Cell death induced by 2-phenylethynesulfonamide uncovers a pro-survival function of BAX

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Footnotes:

Abbreviations: DKO, Double Knock Out; GFP, Green Fluorescent Protein; MEFs, Mouse Embryonic Fibroblasts; MOMP, Mitochondrial Outer Membrane Permeabilisation; PES, 2-phenylethynesulfonamide; SEM, Standard Error of Mean; STS, staurosporine; wt, wild type.

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ABSTRACT

PES (2-phenylethynylsulfonamide) was initially identified as an inhibitor of p53 translocation to mitochondria and named Pifithrin- μ . Further studies showed that PES selectively killed tumour cells and was thus a promising anticancer agent. PES-induced cell death was characterised by a non-apoptotic, autophagosome-rich phenotype. We observed this phenotype via electron microscopy in wild type (wt) and double *Bax*^{-/-} *Bak*^{-/-} (DKO) mouse embryonic fibroblasts (MEFs) treated with PES. We excluded the involvement of effector caspases, BAX and BAK, in causing PES-triggered cell death. Therefore, apoptosis was ruled out as the lethal mode of action of PES. Surprisingly, MEFs containing BAX were significantly protected from PES treatments. BAX overexpression in *Bax*^{-/-} MEFs confirmed this pro-survival effect. Moreover, this protective effect required the ability of BAX to localise to mitochondrial membranes. Conversely, mitochondrial fusion induced by treatment with Mdivi-1 conferred increased resistance to MEFs subjected to PES treatment. The involvement of BAX in the regulation of mitochondrial dynamics has been reported. We propose the promotion of mitochondrial fusion by BAX to be the pro-survival function attributed to BAX.

KEYWORDS: Cell death / Mitochondrial dynamics / BAX / BAK / Pifithrin- μ / Mdivi-1

1. Introduction

Pifithrins are small synthetic molecules that can inhibit the apoptosis-inducing functions of the p53 protein. Pifithrins were created to reduce the toxicity associated with radiation and chemotherapy in the context of cancer treatment, which is largely mediated by p53. Pifithrin- α was the first compound identified and characterised as an inhibitor of the transcriptional activity of p53 [1]. However, p53 can cause apoptotic cell death via mechanisms that are independent of transcription. For instance, p53 can translocate to mitochondria, interact with the anti-apoptotic members of the Bcl-2 family of proteins, neutralise them and cause apoptosis [2]. This fact prompted new analyses of chemical libraries and the discovery of pifithrin- μ . Pifithrin- μ can disrupt the interactions between the p53 and Bcl-2 proteins without modifying the transcriptional activity of p53. Consistently, it conferred a partial protection to mice subjected to γ -radiation [3].

Further studies demonstrated that pifithrin- μ was an effective inducer of cell death and that cancer cells are more sensitive to this drug [4,5]. The characterisation of this death process showed it to be independent of p53 and caspases. The cell morphology was characterised by increased vacuolisation and abundant autophagic vesicles. However, this change was not due to the promotion of autophagy but rather to the blockage of the autophagic flux. Therefore, proteotoxic stress, defined as the accumulation of misfolded proteins, was proposed as the mechanism of pifithrin- μ -induced cell death [4,6]. Moreover, in addition to p53, pifithrin- μ targeted the heat-shock protein 70 (HSP70) family of proteins [4,7]. The subsequent disruption of its chaperone function was very consistent with the resulting proteotoxicity. The identification of targets different from p53 prompted the renaming of pifithrin- μ to PES (the acronym of 2-phenylethynylsulfonamide) [4]. We adhere to this naming convention hereafter.

Although HSP70 and proteotoxicity explained the lethal properties of PES quite satisfactorily, controversy arose about the type of cell death this drug induced. Some researchers were clearly finding a caspase-independent, non-apoptotic, type of cell death [4,7]. On the contrary, other researchers were

