Two aspects of cisplatin-induced apoptosis: potentiation by energy modulators and apoptosis inhibition by high concentrations
SUMMARY

Tumour cells have a tendency to alter their energy metabolism; they switch from oxidative phosphorylation to aerobic glycolysis and fatty acid β-oxidation. This property can probably be used in future cancer treatments, where the tumour cells could be treated with pharmacological modulators of energy metabolism (MEMs) that either block glycolysis or β-oxidation or otherwise affect energy metabolism. MEMs in combination with different chemotherapeutic drugs are hypothesized to induce apoptosis in cells that have become resistant to chemotherapeutic drugs. Here, the apoptotic doses for the different MEMs were determined as well as how well they potentiated apoptosis in combination with low doses of chemotherapeutic drugs, in particular cisplatin.

My co-workers have shown earlier that in HCT116 colon cancer cells treated with 30 μM cisplatin, pore-like Bak complexes in the mitochondrial outer membrane allow release of cytochrome c and the subsequent activation of caspases. Treatment with 90 μM cisplatin, however, was shown to abrogate all cell death for unknown reasons. The relationship between the apoptosis-related proteins Bak and Bax was examined in this study, which indicated that despite inhibited cell death Bak complexes are present in cells treated with 90μM cisplatin. This means that 90 μM cisplatin does not inhibit formation of Bak complexes, as inhibition of formation of these complexes was earlier thought to be one of the reasons for inhibited cell death.
INTRODUCTION

Tumour cells produce half of their ATP through oxidative phosphorylation, which differs from normal cells that produce 95% of their ATP through the same process. The rest of the required ATP in tumour cells is produced through aerobic glycolysis, and fatty acid β-oxidation also supports non-glucose fuel oxidation and production of reducing equivalents (NAD(P)H). Tumour cells switch to glycolysis even in presence of oxygen, a phenomenon called the Warburg effect (Xu et al., 2005, Harper et al., 2002, Shaw, 2006).

Tumour cells can become resistant to chemotherapeutic drugs, but recent studies have shown that different pharmacological modulators of energy metabolism (MEMs) in combination with different chemotherapeutic drugs can induce apoptosis in those cells that have become resistant to chemotherapeutic drugs (Xu et al., 2005, Ihrlund, Khan, Hernlund, Castro, Panaretakis and Shoshan, personal communication).

Apoptosis and necrosis

Apoptosis is a process of programmed cell death, and is an important defence mechanism for the body. It involves a series of biochemical events that lead to breakdown of the cell into membrane-bound apoptotic bodies and to cell death. Apoptosis is executed in such a way as to safely dispose of cell fragments and thereby not affect the tissue negatively (Linder et al., 2004).

In case of cancer, the cell death is enhanced and the clearance mechanisms may not be able to clear out all the cell fragments present. Apoptotic bodies that are not engulfed by macrophages will dissolve and their contents may reach the circulation (Linder et al., 2004). Cytokeratins are intermediate filament proteins that are expressed by most types of tumour cells. During apoptosis and necrosis these proteins can be released into the circulation due to impaired cell membrane integrity. However, it is only during apoptosis that cytokeratins are
cleaved by caspases, whereupon they are released into the circulation. Caspase-cleaved fragments of cytokeratin 18 can be detected by a monoclonal antibody that recognizes only the caspase-cleaved fragments and not the intact protein. Therefore, elevated levels of caspase-cleaved cytokeratin 18 is associated with higher apoptosis (Linder et al. 2004). Necrosis is a pathological cell death. In contrast to apoptosis, necrosis is less orderly and causes inflammation due to release of intracellular content. It is accompanied by mitochondrial swelling and increased plasma membrane permeability. The intracellular content is released because it is difficult for the immune system to locate and recycle dead cells, since necrosis usually does not send out any signals to the phagocytes that should engulf the dead cells (Wu et al., 2001).

One of the cytosolic proteins that is released into the circulation following membrane damage is lactate dehydrogenase (LDH). It is often used as a marker for measuring necrosis levels. However lactate dehydrogenase can sometimes be released in small amounts during late apoptosis, due to damage to the cell membrane before the cells were cleared. Therefore, lactate dehydrogenase can sometimes also be used as a marker for apoptosis measurement (Moran and Schnellmann, 1996).

