(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 August 2005 (11.08.2005)

PCT

(10) International Publication Number
WO 2005/073720 A2

(51) International Patent Classification[7]: G01N 33/53

(21) International Application Number:
PCT/GB2005/000356

(22) International Filing Date: 2 February 2005 (02.02.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0402269.5 2 February 2004 (02.02.2004) GB

(71) Applicant *(for all designated States except US)*: **ASTELLAS PHARMA INC.** [JP/JP]; 4-7, Doshomachi 3-chome, Chuo-ku, Osaka-shi, Osaka 541-8514 (JP).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SHARKEY, John** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB). **KERR, Lorraine, Elizabeth** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB). **SHORT, Duncan, Matthew** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB). **BIRSE-ARCHBOLD, Jui-Lee, Andrea** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB). **THOMPSON, Jane, Victoria** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB). **MCGREGOR, Ailsa, Lynn** [GB/NZ]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (NZ). **MCCULLOCH, James** [GB/GB]; Fujisawa Institute of Neuroscience, University of Edinburgh, 1 George Square, Edinburgh, Lothian EH8 9JZ (GB).

(74) Agents: **GAUNT, Robert** et al.; Stevens Hewlett & Perkins, Halton House, 20/23 Holborn, London, Greater London EC1N 2JD (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF SCREENING FOR NEW DRUGS

(57) Abstract: A method for identifying compounds that are effective in modulating the binding of nucleophosmin to Bax (Bcl-2-associated X protein). Nucleophosmin has been found to regulate the activity of Bax. The interaction between Bax and nucleophosmin is used in a method for the screening of new drugs for the treatment of disorders that involve Bax-mediated cell death. A kit for assaying the interaction between Bax and nucleophosmin is also provided. Compounds identified by the aforementioned method are used for the manufacture of medicaments for treating cerebral and myocardial ischaemic injury, neonatal hypoxic brain injury, traumatic brain injury and other neurological disorders.

WO 2005/073720 A2

METHOD OF SCREENING FOR NEW DRUGS

This invention relates to the discovery that nucleophosmin regulates the activity of Bax and, more particularly, to the use of this interaction between nucleophosmin and Bax in a method for the screening of new drugs for their suitability for the treatment of neurological disorders that involve Bax-mediated cell death.

Bax (Bcl-2-associated X protein) is a 21kDa death promoting member of the Bcl-2 family which was first identified by its association with Bcl-2 (Oltvai *et al*, 1993). It exists as a cytosolic protein in healthy living cells. However upon induction of apoptosis, Bax translocates and inserts into the mitochondrial membrane, thereby causing the release of cytochrome c and other apoptotic proteins consequently resulting in cell death (Hsu *et al*, 1997; Wolter *et al*, 1997; Gross *et al*, 1998). The molecular mechanisms regulating the activation and translocation of Bax and its role in regulating cell death remain unknown and are currently the subject of much controversy and investigation.

The structure of inactive cytosolic Bax was recently determined using nuclear magnetic resonance (NMR) spectroscopy (Suzuki *et al*, 2000) and it was demonstrated that Bax consists of nine α helices (α1-α9). The tertiary structure of Bax closely resembles that of Bcl-x_L (Muchmore *et al*, 1996) and Bid (Chou *et al*, 1999; McDonnell *et al*, 1999) with eight amphipathic α helices clustered around one central hydrophobic α helix (α5). The last 21 amino acids of Bax at the C terminus are thought to have a role in targeting Bax to mitochondria during apoptosis as deletion of this hydrophobic sequence abrogates the ability of Bax to insert into the mitochondria during apoptosis (Wolter *et al*, 1997). Moreover, the serine residue at position 184 has been identified as a particularly important amino acid in the regulation of Bax C-terminal conformation and subsequently Bax subcellular localisation. Studies have shown that replacing this serine with a valine (Bax S184V) or deleting the

serine altogether (Bax ΔS184) causes Bax to constitutively localise to the mitochondria (Nechushtan *et al*, 1999; Suzuki *et al*, 2000). Indeed fusing the 20/21 amino acid C-terminal sequence of Bax ΔS184 or S184V to the reporter gene GFP is sufficient to constitutively target GFP to mitochondria (Nechushtan *et al*, 1999). Structural studies have shown that in cytosolic inactive Bax, the C terminal α9 helix is located within its own BH3 binding hydrophobic pocket in a similar manner to the way the Bak BH3 peptide has been shown to bind to Bcl-x_L (Suzuki *et al*, 2000). This conformation would prevent dimer formation with other Bcl-2 family proteins as it is very unlikely that a BH3 domain of another Bcl-2 family member could compete with the C-terminal α9 helix of Bax for binding to its BH3 pocket. However upon activation, Bax undergoes a major conformational change that is suggested to result in the disengagement of helix α9 and exposure of the BH3 binding pocket. This model would allow for the translocation of Bax from the cytosol to mitochondria (targeting via exposure of α9 helix) and the subsequent oligomerisation of Bax (via exposure of its BH3 binding pocket; Antonsson *et al*, 2001). However the nature of the stimulus and the details of Bax conformational change upon apoptotic stimulus have yet to be elucidated.

Based on this information, a strategy to identify mitochondrial receptors and chaperones of Bax translocation was initiated. As the C-terminal 21 amino acids of mutant Bax (ΔS184 or S184V) are sufficient to target molecules to mitochondria, wild type and mutant peptides were synthesised and affinity chromatography performed. Proteins binding to the peptides from cell lysates were isolated and identified using MALDI-TOF mass spectrometry.

Bax activation and translocation to the mitochondria is a critical event following a number of different apoptotic stimuli (Wolter *et al*, 1997; McGinnis *et al*, 1999; Antonsson *et al*, 2001). Bax has been implicated in cell death in a wide variety of diseases including focal cerebral ischaemia (Cao *et al*, 2001), Parkinson's disease (Hassouna *et al*, 1996;

Fiskum *et al*, 2003; Lev *et al*, 2003), Huntington's disease (Vis *et al*, 2001; Antonawich *et al*, 2002), Alzheimer's disease (Paradis *et al*, 1996; Su *et al*, 1997; Tortosa *et al*, 1998; Giannakopoulos *et al*, 1999) and amyotrophic lateral sclerosis (ALS; Gonzalez de Aguilar *et al*, 2000; Sathasivam *et al*, 2001; Guegan and Przedborski, 2003).