finding canonical apoptotic processes [5,8,9]. Moreover, in pancreatic cell lines, a mixture of caspase-dependent and independent processes were identified [10]. Most models of apoptosis depend on mitochondrial outer membrane permeabilisation (MOMP), an event mediated by the BAX and BAK proteins. These proteins define the intrinsic or mitochondrial pathway of apoptosis. Cells deficient in both proteins become totally resistant to apoptosis induction via the intrinsic pathway [11,12]. Double *Bax*^{-/-} *Bak*^{-/-} (DKO) mouse embryonic fibroblasts (MEFs) and their wild type (wt) counterparts provide the most conclusive system for assessing the implication of the mitochondrial mechanisms that trigger apoptosis. Our aim was to explore the lethal effects of PES on this system. Unexpectedly, we found that more wt MEFs survived than DKO MEFs after a treatment with PES. PES did not activate either the effector caspases or the intrinsic apoptotic pathway. Therefore, apoptosis could be excluded as the type of cell death triggered by PES. The protein BAX was identified as promoting the increase in cell survival in wt MEFs. Upon induction of mitochondrial fusion, we found that MEFs became more resistant to PES-induced cell death. We hypothesised that the pro-survival action of BAX was based on the modulation of mitochondrial dynamics.

2. Materials and methods

2.1 Cell lines and drug treatments

Immortalised embryonic fibroblasts derived from mice (MEFs) knocked out for *Bax* (*Bax*^{-/-}), *Bak* (*Bak*^{-/-}), both genes (DKO) and their wild type (wt) counterparts were obtained from the laboratory of the late Prof. Korsmeyer [11,12]. These MEFs have been an important tool in our previous pharmacological studies [13,14]. The HeLa cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell types were grown in 2 mM L-glutamine-supplemented DMEM medium that contained 10% of foetal calf serum (FCS). The media and FCS were provided either by Invitrogen or Lonza (Barcelona, Spain). Plasmocin™ (5µg/ml) was used as the antibiotic in the medium (InvivoGen, San Diego, CA, USA). The general culturing conditions were 37°C and a water-saturated, 5% CO₂

atmosphere. The culture dishes and other plastic disposable tools were supplied by Bibby Sterilin (Staffordshire, UK) and Becton Dickinson (Franklin Lakes, NJ, USA). PES (2-phenylethynesulfonamide), i.e., pifithrin- μ , staurosporine (STS) and Mdivi-1 were purchased from Sigma (St. Louis, MO, USA). The stock solutions were prepared in DMSO. The drugs were serially diluted from these stock solutions or delivered to the media. The final concentrations are reported in the text and figures.

2.2 Cell death and caspase activity assessment

To quantify the ratios of cell death in the culture plates, the Cell Titer 96® and Cytotox 96® kits were used. Cell Titer 96® measured the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). Cytotox 96® measured the amount of lactate dehydrogenase (LDH) released from dying cells. Both kits were supplied by Promega Biotech Ibérica, SL (Barcelona, Spain). Because MTS reduction alone can lead to misleading interpretations due to a drug-induced cessation in cell proliferation, we supplemented the MTS data with LDH measurements. The combination of both procedures allows a robust determination of cell death, notwithstanding the type of death [13–15]. Apoptosis is a phenotype of cell death that depends on effector caspase activation. This activation can be quantified based on the cleavage of a synthetic substrate (Ac-DEVD-afc) and the subsequent fluorescence released (DEVDase activity), as we have reported previously [16].

2.3 Protein extraction and western blotting

The cells were lysed in a buffer containing 100 mM Tris/ClH pH 6.8, 1% SDS, 1 μ M EDTA, plus a cocktail of protease inhibitors from Sigma and subjected to sonication. The protein extract was obtained after a centrifugation at 12000 g for 15 minutes. The protein concentration was determined, and an equal amount was loaded per lane for SDS 12%-polyacrylamide gel electrophoresis. Following electrotransfer to 0.45 μ m PVDF membranes (Immobilon™) from Merck Millipore (Barcelona, Spain),

immune detection was performed with the following primary antibodies: anti-BAX rabbit polyclonal IgG (Upstate catalog 06-499) and anti-BAK rabbit polyclonal IgG (Upstate Catalog 06-536), which were both diluted to 2 µg/ml and supplied by Merck Millipore (Barcelona, Spain). Glyceraldehyde-3-P-dehydrogenase (GAPDH) was detected with the peroxidase-conjugated monoclonal antibody from Sigma. This monoclonal antibody was employed at a dilution of 1:4000 to assess the amount of protein loaded per lane. The Immobilon™ reagent from Merck Millipore was employed as the chemiluminescent peroxidase substrate.

2.4 Transient transfections

DKO MEFs were plated in p35 wells at 60-80% confluence. After 24 hours, 4 µg of plasmid DNA was transfected using the TurboFect reagent (Fisher Scientific, Barcelona, Spain) according to the manufacturer's instructions. The plasmids employed were hBax C3-EGFP (Addgene #19741), hBax S184E C3-EGFP (Addgene #19743), EGFP-Bak (Addgene #32564) and the empty vector (pEGFP-C3). These plasmids were originally used to explore the functional domains of the BAX protein [17]. The cells were then allowed to recover for an additional 24 hours, harvested, plated at 6000 cells/well in M96 plates and subjected to drug treatment.

