Cell death induction by BIRO1

BIRO-1, a Cell Permeable BH3 Peptide, Promotes Mitochondrial Fragmentation and Death of Retinoblastoma Cells

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Conflict of Interest
The authors confirm that the findings described in this manuscript have no conflict of interest.
Cell death induction by BIRO1

Abstract

Retinoblastoma is the most common pediatric intraocular neoplasm. While retinoblastoma development requires the inactivation of both alleles of the RB tumor-suppressor gene (RB1) in the developing retina, additional genomic changes are involved in tumor progression, which progressively lead to resistance of tumor cells to death. Therapeutics acting at very downstream levels of death signaling pathways should therefore be interesting in killing retinoblastoma cells. The BH3 only proteins promote apoptosis by modulating the interaction between the pro- and anti-apoptotic members of the BCL-2 protein family, and this effect can be recapitulated by the BH3 domains. This report analyzes the effect of various BH3 peptides, corresponding to different BH3 only proteins, on two retinoblastoma cell lines, Y79 and WERI-Rb, as well as on the photoreceptor cell line 661W. The BH3 peptide BIRO-1, derived from the Bim death domain, was very effective in promoting Y79 and WERI-Rb cell death without affecting the 661W photoreceptor cells. This cell death was efficient even in absence of Bax and was shown to be caspase-independent. While ROS production or AIF release was not detected from mitochondria of treated cells; BIRO-1 initiated mitochondria fragmentation in a short period of time following treatment.

Implications: The BIRO-1 peptide is highly effective at killing retinoblastoma cells and has potential as a peptidomimetic.
Introduction

Retinoblastoma is a malignant tumor of the developing retina that manifests in early childhood, affecting 1 in 15,000 live births. Retinoblastoma is initiated by inactivation of both alleles of the \textit{RB1} tumor suppressor gene in the developing retina (1). Loss of both alleles appears however to be insufficient to initiate tumor formation, and further genomic changes may drive to highly proliferative retinoblastoma, exhibiting altered gene copy numbers and modulated expression of oncogenes (MYCN, E2F3, DEK, KIF14 and MDM4) and tumor suppressor genes (CDH11, p75NTR) (2-5).

Much progress has been done in the treatment of retinoblastoma and the survival rate in developed countries is over 95% with appropriate treatment, i.e. through chemotherapy with adjuvant local treatments. The incidence of metastatic disease is low. Unfortunately, when retinoblastoma is detected at later stages of the disease, especially in children representing the first case in a family or in developing countries, the prognosis is poor for visual function and even for survival, as only 50% of the children survive (6). In these situations, aggressive treatments, including enucleation of the most affected eye, radio/brachy/cryotherapy and sometimes systemic or local chemotherapy are involved. These treatments, in addition to having a poor efficiency, have major drawbacks in pediatric patients, such as bone marrow suppression, second cancer occurrence, cataracts and retinopathy. Therefore finding safer and more efficient treatments remains a major challenge.

During retinoblastoma spreading, \textit{RB1} inactivation is followed by additional genomic modifications which progressively lead to resistance of tumor cells to death. To counteract any tumoral modification and death resistance, drugs that act at downstream levels of death signaling pathways should be effective in killing retinoblastoma cells. The emerging
small anticancer drugs which target the anti-apoptotic members of the mitochondrial machinery offer a possibility to overcome the death resistance of cancer cells. Mitochondria is a key downstream element in the different programmed cell death pathways, i.e., apoptosis, autophagy and necrosis. Mitochondrial integrity is under the control of the proteins of the BCL-2 family (7). These proteins function as a life/death switch that integrates diverse inter- and intracellular events to determine whether the apoptotic pathway should be activated (8). The switch operates through the interactions between pro- and anti-apoptotic members of the family. The pro-survival family members (BCL-2, BCL-XL, BCL-W, MCL-1, A1) are critical for cell survival, since loss of any of them causes cell death in certain cell types (9). Consistently, BCL-2 overexpression induces drug resistance in various tumors cell types. The pro-apoptotic BCL-2 family members are divided into two classes: the BAX-like proteins (BAX, BAK and BOK) and the BH3-only proteins (BIM, BID, PUMA, NOXA, BMF, BAD, HRK and BIK) (10-11). Structural studies have revealed that the 20 amino acids BH3 domain of the BH3-only proteins is able to interact with specific residues of the BH1, BH2 and BH4 domains of the pro-survival members (9), leading to their neutralization. Relating to BAX and BAK, they have been shown to be essential to the pro-apoptotic function of BH3-only proteins (12). The lack of both Bax and Bak induces perinatal lethality in mice, while cells knockdown for both these proteins are resistant to overexpression of BH3-only proteins as well as to various stimuli known to activate the intrinsic apoptotic pathway.

Manipulating the balance between the pro- and anti-apoptotic BCL-2 family members presents a great opportunity to restore apoptosis in cancer cells (13-21). BH3-peptide-based approach utilizes the prodeath BH3 minimal death domains to re-establish mitochondrial sensitivity in tumoral cells (13-14).
Cell death induction by BIRO1

domains can retain an α-helical structure (14) and are able to recapitulate pro-apoptotic
effects. In cell-free assays consisting of isolated mitochondria, BH3 peptides disrupt
complexes formed between pro-apoptotic and anti-apoptotic BCL-2 family proteins (22),
induce oligomerization of BAX and BAK followed by mitochondrial membrane
permeabilization, and release of cytochrome c. These effects could be abolished by using
BAK−/− mitochondria or by BCL-2 overexpression (22). BH3 peptides delivered into tumor
cells have been shown to engage apoptosis through BAX activation and cytochrome c
release (18, 23). Therefore, BH3 peptides can provide molecular targeting of anti-apoptotic
members of BCL-2 protein family and potentially improve traditional therapy of cancer.