Nucleophosmin (NPM also known as B23, numatrin, NO38) is a mobile nucleolar phosphoprotein that engages in nucleocytoplasmic shuttling (Borer *et al*, 1989) which is related to another nuclear chaperone nucleoplasmin (Shackleford *et al*, 2001). Many roles for nucleophosmin have been suggested including a role in ribosome assembly (Szebeni and Olson, 1999; Hingorani *et al*, 2000; Shackleford *et al*, 2001), as a genotoxic stress-responsive RNA-binding protein (Yang *et al*, 2002), as a histone chaperone protein (Okuwaki *et al*, 2001) and as a molecular chaperone that prevents protein aggregation in the nucleolus (Chan *et al*, 1999). Nucleophosmin has been reported to translocate from the nucleoli into the nucleoplasm during the stationary growth phase (Perlaky *et al*, 1997), during treatment with certain anti-tumour drugs (Yung *et al*, 1985; Yung *et al*, 1990; Wu *et al*, 1995), serum starvation (Chan *et al*, 1985), actinomycin (Yung *et al*, 1985) and luzopeptin A (Yung *et al*, 1986).

The tumour suppressor, p53 is a key regulator of cell cycle arrest and apoptosis and approximately half of all human tumours carry mutant p53 (Bykov *et al*, 2003; Gostissa *et al*, 2003). It has recently been demonstrated that nucleophosmin binds to p53 and nucleophosmin was shown to be crucial for stabilization and activation of p53 in response to different types of stress (Colombo *et al*, 2002). The mechanism of action of nucleophosmin in the regulation of p53 is currently unclear.

Nucleophosmin has a role in a number of diseases including:-

i) Anaplastic large cell lymphoma (ALCL) (non-Hodgkin's lymphoma)

- exhibits t(2;5)(p23;q35) translocation (Morris *et al*, 1994)
- characterised by resulting expression of oncogenic fusion protein nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) gene

product (Morris *et al*, 1994)

- NPM-ALK chimeric protein shows phosphotransferase activity and via interactions with various ALK-adaptor proteins, induces cell transformation and increases cell proliferation in vitro (Bonvini *et al*, 2002).

ii) Hodgkin's lymphoma

- t(2;5)(p23;q35) NPM1-ALK translocation rare but also detected in some cases of Hodgkin's lymphoma (Trumper *et al*, 1997)

iii) Acute promyelocytic leukaemia (APL)

- t(5;17)(q32;q11) RARA-NPM translocation (Redner *et al*, 1996)
- characterised by resulting expression of oncogenic fusion protein nucleophosmin-retinoic acid receptor (NPM-RARA) gene product.

This invention results from the discovery by the inventors that nucleophosmin is a regulator of Bax induced cell death. The translocation of Bax from the cytosol to the mitochondrial initiates a cascade of events which results in cell death. By interfering with the translocation of Bax, inter alia, nucleophosmin can prevent cell death. Further, by evaluating the effect of different compounds and conditions on the interaction between Bax and nucleophosmin, new drugs and therapies can be identified for the treatment of ischaemia and neurological disorders.

According to the present invention there is provided a method for identifying a compound that is effective in modulating the binding of nucleophosmin to Bax.

According to a further embodiment, the present invention relates to the use of a compound that is effective in modulating the binding of nucleophosmin to Bax for the manufacture of a medicament for treating cerebral (including spinal cord) and myocardial ischaemic injury, neonatal hypoxic brain injury, traumatic brain injury and other neurological disorders, including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and Parkinson's disease. Such compounds may be, but need not necessarily have been, identified by the aforementioned method of this

invention.

According to a still further embodiment of the present invention there is provided a kit for assaying the interaction between Bax and nucleophosmin, wherein the interaction is detected with high sensitivity and specificity together with high signal/background ratios by the amplified luminescent homogeneous proximity assay using, for example the multi-well plate comprising a 4-, 6-, 12-, 24-, 48-, 96-, 384-, or 1536-well design.

In the method of this invention, a compound that is effective in modulating the binding of nucleophosmin to Bax may be identified, for example, by assessing its inhibitory effect on the interaction between Bax and nucleophosmin. The interaction may be assessed, for example, by an amplified luminescent homogeneous proximity assay. Typically, such an assay uses the following components:

(a) recombinant Bax protein, or fragment thereof, whether native protein or tagged with histidine, biotin or Gluthathione-S-Transferase (GST), and/or directly conjugated to acceptor or donor beads; and

(b) recombinant nucleophosmin protein, or fragment thereof, whether native protein or tagged with histidine, biotin or Gluthathione-S-Transferase (GST), and/or directly conjugated to acceptor or donor beads; and/or:

(c) either monoclonal or polyclonal nucleophosmin antibody conjugated to donor or acceptor beads; and/or

(d) either monoclonal or polyclonal Bax antibody conjugated to donor or acceptor beads; and/or

(f) streptavidin-coated donor or acceptor beads.

Alternatively, the interaction between Bax and nucleophosmin is assessed by a method comprising the steps of:

(I) combining the following component (a) with (b):

(a) recombinant Bax protein, or fragment thereof, directly or indirectly conjugated to donor beads

(b) recombinant nucleophosmin protein, or fragment thereof, directly or indirectly conjugated to acceptor beads; or

(I') combining the following component (c) with (d):

(c) recombinant Bax protein, or fragment thereof, directly or indirectly conjugated to acceptor beads

(d) recombinant nucleophosmin protein, or fragment thereof, directly or indirectly conjugated to donor beads;

and the method further comprising the step of:

(II) determining whether the proximity-dependent chemical energy transfer occurs between the acceptor and donor beads.

In a further embodiment of the method of this invention for identifying a chemical substance which has an inhibitory effect on the interaction between Bax and nucleophosmin, the method comprises the steps of:

(a) assaying the interaction between Bax and nucleophosmin, wherein the interaction is measured according to the method of the preceding paragraph in the presence of a test compound; and

(b) assaying the interaction between Bax and nucleophosmin, wherein the interaction is measured according to the method of the preceding paragraph in the absence of any test compounds; and

(c) selecting a test compound showing a lower affinity level of interaction in the step (a) compared with that in the step (b).

In a still further embodiment of the method of this invention for identifying a chemical substance which has an inhibitory effect on the interaction between Bax and nucleophosmin, the interaction between Bax and nucleophosmin is detected with high sensitivity and specificity together

with high signal/background ratios by the amplified luminescent homogeneous proximity assay using the multi-well plate comprising a 4-, 6-, 12-, 24-, 48-, 96-, 384-, or 1536-well design.

Bax for use in the method of this invention may be selected from, for example, any one of the following:

(a) full-length native Bax protein; or

(b) full-length recombinant Bax protein; or

(c) an N-terminal or C-terminal truncated Bax peptide; or

(d) a fragment derived from Bax protein; or

(e) recombinant Bax protein or fragment thereof tagged with histidine; or

(f) recombinant Bax protein or fragment thereof tagged with biotin; or

(g) recombinant Bax protein or fragment thereof tagged with Glutathione-S-Transferase (GST); or

(h) any of the above Bax peptides or fragments thereof containing point mutations.