2.5 Microscopic morphological studies

For electron microscopy, cells from treated or untreated cultures were collected, washed twice in PBS (150 mM ClNa, 2.7 mM ClK, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and fixed for 30 minutes at 4°C in 100 mM phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. After rinsing the pellets twice with PBS at 4°C, the cells were post-fixed in buffered OsO₄, dehydrated in graded acetone and embedded in Durcupan® ACM resin (Fluka, Buchs, Switzerland). Ultrathin sections mounted on copper grids were counterstained with uranyl acetate and lead citrate. A transmission electron microscope (Zeiss EM 910) was employed to visualise the cellular ultrastructure.

For the fluorescence microscopy of mitochondria, plated cells were placed in PBS containing MitoTracker® Red (Life Technologies, Barcelona, Spain) at a concentration 50 nM for 30 minutes. After rinsing, the plated cells were maintained in complete medium and observed with an inverted fluorescence microscope.

3. RESULTS

3.1 Cell death induction by PES was caspase-independent and more prominent in the DKO MEFs

To circumscribe the role of BAX and BAK in the cell death process triggered by PES, we characterised its lethal effects in DKO MEFs and their wt counterparts. Both cell lines were treated with increasing concentrations of PES, and the cell viability was determined after 8 hours of treatment. The cell viability was measured using the MTS procedure and found to decrease in a concentration-dependent manner (Fig. 1A). To confirm this result, both cell lines were subjected to identical PES concentrations, and the cell death was measured with the LDH procedure after 24 hours of treatment (Fig. 1B). The cell death and viability values were complementary and consistently proved the greater resistance of wt MEFs. In parallel, these cell lines were treated with staurosporine (STS) at a concentration of 1 μ M for 8 hours (MTS assay) and 24 hours (LDH assay). STS is one of the most widely accepted inducers of the intrinsic or mitochondrial pathway of apoptosis, i.e., the pathway mediated by BAX and BAK. As expected, DKO cells but not the wt MEFs were resistant.

Because apoptosis can also be triggered in a BAX- and BAK-independent manner via the extrinsic pathway, we studied the activation of effector caspases in wt MEFs treated with PES (fig. 1C). The cells were treated for 12 hours with increasing concentrations of PES and STS (200 nM) to control apoptosis and caspase activation. STS was observed to increase the caspase activity more than 10-fold compared to the basal value of the untreated cells. Conversely, the PES-induced values did not surpass the basal level, irrespective of the concentration employed. Moreover, they fell below the basal level in a concentration-dependent manner. In conclusion, the lack of effector caspase activation and non-

involvement of BAX and BAK rule out apoptosis as the lethal mode of action of PES. In addition, PES highlighted a pro-survival effect for BAX, BAK, or the combination of these proteins.

3.2 BAX protein was protecting MEFs from PES-induced cell death

We performed a new series of experiments in order to determine the involvement of BAX, BAK or the combination of these proteins in protecting cells from PES. The effects of PES on *Bax*^{-/-}, *Bak*^{-/-}, DKO and wt MEFs were tested in parallel (Fig. 2A). *Bax*^{-/-} and DKO MEFs were similarly sensitive and displayed the greatest susceptibility to PES lethality. Conversely, *Bak*^{-/-} and wt MEFs were clearly more resistant to PES. To better quantify the sensitivity of these cell lines to PES, we calculated the drug concentration required to kill 50% of the cell population (LC₅₀). Based on our data (Fig. 2A), the LC₅₀ was 6.5 μ M for DKO, 7.25 μ M for *Bax*^{-/-}, 14.5 μ M for wt and 21.5 μ M for *Bak*^{-/-} MEFs. Therefore, wt MEFs doubled and *Bak*^{-/-} MEFs approximately tripled the values of their *Bax*^{-/-} and DKO counterparts. In conclusion, the presence of BAX seemed to be the crucial element correlating with resistance to PES-induced cell death.