In this report, we studied the death potential of different BH3 derived peptides on two
human retinoblastoma cell lines, Y79 and WERI-Rb, as well as on primary mouse
retinoblastoma. A massive cell death was promoted by the BH3 peptide from BIM, through
a mechanism that is caspase-independent, and that has necrotic characteristic.

**Material and Methods**

**Chemicals**

The different BH3 peptides were synthetized by NeoMPS (Strasbourg, France) and Auspep
(Parkville, Australia). zVAD was purchased from Promega, 3-MA and Necrostatin-1 were
from Calbiochem, and butylated hydroxyanisole (BHA) was from Sigma-Aldrich. The primary
antibodies used for western blotting, immunoprecipitation and immunofluorescence
experiments were as followed, Bax (Santa-Cruz sc-493), Bax conf (BD Pharmingen, 556467),
Bak (Cell Signaling, 3814), Bcl-2 (Cell Signaling, 2876), Bcl-XL (Cell Signaling, 2764), Bim (Cell
Signaling, 2819), Bid (Cell Signaling, 2002), Mcl-1 (Cell Signaling, 4572), A1 (sc-8351), Noxa
(sc-30209), Bad (sc-942), Puma (Cell signaling 4976), VDAC (Abcam, Ab16816-100) and actin
Cell death induction by BIRO1

(Sigma, A5441), PARP (Santa-Cruz sc-7150), AIF (Santa-Cruz sc-5589), DRP1 (BD Bioscience, 611113), LC3 (Cell Signaling, 2775), PGAM5 (Santa-Cruz sc-161156), Ezrin (sc-20773), OPA1 (BD Bioscience 612606).

WERI-Rb, Y79 and 661W Cell Cultures

All the cell lines were cultured in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 1 mM sodium pyruvate, 2 mM glutamine and 10% fetal calf serum (20% for Y79). 661W cells were generously provided by Dr. M. Al-Ubaidi (University of Oklahoma, Oklahoma City, USA).

Isolation of primary tumoral cells

The SV40-LT transgenic mice developing retinoblastoma are a gift of JM O’Brien (Salk Institute for Biological Studies, La Jolla, California) (24). The eye of sick mice was removed and tumor material was isolated. Primary tumor cells were immediately transferred into RPMI-1640 without FBS, minced, and washed with PBS to remove any residual blood. The washed cells were then cultured in RPMI-1640 containing 10% fetal bovine serum as above.

Cell Viability Assays

For viability assays, cells were seeded at a density of 10,000 cells per well in 96-well plates, incubated overnight in 10% FBS/RPMI (20% for Y79), then treated for varying lengths of time with increasing doses of ABT-737. Following treatment, ATP content was measured using the ATPlite assay, a luminescence ATP detection assay system developed by PerkinElmer using a microplate reader (Bio-Tek Instruments).

Hoechst/PI Staining

Dying cells were discriminated from normal cells by the Hoechst/propidium iodide (PI) technique. Briefly, tumors were incubated with Hoechst 33342 (12.5 μg/mL) and PI (5 μg/mL) for 5 min before visualization under a fluorescence microscope.
FACS Analysis

Cell viability was evaluated by the annexin V/propidium iodide (BD Biosciences) double staining assay following the manufacturer's instructions. Y79 cells were centrifuged at the end of treatment, rinsed twice with PBS, and stained with Annexin V-FITC apoptosis detection kit I (BD Biosciences). Analysis was performed on the FACS Calibur using CellQuest software.

Caspases-7- and -3 activation

Cells were seeded at a density of 10,000 cells per well in 96-well plates, incubated overnight in 10% FBS/RPMI (20% for Y79), then treated for varying lengths of time with increasing doses ABT-737 (1, 3 and 10 μM), in presence or absence of cisplatin and etoposide. Following treatment, caspase-3 and -7 activity was measured using the the Caspase-Glo® 3/7 assay from Promega.

The cleavage of PARP, a downstream substrate of caspase-3, was studied by western blotting experiment using an antibody that recognize the full length protein (105 kDa), as well as the cleaved form (85 kDa form).

Measurement of ROS level

Following exposure to ABT-737, cells were stained with either 10 μM of the dye dihydroethidium (HE, Invitrogen) to measure intracellular superoxide or 10 μM of the dye dihydrodichlorofluorescein-diacetate (H2DCFDA, Invitrogen) in 1 ml media to measure intracellular hydrogen peroxide. Cells were stained for 30 min at 37°C and fluorescence was measured using an EnVision 2103 Multilabel Reader (PerkinElmer).

Whole cell lysates

Cells were washed once in cold Phosphate Buffered Saline (PBS) and recovered by centrifugation. Briefly, cell pellets were dislodged into cold lysis buffer (20mM Tris-acetate
Cell death induction by BIRO1

pH 7.0, 0.27M sucrose, 1mM EDTA, 1mM EGTA, 50mM sodium fluoride, 1% Triton X-100, 10mM β-glycerophosphate, 1mM DTT, 10mM p-nitrophenyl-phosphate, and antiproteases), and centrifuged at 15,000 rpm for 20 minutes. Supernatants were recovered and stored at −70°C until use. Total protein in cell lysates was quantified by Bradford method. Cell lysates used for immunoprecipitation were prepared in 0.1% Triton X-100 lysis buffer (standard lysis buffer with only 0.1% Triton X-100).