The above-listed sources (a)-(h) of the Bax component may be prepared, for example, using any one of the following systems:

(a) cell based expression systems; or

(b) *in vitro* cell-free procaryotic expression systems based on coupled transcription and translation; or

(c) *in vitro* cell-free eucaryotic expression systems based on coupled transcription and translation.

Nucleophosmin for use in the method of this invention may be selected from, for example, any one of the following:

(a) full-length native nucleophosmin protein; or

(b) full-length recombinant nucleophosmin protein; or

(c) a fragment derived from nucleophosmin protein; or

(d) recombinant nucleophosmin protein or fragment thereof tagged with histidine; or

(e) recombinant nucleophosmin protein or fragment thereof tagged with biotin; or

(f) recombinant nucleophosmin protein or fragment thereof tagged with Glutathione-S-Transferase (GST); or

(g) any of the above nucleophosmin peptides or fragments thereof containing point mutations.

The above-listed sources (a)-(g) of the nucleophosmin component may be prepared, for example, using any one of the following systems:

(a) cell based expression systems; or

(b) *in vitro* cell-free procaryotic expression systems based on coupled transcription and translation; or

(c) *in vitro* cell-free eucaryotic expression systems based on coupled transcription and translation.

In another embodiment of the method of this invention, the interaction between Bax and nucleophosmin is evaluated by assaying the interaction between a C-terminal truncated Bax peptide and a histidine-tagged nucleophosmin protein using the following components (l) to (o) for an amplified luminescent homogeneous proximity assay:

(l) a biotinylated C-terminal truncated Bax peptide; and

(m) a histidine-tagged nucleophosmin protein; and

(n) nucleophosmin monoclonal antibody-conjugated acceptor beads; and

(o) streptavidin-coated donor beads.

The invention thus provides a valuable new screening system for identifying and evaluating apoptosis-preventing compounds. It will be understood that the method of this invention may also be used to identify and evaluate apoptosis-inducing compounds which could be effective for treating certain types of cancer. It will now be described in more detail in the following Examples and by reference to the accompanying Figures, in which:-

Figure 1 shows immunoprecipitation of active Bax from ischaemic mouse brain. Bax was immunoprecipitated from mouse brain samples (cortex, striatum and cerebellum) which had been subject to either 30 minutes MCA occlusion followed by 2.5 hours reperfusion or a sham operation, using a monoclonal antibody specific for activated Bax. Immunoprecipitated samples (B) and depleted supernatant (S) were analysed by Western blotting with a polyclonal antibody which recognises Bax irrespective of its activation state. Active Bax was detected in ipsilateral cortex and striatum.

Figure 2 shows analysis of column eluates by SDS-PAGE. Affinity chromatography was performed using Bax wild type or mutant (S184V) peptide with control SH-SY5Y cell lysates. Eluates were analysed by SDS-PAGE and silver stained. Three proteins (of approximately 92kD, 68kD and 48kD) were eluted from the wild-type peptide affinity column and five (92kD, 68kD, 48kD, 41kD and 26kD) from the S184V mutant peptide affinity column. Subsequent mass spectrometric analysis identified the 92kD band as Hsp90, the 68kD band as Hsp60 and the 48kD band as β-actin. Two smaller bands of 41kD and 26kD were isolated only with the mutant peptide. The 41kD band was identified as nucleophosmin while the identity of the 26kD band has still to be established.

Figure 3 shows co-immunoprecipitation of Bax and nucleophosmin. Nucleophosmin was immunoprecipitated from control or

apoptotic (staurosporine-treated) SH-SY5Y cell lysates. Immunoprecipitated complexes were analysed by SDS-PAGE and Western blotting with an anti-Bax antibody.

Figure 4 shows specificity of Bax-nucleophosmin interaction. Immunoprecipitation from SH-SY5Y cells was performed using monoclonal antibodies against survival motor neuron (SMN) or nucleophosmin (NPM) and precipitated complexes analysed by Western blotting for Bax. Bax specifically co-precipitated with nucleophosmin but not SMN.

Figure 5 shows specific knock down of nucleophosmin expression. SH-SY5Y cells were transfected with nucleophosmin or control shRNA expression vectors containing the H1 promoter. Control cells were either not transfected or given transfection reagent alone. Apoptosis was induced with staurosporine 24 hours post transfection and nucleophosmin expression was analysed by Western blotting 4 hours after the receipt for the apoptotic stimulus. A reduction in nucleophosmin expression was detected in cells treated with NPM shRNA but not in cells treated with control shRNA or transfection reagent alone.

Figure 6 shows reduction of apoptotic cytochrome c release after knock down of nucleophosmin expression. SH-SY5Y cells were transfected with nucleophosmin or control shRNA expression vectors. Control cells were treated with transfection reagent alone. Apoptosis was induced with staurosporine 24 hours post transfection and cytochrome c release analysed by ELISA 4 hours after the receipt for the apoptotic stimulus. A reduction in apoptotic cytochrome c release was detected in cells treated with NPM shRNA but not in cells treated with control shRNA.

Figure 7 shows reduction in cleaved caspase 3 detected after knock down of nucleophosmin expression. SH-SY5Y cells were transfected with nucleophosmin or control shRNA expression vectors. Control cells were either not transfected (medium) or given transfection reagent alone. 24 hours later, apoptosis was induced with staurosporine and cleaved caspase 3 detected by ELISA 3 hours after the receipt of the

apoptotic stimulus. A reduced amount of cleaved caspase 3 was detected in cells treated with NPM shRNA.

Figure 8 shows Bax cellular distribution in the ischaemic cortex does not alter following 3 hours transient MCAo. MCAo (30 minutes occlusion followed by 2.5 hours reperfusion) was performed in a group of 6 mice. Sham operated mice were used as controls. Samples of ischaemic cortex were fractionated into nuclear, mitochondrial and cytosolic fractions were isolated and analysed by Western blotting for Bax (A). The relative distribution of Bax within the cell in these organelles was calculated (B). No change in relative Bax subcellular distribution was observed following 3 hours transient MCAo.

Figure 9 shows nucleophosmin redistribution within the cells of the ischaemic cortex following 3 hours transient MCAo. MCAo (30 minutes occlusion followed by 2.5 hours reperfusion) was performed in a group of 6 mice. Sham operated mice were used as controls. Samples of ischaemic cortex were fractionated into nuclear, mitochondrial and cytosolic fractions were isolated and analysed by Western blotting for nucleophosmin (A). The relative distribution of nucleophosmin within the cell in these organelles was calculated (B). Nucleophosmin translocated from the nuclear fraction and accumulated in both the cytosolic and mitochondrial fractions 3 hours following MCA occlusion.

Figure 10 shows co-immunoprecipitation of eukaryotically expressed Bax and nucleophosmin. Recombinant Bax and nucleophosmin were expressed in vitro and the protein solutions mixed. Nucleophosmin was immunoprecipitated (IP) from the mixture and analysed by Western blotting for the co-immunoprecipitation of Bax. All detectable nucleophosmin was precipitated while only a small fraction of Bax co-precipitated.

