2. MATERIAL AND METHODS

2.1.MATERIALS

2.1.1. Reagents

Salts and buffer reagents were purchased from Invitrogen, (Karlsruhe, D), Roth (Karlsruhe, D), Merck (Darmstadt, D) or Sigma (Munich, D). Solutions and solvents were purchased from J.T.Baker (Deventer, NL), Merck (Darmstadt, D), Roth (Karlsruhe, D) or Sigma (Munich, D).

Special agents were purchased as below:

Bisphosphonates:

dichloroydromethylene-diphosphonic acid (clodronate)	Sigma (Munich, D).
pamidronate (Aredia)	Novartis Pharma (Nürnberg, D)
zoledronate (Zometa)	Novartis Pharma (Nürnberg, D)

All drugs were dissolved in water and stored at -20 °C

crystal violet	ICN (Eschwege, D)
hydroxyurea	Sigma (Munich, D)
propidium iodide	Sigma (Munich, D)
ribonuclease A	Sigma (Munich, D)

Consumable goods :

nitrocellulose membrane	Schleicher & Schwell (Dassel, D)
scientific imaging films	Kodak (Berlin, D)

2.1.2. Cell culture materials

Cell culture flasks, centrifuge tubes	Nunc (Wiesbaden, D)
50 ml Falcon tubes, serological pipettes	Becton Dickinson Labware (Franklin Lakes NJ, USA)

6-, 24-wells plates	Corning (New York, NY, USA)
Sterile filters	B.Braun (Melsungen, D)
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen (Karlsruhe, D)
Foetal Calf Serum (FCS)	Seromed-Biochrom (Berlin, D)
Penicillin/ Streptomycin	Seromed-Biochrom (Berlin, D)
Phosphate buffered saline (PBS)	Seromed-Biochrom (Berlin, D)
Trypsin	Seromed-Biochrom (Berlin, D)
Geneticin G-418 sulphate	Invitrogen (Karlsruhe, D)

2.1.3. Antibodies

Anti-cytochrome c monoclonal antibody (mouse)	BD-PharMingen (Hamburg, D)
Anti-β-actin monoclonal antibody (mouse)	Sigma (Munich, D).
Anti-mouse antibody coupled with horseradish-peroxidase (goat)	Dako (Hamburg, D)

2.1.4. Cell lines

A375 melanoma cell line, originating from a primary melanoma was purchased from American Type Culture Collection (ATCC) (CRL-1619). M186 melanoma cell population was obtained by surgical intervention from a patient with histological confirmed melanoma metastasis.

The melanoma cell line A375/Bcl-2, which stably overexpresses Bcl-2 was obtained in our laboratory. The construction of pIRES/mBcl-2 has been described previously (MÜLLER-WIEPRECHT *et al.*, 2000; RAISOVA *et al.*, 2001). After a period of approximately 10 weeks of selection with geneticin, individual cell clones were isolated. Bcl-2 overexpression in these cell lines was confirmed by Western blot analysis (data not shown).

2.1.5. Equipment

centrifuges	Biofuge pico, Heraeus (Osterode, D)
	Biofuge fresco, Heraeus (Osterode, D)
	Laborfuge 400E, Heraeus (Osterode, D)
	Varifuge RF, Heraeus (Osterode, D)
	Centrifuge J2-21, Beckman Instruments (Glenrothes, GB)
imaging densitometer	Model GS-700, Bio-Rad (Munich, D)
CO_2 -incubator	BB16, Heraeus (Osterode, D)
drying oven	T6 Heraeus (Osterode, D)
electrophoresis -equipment	Mini Protean II, Bio-Rad (Munich, D)
electrophoresis-power supplies	Model 1000/500, Bio-Rad (Munich, D)
ELISA photometer	Model 550, Bio-Rad (Munich, D)
flow cytometry system	FACSCalibur, BD Biosciences (Heidelberg, D)
heating bath	1.002, GFL (Burgwedel, D)
incubation shaker	Aerotron AI 18 Infors (Bottmingen, CH)
laminar flow bench	BSB 4A, Gelaire Flow Laboratories (Opera, I)
magnetic stirrer	MR 2000, Heidolph (Kehlheim, D)
microscopes	Diavert, Leitz (Wetzlar, D)
	BX60F5, Olympus (Berlin,D)
mixers	Thermomixer comfort, Eppendorf (Hamburg, D)
	Vortex IKA VF2, Jahnke und Kunkel (Staufen i. BR., D)
pH- meter	pH 526, WTW (Weilheim i.OB, D)
precision balances	Kern 474, Gottl Kern& Sohn (Albstadt, D)
	Type 2662, Sartorius (Göttingen, D)
western blot transfer unit	Mini-Trans-Blot, Bio-Rad (Munich, D)