There are two apoptotic pathways, the intrinsic and the extrinsic pathway. The intrinsic pathway is the mitochondrial pathway, which is activated as a response to, e.g., DNA damage. A family of proteins important for the intrinsic pathway is the Bcl-2 (B-cell/lymphoma-2) family. These proteins regulate the initiation of apoptotic response. Two proteins, Bak and Bax, from this family are required to form mitochondrial pores for release of cytochrome c from mitochondria, leading to caspase activation (Ihrlund-Strandberg, 2007). The extrinsic pathway is the death receptor pathway, activated by binding of ligands to death receptors that belong to the Tumour necrosis factors receptor family (Nelson and White, 2004). Both pathways lead to activation of caspases, which in turn lead to proteolysis of nuclear and
cytoskeletal components that gives the cells a characteristic apoptotic phenotype (Ihrlund-Strandberg, 2007).

Many chemotherapeutic drugs and irradiation induce a MAPK (mitogen-activated protein kinase) family member, C-Jun N-terminal kinases (JNK), that regulates Bax activity, and induces apoptosis by helping Bax to translocate to mitochondria (Tsuruta et al, 2004). However, JNK is not involved in activation of Bak, but stimulates already activated Bak to form complexes that are important for apoptosis (Ihrlund et al, 2006). In other words, activated Bak by itself does not induce apoptosis, but needs to form complexes for apoptosis to take place. Cisplatin, a chemotherapeutic drug (Adams and Cory, 1998, Ihrlund et al, 2006), can induce JNK-stimulation of activated Bak, and make Bak form apoptosis-related complexes (Ihrlund et al, 2006) (Figure 1).

**Bis-maleimidohexane and Bis(Sulfosuccinimidyl)suberate crosslinking**

Both Bak and Bax expose their N-terminal epitope when they are activated. This property has been seen after treatment with cisplatin (Adams and Cory, 1998, Ihrlund et al, 2006). BMH (Bis-maleimidohexane) can crosslink molecules that are included in Bak and Bax-containing protein complexes, which are present in mitochondria-enriched heavy membrane fractions. Crosslinking of the Bak molecules protects the complexes from being dissociated by sodium dodecyl sulphate (SDS) (which is a detergent), and makes it possible to detect the complexes using western blotting. BMH (Figure 2) has a spacer arm of 16.1 Å, which is enough to crosslink the inactive Bak and fix it in a specific position, while at the same time it also crosslinks the molecules included in the Bak complexes (Ihrlund-Strandberg, 2007). Another crosslinker, BS³ [Bis(Sulfosuccinimidyl)suberate], has a spacer arm of 11.4 Å (www.piercenet.com).
When western blotting is done, using the anti-Bak (rabbit) antibody for detection, the inactive Bak will be seen at 21 kDa while the pore-forming complexes of Bak will be seen as slower-migrating multimers.

![Diagram of JNK-stimulation of activated Bak](image)

Figure 1. Cisplatin induces JNK-stimulation of activated Bak and makes it form complexes that promote apoptosis by allowing cytochrome c release (Ihrlund et al., 2006).

![Bis-maleimidohexane structure](image)

Figure 2. *Bis*-maleimidohexane (www.piercenet.com).

One of my co-workers has found that human colon cancer HCT116 wt-p53 cells treated with 90 μM cisplatin did not undergo apoptosis, but were healthy and increased in number, while cells treated with 30 μM cisplatin underwent apoptosis as expected. The question is what happened when cells were treated with 90 μM cisplatin, and how it is possible that the cells even proliferated during this treatment. Experiments showed that active Bak and active JNK were present in the cells, and the only difference from cells treated with 30 μM cisplatin
was that Bax was not activated at all. It is also known that BAPTA-AM (which is a calcium-specific chelator) inactivates the Bax that is present in cells treated with 30 μM cisplatin.