Figure 11 shows co-immunoprecipitation of procaryotically expressed His-Bax and His-nucleophosmin. Recombinant His-tagged Bax and His-tagged nucleophosmin were expressed in vitro and the protein

solutions mixed. His-tagged Bax was immunoprecipitated (IP) from the mixture and analysed by Western blotting for the co-immunoprecipitation of His-tagged nuclephosmin. All detectable His-tagged Bax was precipitated and a substantial fraction of His-tagged nucleophosmin was co-precipitated.

Figure 12 shows AlphaScreen™: Experimental design and assay verification. To evaluate the interaction between His-Bax and His-nucleophosmin, acceptor beads (A) were conjugated with mAb nucleophosmin to recognise His-nucleophosmin and streptavidin-coated donor beads (D) were bound to a biotinylated mouse 2°Ab IgG and a mAb Bax to recognise His-Bax. Lower figure: Two separate assays were designed to verify interaction of both left and right arms - mAb Bax and biotinylated mouse IgG and conjugated mAb NPM acceptor beads.

Figure 13 shows interaction of biotinylated mouse secondary antibody IgG with a monoclonal Bax antibody. Streptavidin-coated donor beads were bound to the biotinylated mouse secondary antibody and anti mouse IgG coated acceptor beads to monoclonal Bax antibody. Interaction between the two antibodies could clearly be seen compared to the negative control. Increasing CHAPS concentration did not affect the interaction.

Figure 14 shows interaction between biotinylated mouse secondary antibody and monoclonal nucleophosmin antibody conjugated acceptor beads. Streptavidin-coated donor beads were bound to the biotinylated mouse secondary antibody and monoclonal nucleophosmin antibody conjugated acceptor beads. Interaction between the biotinylated mouse secondary antibody and the monoclonal nucleophosmin antibody conjugated acceptor beads could clearly be seen compared to the negative control (A). Increasing CHAPS concentration did not enhance the interaction (B).

Figure 15 shows AlphaScreen™ experimental assay for C-terminal truncated Bax peptide interaction with His-nucleophosmin. Streptavidin-coated donor beads were bound to the biotinylated C-terminal

truncated Bax peptide and monoclonal nucleophosmin antibody conjugated acceptor beads to His-nucleophsmin.

Figure 16 shows interaction of His-nucleophosmin with a C-terminal truncated Bax peptide. Interaction between C-terminal truncated Bax peptide and His-nucleophosmin was shown. Both proteins were serially diluted to produce a standard titration curve. Optimisation of the signal was achieved by reducing the Tween concentration.

Example 1

Aim:

To establish that Bax is activated and undergoes a conformational change after focal cerebral ischaemia.

Methods:

Surgery was performed in male C57BL/6J mice (25-30g). Occlusion of the middle cerebral artery (MCA) was performed under surgical anaesthesia (halothane in N_2O/O_2) using a modification of the monofilament occlusion model described by Hata *et al*, 1998. Briefly, the common carotid artery and its branches were exposed via a 10mm midline cervical incision; an 8-0 silicone-coated monofilament (diameter 220μm) was introduced into the lumen of the common carotid artery and advanced through the internal carotid artery so as to occlude the origin of the MCA. The monofilament was completely withdrawn after 30 minutes to allow reperfusion and tissues were dissected from regions of interest (cortex, striatum and cerebellum) at 3 hours. In the cases of sham-operated controls, mice were subjected to monofilament insertion immediately followed by withdrawal, and sacrificed after 3 hours. Tissues were processed immediately for immunoprecipitation and subsequent Western blotting analysis by homogenisation in ice cold extraction buffer (150mM NaCl, 10mM HEPES-KOH pH 7.6, 1.0% CHAPS, 1mM PMSF, 10μg/ml each aprontinin, pepstatin A and leupeptin). Homogenates were centrifuged (10000rpm for 5 minutes at 4°C) to pellet unbroken cells and

nuclei. The protein concentrate of the lysate (supernatant) was adjusted to 2mg/ml and incubated with the precipitating antibody (anti-Bax monoclonal, 6A7, BD Biosciences; 5µg antibody to 100µg protein) which is reported to bind to Bax only in its conformationally activated form (Hsu and Youle, 1998), overnight at 4°C. Immune complexes were isolated by incubation with protein G-conjugated sepharose beads for 1 hour with constant agitation at room temperature. Beads were collected by centrifugation and washed 3 times in excess extraction buffer then eluted directly by boiling in SDS-PAGE sample buffer. Depleted supernatants (30µg protein) and immunoprecipitated samples were separated by SDS-PAGE and analysed by Western blotting using a polyclonal anti-Bax antibody (Bax NT, Upstate Biotechnologies) which recognises Bax irrespective of its activation state.

Results:

The monoclonal antibody 6A7 displays conformational-dependent activity and only recognises Bax in its conformationally-altered active state. This antibody was therefore used to immunoprecipitate active Bax to determine whether an ischaemic insult (occlusion of the MCA) leads to bioactivation of Bax. Tissue from occluded and sham-operated mouse brain was extracted in CHAPS-containing buffer and reacted with the 6A7 antibody then analysed by Western blotting using an anti-Bax antibody which recognises Bax irrespective of its activation state. The ischaemic insult resulted in the activation of a proportion of the Bax protein in cortical and striatal samples as assessed by 6A7 activity (Figure 1). Bax activation was detected in ipsilateral cortex and striatum and contralateral striatum in occluded animals but not in sham operated animals. No Bax activation was detected in samples taken from the cerebellum, an area of the brain outwith the territory of the MCA.

Example 2

Aim:

To identify proteins binding to the mitochondrial targeting domain of Bax.

Methods:

Total cell lysates from SH-SY5Y cells (ECACC number 94030304), a human neuroblastoma cell line, were prepared under control and apoptotic conditions. Apoptotic cells were treated with 0.5μM staurosporine for 5 hours prior to collection and extraction in 150mM NaCl, 10mM HEPES-KOH pH7.6, 1% CHAPS, 1mM PMSF, 10μg/ml each aprotinin, pepstatin A and leupeptin. Control cells received no staurosporine. Cells were homogenised on ice using a tight-fitting Dounce glass homogeniser (15 strokes). After an initial centrifugation step (12000g for 5 minutes) to remove the insoluble material, the soluble material (total cell lysate) was assessed for total protein content (BCA protein assay, Pierce). An aliquot of each lysate was analysed by SDS-PAGE (30μg total protein loaded per lane) followed by Western blotting with anti-active caspase 3 antibody (New England Bioloabs, 1/1000) to confirm induction of apoptosis in the staurosporine treated cells.