2.2. METHODS

2.2.1. Cell culture

2.2.1.1. Cell culture media and solutions

For growth and cultivation of melanoma cell lines following media and solutions were used:

Dulbecco's modified Eagle' medium (DMEM / 10 % FCS)

DMEM (incl. 4 mM L-glutamine, 25 mM glucose, without sodium pyruvate)	500 ml
heat inactivated fetal calf serum (FCS)	50 ml
10,000 IE penicillin/ 10,000 µg/ ml streptomycin (stock solution)	5 ml

The complemented medium can be stored for four weeks at 4 °C.

Trypsin solution

trypsin	1.5 g
phosphate buffered saline (PBS)	500 ml

The solution is stored in aliquots of 10 ml at -20 °C.

Freezing solution for melanoma cells

DMEM	20 ml
heat-inactivated fetal calf serum	20 ml
dimethylsulfoxid (DMSO)	10 ml

The solution is stored in aliquots of 10 ml at -20°C.

2.2.1.2. Cultivation of cells

Melanoma cells were grown in 12 ml DMEM / 10 % FCS in 75 cm^2 tissue culture flasks, in a humidified atmosphere with 5 % CO_2 at 37 °C. All melanoma cell lines grow adherent on the ground of the flasks. Every third day the medium was removed, cells were rinsed with 6 ml PBS to remove dead cells and catabolites, and supplemented with fresh medium. For passaging, 80 % confluent cells were rinsed with PBS, and then incubated with 3 ml trypsin solution at 37 °C for 5 minutes. After detachment from the flask's ground, cells were harvested in 10 ml DMEM/10 % FCS. FCS was used to stop the proteolytic activity of trypsin. The cells were then pelleted at 200 x g for 5 minutes. The supernatant was aspirated, the cells were resuspended in DMEM/10 % FCS and then counted in a hemocytometer. The cells were then seeded with 10,000 cells/cm^2 in new flasks. The first change of medium occurred after 36 hours.
The A375/Bcl-2 and A375/pIRES were maintained in culture medium supplemented with geneticin (G-418) (870 μM).

2.2.1.3. Freezing and thawing of cells

Confluent cells were washed in 6 ml PBS and trypsinized as described previously (2.2.1.2). The supernatant was then aspirated and cells resuspended in 1 ml freezing solution. The cell suspension was then transferred in 1 ml cryotubes. The presence of DMSO in the freezing solution prevents the crystal growth within cells. Freezing of cells has to be a slow process, so that the freezing tubes were packed in tissue paper and kept for 6 hours at -20 °C, then transferred at -80 °C, where the cells can be stored for up to 6 months. Subsequently the cells were stored in liquid nitrogen (-196 °C), where they can be stored unlimited.

Thawing of the cells should be in contrast fast. Therefore the cells are thawed at 37 °C in an water bad, resuspended in 20 ml of DMEM/10 % FCS previously warmed at 37 °C, and then centrifuged at 200 x g for 5 minutes. Supernatant is aspirated, the cell pellet resuspended in 24 ml culture medium and then distributed in two culture 75 cm^2 flasks.

2.2.2. Cell biological techniques

2.2.2.1.Measurement of cell proliferation

Proliferation of cells can be considered as an increase in the cell number. Thus the estimation of cell number is a straightforward method to quantify proliferation. Adherently growing cells detach from the ground of culture flask as they die and can be washed off. This phenomenon represents the basis of the method described by Gillies et al (1986). According to this method, after washing away dead cells, the adherent cells are fixed with glutaraldehyde and subsequently stained with crystal violet. The bound dye can be then released using Triton -X100 and the dye amount released in this solution is measured with an ELISA photometer.

Solutions used:

Fixing solution

components	working concentration	quantity
50 % Glutaraldehyde	0.1 M	250 µl
PBS		12.25 ml

The solution has to be freshly prepared.

Crystal violet stock solution

components	working concentration	quantity
crystal violet	2 mM	15 mg
PBS		15 ml

The solution is sterile filtered and stable at room temperature for 2 months.

