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Mitochondrial fragmentation and neuronal cell death in response to the Bcl-2/Bcl-x_L/Bcl-w antagonist ABT-737

Kenneth W. Young [a,*,1], Lucia G.P. Piñón [a,1], Dalbir Dhiraj [a], Davina Twiddy [a], Marion MacFarlane [a], John Hickman [b], Pierluigi Nicotera [a,2]

[a] MRC Toxicology Unit, University of Leicester, Hodgkin Building, Lancaster Road, Leicester LE1 9HN, UK
[b] Institut de Reserches Servier, Croissy sur Seine, France

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ABSTRACT

Inhibition of pro-survival Bcl-2 family proteins by BH3-only proteins is a key initial step leading to apoptotic cell death. In neurons, investigating cell death pathways is often hampered by the multi-factorial nature of the stress stimuli employed. Here we investigate the action of ABT-737, a small molecule inhibitor which specifically targets the BH3-protein binding domain of pro-survival Bcl-2, Bcl-X_L and Bcl-w. ABT-737 produced a time- and concentration-dependent neuronal cell death which displayed the classical hallmarks of apoptosis. Cell death was maximal by around 4 h ABT-737 treatment, and the effect of ABT-737 could be delayed by the broad spectrum caspase inhibitor zVADfmk. Examining, using real-time confocal microscopy, the molecular basis for the onset of response demonstrated recruitment of pro-apoptotic Bax to specific mitochondrial foci, followed by mitochondrial fragmentation. Treatment of neurons with ABT-737 also produced cleavage of Bid, a BH3-only protein known to be a caspase substrate. Interestingly, cleaved Bid translocated to mitochondria but did not colocalise with Bax foci. zVADfmk inhibited Bid cleavage and slowed the rate of fragmentation, suggesting a role for cleaved Bid in the amplification of the apoptotic response. siRNA-mediated knockdown of Bax significantly inhibited ABT-737 induced cell death, whereas knockdown of the BH3-only proteins Bid or Bim had no effect. ABT-737 therefore appears to be a useful tool with which to examine neuronal apoptotic pathways. Our data suggests that caspase-dependent cleavage of Bid may be a downstream amplification event which enhances the rate of mitochondrial fragmentation.

1. Introduction

The Bcl-2 family of proteins, which comprises of both pro-survival and pro-apoptotic members, are key regulators of mitochondrial-mediated programmed cell death (Vaux et al., 1988; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). The critical step in the mitochondrial-dependent cell death pathway involves the multi-domain pro-apoptotic proteins Bax and Bak which form homo-oligomers at the mitochondrial membrane, resulting in the release of cytochrome C and activation of downstream caspases (Ow et al., 2008). Permeabilisation of the outer mitochondrial membrane by Bax or Bak is normally held in check by the pro-survival Bcl-2 family members (such as Bcl-2 and Bcl-x_L). The pathway becomes active in response to a third set of Bcl-2 family proteins, namely the BH3-only proteins, which act as transducers of the cell death stimuli (Youle and Strasser, 2008). Programmed cell death, involving Bcl-2 family proteins, is an essential mechanism employed by the developing nervous system to remove excess or unconnected neurons (Martinou et al., 1994; Greenlund et al., 1995; Merry and Korsmeyer, 1997). However, programmed cell death also becomes activated during various neurodegenerative diseases and therefore remains an important therapeutic target for combating neurodegenerative disorders (Vila and Przedborski, 2003).

Despite a great deal of research, the mechanism via which Bax or Bak become activated remains controversial (Kuwana et al., 2005; Kim et al., 2006; Willis et al., 2007; Chipuk and Green, 2008; Czabotar et al., 2009). A key initial step in the process is the formation of Bax or Bak homo-dimers through the binding of the BH3-domain of one Bax or Bak molecule to a hydrophobic groove of a second Bax or Bak (Dewson and Kluck, 2009). It is generally

* Corresponding author. Fax: +44 116 252 5616.
E-mail address: kwy1@le.ac.uk (K.W. Young).
[1] These authors contributed equally to this work.
[2] present address: German Centre for Neurodegenerative Diseases (DZNE), Bonn, Germany.