**Aims**

My hypothesis was that treatment with 90 μM cisplatin would have a similar effect on cells as treatment with 30 μM cisplatin together with BAPTA. I therefore wished to examine whether Bak complex formation was impaired in cells treated with 90 μM cisplatin and whether this correlated with altered presence of Bax in the mitochondrial fraction. Here I also investigated how well different MEMs induced apoptosis when combined with chemotherapeutic drugs, and at which doses.
RESULTS

1. Potentiation of platinum-induced apoptosis using MEMs

To measure the apoptosis level in human colon cancer HCT116 wt-p53 cells the amount of an apoptosis marker was measured. The cells were treated with chemotherapeutic drugs such as cisplatin, paraplatin and doxorubicin. In order to examine the possible synergetic effects of the different MEMs the chemotherapeutic drugs were combined with MEMs such as 2-deoxyglucose (DG), etomoxir, metformin, rosiglitazone and pioglitazone. 2-deoxyglucose is a glycolysis inhibitor that inhibits the first enzyme of the glycolytic pathway. Etomoxir inhibits the β-oxidation of fatty acids, while metformin, rosiglitazone and pioglitazone are drugs used for treatment of diabetes, but are proven to have antiproliferative effects on cancer cells (Ihrlund, 2007).

Figure 3 shows the dose-response for metformin, pioglitazone and rosiglitazone. The result showed that metformin at 5 mM and 10 mM did not induce apoptosis, and that it induced some apoptosis at 15 mM and 20 mM. The result also showed that pioglitazone did not induce apoptosis at 10 mM, but it did induce apoptosis at 50 mM and 100 mM. Increase in apoptosis induction with 50 μM and 100 μM pioglitazone in combination with 0.75 mM valproic acid was seen. Rosiglitazone induced apoptosis more at 10 mM than at 50 mM, which could be due to more necrosis at 50 mM.

Based on specific assessment of accumulated apoptosis, the results showed that paraplatin might be potentiated by 20 mM of 2-deoxyglucose, although a smaller potentiation was also seen with 10 mM of 2-deoxyglucose (Figure 4). The combination of 12 μM cisplatin and 25 μM pioglitazone had a great synergetic effect on apoptosis, and also 10 mM metformin potentiated the apoptotic response to cisplatin. A small potentiation of doxorubicin-induced apoptosis was seen with 10 mM metformin (Figure 5).
To sum up these results, the highest level of apoptosis potentiation was seen with paraplatin together with 20 mM of 2-deoxyglucose, cisplatin combined with 25 µM pioglitazone and cisplatin combined with 10 mM metformin.

Figure 3. Dose-response for HCT116 p53wt cells.

Dose-response for different MEMs on human colon cancer HCT116 p53wt cells, over a period of 24 hours. Approximately 10,000 cells were seeded in 96-well plates and treated with drugs as indicated, in 37°C. The M30 Apoptosense Assay is a type of ELISA, and was used to measure apoptosis by measuring the accumulation of the CK18Asp396 neo-epitope that is exposed after cleavage of CK18 by caspases. The color intensity was measured at 450 nm. The control samples contain untreated cells.
Figure 4. The response of HCT116 p53 wt cells to different drug treatments.

The apoptotic response of human colon cancer HCT116 p53 wt cells to Doxorubicin per se and to Paraplatin in combination with DG and Etomoxir, over a period of 24 hours. Approximately 10,000 cells were seeded in 96-well plates and treated with drugs as indicated, in 37°C. Apoptosis was measured with the M30 Apoptosense Assay that measures the accumulation of the CK18Asp396 neo-epitope that is exposed after cleavage of CK18 by caspases. The color intensity was measured at 450 nm.
Figure 5. Response of HCT116 p53 wt cells to different drug treatments.

Apoptotic response of HCT116 p53wt cells to combination treatments, over a period of 24 hours. Approximately 10,000 cells were seeded in 96-well plates and treated with drugs as indicated, in 37°C. Apoptosis was measured with the M30 Apoptosense Assay that measures the accumulation of the CK18Asp396 neo-epitope that is exposed after cleavage of CK18 by caspases. The color intensity was measured at 450 nm.

2. Effects of 90 µM cisplatin on formation of Bak complexes in the mitochondria

In order to explain why 30 µM cisplatin induced apoptosis while 90 µM blocked the apoptotic machinery (Hernlund, unpublished data), the mitochondria were extracted from human colon cancer HCT116 p53 wt cells treated with 30 µM and 90 µM. After addition of crosslinkers BMH and BS³ that crosslink the molecules present in Bak and Bax-containing complexes in the isolated mitochondria-enriched heavy-membrane fractions, western blotting was done and the results were recorded using a camera. The results presented here indicate that a larger
amount of approx 80 kDa Bak-containing complexes (i.e., possibly trimers) was formed after treatment with 90 μM cisplatin than after treatment with 30 μM cisplatin, and cisplatin 30 μM in combination with BAPTA-AM 10 μM (Figure 6). The complexes were only found after treatment with 90 μM cisplatin when the molecules were crosslinked with BMH and BS³. BAPTA-AM was used in the experiment because others in the research group had shown that it blocks apoptosis induced by 30 μM cisplatin likely by leading to inactivation of Bax. Treatment with 90 μM cisplatin earlier also has been shown to inhibit Bax activation.