Total cell lysate was diluted to 2mg/ml and 1mg protein was used for affinity chromatography using the biotinylated peptides corresponding to the C-terminal 21 amino acids of wild type and mutant (which constitutively targets to mitochondria) Bax. These peptides were custom synthesized by Albachem (Elvingston Science Centre, East Lothian). The wild type peptide sequence was biotin-TVTIFVAGVLTASLTIWKKMG whereas the mutant sequence had the serine (S) substituted by a valine (V). These peptides were coupled to NeutrAvidin-agarose (Pierce), a commercially available form of streptavidin that has been altered to produce less non-specific binding, and used for affinity chromatography. Captured proteins were washed with extraction

buffer (150mM NaCl, 10mM HEPES-KOH pH7.6, 1% CHAPS) and eluted by boiling in SDS-PAGE sample buffer (1 minute) for subsequent analysis by SDS polyacrylamide gel electrophoresis. Gels were visualised with a mass spectrometry compatible silver stain (Silver Stain Plus, BioRad) or a fluorescent stain (Sypro Ruby, Molecular Probes). Protein bands were excised from the stained acrylamide gels and processed for further anyalysis by mass spectrometry. The SDS was removed by repeated washing in 200mM ammonium bicarbonate in 50% acetonitrile at 30°C (three 30 minute washes). The protein was reduced by treatment with 20mM dithiothreitol, 200mM amminoium bicarbonate, 50% actetonitrile (30°C for 1 hour). Cysteines were alkylated by incubation in 50mM iodoacetamide, 200mM ammonium bicarbonate and 50% acetonitrile for 20 minutes in the dark. Gel slices were coated in acetonitrile and the protein digested with 10μg trypsin in 50mM ammonium bicarbonate overnight at 32°C. Samples were then analysed by MALDI-TOF mass spectrometry (Voyager MALDI-TOF, PerSeptive Biosystems). Resulting MS peptide fingerprints were used to search the NCBI or Swissprot databases using the Protein Prospector MS-Fit peptide mass fingerprinting tool (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). The database was initially interrogated over all species and molecular weights and then refined to species (homo sapiens) if the molecular weight of the match was unrealistic. Matches were recorded as having a MOWSE score of greater than 10^4, a percentage coverage of the protein and closely matched the molecular weight estimated from the SDS-PAGE.

Results:

Bax is a pro-apoptotic Bcl-2 family protein which normally exists in the cytosol but upon receipt of an apoptotic stimulus, it undergoes a conformational change and translocates to the mitochondria. This conformational change results in exposure of previously hidden regions at the N and C-termini. The C-terminal 21 amino acids of Bax (Thr172- Gly192) are thought to play a role in the targeting of Bax to the

mitochondria as deletion of this hydrophobic sequence abrogated the ability of Bax to target to the mitochondria during apoptosis (Wolter *et al*, 1997). A serine residue in this region (at position 184) has been identified as a particularly important amino acid in the regulation of Bax C-terminal conformation and subsequently Bax subcellular localisation. Studies have shown that replacing this serine with a valine (Bax S184V) or deleting this serine altogether (Bax ΔS184) caused Bax to be constitutively localised to the mitochondria (Nechushtan *et al*; Suzuki *et al*, 2000). Indeed fusing the 20/21 amino acid C terminal sequence of Bax ΔS184 or S184V to the reporter gene was sufficient to constitutively target GFP to mitochondria (Nechushtan *et al*, 1999). Therefore biotinylated peptides corresponding to the C-terminal 21 amino acids of wild type and S184V mutant Bax were synthesised and used for affinity chromatography to isolate interacting proteins which may regulate the targeting of Bax. Affinity chromatography with the native Bax peptide and the S184V mutant resulted in the repeated isolation of the same 5 protein bands. Three proteins of approximately 92kD, 68kD and 48kD were eluted from both the wild-type and S184V mutant peptide affinity columns (Figure 2). These bands were cut out from the gel and digested with trypsin. Subsequent mass spectrometric analysis identified the 92kD band as Hsp90, the 68kD band as Hsp60 and the 48kD band as β-actin (Table 1). Two bands of 41kD and 26kD were isolated only with the mutant peptide (Figure 2). The 41kD band was identified as nucleophosmin (also known as NPM, B23, numatrin and NO38) using MALDI-TOF mass spectrometry (Table 1).

Table 1:

Identification of column eluates by MALDI-TOF mass spectrometry.

Affinity chromatography was performed using Bax wild type or mutant (S184V) peptide with control SH-SY5Y cell lysates. Eluates were analysed by SDS-PAGE and silver stained. Three proteins (of approximately 92kD, 68kD and 48kD) were eluted from the wild-type

peptide affinity column and five (92kD, 68kD, 48kD, 41kD and 26kD) from the S184V mutant peptide affinity column. Subsequent mass spectrometric analysis identified the 92kD band as Hsp90, the 68kD band as Hsp60, the 48kD band as β-actin and the 41kD band as nucleophosmin. The identity of the 26kD band isolated only with the mutant peptide, has yet to be established.

Protein	Masses matched/ masses submitted	MOWSE score	Molecular Weight	pI	Accession number
Hsp 90	14/180 18/271	1.9×10^7 4×10^7	84 674 83 265	4.9 5.0	P07900 (alpha) P08238 (beta)
Hsp60	26/168	9.5×10^{10}	61 055	5.7	P10809
β-actin	19/130	5.2×10^9	41 005	5.56	P02570
Nucleophosmin	5/25	1.5×10^4	32 575	4.64	P06748

Example 3

Aim:

To verify the interaction between Bax and nucleophosmin by demonstrating co-precipitation.

Methods:

Total cell lysates from SH-SY5Y cells were prepared under control and apoptotic conditions. Apoptotosis was induced by treating the cells with 0.5μM staurosporine for 5 hours prior to extraction in 150mM NaCl, 10mM HEPES-KOH pH7.6, 1% CHAPS, 1mM PMSF, 10μg/ml each aprotinin, pepstatin A and leupeptin. Control cells received no staurosporine. Cells were homogenised on ice using a tight-fitting Dounce glass homogeniser (15 strokes). After a spin (13000 rpm for 5 minutes) to remove the insoluble material, the soluble material (total cell lysate) was assessed for total protein content (BCA protein assay, Pierce).

Nucleophosmin was immunoprecipitated from these cell lysates using 5μg anti-nucleophosmin polyclonal antibody (Cell Signaling Technology) overnight at 4°C and immune complexes isolated by incubation with protein G-conjugated sepharose beads for 1 hour with constant agitation at room temperature. Beads were collected by centrifugation (13000 rpm for 5 minutes) and washed 3 times in excess extraction buffer then eluted directly by boiling in SDS-PAGE sample buffer. To demonstrate the specificity of this interaction, immunoprecipitation from SH-SY5Y total cell lysates was performed as above using monoclonal antibodies raised against either nucleophosmin (Sigma) or survival motor neuron protein (SMN, BD Transduction Laboratories). Immunoprecipitated samples were separated by SDS-PAGE and analysed by Western blotting using a polyclonal anti-Bax antibody (Bax NT, Upstate Biotechnologies).