Dye solution

components	working concentration	quantity
crystal violet 2 mM	0.2 mM	1.3 ml
PBS		11.7 ml

The solution has to be freshly prepared.

Destaining solution

components	working concentration	quantity
Triton X100	0.2 %	10 µl
PBS		5 ml

Cells were seeded at a density of 30,000 cells/cm^2 in 24-well plates, allowed to adhere over night and treated the next day with the indicated agents or corresponding solvents as control, for the described duration. After the incubation time, supernatant was removed, cells were rinsed in 250 µl PBS/well and then fixed with 500 µl/well of the fixing solution over 30 minutes, at room temperature under moderate shaking. Subsequently, the fixing solution was aspirated and the cells were washed with PBS 500 µl/well. Staining was performed by adding 500 µl of dye solution to each well and incubating for another 30 minutes at room temperature under moderate shaking. Unbound dye was then washed away by submerging the plates in deionised water for 15 minutes. After washing, the water excess was removed from the plates by tapping on tissue paper. To release the bound dye, 200 µl of destaining solution was added to each well and incubated for at least 1 hour, at room temperature under moderate shaking. After incubation, 100 µl of supernatant from each well was transferred into a microtiter plate and the extinction was measured at 570 nM using an ELISA photometer. The extinction values of vehicle treated cells were set as 100 % and the cell number of treated cells was calculated as percents of controls.

2.2.2.2. Cytotoxicity assay

Cell necrosis is characterised by plasma membrane damage and release of the intracellular content in the surrounding medium. Cell death can be evaluated by measuring the uptake or exclusion of vital dyes (such as trypan blue, eosin Y, nigrosine, propidium iodide or ethidium bromide), the release of radioactive isotopes from previously labelled cells or the activity of cytoplasmatic enzymes which are released upon cell death. Lactate dehydrogenase (LDH) is a stable cytoplasmatic enzyme, present in all cells, and which is rapidly released in the culture supernatant after damage of the plasma membrane.

LDH activity was evaluated with the "Cytotoxicity Detection Kit (LDH)" from Roche Diagnostics (Mannheim D), by means of a two-step enzymatic reaction. In the first step NAD^+ is reduced to $NADH/H^+$ by the LDH-catalysed oxidation of lactate to pyruvate. In the second step,

the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nytrophenyl]-5-phenyltetrazolium chloride) which is reduced to red coloured formazan. The increase of the LDH enzyme activity following plasma cell damage correlates to the amount of formazan formed. The quantity of formazan formed can be measured by an ELISA photometer at 490 nm.

Solutions used:

Catalyst solution and dye solutions are ready to use solutions.

Reaction buffer

components	quantity
catalyst solution	2 µl
dye solution	90 µl

Cells were seeded at 40,000 cells/cm^2 in 24-wells plates, and allowed to adhere overnight. The following day they were treated with the indicated compounds or the respective solvents, for the described duration. The cell supernatant was then collected and centrifuged at 300 x g for 5 minutes. 50 µl of the resulting supernatant was transferred into a microtiter plate and 50 µl of reaction buffer were added. The samples were incubated protected from light for 15 to 30 min. at room temperature. The absorption at 490 nm was measured by an ELISA photometer. Absorption values of controls were set at 100 % and the rate of LDH release from the treated cells was calculated as percentage of controls.

2.2.2.3. Detection of apoptosis

Apoptosis can be assessed by various methods, among which the measurement of DNA fragmentation is one of the most reliable. DNA fragmentation represents the cleavage of chromosomal DNA in oligonucleosome-sized fragments and is a biochemical hallmark of apoptosis. Specific endonucleases which become activated at the end of the apoptosis signalling cascade cleave the nuclear DNA at the most accessible, internucleosomal sites, and the resulting mono- or oligonucleosomes are released in the cytoplasm. (ZHANG *et al.*, 2000**).** The resulting DNA fragments are discrete multiples of 180 bp subunit. They remain bound to histone and can be detected using a combination of anti-histone and anti-DNA antibodies. Detection of mono-

and oligonucleosomes in the cytoplasma is possible due to the fact that during apoptosis, DNA fragmentation occurs several hours before plasma membrane breakdown.