Figure 7 shows the same gel as in figure 6, but blotted in 1% methanol. Methanol usually is added to the transfer buffer during the blotting to enhance the transfer efficiency of hydrophobic proteins, since it is difficult to transfer hydrophobic proteins to solid membranes. Generally 10-20% methanol is used, but here 1% methanol was used in order to transfer protein complexes with high molecular weight. Bak complexes here were detected at 200 kDa with the BAPTA + 30 μM treatment. However, these complexes are not correlated with apoptosis (Ihrlund et al., 2006).

The aim of detecting Bax on the membrane in Figure 8 was to see if there were any differences between cells treated with 30 μM cisplatin and cells treated with 90 μM cisplatin. The question was also whether there were any differences between treatment with 90 μM cisplatin and with 30 μM cisplatin plus 10 μM BAPTA. A Bax-containing complex was detected at around 60 kDa, but only after treatment with 90 μM cisplatin. Bax-containing complexes were also seen in cells with the same treatment, in the sample treated only with dimethyl sulfoxide (DMSO), which is not possible in theory, since DMSO is known to have strong protein denaturing properties. Therefore, this indicates a possible mix-up of samples (Figure 8). Unfortunately this experiment was not repeated due to lack of time.
Figure 6. Detection of Bak with western blotting.

Negative image of a 10% Bis-Tris Gel transferred onto a membrane in 10% methanol. The wells were loaded with proteins that were treated with dimethyl sulfoxide (DMSO), BMH or BS³. Bak was detected with the anti-Bak (rabbit) antibody. The membrane was photographed in an Intelligent Dark Box-camera.

1: Only dimethyl sulfoxide added to proteins; 2: BMH crosslinker added to proteins; 3: BS³ crosslinker added to proteins.
Figure 7. Detection of Bak with western blotting.

Negative image of 10 % Bis-Tris Gel transferred onto a membrane in 1% methanol. A small concentration of methanol was used in order to be able to transfer high molecular weight-proteins onto the membrane. The wells were loaded with proteins that were treated with dimethyl sulfoxide (DMSO), BMH or BS\(^3\). Bak was detected with the anti-Bak (rabbit) antibody. The membrane was photographed in an Intelligent Dark Box-camera.

1: Only dimethyl sulfoxide added to proteins; 2: BMH crosslinker added to proteins; 3: BS\(^3\) crosslinker added to proteins.
Figure 8. Detection of Bax with western blotting.

Negative image of a 10% Bis-Tris Gel transferred onto a membrane in 10% methanol. The wells were loaded with proteins that were treated with dimethyl sulfoxide (DMSO), BMH or BS\(^3\). Bax was detected with the anti-Bax (rabbit) antibody. The membrane was photographed in an Intelligent Dark Box-camera.

1: DMSO only; 2: BMH crosslinking; 3: BS\(^3\) crosslinking.

These results showed that Bak-containing complexes (i.e., possibly trimers) were formed after treatment with 90 \(\mu\)M cisplatin. A Bax-containing complex was also detected after treatment with 90 \(\mu\)M cisplatin.
DISCUSSION

Potentiation of platinum-induced apoptosis using MEMs

The results from Figures 4 and 5 show that DG, pioglitazone and metformin induce more apoptosis than rosiglitazone and etomoxir. With a few exceptions, potentiation was not remarkably great, but the possibility that necrosis could be responsible for additional cell death should be taken into consideration. Experiments where necrosis is measured with for example the LDH (lactate dehydrogenase) assay should be done to see if necrosis is responsible. LDH is an enzyme that is released when the cells undergo necrosis. When the LDH reagent is added to cells after the experimental treatment, the medium changes colour in proportion to the LDH that is released from the cells into the medium. The colour intensity is measured in a spectrophotometer.

In my experiments valproic acid was tested in combination with pioglitazone because it had been shown earlier (Annicotte et al, 2006) that a combination of these two resulted in arrest of proliferation and increase of apoptosis. Moreover, it was also shown that a combination of valproic acid and pioglitazone decreased the invasivity of prostate cancer cells more than the each substance individually (Annicotte et al, 2006).