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agreed that pro-survival Bcl-2 family members prevent the formation of Bax or Bak homo-dimers by binding to the BH3-domain of active Bax or Bak, and that BH3-only proteins act, at least in part, by occupying the BH3-binding pocket of the pro-survival proteins. Therefore, when levels of active BH3-only proteins increase, the BH3-binding pockets of the pro-survival proteins become saturated, and Bax or Bak activation is then able to proceed unchecked (Adams and Cory, 2007). However, in addition, certain BH3-only proteins (including Bid and Bim) may be able to directly bind to and activate Bax and Bak (Kuwana et al., 2005; Kim et al., 2006; Brunelle and Letai, 2009). In this model, BH3-only proteins can be classed as either 'activators' if they directly target Bax or Bak, or 'sensitisers' if they only demonstrate binding to pro-survival Bcl-2 proteins.

Differentiated neurons expressive exclusively a truncated form of Bak which is incapable of generating pore-forming units, and this may explain why neuronal apoptosis is almost totally Bax dependent (Uo et al., 2005). Bax oligomerisation is stimulated when specific BH3-only proteins become activated in response to noxious stimuli such as seizure activity (Meller et al., 2003) and oxidative stress (Steckley et al., 2007). There is also considerable evidence linking BH3-only proteins to cell death in stroke models (Ness et al., 2006). Activation of BH3-only proteins in response to ischemic treatments may involve transcriptional upregulation, as is the case for Bim, Noxa and Puma (Inta et al., 2006; Steckley et al., 2007), phosphorylation, as is the case for Bad (Kamada et al., 2007), or caspase-dependent cleavage as is observed with Bid (Plesnila et al., 2001; Yin et al., 2002). However, in the case of ischemic cell death in neurons, investigation of the molecular events which link the BH3-only proteins to Bax activation has been hampered by the multi-factorial nature of the cell death process. We have therefore utilised the small molecule BH3-protein mimetic, ABT-737 (Oltersdorf et al., 2005; Vaux, 2008), which specifically targets the BH3-binding pocket of Bcl-2, Bcl-x_L and Bcl-w, in order to investigate: firstly, whether in neurons, increased occupancy of these BH3-binding pockets is in itself sufficient to initiate a cell death response, and secondly what are the critical events which occur as a result of this occupancy which lead to the onset of the programmed cell death pathway.

2. Methods

2.1. ABT-737 Synthesis

ABT-737 was synthesized according to the method of Oltersdorf et al. (2005). It was dissolved in DMSO and stored at −20 °C until use.

2.2. Primary neuronal cultures

Primary cultures of rat hippocampal neurons, prepared from neonates as described previously (Young et al., 2005), were grown for 9–11 days *in vitro* (DIV) on poly-D-lysine coated glass coverslips or cell culture plates. Primary cultures of rat cerebellar granule neurons were prepared essentially as described by Leist et al. (Leist et al., 1997) and grown for 7–9 DIV. All animal work was performed according to UK Home Office guidelines.

2.3. Assay for cell viability after ABT-737 treatment

Neurons were grown in 12-well culture dishes and treated with ABT-737 at the indicated concentrations for 1–48 h. The nuclear stains: Hoechst 33 342 (Invitrogen, 5 μg/ml) and Sytox orange (Invitrogen, 0.5 μM) were included for the last 20 min of the incubations. Cells were then washed twice with Krebs/HEPES buffer (KHB in mM: 20 HEPES, 130 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, pH 7.4) before cell death was measured via epifluorescence microscopy. Images of Hoechst and Sytox orange were collected using the excitation filters 350/50, and 555/25 and emission filters 455/30, and 600/40, respectively. Loss of cell viability, indicated by nuclear uptake of Sytox orange in Hoechst positive cells, was measured using an automated cell counting assay (Volocity, Improvision). Antagonists were added 20 min before ABT-737, and were included for the duration of the experiment. For experiments investigating the effect of DRP-1^{K38A} expression on ABT-737-mediated cell death, neurons were transfected with eGFP-tagged Drp-1^{K38A} at 7 DiV. After ABT-737 treatment, eGFP-Drp-1^{K38A} expressing neurons were identified via epifluorescence microscopy and cell death measured as above. Loss of cell viability in eGFP-Drp-1^{K38A} expressing neurons was then compared to the surrounding non-transfected neurons in the same culture dish.

2.4. Confocal imaging of mitochondrial fragmentation

At 7 DIV, neurons were transfected with plasmids expressing eGFP-Bax, eGFP-Drp-1, eGFP-Drp-1^{K38A}, mitochondrial-targeted DsRed (mtDsRed), or CFP-Bid-YFP (amino acids 16–194). A total of 1 μg DNA and 2 μl Lipofectamine2000 (Invitrogen) was added to cells for 6 h, before medium was replaced. Neurons were then mounted on a Zeiss LSM510 confocal microscope and maintained at 37 °C in KHB. Time-lapse confocal images were then taken at a frame rate of 2–15 min per z-series using a ×63 objective as indicated in the text. Neurons expressing mtDsRed were excited at 543 nm and emissions collected above 560 nm eGFP and YFP images were excited at 488 nm and collected between 500 and 530 nm 3D reconstructions of neurons were created using volume rendering software (Volocity, Improvision).