DNA fragmentation in melanoma cells was measured using a commercially available kit "Cell Death detection ELISA Plus" from Roche Diagnostics (Mannheim, D). The test is performed on streptavidin-coated microtiter plate. After transfer of cell lysates to the microtiter wells, a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies is added. The anti-histone antibodies recognise the protein fraction of the nucleosomes and immobilise it via streptavidin-biotin interaction on the microtiter plate. The DNA coiled around histones is recognised by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate is added and the absorption is measured with ELISA photometer.

Solution used:

Lysis buffer, incubation buffer and substrate solution are ready to use solutions.

Immunoreagent solution (for one probe):

components	**quantity**
anti-histone-biotin antibody	4 µl
peroxidase-coupled anti-DNA antibody	4 µl
incubation buffer	72 µl

Cells were seeded at 40,000 cells/cm^2 in 24-wells cell culture plates and incubated overnight. Subsequently, they were incubated with the distinct apoptotic stimuli. At the end of treatment the plates were centrifuged for at 300 x g for 5 min. The supernatant was cautiously removed and 200 µl/well of lysis buffer was added and further incubated for 30 min. at room temperature under moderate shaking. The cytosolic supernatant was prepared by centrifugation at 300 x g for 10 min. Subsequently, 20 µl of the respective supernatant is added to a streptavidin-coated microtiter plate. The probes are then incubated for 2 h with 80 µl of the immunoreagent solution, at room temperature under moderate shaking. After washing, the wells are supplied with 100 µl substrate solution/well and incubated 10 min. at room temperature in the dark. Absorption at 405 nm is measured using an ELISA photometer. Absorption of control cells was set at 100 % and the DNA fragmentation in the treated cells was calculated as percent of control.

2.2.3. Protein analysis

2.2.3.1. Determination of protein concentration

Protein concentration is determined using a commercially available kit from Pierce (Weiskirchen, D) which is based on a method by SMITH *et al* (1985). The principle of the method is the biuret reaction, where the amino group of proteins reduces copper II cations to copper I cations in alkaline medium. Copper I cations react with two molecules of bicinchoninic acid (BCA) and a stabile chelate complex of violet colour results, which exhibits maximal absorption at 562 nm. Depending of the incubation temperature, different sensitivities can be achieved:

incubation time	temperature	concentration range
30 min	60° C	5-250 µg/ml
30 min	37° C	20-1200 µg/ml
2 h	room temperature	20-1200 µg/ml

This method has the advantages of the easy handling, the stable working reagent, which can be stored for up to one week, and the low influence of detergents and salts.

Solutions used:

Solution A was purchased from Pierce (Weiskirchen, D).

Solution A:

components	working concentration	quantity
disodium bicinchoninate	26 mM	10 g
disodium carbonate-monohydrate	161 mM	20 g
disodium tatrate-dihydrate	7 mM	1.6 g
NaOH	100 mM	4 g
sodium hydrogen carbonate	113 mM	9.5 g
water, double distilled	ad 1000 ml	

Solution B

components	working concentration	quantity
copper II sulphate-pentahydrate	160 mM	1 g
water, double distilled		ad 25 ml

Both solutions are long time stable at room temperature.

The working reagent is prepared by mixing fifty parts Solution A to one part Solution B. A protein standard rank is prepared by successive dilutions of bovine serum albumin (BSA) in sample buffer. Sample buffer contains usually leupeptin and pepstatin, which are peptides and therefore undergo the biuret reaction. Further components of the sample buffer, such as phenylmethansulfonyl fluoride (PMSF) and dithiothreitol (DTT) act in change as reducing agents. Protein samples are also diluted in sample buffer (1:5). 10 µl of each protein sample and of the standard rank probes are transferred to a microtiter plate in duplicate. 200 µl/well of working solution is added, and the probes are mixed and incubated at the appropriate temperature.

The absorption is then measured at 570 nm with an ELISA photometer. The protein concentration can be calculated using the standard rank curve.

2.2.3.2. SDS-polyacrylamide gel electrophoresis

The discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is performed in slightly modified form as described by LAEMMLI (1970). Most of the analytical electrophoresis of proteins are carried out in polyacrylamide gels, under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Polyacrylamide gels are prepared by free-radical induced polymerisation of acrylamide with N, N'-methylenebisacrylamide as cross-linker. Ammonium persulphate (APS) and N, N, N', N'-tetramethylethylenediamine (TEMED) are added as provider and stabiliser of free radicals respectively.