Although these results give an indication of which combinations work best, more experiments need to be done in order to get a better understanding of the efficiency of different MEMs and chemotherapeutical drugs. For example, the same combinations used in these experiments could be tested on spheroids of HCT116 cells. Spheroids are three-dimensional cell aggregates that have similar properties as tumours in vivo (Mueller-Klieser, 1987). This would increase the physiological relevance of these experiments and perhaps also give an indication of how tumours in vivo would respond to the combinations of chemotherapeutical drugs and different MEMs.
Treatment with 90 µM cisplatin does not affect the formation of Bak complexes

In the second part of the study, cisplatin was used at the very high concentration of 90 µM. An observation was earlier made by my co-workers that while 30 µM cisplatin induces apoptosis, cells treated with 90 µM cisplatin did not undergo apoptosis, even though Bak and JNK were highly activated, and the Bax protein was upregulated. Their data showed (Hernlund, unpublished data) the only difference observed so far, which was that with 90 µM cisplatin, Bax activation was impaired. A similar impairment of Bax activation had been observed when cells were co-treated with 30 µM cisplatin and BAPTA-AM, a calcium chelator. However, Bak and Bax are generally believed to have overlapping functions, and loss of only one of these protein activities usually does not block cell death as completely as 90 µM cisplatin did (M. Shoshan, personal communication).

My starting hypothesis was that the Bak molecules were not able to form apoptosis-related complexes in cells treated with 90 µM Cisplatin. If so, the expected outcome of experiments where cells were treated with cisplatin and BAPTA (in figure 6, 7 and 8) would be that no mitochondrial Bak complexes would be seen in the cells treated with 30 µM cisplatin and BAPTA, or cells treated with 90 µM cisplatin, but only in cells treated with 30 µM cisplatin. However, I found 80 kDa (trimeric) Bak complexes in cells treated with 90 µM cisplatin, but not in any other samples. Since the complexes were seen only in cells treated with 90 µM cisplatin they might represent a particular type of complex that might correlate with the complete blocking of apoptosis in these cells. More experiments are needed to reveal the cause of absence of apoptosis in cells treated with 90 µM cisplatin.

As mentioned before, active Bax that is normally present in cells treated with 30 µM cisplatin is inactivated by the cotreatment with BAPTA. Figure 8 shows a Bax-containing complex at 60 kDa, but only in cells treated with 90 µM cisplatin. The complex was seen in
samples treated with BMH and DMSO. This result resembles the result of Bak-complex detection, i.e. the treatment with 90 μM cisplatin gives different results from the treatment with 30 μM cisplatin + BAPTA (Figure 6, 7 and 8). This Bax-containing complex appeared so prominent that it may have a significant but as yet not understood role. The detection of Bax-containing complex in the sample treated with DMSO was probably due to a mix up of samples, after which the experiment was not repeated due to lack of time.

However, the results only showed that the Bak and Bax complexes were present in cells treated with 90 μM cisplatin, but not how or if they might contribute to the cell death blocking induced by 90 μM cisplatin. Perhaps it is another component of the apoptotic signalling pathway that is not present, or not active. If the treatment with 30 μM cisplatin + BAPTA induces completely different components of apoptosis from the ones induced by 90 μM cisplatin, then there should probably be a difference between the 30 μM cisplatin-treatment and the 90 μM cisplatin-treatment that leads to inhibition of the apoptotic machinery. Since this study indicates that there are Bak-complexes in cells treated with 90 μM cisplatin (and 30 μM cisplatin according to earlier experiments) perhaps the Bak-complexes that are formed are not able to induce the release of cytochrome c from the mitochondria, because some other component that is necessary for this event is inactivated.

However, in order to answer the question why the cells do not undergo apoptosis during treatment with 90μM cisplatin, more studies are needed.
Materials and methods

Cells
HCT116 human colon carcinoma cells were used. The cells were cultured in McCoy’s medium (GIBCO™ McCoy's 5A Medium, Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1% of a penicillin/streptomycin mixture (Invitrogen), until 80% confluency.