Isolation of Mitochondrial Extracts

Cells were lysed by mechanical homogenization using a small pestle, and mitochondrial extraction was performed using the mitochondrial extraction kit from Pierce according to the manufacturer’s instructions.

Western blotting experiments

Equal quantities of total protein lysates were resolved by 8-15% SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes. Nonspecific protein binding was blocked by incubating the membrane with a blocking solution (1x TBS, 0.1% Tween 20, 5% nonfat dried milk powder) for 1 h at room temperature. The blots were then probed with primary antibodies overnight. The immune complex was detected by using a peroxidase-conjugated secondary antibody and the chemiluminescent detection kit according to the manufacturer’s specifications (Millipore). For non-denaturing PAGE-SDS, b-mercaptoethanol was not added to samples.

Pull-down and Immunoprecipitation experiments

Y79 protein extracts (1 mg) were first incubated with a GST protein bound to glutathione–agarose beads for 3 h at 4°C. Following this pre-clearing step, the supernatant was incubated with a GST-BIRO1 fusion protein bound to glutathione–agarose beads for 16 h at
Cell death induction by BIRO1

4°C. After four washes, the proteins pulled down with the GST-BIRO1 were subjected to SDS-PAGE and immunoblotting.

Immunoprecipitations were performed with the PIERCE Co-IP Kit as mentioned by the manufacturer.

**Immunofluorescence Microscopy**

Cells were grown on coverslips at 60% density and fixed in ice-cold acetone: methanol (1:1) for 5 min at room temperature, followed by permeabilization with 0.2% Triton X-100/PBS for 5 min. Cells were then blocked using 2% bovine serum albumin in PBS for 1 h and incubated with the monoclonal anti-ezrin antibody at the dilution of 1:100. Bound antibodies were detected with AlexaFluor-594 (1:4000; Molecular Probes, Inc., Eugene, USA) secondary antimouse antibody.

Mitochondria were detected by staining with Mitotracker Red CM-H2Xros (Molecular Probes) at 200 nM for 30 min. Nuclei were counterstained with the DNA dye Hoechst 33285 (Sigma). Coverslips were mounted with Vectorshield aqueous mountant (Vector Laboratories) and observed and photographed using an Olympus BL51 fluorescence microscope at 400x magnification.

**Results**

**Expression of BCL-2 family members in tumor cell lines**

Analysing apoptosis induced by BH3 domains, we investigated the expression of the different partners involved in mitochondrial outer membrane permeabilization, i.e anti-apoptotic (BCL-2, BCL-XL, and MCL-1), pro-apoptotic (BAX and BAK), and BH3-only pro-apoptotic proteins (BIM and PUMA) in human retinoblastoma tumoral cells Y79, WERI-Rb, in primary mouse retinoblastoma (PR), in Rb4820 and Rb6226, two primary mouse tumoral
Cell death induction by BIRO1

cell lines derived from primary retinoblastoma, in 661W photoreceptor cell line and in non ocular tumoral cells. While BAX was highly expressed in WERI-Rb, Rb4820, Rb6226, and in four different primary retinoblastoma tumors and in 661W cells, it was completely absent in Y79 cells (Fig 1.A). BAK was lacking in Rb4820 and 661W. BCL-XL was abundantly expressed in Y79 and WERI-Rb, while MCL-1 was detected in both these human cells. These data suggest that WERI-Rb, the four PR and Rb6226 should be more sensitive to cell death induction than Y79 and Rb4820.

Induction of cell death in retinoblastoma cell lines using BH3 peptidic compounds

Cellular delivery of peptides derived from the BH3 domain was achieved by linking BH3 sequences to the peptidic transporter TAT_{48-57} from HIV (10 amino acids, GRKKRRQRRR), known to have a good efficacy in crossing cell-membranes from different cell-types (25), and in transducing healthy cells from various compartments of the eye following injection into the vitreous body or subretinal space of adult mice (26). Various peptides were designed based on the chemical and conformational properties of BH3 domains (Table 1). To enhance protease resistance and improve stability, peptides were synthesized in D-retro inverso conformation (25-26). To assess the capacity of D-TAT peptides to cross the cell membrane, cell lines were incubated with the D-TAT-FITC-labeled peptide. As observed in figure 1.B, the fluorescent peptide was able to very rapidly enter (1 h) tumor cells, as well as 661W cells. The entry of BIRO1-FITC, the BH3 labeled peptide derived from BIM, was also tested and its localization was determined under a fluorescent microscope, showing a diffuse repartition in the whole cell without accumulation in any specific compartment (Fig 1.C).
We next examined whether cell-permeable BH3 peptides could induce cell death by exposing Y79 and WERI-Rb to peptides corresponding to the BH3 domains of BIM, BAD, BIK and BOK. BIRO1 exhibited a massive killing activity (80%) (Figure 2.A), while BAD displayed a moderate cell death induction, and the other peptides showed little, if any, killing effect. As a control, cells were exposed to a mutated form of BIRO1, BIRO1 MT, in which the conserved leucine and aspartate residues were mutated to alanine. The mutation of these two critical amino acids has been shown to disrupt interaction with other members of the BCL-2 protein family (19, 22). The pro-death effect of BIRO1 MT was decreased compared to BIRO1, with only 20% of cell death (Fig 2.A). SN50 peptide was used as a negative control, as a peptide unrelated to Bcl-2 proteins family.