The content of BAX and BAK proteins was routinely assessed in the four cell lines using western blotting and found to be consistent with expected values (Fig. 2B). This determination excluded mistakes due to mislabelling or the accidental cross-contamination of the cell lines. However, PES could change the cell content of BAX and BAK proteins by modulating their expression or degradation rates. The wt MEFs were treated with increasing concentrations of PES for 8 hours, and the content of BAX and BAK proteins was assessed with a western blot (Fig. 2C). As shown, PES did not significantly affect the amounts of BAX and BAK proteins. Because epigenetics or other uncontrolled phenomena could influence the response to PES in each type of MEF, we adopted another experimental approach. We transiently transfected DKO MEFs with plasmids to overexpress the BAX, BAXS184E and BAK proteins. BAXS184E contains a Serine 184 to Glutamic mutation in the C terminus transmembrane domain. This mutation is known to impair the location of BAX to mitochondrial membranes [17]. These proteins are

fused with the green fluorescent protein (GFP), and therefore, the efficiency of the transient transfection can be assessed by fluorescence microscopy. Experiments were only continued if the efficiency rated 50% of the cell population. As shown, the non-mutated *Bax* gene conferred a statistically significant protection to DKO MEFs (Fig. 3A). Neither the *BaxS184E* nor *Bak* gene promoted any survival effect after being transfected. The effect of STS, a canonical inducer of apoptotic cell death, was tested in parallel to assess the function of the transfected proteins. The transfection of the *Bax* and *Bak* genes increased cell death in accordance with their apoptosis mediating role. The transfection of the *BaxS184E* mutant was significantly less lethal than that of the *Bax* gene, which agreed with its impaired functionality (Fig. 3B). In conclusion, the BAX pro-survival effect in cells treated with PES was proven and shown to be dependent on its appropriate insertion into the mitochondrial membranes.

3.3 Mitochondrial fusion was observed in wt MEFs treated with PES

In our initial studies of PES-induced lethality, wt and DKO MEFs were treated with 30 μ M PES for 7 hours and subjected to electron microscopy analysis. Healthy, untreated wt MEFs displayed a typical fibroblast morphology with a developed reticulum and abundant mitochondria in the cytoplasm. The nuclei were quite large and contained discernible heterochromatin condensations located preferentially adjacent to the nuclear membrane (Fig. 4A). Upon PES treatment, a prominent vacuolisation was observed in the cytoplasm (Fig. 4B). The nuclei lost their peripheral heterochromatin but remained well preserved. No apoptotic features were detected. When the resolution of the images was increased, abundant multi-lamellar autophagosome structures that characterise autophagy were observed (Fig. 4C, arrows). This cell death phenotype reinforced the occurrence of a non-apoptotic type of cell death and reproduced the type previously reported in other cells [4]. DKO MEFs subjected to PES treatment were also studied and displayed a death pattern similar to the one described for the wt MEFs (not shown). However, in PES-treated cells, branched and fused mitochondrial assemblies

displaying cristae with a normal morphology were only found in wt MEFs (Fig. 4D, arrowheads). This finding seemed to indicate that mitochondrial fusion was more common if the BAX protein was present.

3.4 Increased mitochondrial fusion had a protective effect on MEFs treated with PES.

To assess whether mitochondrial fusion protected cells challenged with PES, we relied on the Mdivi-1 compound as an inducer of mitochondrial fusion [18]. Following a pre-incubation of 6 hours with Mdivi-1 (40 μ M), MEFs became significantly resistant to PES treatment (not shown). The resistance was even greater if Mdivi-1 was maintained throughout the treatment with PES after the pre-incubation period (Fig. 5A). The protective effect of Mdivi-1 was observed in MEFs containing BAX (wt MEFs) and those not expressing this protein (*Bax*^{-/-} and DKO MEFs). However, the Mdivi-1 protection was significantly increased in wt compared to *Bax*^{-/-} or DKO MEFs. To assess the effect of Mdivi-1 on mitochondrial fusion, wt MEFs were subjected to the Mdivi-1 pre-incubation and stained with a mitochondrial dye (MitoTracker®). Control, untreated cells displayed the spotted pattern expected if mitochondria are not fused (Fig. 5B). After 24 hours of Mdivi-1 treatment, a pattern of branched and filamentous mitochondria was observed (Fig. 5C). This pattern indicated an extreme condition of mitochondrial fusion. Finally, HeLa cells were also treated with increasing concentrations of PES, and a decrease in cell viability was observed. As reported above for the MEFs, HeLa cells were also protected from PES toxicity by the 6 hours of pre-incubation and maintenance with Mdivi-1 (Fig. 5D). In conclusion, the degree of mitochondrial fusion correlated with the protection of the cells treated with PES.