The strong anionic detergent SDS is used in combination with heat to dissociate proteins before they are loaded on the gel. Dithiothreitol is added to the sample buffer to reduce disulphide bonds. The amount of SDS bound is nearly proportional to the molecular weight of the polypeptides (about 1.4 g of SDS per 1 g of protein), and independent of the sequence. The

large negative charge of SDS masks the intrinsic charge of proteins, so that complexes of identical charge-to-mass ratio are formed. In consequence, SDS-polypeptide complexes migrate through polyacrylamide gel according to the size of the polypeptide. The molecular weight of unknown polypeptide chains can be estimated by running in parallel markers of known molecular weight.

Solutions used**:**

AA-Bis

components	**working concentration**	**quantity**
acrylamide	5.44 M	386.6 g
N,N-methylenebisacrylamide	87 mM	13.4 g
water, double-distilled		ad 1000 ml

The ratio acrylamide:methylenebisacrylamide is 29:1 (w/w) or 40 % T and 3.4 % C.

10 % APS

components	**working concentration**	**quantity**
ammonium persulphate	0.44 M	0.1 g
water double-distilled		ad 1 ml

The solution is stable 4 weeks at 4 °C.

Running Gel Buffer (5x)

components	working concentration	quantity
Tris	375 mM	113.5 g
sodium dodecyl sulphate	35 mM	2.5 g
hydrochloric acid		ad pH 8.8
water, double-distilled		ad 500 ml

Stacking gel Buffer (4x)

components	working concentration	quantity
Tris	175 mM	16.95 g
sodium dodecyl sulphate	35 mM	0.80 g
hydrochloric acid		ad pH 6.8
water, double-distilled		ad 200 ml

Electrophoresis Buffer (10x) (SDS)

components	working concentration	quantity
Tris	175 mM	60.55 g
sodium dodecyl sulphate	35 mM	20.19 g
glycine	190 mM	285.27 g
water, double-distilled		ad 200 mL

The pH value should not need to be adjusted and should be ~ 8.3.

Loading Buffer (5x)

components	working concentration	quantity
1 M Tris/ HCl pH 6.8	30 mM	750 µl
glycerol	0.7 M	1 250 µl
sodium dodecyl sulphate	35 mM	0.25 g
1,4-dithiothreitol	0.1 M	0.39 g
1 % bromophenol blue	0.14 mM	250 µl
water, double-distilled		ad 5 ml

Pipetting scheme for 2 gels, 80 mm x 60 mm x 1.5 mm size:

Components	Running gel					Stacking gel
	5 %	7.5 %	10 %	12.5 %	15 %	3 %
water double-distilled [ml]	13.4	12.5	10.9	9.65	8.4	-
5x running gel buffer [ml]	4	4	4	4	4	-
4x stacking gel buffer [ml]	-	-	-	-	-	2.0
acrylamid (40 %) [ml]	2.5	3.75	5.0	6.25	7.5	0.6
TEMED [µl]	5	5	5	5	5	6
10 % APS [µl]	95	95	95	95	95	55

The running gel solution is prepared following the scheme given above. Polymerisation starts with the addition of TEMED and ammonium persulphate (APS). The solution is carefully mixed and poured into the gap between two glass plates of the slab gel unit and then the running gel is cautiously overlaid with water-saturated 1-butanol. When polymerisation is completed (30-45 min) the overlay is poured off, and the excess is completely removed with the edge of a Whatman 3 MM paper. The stacking gel solution is then poured directly to the surface of the polymerised running gel, and a clean plastic comb is inserted immediately, avoiding the formation of air bubbles. After complete polymerisation, the comb is removed and the slab gel unit is inserted in the electrophoresis chamber, filled with the electrophoresis buffer.

The samples are adjusted to equal protein concentrations through dilution in the corresponding lysis buffer. The solution is completed by adding one fourth of the volume loading buffer (5x).

The proteins are then denaturated for 5 min. at 95 °C and loaded with a Hamilton syringe to the stacking gel wells.

Electrophoresis is performed at 35 mA per gel for 15 min, and subsequently at 20 mA per gel. Voltage is maintained at 150 V. When the bromphenol blue reaches the bottom of the running gel, electrophoresis is completed. The gel slab units are disassembled and the gels are prepared for western blotting.