To assess the kinetics of ATP content decrease in Y79 and WERI-Rb, cells were exposed to BIRO1 for 48 h and ATP content was measured at 4, 8, 24 and 48 h. As shown in figure 2.B, ATP content was already massively decreased 4 h post-treatment in WERI-Rb (70%) and this down-regulation was already well promoted in Y79 (40%). A rapid reduction in cellular ATP content was detected in cells undergoing necrosis. To discriminate between necrosis and apoptosis, annexin V-FITC/PI double staining analysis was performed by flow cytometry on Y79 cells treated with BIRO1. After only 2 h of BIRO1 treatment, Y79 culture showed 25% of PI positive cells and 52% of cells positive for double staining (annexin V-FITC and PI), which suggests the activation of a necrotic death pathway rather than apoptosis (Figure 2.C).

In necrosis cell death, cell swelling occurs with membrane blebbing leading to membrane rupture. To observe the impact of BIRO1 on cell membrane behaviour, we determined the localization of Ezrin (EZR) by immunofluorescence. EZR is a protein localized just beneath the plasma membrane, playing the role of linker between the plasma membrane and actin
Cell death induction by BIRO1
cytoskeleton. As shown in figure 2D, the treatment of Y79 with BIRO1 quickly induced
outgrowths followed by cell membrane breakdown.
In order to investigate whether BIRO1 killing effect was restricted to Y79 and WERI-Rb cell
lines, we tested cell death induction in 661W cells, as well as in non ocular tumoral cells.
MDA-465 cells were the only one to display cell death (60%) after exposure to 10 μM BIRO1
(Fig 2.E). At higher peptide concentration (20 μM), U2OS and HCT-116 showed 80% and 50%
cell death. As a control for toxicity of high peptidic concentration (>10 μM), cells were
exposed to 20 μM of the mutated form of BIRO1. No toxic effect was observed.

Effect of BIRO1 on primary mouse retinoblastoma
As the killing activity of BIRO1 was highly variable from one cell line to another, the effect of
the peptide on primary retinoblastoma tumors was difficult to predict. Primary mouse
retinoblastoma were isolated from the SV40-LT transgenic mice (24) and their sensitivity to
BIRO1 was tested. Retinoblastoma tumors were very sensitive to BIRO1, as observed by
Hoechst/PI staining, while BIRO1 MT had a weak effect (Fig 2.F). These results instigate us to
further examine BIRO1 efficacy in vivo in mouse retinoblastoma models.

Cell death induced by BIRO1 is caspases independant
The rapid ATP content decrease and the early PI staining observed in Y79 and WERI-Rb
treated with BIRO1 suggested that a mechanism other than apoptosis was implicated in the
cell death program. To assess whether caspases were involved, the broad-spectrum caspase
inhibitor Z-VAD-FMK was added to Y79 and WERI-Rb exposed to BIRO1. The BH3 peptide
induced cell death in the presence of Z-VAD-FMK (Fig 3.A). In addition, we were unable to
measure any caspase activation by western blotting (data not shown) or fluorescent assay in
Cell death induction by BIRO1

Y79 and WERI-Rb exposed to BIRO1 (Fig. 3.B), while treatment with etoposide or cisplatine triggered caspases activation. Caspases activation can also be visualized by the cleavage of caspases substrate proteins. As shown in figure 3.C, Y79 and WERI-Rb exposed to BIRO1 did not lead to PARP cleavage, while cisplatine did. In agreement with a non-apoptotic death program, we were unable to detect any Bax translocation (data not shown), cytochrome c or AIF release in both human retinoblastoma cell lines (data not shown). Checking for DNA fragmentation, we noticed chromatin cleavage in WERI-Rb exposed to BIRO1 as well as to cisplatine (data not shown), while no DNA fragmentation was detected in Y79. These results suggest that a small fraction of WERI-Rb cells entered into apoptosis, while the majority of the cells died through necrosis. Relating to Y79, the absence of Bax most likely repressed any apoptotic program through the mitochondria, leading to a necrotic death process.

Cell death induced by BIRO1 did not implicate autophagy or necroptosis

To determine whether the autophagy or the necroptotic machinery were modulated in Y79 and WERI-Rb exposed to BIRO1, cells were pretreated with 3-methyladenine (3-MA) and necrostatin (Nec-1), an inhibitor of autophagy and necroptosis respectively. Neither 3-MA, nor Nec-1 did protect Y79 and WERI-Rb against cell death induced by BIRO1 (Fig 3.A). To further confirm an autophagy-independent killing pathway, the absence of conversion of LC3I to the lipidated form LC3II was assessed (27-28) (Fig 3.D). Our data suggested therefore that BIRO1-induced cell death in Y79 and WERI-Rb was not mediated by autophagy or necroptosis.

Cell death induced by BIRO1 did not induce calpains activation or reactive oxygen species (ROS) production
Calpains are important players in programmed necrosis. These calcium-activated proteases initiate lysosomal disruption (29) followed by the release of cathepsins and the rearrangement of the actin cytoskeleton (30). To clarify whether BIRO1 induced modulation of calpains activity and downstream release of lysosomal proteases, we tested whether calpains inhibitors (calpastatin, ALLN) as well as cathepsin B and L inhibitors (ALLN) decreased BIRO1 effect on cell viability. Both calpastatin and ALLN failed to affect cell death induced by BIRO1 (Fig 3.A).