2.5. Western blotting

Cerebellar granule neurons were washed in phosphate buffered saline (PBS) then lysed in an ice-cold RIPA buffer (in mM: 150 NaCl, 10 Tris–HCl, 5 EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) supplemented with protease inhibitor cocktail (Complete, Roche). Cells were then sonicated for 10 s, centrifuged (4000 rpm at 4 °C) for 10 min and stored at −20 °C until required. Protein concentrations were determined using a Bradford protein assay (Bio-Rad). Samples were heated to 95 °C for 5 min and 15 μg of protein was loaded onto a 13% polyacrylamide gel for electrophoresis. Separated protein bands were then transferred to a nitrocellulose membrane (Geneflow,UK), and blotted with a specific antibody for cleaved caspase 3 (1:1000, Cell Signaling Technology), or GAPDH (1:10 000, Santa Cruz). Chemiluminescence was detected using ECL-plus (Amersham) and analysed using Image J software. For measurements of mitochondrial cytochrome C release, granule neurons were collected using 0.05% trypsin/0.5 mM EDTA, centrifuged at 200 × g for 3 min, and then lysed for 5 min on ice (lysis buffer in mM: 250 sucrose, 20 HEPES, 5 $MgCl_2$, 10 KCl, 1 EDTA, 1 EGTA, 0.03% digitonin and protease inhibitor cocktail (Roche), pH 7.4). The cells were centrifuged at 13 000 rpm at 4 °C for 3 min and the supernatant (cytosolic fraction) collected. A total of 5 μg of protein was loaded onto a 10% polyacrylamide gel. Cytochrome C was identified using a rabbit anti-cytochrome C antibody (1:1000, Cell Signalling).

To assess protein levels of Bax, Bid and Bim after siRNA down-regulation (see Section 2.7), rat hippocampal neurons were washed 3 times with PBS and then lysed with 50 μl of loading buffer (70 mM Tris–HCl pH 6.8, 5% glycerol, 1.25% SDS, 0.05% bromophenol blue, and 25 mM DTT). Samples were heated to 95 °C for 5 min and loaded onto a 15% polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with appropriate antibodies: Bax (1:1000, Cell Signalling), Bid (1:12 000, Millipore), Bim (1: 1000, Stressgen) and GAPDH (1:5000, Santa Cruz).

2.6. Immunocytochemistry

Cerebellar granule neurons, cultured on glass coverslips, were washed twice in phosphate bufferd saline (PBS, Invitrogen) and fixed using 4% paraformaldehyde for 20 min at room temperature. The fixative was removed by 3 washes with PBS and then cells were permeabilised using 0.5% Triton X-100 for 15 min at room temperature. The neurons were then blocked with 5% normal goat serum containing 0.1% Triton in PBS for 1 h. Mouse anti-cytochrome C monoclonal antibody (1:1000 dilution, BD Pharmingen) was incubated over-night at 4 °C. Neurons were then washed 3 times in PBS and incubated with Alexafluor goat anti-mouse IgG 488 (Invitrogen) for 1 h at room temperature supplemented with 0.5 μg/ml Hoechst-33342 for 10 min. Images were then collect on a Zeiss LSM510 confocal microscope.

2.7. RNAi mediated down-regulation

Rat hippocampal neurons were transfected six days after plating using 2–3 μM of a pool of four short interfering RNAs (siRNAs) targeting rat Bid or Bim (Accell siRNA SMARTpool; Thermo Scientific Dharmacon), or a single custom-made Accell siRNA targeting Bax (target sequence: NNCUGACAUGUUUGCUGAUGG), according to the manufacturers guidelines. A non-targeting Accell pool consisting of four siRNAs was used as a negative control (Scr). Three days after transfection, cells were lysed for RNA extraction, used for a cell viability assay, or collected for western blot analysis.

2.8. RNA extraction and real-time PCR

Effectiveness of RNA knockdown was assessed using RT-PCR. Isolation of total RNA was performed using the RNeasy Plus Min System (QIAGEN). Quantitative real-time RT-PCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystem), using SYBR Green system from Applied Biosystem. Reactions were performed in a mixture containing 12.5 μl Master Mix, 900 nM of each primer, 1 μl DNA sample from