Results:

Bax was co-immunoprecipitated with nucleophosmin from both control and apoptotic cell lysates (Figure 3). More Bax was precipitated with nucleophosmin under apoptotic conditions than under control conditions. The specificity of the interaction was demonstrated by immunoprecipitation with two monoclonal antibodies raised against either nucleophosmin or SMN (Figure 4). Although Bax co-precipitated with nucleophosmin, no co-precipitation with SMN was detected.

Example 4

Aim:

To examine the effect of nucleophosmin knock down on Bax mediated apoptosis.

Methods:

Four target sequences were chosen from within the nucleophosmin coding sequence suitable for cloning into a siRNA expression vector, pSilencer 3.1-H1 hygro (Ambion, see Table 2 for sequences). The specificity of target sequences was verified by Blast search. Short hairpin RNA (shRNA) oligonucleotides were synthesized (Invitrogen Life Technologies), the corresponding sequences annealed (3 minutes at 90°C followed by 1 hour at 37°C) and ligated into the precut vector (2 hours at 37°C using T4 DNA ligase). Ligated products were transformed into competent DH5α *E. coli* cells and cultured on LB-ampicillin (100μg/ml) plates overnight. Cells were also transformed with ready-made pSilencer 3.1-H1 hygro vector containing a negative control shRNA insert with no homology to any known mammalian gene (Ambion). Three colonies were picked from each transformation, DNA mini-preps (Promega) performed and clones successfully validated using insert-specific restriction enzymes.

SH-SY5Y cells were seeded (5 x 10^4 cells/well) in 24 well culture plates then allowed to attach for 24 hours prior to transfection. Equal amounts of each of the four nucleophosmin shRNA clones were mixed and a total of 1ug of DNA per well was transfected into SH-SY5Y cells over a four-hour period using Transmessenger transfection reagent (Qiagen) in accordance with the manufacturer's instructions. Analysis for nucleophosmin expression was performed 24-72 hours later by Western blotting. Twenty four hours post transfection, apoptosis was induced with staurosporine (0.5μM, 3-4 hours) while control cells received DMSO vehicle. Cells were subsequently extracted for measurement of nucleophosmin, cytochrome c or cleaved caspase 3. For nucleophosmin

or caspase 3 measurement, cells were extracted in 10mM HEPES KOH pH7.4, 2mM $MgCl_2$, 5mM EGTA, 1% CHAPS, 50mM KCl, 1mM PMSF, 10µg/ml each leupeptin, pepstatin A and aprotinin. For cytochrome c analysis, cells were extracted in 3.125µM digitonin, 40mM HEPES, 140mM KCl, 20mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 0.566mM $CaCl_2$, 5mM ATP (to permeabilise the cell membrane but not the mitochondria) or the same buffer also containing 1% CHAPS to allow comparison of cytosolic content with total cellular content of cytochrome c (Sparagna *et al*, 2000) (Kirchhoff *et al*, 2002). Cells were then scraped, collected lysates centrifuged and supernatants stored at -70°C. The cleaved caspase 3 and cytochrome c content of the supernatants were measured by ELISA (BD Biosciences and Caltag MedSystems respectively) while nucleophosmin was analysed by Western blotting using a monoclonal antibody (anti-B23 nucleophosmin/NPM, Sigma).

Results:

Specific knockdown of nucleophosmin expression, corresponding to an approximate 50% decrease, was obtained in cells transfected with nucleophosmin shRNA but not control shRNA (Figure 5). After knockdown of nucleophosmin, a corresponding decrease in the release of mitochondrial cytochrome c into the cytosol in response to an apoptotic stimulus (4 hours staurosporine) was detected (Figure 6). Similarly, a reduction in the amount of cleaved caspase 3 was detected by ELISA following induction of apoptosis in nucleophosmin shRNA treated cells but not in control shRNA treated cells (Figure 7). These data suggest that knockdown of nucleophosmin expression, decreased the sensitivity of cells to apoptosis induced by staurosporine treatment.

Table 2:

Details of sequences used to construct nucleophosmin shRNA expression clones.

The nucleophosmin cDNA sequence (Accession number

M28699) was searched for suitable 21 nucleotide target oligonucleotides. Four suitable sequences spaced along the length of the open reading frame and specific to nucleophosmin by BLAST search were chosen to increase the chances of successful knockdown of protein expression. Target sequences and their position with respect to the human sequence are detailed in the right hand column. For each target, a 'sense' 55-60 mer hairpin template was drawn up containing the 19-mer target sequence as its stem separated by a 9 nucleotide loop sequence, a 5-6 nucleotide poly(T) tract and a 5' overhang forming the BamHI and HindIII restriction sites. For each sequence, a sense and antisense hairpin template were designed and subsequently annealed together prior to cloning into the expression vector.

Sequence number	shRNA sequence 5'-3'	Targeted sequence
1 sense	GAT CCC GCA GAG GCA ATG AAT TAC GTT CAA GAG ACG TAA TTC ATT GCC TCT GCT TTT TTG GAA A	Nucleotides 182-202 of human NPM AAG CAG AGG CAA TGA ATT ACG
2 anti-sense	AGC TTT TCC AAA AAA GCA GAG GCA ATG AAT TAC GTC TCT TGA ACG TAA TTC ATT GCC TCT GCG G	
3 sense	GAT CCC GAT GCA GAG TCA GAA GAT GTT CAA GAG ACA TCT TCT GAC TCT GCA TCT TTT TTG GAA A	Nucleotides 362-382 of human NPM AAG ATG CAG AGT CAG AAG ATG
4 anti-sense	AGC TTT TCC AAA AAA GAT GCA GAG TCA GAA GAT GTC TCT TGA ACA TCT TCT GAC TCT GCA TCG G	
5 sense	GAT CCC GGA CCT AGT TCT GTA GAA GTT CAA GAG ACT TCT ACA GAA CTA GGT CCT TTT TTG GAA A	Nucleotides 470-490 of human NPM AAC TTG CTG CTG ATG AAG ATG
6 anti-sense	AGC TTT TCC AAA AAA GGA CCT AGT TCT GTA GAA GTC TCT TGA ACT TCT ACA GAA CTA GGT CCG G	
7 sense	GAT CCC GAT GCA GAG TCA GAA GAT GTT CAA GAG ACA TCT TCT GAC TCT GCA TCT TTT TTG GAA A	Nucleotides 716-736 of human NPM AAG GAC CTA GTT CTG TAG AAG
8 anti-sense	AGC TTT TCC AAA AAA GAT GCA GAG TCA GAA GAT GTC TCT TGA ACA TCT TCT GAC TCT GCA TCG G	

Example 5

Aim:

To examine the subcellular localisation of Bax and nucleophosmin following experimental stroke.