ROS is another effector in cell death signaling pathways including apoptosis and necrosis. To explore its potential role in BIRO1-induced cell death, Y79 and WERI-Rb were pre-treated with the antioxidant BHA before exposure to BIRO1. As shown in Fig. 4A, BHA did not attenuate retinoblastoma cell death. We also evaluated BIRO1 effect on intracellular ROS production, i.e. hydrogen peroxide and superoxide, using two different fluorescent probes (HE or H2DCFDA). Exposure of WERI-Rb to BIRO1 did not result in an increase in intracellular ROS production, neither hydrogen peroxide (Fig 4.B) nor superoxide (data not shown), as measured by fluorescence emission. At the same time the BH3 mimetics ABT-737, known to induce WERI-Rb apoptosis (21) induced hydrogen peroxide production (Fig 4.B).

BIRO1 promoted mitochondrial fragmentation in Y79

Under physiological conditions, mitochondria are elongated and filamentous. Upon stress conditions or apoptotic stimuli, mitochondria become fragmented. Mitochondrial fission implicates the constriction and cleavage of mitochondria by fission proteins that mediate the remodeling of the outer and inner mitochondrial membranes (31). It has previously been shown that $\text{Bax}^{-/-}/\text{Bak}^{-/-}$ MEF cells exposed to a high concentration of a BID BH3 peptide (100 $\mu$M) displayed mitochondrial fragmentation characterized by spherical mitochondria
Cell death induction by BIRO1 (32). To assess whether any morphological changes in mitochondria was generated in Y79 following exposure to BIRO1, cells were stained with the fluorescent dye Mitotracker Red CM-H2Xros, and mitochondrial morphology was examined (Fig 5.A). Untreated cells exhibited elongated tubular mitochondria, while mitochondria of Y79 treated with BIRO1 displayed a conversion of tubular fused mitochondria into isolated small punctuated organelles after 1 h only (Fig 5.A), indicative of mitochondrial fragmentation.

Mitochondrial fragmentation can result either from an increase in fission activity or an inhibition of fusion (33-36). To address whether BIRO1 induced fragmentation by recruiting the key fission protein DRP1, we investigated the expression and localization of DRP1 in Y79. Under normal conditions, the main fraction of DRP1 was cytosolic (Fig 5.B), with a weak portion localized at mitochondria. Following exposure to BIRO1, we were unable to detect any translocation of DRP1 to mitochondria or DRP1 dimerization (Fig 5.C). Furthermore, the treatment of Y79 cells with the DRP1 inhibitor mdivi1, which attenuates Drp1 self-assembly, showed no protective effect following BIRO1 treatment (Fig 5.D). These results suggested that mitochondrial fission induced by BIRO1 in Y79 was DRP1-independent, and might implicate unidentified regulators.

Another possible pathway leading to mitochondrial fragmentation is the proteolytic processing and inactivation of OPA1. BIRO1 had no effect on OPA1 (Fig 5.E), indicating that BIRO1-induced mitochondrial fragmentation was not the side effect of the inhibition of mitochondrial membrane fusion.

The impact of BIRO1 on factors involved in mitochondria dynamics

While Y79 mitochondria fragmentation and cell membrane blowout were very early events following BIRO1 treatment (60-90 min, Fig 5.A and 2.D), we measured a stable content in
Cell death induction by BIRO1

Various proteins, including proteins implicated in mitochondria dynamics and mitochondria-dependent cell death, i.e. voltage-dependent anion channel (VDAC), hexokinase-2 (HK2), OPA1 and BCL-2 proteins (Fig 6.A). A downregulation of BID, PUMA and DRP1, as well as the cleavage of MCL1 were observed 8 h post-treatment. An increase in the cleaved form of phosphoglycerate mutase family member 5 (PGAM5) isoform 1 was observed 8 h post-treatment.

BIRO1 interacted with BCL-X\textsubscript{L}

BIRO1 derived from the BH3 domain of BH3-only proteins induced necrosis in Y79 and WERI-Rb which implicated mitochondria. To determine which mechanism was responsible for the high Y79 and WERI-Rb sensitivity to BIRO1, we investigated the potential interaction between BIRO1 and anti-apoptotic members of the BCL-2 proteins family. We detected an interaction of BIRO1 with BCL-X\textsubscript{L} in Y79 by pull-down experiments, while BIRO1 was unable to bind BCL-2 and MCL-1 (Fig. 6B). We also verified whether other factors playing a role in mitochondria dynamics were able to interact with BIRO1 (Fig. 6B). None of these factors were found to bind to BIRO1 in Y79. The mutated form of BIRO1 was unable to interact with BCL-X\textsubscript{L} (data not shown).

Our hypothesis was therefore that BIRO1/BCL-X\textsubscript{L} interaction induced necrosis by releasing a deleterious factor from BCL-X\textsubscript{L}. BCL-X\textsubscript{L} is known to bind different factors and we focused on the interaction of BCL-X\textsubscript{L} with BID, PUMA, DRP1, HK2, VDAC and PGAM5 in Y79 (Fig 6.C). BCL-X\textsubscript{L} was found to interact with BID, HK2 and VDAC. Following treatment of Y79 with BIRO1, the BCL-X\textsubscript{L}/BID interaction was decreased while the BCL-X\textsubscript{L}/HK2 and BCL-X\textsubscript{L}/VDAC interaction were increased (Fig 6.D). A similar experiment was performed with BIRO1 MT and no effect was observed (data not shown).
Cell death induction by BIRO1

Discussion

Specific modulations of various pathways, i.e. PI3K/AKT, JAK-STAT, p53, progressively lead to resistance of retinoblastoma cells to death (2-5). The emerging small anticancer drugs which target BCL-2 family proteins offer a way to overcome the death resistance of cancer cells (13-21).