DISCUSSION

The main purpose of this work was to assess the role of BAX and BAK proteins in the death process induced by PES. MEFs defective in BAX, BAK, both proteins and their wt counterparts were used as experimental tools. We concluded that neither protein was necessary for PES to cause cell

death. Moreover, PES-induced cell lethality occurred without the activation of effector caspases. Therefore, apoptosis could be excluded as the mechanism of cell death triggered by PES in our model. Unexpectedly, BAX significantly protected cells from PES treatment. This result suggests that the cellular functions of BAX differ from those involved in mitochondrial outer membrane permeabilisation (MOMP) and apoptosis induction. Although somewhat unexpected, a pro-survival function for BAX has previously been reported. For example, hypoplasia due to cell death was observed in specific tissues in early studies of *Bax*^{-/-} mice [19]. A survival effect for BAX was also observed in specific neuronal populations undergoing neurotrophic factor deprivation or Sindbis virus infection [20,21]. Finally, BAX overexpression reduced the ratios of cell death triggered by chemical compounds, such as Nigericin and 6-hydroxydopamine [22,23]. However, these reports do not provide consistent evidence about the nature of the pro-survival effect of BAX, and the issue remains very obscure.

We have shown that BAX needed to be inserted into the mitochondrial membranes in order to protect cells from PES treatment. Moreover, we demonstrated the ability of mitochondrial fusion caused by Mdivi-1 to counteract the toxic effects of PES. Based on these facts, we propose that BAX exerts a pro-survival effect via mitochondrial fusion. In support of our hypothesis, BAX is known to interact with the molecular machinery that controls mitochondrial fusion in healthy cells [24]. However, BAX is also located at the fission sites and involved in the extreme fragmentation of mitochondria observed in apoptosis [25,26]. Therefore, the involvement of BAX in mitochondrial dynamics is clear, but the final outcome seems controversial. Moreover, most members of the Bcl-2 family seem to participate in the regulation of mitochondrial dynamics, but a clear picture cannot yet be drawn regarding their precise involvement [27–29]. In Fig. 5A, we showed that Mdivi-1 more effectively prevented PES lethality when the BAX protein was present (wt MEFs), but BAX was not required for the protective effect of Mdivi-1 (*Bax*^{-/-} or DKO MEFs). Mdivi-1 can likely promote mitochondrial fusion in the absence of BAX, although less efficiently. Finally, the protective effect of Mdivi-1 is not circumscribed to MEFs because it was also found in HeLa cells (Fig. 5D).

PES is believed to generate non-apoptotic, non-autophagic cell death by promoting proteotoxic stress [4,6]. In this context, we found BAX and mitochondrial fusion converging to prevent this type of cell death. In a previous report, BAX and mitochondrial fusion mediated by Mdivi-1 promoted necrotic cell death. In this report, ionomycin and the subsequent overload of cytoplasmic Ca^{2+} were the inducers of necrosis. [30]. How can these opposite findings be conciliated? We propose that mitochondrial fusion generates opposite results depending on the death stimuli. The mitochondrial dynamics need to be studied further. For example, its role as a modulator of the cellular response to toxic insults awaits a systematic characterisation.

Normal T lymphocytes were found to be more resistant to PES than were leukemic ones [9]. Similarly, non-transformed fibroblasts were far more resistant than the 10 tumour-derived cell lines tested in parallel [4]. The DKO MEFs studied herein are known for their resistance to chemotherapy. Therefore, they can be considered to display an aggressive oncogenic phenotype [31]. We have herein reported the increased sensitivity of *Bax*^{-/-} and DKO MEFs to PES. The deficiency of BAX becomes a crucial factor to explain this increased sensitivity. In conclusion, we are expanding the profile of PES as a promising drug for its anticancer selectivity. Specifically, cancer cells harbouring oncogenic BAX deletions or mutations are expected to preferentially succumb to PES.

Conflict of interest

No conflicts of interest are disclosed.

Acknowledgements

This work was supported by the Spanish Government, MINECO (Ministerio de Economía y Competitividad), project SAF2011-29730. Paolo Mattiolo was supported by a FI fellowship from AGAUR (Generalitat de Catalunya).

