes mitochondrial calcein fluorescence. The validation of this method allows for a qualitative as well as a quantitative approach to mPTP opening in SU-DHL-4 in response to BIRD-2. This sets the stage for identifying downstream effects of BIRD-2 treatment at the level of the mitochondria.

CONCLUSION

The working mechanism of BIRD-2 requires further study. In this paper, we highlighted the existence of distinct functions of Bcl-2 at different subcellular compartments in "Bcl-2-addicted" cancers via a caspase-3 activity assay in response to BIRD-2. Furthermore, Ca^{2+} signaling was shown to be an integral part of the working mechanism of BIRD-2. The differences between BIRD-2-sensitive and -resistant cell lines considering protein expression levels in ER-Ca^{2+} transport, in particular of VDAC1 and IP_3R2, may hint towards the involvement of the mitochondria as downstream effectors of BIRD-2. One of these effects could be the opening of the mPTP opening. To assess this event, we validated a calcein-AM/$CoCl_2$ staining assay in DLBCL cancer cells. This sets the stage for further research towards the role of the mitochondria in BIRD-2-induced cell death.

ROLE OF THE STUDENT

The experiments were performed in early 2015 by the student after a training period by Dr. Rita La Rovere to acquaint the different experimental procedures. At regular times, discussions were held with Prof. Geert Bultynck, Dr. Rita La Rovere and other group members of the Laboratory of Molecular and Cellular Signaling about the acquired data and future perspectives, in which the student actively participated and contributed.

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