Methods:

Briefly, all studies were performed by licensed personnel and under the authority of the UK *Animal Scientific Procedures* act of 1986. Mice were anaesthetised (Induction: 5% halothane in nitrous oxide/oxygen 70:30 v/v and maintained in 2-2.5% halothane in nitrous oxide/oxygen). Rectal temperature was maintained at 37±1°C throughout the surgical procedure by means of a heating blanket. After midline incision, the left common carotid and external carotid arteries were isolated and ligated and a microvascular clip placed over the internal carotid artery. An 8/O nylon monofilament (Ethicon) coated with Xantopren silicon resin (Bayer) was inserted into the common carotid and advanced 9mm from the carotid bifurcation so that the tip of the filament lay across the origin of the middle cerebral artery. The monofilament was secured and the wound sutured before the animal was placed in an incubator to recover from anaesthesia. In sham operated mice, the monofilmant was introduced and immediately withdrawn. Thirty minutes after the onset of ischaemia, the mouse was re-anaesthetised and the monofilament removed to allow reperfusion of the MCA vascular territory. The wound was again sutured, anaesthesia discontinued and the mouse returned to the incubator to recover. After 150 minutes of reperfusion the mouse was decapitated, the brain removed and the ipsilateral region of cortex vulnerable to ischaemic damage dissected out and weighed (~20 mg). Samples were each disrupted in 40µl EB(10mM HEPES-KOH, pH 8.0, 0.32M sucrose, 1mM EGTA, 25mM KCl, 5mM $MgCl_2$, 0.1mM DTT, 1mM DNase I, 1mM PMSF, 10µg/ml each aprotinin, pepstatin A and leupeptin) using a hand held tissue disruptor. After 1 hour on ice (minimum time required to perform MCA occlusion of 6 mice), cortical and striatal tissue from all 6 mice was pooled, homogenised

in a total volume of 240µl EB using 9 'loose' up/down strokes of a glass Dounce homogeniser on ice and passed through gauze (125µm clearance) to remove unbroken cells and cell debris. The nuclear fraction was obtained using a modification of the method described by Muramatsu and co-workers in 1963 (Muramatsu et al, 1963, Cancer Res 25:693-697). Briefly, the homogenate was spun at 600g for 10 minutes to pellet out the crude nuclear fraction, which was washed twice in EB (200g for 5 minutes) to pellet out unbroken cells. The pellet was re-suspended in 3ml nuclear buffer 1 (NE1; 0.25M sucrose, 10mM $MgCl_2$) and layered over 3ml NE2 (0.35M sucrose, 0.5mM $MgCl_2$), spun at 1,450g for 5 minutes and the creamy middle layer of the pellet re-suspended in EB containing 2% CHAPS. The supernatant fraction from the original 600g spin was further homogenised by 15 'tight' up/down strokes and spun at 10,000g for 30 minutes. The pellet and supernatant represented the crude mitochondrial and cytosolic fractions, respectively. The mitochondrial fraction was washed twice in EB (10,000g for 30 minutes), subjected to a final 600g spin for 10 minutes to pellet out any insoluble material and resuspended in 50µl EB containing 2% CHAPS. The crude cytosolic fraction was spun at 10,000g for 30 minutes to pellet out mitochondria and the supernatant spun at 100,000g for 1 hour. The supernatant was retained as the soluble cytosolic (S100) fraction. Samples were maintained at 4°C throughout. The purified cytosolic and enriched nuclear, mitochondrial and cytosolic fractions were analysed by SDS-PAGE and Western blotting. Procaspase-3, VDAC and c-jun were used as cytosolic, mitochondrial and nuclear protein markers respectively to confirm fraction purity. The total protein contents of the nuclear, mitochondrial and cytosolic fractions of sham and occluded cortex were determined (BCA protein assay, Pierce) and analysed by SDS PAGE (20µg per lane). Primary antibodies used were mouse monoclonal anti-c-jun (BD Biosciences, 1:500) or rabbit polyclonal antibodies, anti-VDAC (Ab-1, Oncogene, 1/500), anti-procaspase-3 (Cell Signalling Technology, 1/1000), anti-Bax NT (Upstate, 1/1000) or anti-NPM

(New England BioLabs, 1/1000). Bound antibodies were detected using anti-rabbit or anti-mouse immunoglobulin coupled to horseradish peroxidase (Amersham, 1/2000) and enhanced chemiluminescence (ECL, Amersham).

Results:

There was no evidence of Bax accumulation at the mitochondria 3 hours following MCA occlusion (Figure 8). Although previous data demonstrated that Bax undergoes a conformational change within 3 hours after the onset of focal cerebral ischaemia in the mouse (Figure 1). In contrast, nucleophosmin translocated from the nuclear fraction and accumulated in both the cytosolic and nuclear fractions 3 hours following MCA occlusion (Figure 9). Taken together, these studies reveal that, following occlusion of the middle cerebral artery, nucleophosmin translocation from the nucleus occurs prior to Bax translocation, but co-incident with conformational changes in Bax.

Example 6

Aim:

To investigate the interaction between recombinant Bax and nucleophosmin using prokaryotic and eukaryotic expression systems.

Methods:

Cell-free expression and purification of recombinant native and His-tagged Bax and nucleophosmin

Full-length native and His-tagged Bax and nucleophosmin were expressed using *in vitro* cell-free expression systems. Eucaryotic expression (TnT® quick coupled transcription/translation with rabbit reticulocyte lysate; Promega) in accordance with the manufacturer's instructions. Following *in vitro* expression, His-tagged Bax and nucleophosmin were purified from the rabbit reticulocyte lysate using MagZ beads. Protein samples were incubated with the beads for 15 minutess at room temperature. Beads were captured using a magnetic stand, and bound protein washed 4 times.

Protein was then eluted in 500mM imidazole. Further dialysis of the proteins against a Tris Buffer was carried out using Slide-A-Lyzer cassettes (10KDa MWCO) (Pierce) to remove imidazole.

Procaryotic expression was performed using the Expressway™Plus Expression System (Invitrogen). Full length ORF's for native and His-tagged Bax and nucleophosmin previously subcloned from the pENT™221 vector into pCRT7/NT-TOPO vector were used. Expression was driven from the T7 promoter using a quick coupled transcription/translation system with *E.coli* lysate. Expressed proteins were analysed by Western blotting using protein specific antibodies. Immunoprecipitation of recombinant His-tagged Bax and nucleophosmin was performed as previously described below.