2.2.3.3. Western blotting

Different proteins previously separated by SDS-PAGE can be identified by binding of specific antibodies. An antigen-antibody reaction can not properly occur inside the polyacrylamide gel, so that the proteins have to be first transferred to a nitrocellulose membrane before exposure to the antibodies. The transfer of proteins to the membrane is performed immediately after electrophoresis, using an electrical field (TOWBIN *et al*., 1979). The proteins are negatively charged at the transfer pH, due to the bound SDS, so they will migrate from the cathode to anode.

The membrane loaded with proteins is then incubated with the specific antibodies. The bound antibodies are subsequently labelled with secondary anti-IgG antibodies coupled with horseradish peroxidase, which allows the visualisation of the protein-antibody complexes on addition of a chromogen substrate.

Solutions used:

Transfer buffer (blotting buffer) (2x)

components	**working concentration**	**quantity**
Tris	25 mM	12.11 g
glycine	192 mM	57.65 g
methanol	4.9 M	800 ml
water double-distilled		ad 2000 ml

The pH value should be 8,3 without adjusting.

PBS (10x)

components	working concentration	quantity
potassium chloride	2.68 mM	4 g
potassium dyhidrogen phosphate	1.47 mM	4 g
sodium chloride	137 mM	160 g
disodium hydrogenphosphate dihydrate	8.09 mM	28.8 g
water, double-distilled		ad 2000 ml

pH should be 7.4 and should be not adjusted. Osmolarity is about 300 mosmol.

Staining solution:

components	working concentration	quantity
Ponceau S	13 mM	10 g
glacial acetic acid	0.9 M	50 ml
water, double-distilled		ad 1000 ml

Blotting follows immediately after SDS-PAGE. Whatman papers and nitrocellulose filters are cut in the size of the gels and soaked in transfer buffer for 5 min. The transfer sandwich is assembled in transfer buffer: on the black side of the transfer cassette, adjacent to the cathode are placed in order: a porous pad, a sheet of 3 MM paper, the gel, the blotting membrane, and another sheet of 3 MM paper. Air bubbles are removed by rolling a glass pipette over the sandwich. Finally a last porous pad is placed on top and the cassette is folded together and inserted in the blotting chamber, with the membrane towards the anode. The chamber is filled with transfer buffer to completely cover the electrode panels. A magnetic stirrer is used to mix the buffer and the chamber is placed into a box with ice during the transfer, for cooling. The transfer lasts 1.5 h at 250 mA and 100 V. After blotting, the successful transfer of proteins onto the membrane is proved by staining the membrane with Ponceau solution, which provides a transient staining of protein bands and do not interfere with the subsequent antibody binding. The filters are incubated for 2-5 min. with Ponceau S under gentle shaking at room temperature. When the protein bands become visible, the filters are washed several times in double distilled water and prepared for immunodetection of proteins.

2.2.3.4. Immunodetection of blotted proteins

After transfer to the membrane, the proteins are probed with the primary antibodies, which are specific for each sampled protein. Subsequently they are incubated with the secondary antibodies, specific for the common structure of the primary antibodies. The secondary antibodies are coupled to a peroxidase, which is used to produce a chemoluminiscent signal. Peroxidase can reduce hydrogen peroxide to water, using luminol as reducing agent. An enhancing agent stabilises the excitation state of luminol acquired by the reduction. Upon returning to the basal state of luminol, light is emitted and is detected using an X-ray film.

Solutions used:

PBS-T (1x)

components	**working concentration**	**quantity**
Tween-20	0.1 %	500 µl
PBS 10 x		50 ml
water, double distilled		ad 500 ml

Blocking buffer

components	**working concentration**	**quantity**
skim milk powder	0.3 %	3 g
PBS-T (1x)		100 ml

Super Signal working solution

components	**quantity**
Luminol/ enhancer solution	5 ml
stable peroxide solution	5 ml

The working solution is stable for approximately 24 h. The luminol/ enhancer solution and the stable peroxide solution are purchased from Perbio (Weiskirchen, D). The supplier provided no information about the ingredients.

Stripping Puffer:

components	final concentration	quantity
glycin	0.2 M	15 g
SDS	35 mM	10 g
Tween 20	0.1 %	1 ml
HCl		ad pH 2.3
water, double distilled		ad 1000 ml
Sodium azide 5 M	10 mM	2 ml

Sodium azide was added shortly before use. The buffer is stable at room temperature at least 1 month.