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Figure legends

Fig. 1. MEFs defective in both, BAX and BAK proteins, displayed increased sensitivity to PES toxicity. As stated, wt and DKO MEFs were treated with increasing concentrations of PES and STS (1 μ M). Cell viability was determined using the MTS procedure after 8 hours of treatment. The bar value is the mean $\pm$ SEM, n = 4, each n = 5-6 replicates (A). Cell death was determined by the LDH procedure after 24 hours of treatment. The bar value is the mean $\pm$ SEM, n = 4, each n = 3 replicates (B). PES did not activate effector caspases in wt MEFs. As stated, wt MEFs were treated with increasing concentrations of PES and STS (200 nM). After 12 hours of treatment, the cells were lysed and the DEVDase activity

quantified in arbitrary fluorescent units (a.f.u.) The bar value is the mean $\pm$ SEM of 6 replicates (C). Student's t-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. MEFs deficient in BAX protein displayed increased sensitivity to PES. DKO, *Bax*^{-/-}, *Bak*^{-/-} and wt MEFs were subjected to increasing concentrations of PES as stated in the graph. Cell viability was measured using the MTS procedure after 8 hours of treatment. The values are the mean $\pm$ SEM, $n = 6$, each $n = 5-6$ replicates. Student's t-test is referred to DKO values: * $P < 0.05$, ** $P < 0.01$ (A). BAX and BAK content assessment. Protein extracts were obtained from DKO, *Bax*^{-/-}, *Bak*^{-/-} and wt MEFs and analysed using western blots with antibodies recognising BAX and BAK protein, as indicated (B). PES treatment did not affect the BAX and BAK contents. As stated, wt MEFs were treated for 8 hours with increasing concentrations of PES, and the proteins were extracted and analysed by western blotting as stated above (C) The amount of GAPDH was detected via immunoblotting to assess the amount of protein loaded per lane.

Fig. 3. BAX protein conferred resistance to PES-induced lethality to the cells. DKO MEFs were transiently transfected with the genes indicated in the x-axis. The efficiency of transfection approached 50% of the cells in culture. After a treatment with PES (7.5 μ M) for 24 hours, the cell viability was assessed using the MTS procedure (A). As a functional control, the cells were treated in parallel with STS (100nM) for 24 hours and subjected to the MTS test (B). The bar value is the mean $\pm$ SEM, $n = 1$, $n = 5-6$ replicates, representative of 3 independent experiments. Unless otherwise indicated, Student's t-test is referred to control: *** $P < 0.001$.

Fig. 4. Transmission electron microscopy of wt MEFs treated with PES. Control morphology of untreated wt MEFs. Nu: Nucleus, h: heterochromatin, m: mitochondria. Bar = 1.2 μ m (A). Cell in an intermediate stage of the death process triggered by PES; the cell membrane remained preserved, but

the cytoplasm was severely disrupted by vacuolisation, as indicated with asterisks. Nu: Nuclear chromatin. Bar = 1.8 μm (B). PES promoted the accumulation of autophagic vesicles (asterisks) with a characteristic multilamellar content (arrows). Bar = 0.16 μm (C). Fused and elongated mitochondria with preserved cristae were found after PES treatment (circumscribed by arrowheads). Bar = 0.36 μm (D).

Fig. 5. Mdivi-1 induces mitochondrial fusion and cell resistance to death induction by PES. DKO, *Bax*^{-/-} and wt MEFs were pre-incubated for 6 hours in Mdivi-1 (40 μM) (white bars) or DMSO vehicle (black bars). PES was then added for 12 hours, and the cell viability measured using the MTS procedure. The concentration of PES was 8 μM in *Bax*^{-/-} and DKO MEFs. To equalise the cell viability rates in the absence of Mdivi-1, the concentration of PES was 12.5 μM in wt MEFs. The bar value is the mean $\pm$ SEM, $n = 2$, each $n = 3$ replicates. Student's t-test: *** $P < 0.001$ (A). Control untreated wt MEFs stained with MitoTracker® dye. Bar = 100 μm (B). Fused mitochondria in wt MEFs, incubated in Mdivi-1 (40 μM) and stained with MitoTracker®. Bar = 100 μm (C). HeLa cells were also pre-incubated for 6 hours with Mdivi-1 (white bars) or DMSO vehicle (black bars) as stated above. The cells were then treated with PES at the indicated concentrations, and the cell viability was determined. The bar value is the mean $\pm$ SEM of 3 replicates. Student's t-test: ** $P < 0.01$, *** $P < 0.001$ (D).

Fig. 1.

Fig. 2.

A

B

C

Fig. 3.

A

B

Fig. 4.

Fig. 5.