Inhibitors
The cells were treated with 2-deoxyglucose, metformin, etomoxir, paraplatin, doxorubicin, valproic acid, cisplatin, rosiglitazone and pioglitazone. 2-deoxyglucose, metformin, etomoxir, paraplatin, doxorubicin and valproic acid were purchased from Sigma-Aldrich Sweden AB. Cisplatin was purchased from Bristol-Myers Squibb, while pioglitazone and rosiglitazone were a gift from professor Åke Sjöholm, Karolinska Institute, Södersjukhuset, Stockholm, Sweden.

Antibodies
Mouse cytokeratin18 monoclonal antibody “M5” (PEVIVA AB, Sweden)
Horseradish peroxidase conjugated anti-cytokeratin 18 M30 monoclonal antibody (PEVIVA AB, Sweden)
Rabbit anti-BAK (Upstate-Cell Signalling Solutions)
Anti-rabbit-IgG (Upstate-Cell Signalling Solutions)
Rabbit anti-Bax (Santa Cruz Biotechnology)

Apoptosis assay
The M30-Apoptosense™ ELISA Kit (PEVIVA AB, Sweden) is sandwich/two site ELISA that measures accumulation of a specifically caspase-cleaved fragment of cytokeratin-18
The cleavage site is C-terminal to the aspartic acid residue 396 (CK18Asp396) and cleavage creates a neo-epitope on the molecule (CK18Asp396 neo-epitope) that is specifically recognized by the M30 antibody.

The principle of this type of ELISA is to coat the plate with a capture antibody (an antibody that is directed against Cytokeratin18), which binds to caspase-cleaved Cytokeratin18. This antibody is conjugated to an enzyme called HRP (horseradish peroxidase). Adding the substrate makes colour develop in proportion to the bound analyte.

The assay was performed according to the manufacturer’s instructions (PEVIVA AB, Sweden). Approximately 10,000 cells were seeded in 96-well plates and treated with drugs as indicated. To lyse the cells, 2 µl of 10% Nonidet P-40 was added to each well that contained the treated cells, on a 96-well plate. 25 µl of each standard (A, B, C, and D standard, 50, 125, 500 and 1,000 µg/L, provided with the kit) was added to the wells on the microstrips coated with mouse anti-cytokeratin18 monoclonal antibody “M5”. The samples (25 µl each) were also transferred to the microstrips and 75 µl of horseradish peroxidase conjugated to anti-cytokeratin 18 M30 monoclonal antibody was added. The plate was covered with parafilm and incubated at room temperature on a shaker for 4 hours. After the incubation the wells were emptied and washed five times with 250 µl washing solution (PBS-Tween) (0.014 M phosphate buffer pH 7.4, 0.15M sodium chloride, 0.1 % Tween 20), to remove unbound conjugate. An amount of 200 µl of TMB (3,3',5,5’-Tetramethyl-benzidine) (PEVIVA AB) substrate solution was added to each well for the colour to develop in proportion to the bound analyte. The samples were incubated for 20 min at room temperature and the colour development was stopped with 50 µM stop solution (1.0 M sulphuric acid). The intensity of the colour was measured in a spectrophotometer at 450 nm absorbance.
**Mitochondria extraction:**

The medium from four 15-cm plates with cells was collected and transferred to 4 Falcon tubes (50 ml) in order to collect floating cells. In order to isolate mitochondria-enriched heavy-membrane fractions the following procedure was done:

The attached cells were trypsinized and transferred to the corresponding Falcon tube, and everything was centrifuged for 5 minutes (1400 x g, 4°C). The pellets were washed in ice cold TD buffer (135 mM NaCl, 5 mM KCl, 25 mM Tris-HCl (pH 7.6, ice-cold), resuspended in 1 ml CaRSB buffer (10 mM NaCl, 1.5 mM CaCl₂, 10 mM Tris-HCl (pH 7.5, ice-cold) and transferred to 14 ml Falcon tubes for incubation on ice for 20 minutes. The cells were broken by repeated flushing through a small syringe, about 12 times/tube. An amount of 1 ml of mitochondrial buffer (250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES pH 7.2, and 0.1 mM phenylmethanesulphonylfluoride) was added to each tube, and they were centrifuged for 10 minutes (2000 x g, 4°C). The supernatants were transferred to eppendorf tubes and centrifuged for 30 minutes at maximum speed (25 000 x g, 4°C). Each pellet was resuspended in 65 μl HEPES buffer (2 mM KH₂PO₄, 10 mM HEPES, 250 mM sucrose, 5 mM sodium succinate, 25 mM EGTA).