After staining with Ponceau S, the membranes are washed 2-3 times in doubled distilled water, and then incubated for 1 h in blocking buffer 10 ml/ membrane, at room temperature under moderate shaking. The blocking buffer is then removed, and primary antibodies diluted as indicated, in 10 ml blocking buffer are added to the membranes. The filters are incubated with the primary antibody solution for 2 h at room temperature, or alternatively, over night at 4 °C, under gentle agitation.

Primary antibody	Primary antibody dilution	Secondary antibody	Secondary antibody dilution
Anti-β-actin (mouse)	1: 200,000	Anti-mouse (goat)	1: 40,000
Anti- cytochrome c (mouse)	1:500	Anti-mouse (goat)	1: 10,000

Subsequently the membranes are washed 3 times for 5 min. in PBS-T and then incubated with the secondary antibodies diluted in blocking buffer as indicated, for 1 h at room temperature under gentle agitation.

After that, the secondary antibody solution is discarded, the membranes are washed 2 times in PBS-T for 5 min., and once in PBS for 5 min. For the chemoluminiscence reaction the filters are incubated 5 min in Super Signal working solution at room temperature under moderate shaking. Following, the membranes are placed in a protective plastic sheet, excess of substrate solution and air bubbles are removed by gentle pressing and then the membranes together with

an X-ray film are enclosed in a film exposure cassette. The duration of the film exposure can vary, according to the intensity of the signal.

The membranes can be thereafter probed for other proteins. To this purpose, membranes are first stripped in stripping buffer 30 ml /membrane for 16 h at room temperature. Afterwards they are washed in PBS-T and the protein immunodetection procedure is repeated as described using new primary antibodies specific for the new proteins to be sampled.

2.2.4. Cell fractionation

Subcellular fractionation, with separation of mitochondrial and cytosolic compartments is used for studying apoptotic and signal transduction pathways, in order to detect translocation of different factors between the two compartments. The separated fractions can be subsequently analysed by means of Western blot or ELISA techniques.

For the cell fractionation, the "Mitochondrial /Cytosol Fractionation Kit" purchased from Bio Vision, Inc (Heidelberg, D) was used as described by the manufacturer.

Solution used:

Kit Components:

Mitochondrial Extraction Buffer
Cytosol Extraction Buffer (5x)
Protease inhibitor cocktail
DTT

No information about the components of the buffers was provided. The Mitochondrial Extraction Buffer and the 5x Cytosol Extraction Buffer were stored at 4 °C. Protease inhibitors cocktail and DTT were stored at -20 °C. Protease inhibitor cocktail was prepared by adding 250 µl DMSO to the vial supplied with the kit shortly before use. No detailed information about the composition of the cocktail was provided.

Cytosolic Extraction Buffer (1x)

components	working concentration	quantity
Cytosolic Extraction Buffer (5x)	1x	20 ml
water, double distilled		80 ml

Cytosol Extraction Buffer Mix

components	quantity
Cytosolic Extraction Buffer (1x)	1 ml
Protease inhibitor cocktail	2 µl
DTT	1 µl

Mitochondrial Extraction Buffer Mix

components	quantity
Mitochondrial Extraction Buffer	1 ml
Protease inhibitor cocktail	2 µl
DTT	1 µl

Cytosol Extraction Buffer Mix and Mitochondrial Extraction Buffer Mix were prepared shortly before use and kept on ice all through the experiment.

Cells were seeded at 2,000,000 cells /flask in 150 cm^2 culture flasks and left 48 h to adhere. Afterwards, they were incubated with pamidronate or vehicle, for the indicated duration. At the end of treatment, cells were washed once in PBS, detached from the culture flasks with trypsin and harvested in culture medium. The cell suspension was then centrifuged at 200 x g for 5 minutes, the cells were washed with ice-cold PBS and counted in a hemocytometer chamber. 5 x 10^7 cells of each probe were collected and centrifuged at 600 x g for 5 min.
The supernatant was removed, and the cell pellet was resuspended in 1 ml of 1 x Cytosol Extraction Buffer Mix (containing DTT and protease inhibitors cocktail as described).
The probes were incubated on ice for 10 minutes. Subsequently the cells were homogenised in an ice-cold dounce tissue grinder. The homogenisation was performed on ice by passing the cells 60-80 times through the grinder. Efficacy of homogenisation was controlled with trypan blue staining under the microscope and grinding was occasionally repeated.