In this report, we investigated the minimal death domain of different BH3-only proteins for their capacity to induce cell death in human retinoblastoma cell lines Y79 and WERI-Rb, as well as in primary mouse retinoblastoma. Among the different cell penetrating peptides tested, BIRO1 derived from the BH3 domain of BIM was the most effective in killing both Y79 and WERI-Rb, while it was nontoxic for the photoreceptors 661W cell line. Among the other non-ocular tumoral cell lines exposed to BIRO1, cell response was heterogeneous, with MDA being relative sensitive and all others being more resistant. Interestingly, primary mouse retinoblastoma was shown to be sensitive to BIRO1. The high killing effect of BIRO1 compared to the other peptides suggests that not only BCL-2 and BCL-XI must be inhibited, but also other anti-apoptotic proteins such as MCL-1. Indeed, while the BH3 domain of BIM is able to interact and inhibit all the anti-apoptotic members of BCL-2 family proteins, the BH3 domains of BAD and BIK inhibit only BCL-2, BCL-XI, Bcl-w and Mcl-1, A1 respectively.

The high sensitivity of Y79 to BIRO1 despite the absence of BAX questioned us about the molecular mechanism leading to Y79 cell death. Indeed, BAX is known to be necessary to trigger apoptosis. Investigating markers of apoptosis after exposure to BIRO1 demonstrated that the apoptotic cell death was not involved in Y79 and weakly triggered in WERI-Rb. BIRO1 did not activate caspase-3/7 in both Y79 and WERI-Rb, as observed by direct assays or indirectly by using caspases inhibitors or examining caspase-3 substrate cleavage (PARP). BIRO1 did not induce DNA fragmentation in Y79, while it did in WERI-Rb. This difference in
one of the last step of the apoptotic process suggests that a small part of the WERI-Rb cells are able to initiate apoptosis. We also examined the effect of BIRO1 on mitochondria. BIRO1 promoted mitochondria fragmentation very rapidly (1 h), suggesting an early mitochondrial function in BIRO1 induced cell death.

As BIRO1 did not kill retinoblastoma cells by apoptosis, we assessed the potential roles of necrosis and autophagy. While autophagy can participate to cell death, its activation may also establish a cellular protection against certain stress, in particular following deprivation of nutrients, and therefore against cell death (37). The BCL-2 protein family are implicated in controlling autophagy, although autophagic mechanisms are quite different from apoptotic ones, and require distinct regulator proteins (38-40), including Beclin-1. We therefore tested whether BIRO1 was able to modulate the interaction of Beclin-1 with BCL2 and/or BCL-X\textsubscript{l} to induce autophagy. BIRO1 had no effect on the autophagic process in Y79 and WERI-Rb as observed by LCIIIB absence following peptide treatment.

In necrosis, studies have shown that mitochondria may constitute a regulatory element. Necrosis has long been considered as a passive process devoid of controlled signaling events. However, evidences for a regulated necrosis mechanism are accumulating especially from studies of death receptors leading to TNF-induced necrosis, called necroptosis. In addition to the features of necrosis in BIRO1-treated Y79 and WERI-Rb, i.e., absence of caspases activation and DNA fragmentation, other observations support the necrotic cell death pathway. ATP content in both Y79 and WERI-Rb dropped rapidly and cell membranes were quickly permeabilised. However, other events involved in necrotic cell death were not seen in BIRO1 induced cell death, such as calpain activation or ROS production. In necroptosis, the kinase RIP1 disrupts the interaction of the adenine nucleotide translocase (ANT) with cyclophilin D at the mitochondrial inner membrane, causing mitochondrial dysfunction and
Cell death induction by BIRO1

cell death (41). We have examined whether RIP1 was involved in BIRO1 cell death using Nec-1, an allosteric inhibitor of RIP1 (42) able to protect cells from necroptosis. This inhibitor had no protective effect, suggesting therefore that BIRO1 induces a programmed necrosis different from necroptosis.

BIRO1-induced programmed necrosis directly implicated mitochondria. Indeed, the Y79 mitochondria, which displayed an elongated filamentous morphology under basal conditions, showed an altered fragmented shape and size following BIRO1 exposure. Mitochondrial fission is a process associated with apoptosis, as well as with programmed necrosis or mitophagy. DRP1 has been shown to be involved in mitochondrial fission (33, 36), its translocation from cytosol to mitochondria inducing mitochondria constriction. In Y79, we were unable to detect any DRP1 translocation to mitochondria, suggesting that unidentified regulators of fission machinery might contribute to cell death induced by BIRO1.

PGAM5 seems also to play an important role in mitochondrial dynamics at the interphase of apoptosis, necrosis and autophagy, as reported (43). The PGAM5 gene encodes two protein isoforms, PGAM5-L (32 kDa) and PGAM5-S (28 kDa), both expressed at the level of the outer mitochondrial membrane (OMM). The accumulation of the PGAM5-S results in the formation of fragmented mitochondria (43). PGAM5 has been shown to be activated in necroptosis triggering DRP1 dephosphorylation and translocation to mitochondria leading to mitochondria fragmentation (44). In addition, upon mitochondrial membrane potential loss, PGAM5 is cleaved (45), being therefore a good marker for this event. We observed that BIRO1 induced an accumulation of the cleaved PGAM-L isoform. As PGAM5 has been shown to interact with BCL-XL (46), we verified whether BIRO1 acted in Y79 by modulating PGAM5/BCL-XL interaction. This was not the case (Fig 6.D). We were then unable to
determine whether BIRO1 had a direct effect on PGAM5 or whether PGAM5 cleavage was a secondary effect of BIRO1 on mitochondria. This point needs additional clarification.