**Crosslinking:**

The crosslinkers, BMH (1,6 Bis-maleimidohexane) and BS³ [Bis(Sulfosuccinimidyl) suberate] from PIERCE Technologies, were dissolved in dimethyl sulfoxide to stock solutions of 100 mM. Each mitochondrial sample was split in three, and treated with dimethyl sulfoxide, BMH (10mM) or BS³ (10mM), respectively, as follows, and based on earlier experience:

1. 10 μl of sample + 1 μl of DMSO
2. 25 μl of sample + 2.5 μl of BMH
3. 25 µl of sample + 2.5 µl of BS³

In order to make a subsequent protein assessment, 5 µl of each sample was also removed. The samples were incubated at room temperature for 30 minutes, and the reaction was stopped by adding NuPAGE® LDS sample buffer (Invitrogen). After the incubation the samples were put in a freezer for later analysis by western blot.

**Protein measurement:**

BSA standards (125, 250, 500 and 1000 µg/ml) were used. An amount of 795 µl of dH₂O was put in 9 eppendorf tubes (5 tubes for the standards incl. the blank, and 4 tubes for the samples). To the tube with the blank 5 µl of dH₂O was added, while 5 µl of each standard was added to its respective tube. To the rest of the tubes, 5 µl of respective sample was added. To all tubes 200 µl of Bio-Rad Protein Assay Reagent was added and the tubes were vortexed. After 10-15 minutes the contents of the tubes were transferred to plastic cuvettes and examined in a spectrophotometer, at a wavelength of 450 nm.

**Western blotting:**

An amount of each sample was diluted with dH₂O to get equal concentrations of protein in every sample, and 1 µl of blue sample buffer (Invitrogen) was added to them. The samples were boiled for 10 minutes and loaded onto a NuPAGE® 10 % Bis-Tris gel (Invitrogen) with 15 wells (1.0 mm/well). The gel was run with NuPAGE® MOPS running buffer (Invitrogen) for 1 hour on 200 V. For the blotting procedure, a few nylon pads and 2 filter papers were soaked in NuPAGE® transfer buffer (Invitrogen) with 10 % MeOH. A piece of polyvinylidene fluoride (PVDF) membrane was activated for 3 seconds in 100 % MeOH and placed on the back side of the gel. One of the filter papers was placed on the PVDF membrane and the other was placed on the front side of the gel. Half of the soaked pads were put in the
bottom of a blotting module, and the gel was placed on them with the membrane on top. The rest of the soaked pads were put on top of the gel and the module was placed in an XCell II Blot Module and filled with NuPAGE® transfer buffer (Invitrogen) containing 10 % MeOH. The outside of the module was filled with de-ionized water. The blotting was run for 1 hour, on 30 V. The blotting was then repeated, with a new PVDF membrane and NuPAGE® transfer buffer (Invitrogen) with 1% MeOH instead, to be able to blot the larger proteins that are between 100 kDa and 200 kDa in size.

The resulting membranes were blocked with 5% dry milk in PBS-Tween (0.014 M phosphate buffer pH 7.4, 0.15M sodium chloride, 0.1 % Tween 20) for 1 hour and then washed three times in PBS-Tween. They were then incubated overnight on shaking at 4°C with a primary antibody in milk (rabbit anti-BAK Upstate-Cell Signalling Solutions) at a dilution of 1:1500. After washing three times in PBS-Tween, the membranes were incubated with the secondary antibody in milk (anti-rabbit, Upstate-Cell Signalling Solutions) for 1 hour at room temperature. After washing, each membrane was incubated in 2.5 ml of SuperSignal West Pico Chemiluminescent Substrate (PIERCE Technologies) for 1 minute and the results were recorded using a camera (FUJIFILM Intelligent Dark Box, LAS-1000), with an exposure time of 5 minutes.

The Bak antibody was removed by treating the membranes with stripping buffer (Restore Western Blot Stripping Buffer, PIERCE Technologies) for 15 minutes. After washing 3 times with PBS-Tween, the membranes were incubated again with a primary antibody (rabbit anti-Bax Santa Cruz Biotechnology), and after the second washing they were incubated in secondary antibody (anti-rabbit, Upstate-Cell Signalling Solutions) and treated as before for signal development and recording.
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