Subsequently the homogenate was transferred to 1.5 ml microcentrifuge tubes, and centrifuged at 700 x g for 10 min at 4 °C. The resulting supernatant was transferred into new 1.5 ml centrifuge tubes and centrifuged at 10,000 x g for 30 min at 4 °C.
The final supernatant was collected as cytosolic fraction. The pellet was resuspended in 0.1 ml Mitochondrial Extraction Buffer Mix (containing DTT and protein inhibitors cocktail as described), vortexed for 10 sec and stored as mitochondrial fraction. Both fractions were stored at -70 °C.

The cytochrome c content of the two fractions was analysed by Western blot as previously described.

2.2.5. Cell cycle analysis

Principle of method

DNA content is a marker of cellular maturity in the cell cycle. At the beginning of the cell cycle, in G1 phase, most of cells have a diploid DNA content (2n). During S phase, the cells replicate the DNA and the DNA content increases in proportion to the progression through S phase until it becomes double (4n), whereupon the cells enter the G2 phase. Eventually cells enter in mitosis and divide to recommence the cell cycle. Measuring the DNA content of a cell allows thus to determine in which phase of the cell cycle the cell is.

The univariant analysis of cellular DNA content is therefore the simplest and most commonly used method to reveal the distribution of the cells in the phases of the cell cycle and is one of the major applications of the flow cytometry. The DNA content analysis requires staining of the cells with a nucleic acid-specific fluorochrome such as propidium iodide (PI) or 4´6´-diamidino-2-phenylindole (DAPI). Propidium iodide is the most widely used dye (DARZYNKIEWICH, 1994). It binds stoichiometrically to DNA and in bound state fluorescences red when excited with blue light (488 nm). Because it is excluded by viable cells, cells must be fixed or permeabilised before staining. In the same time, because PI can also bind RNA, it is necessary to remove RNA from the cells by incubation with RNAase, and sufficient time should be allow for the RNAase to eliminate all double-stranded RNA.

Within a flowcytometer, the stained cells are then exposed, one at the time, to a beam of laser light. The laser light excitation will cause the PI particles in each cell to fluorescence, and the intensity of the fluorescent signal emitted will be proportional to the amount of fluorescent dye present in the cell. Results of fluorescence measurements are displayed as cellular DNA content histograms. The histograms show the proportion of cells in each phase of the cycle, based on differences in fluorescence intensity. The actual percentage of G0/1, S and G2/M cells are obtained by deconvoluting the histograms (i.e. integrating the areas under the peaks and the S-phase ridge) using different mathematical procedures. For this purpose different types of software are commercially available.

Preparation of samples

The cells were seeded at 80,000 cells / well in 6 well plates. After 48 h they were treated with the indicated drugs or corresponding vehicles for 24h. At the end of treatment, cells were washed in PBS, detached with trypsin from the culture flask floor, and harvested in culture medium. The cell suspension was centrifuged at 200 x g for 5 min. The cell pellet was resuspended in PBS and the cells were counted in a hemocytometer chamber. 1 x 10^6 cells were collected and centrifuged at 400 x g for 5 minutes. The pellet was washed in cold-ice PBS and the centrifugation was repeated. The pellet was carefully resuspended in ice-cold ethanol 70 % in PBS (v/v) and transferred in 1.5 ml microcentrifuge tubes. The cells were fixated in ethanol at -20 °C over night.

Measurement of DNA content

The samples were thawed and centrifuged at 400 x g for 5 min. The pellet was washed with 1 ml PBS, and the centrifugation was repeated. The supernatant was then removed, the pellet was resuspended in 1 ml Propidium Iodide Solution, and incubated in dark for at least 30 min. Afterwards the cell suspension was transferred in 5 ml polystyrene round-bottomed tubes and the samples were analysed for red fluorescence with a FACSCalibur flowcytometer (Becton Dickinson, D). The DNA histograms were created using the Cell Quest ^{TM} software, version 3.0 for Macintosh (Becton Dickinson). 20,000 events /sample were analysed.
The procentual distribution of cells in the phases of the cell cycle was calculated with ModFit LT, version 2.0 for Macintosh (Becton Dickinson).

Solutions used:

Propidium Iodide solution (for 4 samples)

components	**working concentration**	**quantity**
PBS		ad 5 ml
10 % Triton-X 100 in PBS	0.1 %	50 µl
RNase A (DNAase free) 10 mg/ml	20 %	100 µl
Propidium Iodide 10 mg/ml	2 %	10 µl