VDAC is another abundant protein of the outer mitochondrial membrane playing a role in mitochondria-mediated cell death (47, 48) when it sets up channels in the OMM. The conductance throughout these channels is modulated by various factors including VDAC/BCL-X<sub>L</sub> interaction, which allows the channel to be in an open state, maintaining adenine nucleotide flux and the outer membrane permeability. The disruption of the VDAC/BCL-X<sub>L</sub> interaction triggers mitochondrial permeability modulation and cell death (49-50). In Y79, BIRO1 seemed to strengthen the VDAC/BCL-X<sub>L</sub> binding (Fig 6D), which is in discrepancy with the protective effect of the VDAC/BCL-X<sub>L</sub> interaction.

Our study demonstrated that BH3 peptides enhanced cell death in retinoblastoma cells. Our finding is supported by a recent report (21) showing that retinoblastoma cells are sensitive to another BH3 mimetics, ABT-737. Further investigations are however necessary to clarify the exact mechanisms involved in the cell death process induced by BIRO1. From our study, it appears that an unknown programmed necrotic mechanism is implicated.
Acknowledgements

The authors wish to thank Carole Herkenne, Angélique Schmid and Céline Agosti for precious technical assistance, and Dr. M. Al-Ubaidi for providing the 661W cell line. The SV40-LT transgenic mice are a gift of JM O’Brien.
References


Cell death induction by BIRO1


Cell death induction by BIRO1


Cell death induction by BIRO1


Cell death induction by BIRO1

Table 1. Sequence of the different BH3 peptides

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<tr>
<td>BIRO1 MT</td>
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<td>BAD</td>
<td>RYGRELRRMSDEFVDSFKG</td>
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Figures

Figure 1. BCL-2 family members content and Cellular import of the D-TAT-FITC labelled peptide

A. Protein expression of BCL-2 protein family members was determined by western analysis in Y79, WERI-Rb, 661W, primary mouse retinoblastoma (PR1-4), as well as in cell lines derived from these primary tumors (Rb4820, Rb6226) and in non-ocular tumoral cell lines BT-549 and MDA-465 (human breast cancer cells), HCT116 (human colon cancer cells), U2OS and SaOS (human osteosarcoma).

B. Various cell lines were incubated with the D-TAT-FITC labelled peptide (5 μM) for 1 h and were visualized using live cell fluorescent microscopy.

C. Y79 cells were exposed 1 h to 10 μM BIRO1-FITC and localization of the green fluorescence was evaluated using live cell fluorescent microscopy. Mitochondria were visualized after staining with a MitoTracker (red).

Figure 2. Effect of BH3 peptides on WERI-Rb and Y79 and on primary mouse retinoblastoma

A. The BIRO1 peptide induces cell death in WERI-Rb and Y79 retinoblastoma cells. Y79 and WERI-Rb cells were exposed to different BH3 cell permeable peptides (3 and 10 μM) and to a peptide unrelated to Bcl-2 proteins family (SN50)
Cell death induction by BIRO1

as well as to cisplatin (100 μM) and etoposide (10 μM) for 24 h. Cell viability was determined by measuring ATP content of the cells using a luminescence assay. Independent experiments were repeated five times. Dark grey column, Y79; grey column, WERI-Rb; B-MT, mutated BIRO1.

B. The cell death promoted by BIRO1 is a very rapid process. Y79 and WERI-Rb cells were exposed to various concentrations of BIRO1, and cell viability was determined at different time point by measuring the ATP content of the cells using a luminescence assay. Independent experiments have been repeated five times. Grey lines, Y79; dark lines, WERI-Rb; circle, 10 μM BIRO1; triangle, 3 μM BIRO1; square, no BIRO1.

C. Flow cytometry analysis of plasma membranes with annexin V-FITC/PI double staining. Y79 cells were incubated either in the absence of BIRO1 for 5 h (control), or in the presence of 10 μM BIRO1 for 2 and 5 h. Undamaged cells were stained with negative annexin V-FITC/PI (bottom left quadrant). After incubation with BIRO1 for 2 h, a significant number of cells were stained with positive annexin V-FITC and positive PI (upper right quadrant).

D. Y79 cells were left untreated (Control) and treated with BIRO1 10 μM for 45 and 90 min. Cells were then immunostained with Ezrin monoclonal primary antibody and Alexa-fluor anti-mouse secondary (595-red) antibody. Asterix, membrane swelling; dark arrow, membrane blowout.

E. Differential death response of various tumoral and non-tumoral cell lines to BIRO1 peptide. In addition to WERI-Rb and Y79 cells, other tumoral cell lines and 661W were exposed to increasing concentrations of BIRO1 and to 20 μM of the inactive BIRO1 MT for 24 h, and cell viability was determined at different time point by measuring the ATP content of the cells using a luminescence assay. Independent experiments have been repeated three times.
Cell death induction by BIRO1

F. Retinoblastoma that developed in SV40-LT transgenic mice were isolated at three months of age. Primary tumoral cells were left untreated for 48 h before treatment for 16 h with BIRO1 10 μM or with the mutated form of the peptide (BIRO1 MT). Photographs showing Hoechst 33342 (blue)/propidium iodide (red, dead cells) staining is representative of results obtained in three different primary retinoblastoma.

Figure 3. BIRO1 induced cell death is not apoptosis and is not related to autophagy activation.

A. BIRO1 induced cell death is caspases-independent and does not rely on autophagy or necroptosis activation. Y79 and WERI-Rb were treated with 10 μM BIRO1 for 24 h. Prior to BIRO1 treatment, cells were exposed for 1 h to 10 μM zVAD (a broad range caspase inhibitor), as well as to 0.5 mM 3-MA (autophagy inhibitor), 50 μM Nec-1 (RIP-1 inhibitor) and 10 μM Calpastatin and ALLN (calpains inhibitors). Cell viability was determined by measuring ATP content of the cells using a luminescence assay. Independent experiments were repeated four times. Dark grey column, Y79; grey column, WERI-Rb.

B. Y79 and WERI-Rb cells were treated with 10 μM BIRO1 and caspase-3/-7 activity was measured using a homogeneous and luminescent assay. Independent experiments have been repeated three times.

C. Following exposure of Y79 and WERI-Rb to BIRO1 and Cisplatine, the cleavage of PARP, a downstream substrate of caspase-3, was measured by western blotting experiment using an antibody that recognize the full length protein (105 kDa), as well as the cleaved protein (85 kDa). Independent experiments have been repeated three times.

D. Y79 and WERI-Rb were exposed to BIRO1 for 3 h and LC3II formation was assessed by western blotting during this period of time.
Figure 4. The cell death process induced by BIRO1 is not combined with ROS production in retinoblastoma cell lines.

A. Y79 and WERI-Rb were treated with 10 μM BIRO1 for 24 h. Prior to BIRO1 treatment, cells were exposed for 1 h to 100 μM BHA (antioxidant). Cell viability was determined by measuring ATP content of the cells using a luminescence assay. Independent experiments have been repeated three times. Dark grey column, Y79; grey column, WERI-Rb.

B. WERI-Rb cells were treated with 10 μM BIRO1 during 1 h and further stained for 30 min with H2DCFDA, a cell-permeable indicator for ROS. Changes in overall fluorescent intensity observed after exposure to BIRO1 was measured and quantified using an alpha-screen apparatus. As a positive control, cells were exposed to 1 μM ABT-737 and 100 mM H2O2, which caused a rapid 50% and 100% increase of ROS respectively. Independent experiments have been repeated three times.

Figure 5. BIRO1 induces mitochondrial fragmentation in Y79.

A. Y79 were left untreated or treated with 10 μM BIRO1 for 1, 2 and 3 h. Mitochondria were stained with Mitotracker Red CM-H2Xros (200 nM) and fluorescence microscopy was performed.

B. Mitochondrial and cytosolic fractions were isolated from Y79 cells. DRP1 level in these two fractions was analyzed by western blotting using corresponding antibodies. The mitochondrial fractionation was verified with the mitochondrial marker VDAC.

C. Y79 cell extracts were separated by SDS-gel electrophoresis in the presence (denaturing conditions) or absence (non-denaturing conditions) of b-mercaptoethanol followed by western blot with anti-DRP1 antibody. Under non-reducing conditions, a DRP1 oligomer (*) was observed in addition to the monomer form.
D. Y79 were pretreated with the mitochondrial fission inhibitor mdivi-1 for 60 minutes followed by treatment with of BIRO1 (10µM) for 16 h. Cell viability was determined by measuring ATP content of the cells using a luminescence assay. Dark grey column, Y79; grey column, WERI-Rb.

E. The content of OPA1 playing a role in mitochondrial fusion was determined by western analysis in Y79 following BIRO1 treatment (10 µM).

Figure 6. Effect of BIRO1 on proteins involved in mitochondria dynamics.

A. The content of factors playing a role at the mitochondrial level was determined by western analysis in Y79 following BIRO1 treatment (10 µM).

B. The interaction of the GST-BIRO1 protein with potential partners was determined by pull-down experiments using Y79 proteins extracts. The GST alone was used as a negative control. (WE : Whole extracts, PD : Pull-down, SN : Supernatant).

C. The proteins able to interact with Bcl-XI in Y79 cells were estimated by co-immunoprecipitation using an antibody against Bcl-XI. (IP : Immunoprecipitated, SN : Supernatant, WE : Whole extracts).

D. The modulation of the interaction of Bcl-XI with its partners following BIRO1 exposure (10 µM, 4h) was estimated by co-immunoprecipitation in Y79 and compared to untreated cells (C). (IP : Immunoprecipitated, WE : Whole extracts).
Figure 1.A
Figure 1.B

Y79  WERI  661W  MDA
SaOS  U2OS  HCT  BT

Figure 1.C

Y79
Figure 2.A

Figure 2.B
Figure 2.C

![Figure 2.C](image)

Figure 2.D

![Figure 2.D](image)
Figure 2.E
Figure 3.D

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Figure 4.A

![Figure 4.A](image)

Figure 4.B

![Figure 4.B](image)
Figure 5.A

![Images showing cellular structures labeled Ctrl, BIRO1, 1h, BIRO1, 2h, and BIRO1, 3h.]

Figure 5.B

![Graphs showing protein levels labeled a-DRP1 and a-VDAC for Ctrl and BIRO1 in cytoplasm (Cyto) and mitochondria (Mito).]

Figure 5.C

![Graphs showing protein levels labeled a-DRP1, a-Actin, and a-DRP1 (Non-denat.) for Ctrl and time points 1h and 3h.]
Figure 5.D

![Bar graph showing relative ATP content at different concentrations of MDV1610 and BIRO.]

Figure 5.E

![Western blot images showing a-OPA1 and a-Actin].
Figure 6.A

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Molecular Cancer Research

BIRO-1, a Cell Permeable BH3 Peptide, Promotes Mitochondrial Fragmentation and Death of Retinoblastoma Cells

Nathalie Allaman-Pillet, Anne Oberson and Daniel Francis Schorderet

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