Immunoprecipitation

In vitro expressed recombinant Bax and nucleophosmin were mixed for 30 minutes at room temperature prior to co-immunoprecipitation. Protein G-conjugated sepharose beads (Sigma) were washed and equilibrated in 150mM NaCl, 10mM HEPES-KOH pH7.6. Samples to be immunoprecipitated were pre-cleared by incubation with protein G-conjugated sepharose beads to remove non-specific binding. After 30 minutes incubation, the beads were pelleted by centrifugation (8000rpm for 2 minutes), washed with buffer as above and the non-specifically bound proteins eluted by boiling in SDS-PAGE sample buffer for 5 minutes (pre-clear elution). Nucleophosmin and Bax were immunoprecipitated from the pre-cleared supernatant samples using either 2.5µg anti-nucleophosmin monoclonal antibody (Sigma) or 4µg anti-Bax polyclonal antibody (Bax NT; Upstate Biotechnology) overnight at 4°C. Immune complexes were isolated by incubation with 30µl protein G-conjugated sepharose beads for 1 hour at room temperature with gentle agitation. Beads were collected by centrifugation and washed three times in excess buffer before elution by

boiling in SDS-PAGE sample buffer containing 0.1M DTT. The depleted supernatant was also retained for further analysis. Samples were separated by SDS-PAGE and analysed by Western blotting using polyclonal anti-Bax (Upstate Biotechnology) and anti-nucleophosmin (Cell Signaling Technology) antibodies.

Subcloning of Bax and nucleophosmin

Full length human Bax and nucleophosmin were subcloned from the Gateway® entry vector pENTR™221 into the pDEST™8 and pDEST™10 destination vectors as recommended by the manufacturer (Invitrogen). Clones were transformed into competent TOP10 *E. coli* cells (Invitrogen) and plasmid DNA purified (Wizard Plus SV miniprep system; Promega). Clones were confirmed by restriction enzyme digestion using Bsu36I, BamHI and Ecoo91I (all New England Biolabs) following standard molecular biology procedures. Clones were also subjected to sequence verification (University of Oxford DNA sequencing facility) using the primers detailed in Table 3.

Primer name	**Sequence (5'-3')**	**Detail**
FN171	ATGGACGGGTCCGGGGAGCA	Bax gene-specific forward primer
FN174	ATGGAAGATTCGATGGAGATGG	NPM gene-specific forward primer
FN177	GTTCTAGTGGTTGGCTACGTATA	pDEST8/10 Vector-specific forward primer

Table 3:

Details of primer sequences used to verify Bax and nucleophosmin subclones in pDEST™8 and pDEST™10.
A vector-specific forward primer (FN177) together and gene-specific forward primers (FN171 and FN174 for Bax and nucleophosmin clones

respectively) were used to sequence-verify Bax and nucleophosmin subclones in pDEST™8 and pDEST™10. The ATG start codons of Bax and nucleophosmin open reading frames (ORF) in primers FN171 and FN174 are shown.

Results:

i) Procaryotic and eucaryotic expression studies of human Bax and nucleophosmin were established in cell-free (coupled transcription and translation) with further eukaryotic expression using a cell based (insect cell) system.

ii) Recombinant Bax and nucleophosmin (both native and histidine-tagged proteins) were successfully expressed *in vitro* and recognised by their respective antibodies. To investigate the interaction between these two proteins further, recombinant native and His-tagged Bax and nucleophosmin were used to perform co-immunoprecipitation studies. Recombinant nucleophosmin was immunoprecipitated from the mixture sample and a small proportion of the Bax present in the mixture co-precipitated with the nucleophosmin-immune complex (Figures 10 and 11). Similarly, recombinant His-Bax was immunoprecipitated and a small proportion of the His-nucleophosmin present in the mixture was co-precipitated.

Example 7

Aim:

To investigate protein-protein interactions between Bax and nucleophosmin using an Amplified Luminscent Proximity Homogeneous Assay (AlphaScreen™).

Methods:

Conjugation of acceptor beads

A standard amination procedure was performed to conjugate

acceptor beads to the nucleophosmin monoclonal antibody (Sigma; Cat. No. B0556). Nucleophosmin monoclonal antibody (0.01mg) was added to 0.3mg of unconjugated acceptor beads (PerkinElmer Cat. No 6762001). Sodium cyanoborohydride (to yield a 5% final concentration) and 2-[N-Morpholino] ethanesulfonic acid (MES: 0.02M pH6.0) was added to the antibody/bead mix. The reaction mix was incubated for 48 hours at 37°C in the dark. Following incubation a block was performed with carboxymethoxylamine-hemihydrochloride) (CMO; Sigma) for 1 hour. Purification was then carried out with 3 washes in 0.1M Tris Buffer pH8.0. Conjugated beads were finally prepared as a 5mg/ml solution by resuspending in 1x Buffer (Perkin Elmer).

Biotinylated mouse secondary antibody interaction with Bax monoclonal antibody

An AlphaScreen™ mouse IgG detection kit (PerkinElmer 6760606C) was used to confirm the interaction between the biotinylated secondary antibody and the monoclonal Bax antibody (Santa Cruz). Each verification assay was performed in reaction volumes of 25ul. Donor beads and acceptor beads were added at a final concentration of 20ug/ml. Streptavidin donor beads were added to wells of a 384 well optiplate (PerkinElmer Cat. No. 6007290). Logarithmic serial dilutions of both antibodies were added and incubated with streptavidin donor beads for 30 minutes at room temperature. Thereafter, anti mouse IgG beads were added to the 384-well plate and incubated for a further hour at room temperature. Plates were read using the PerkinElmer AlphaScreen™ system.

Biotinylated mouse secondary antibody interaction with conjugated nucleophosmin monoclonal antibody acceptor beads

To confirm interaction between the biotinylated secondary

antibody and the nucleophosmin monoclonal antibody conjugated acceptor beads, streptavidin donor beads were added to wells of a 384 well optiplate (PerkinElmer Cat. No. 6007290). Logarithmic serial dilutions of the biotinylated mouse secondary antibody were added and incubated with streptavidin donor beads for 30 minutes at room temperature. Following incubation, nucleophosmin monoclonal antibody conjugated acceptor beads were added to the plate and incubated for a further hour at room temperature. Plate was read using AlphaScreen™.

AlphaScreen™ of C-terminal truncated Bax peptide and His-nucleophosmin interaction

His-tagged nucleophosmin was produced by eucaryotic *in vitro* expression, and purified using MagZ beads to remove the protein from the hemin containing rabbit reticulocyte lysate (Promega). Purified protein was dialysed at 4°C overnight against Tris-Buffer. C-terminal truncated Bax peptide was reconstituted in Bax-EB buffer (0.3% Tween 20). Both proteins were prepared in 10 fold serial dilutions for analysis by homogeneous proximity assay (AlphaScreen™). The assay contained 20ug/ml of nucleophosmin monoclonal antibody conjugated acceptor beads, 20ug/ml of streptavidin-coated donor beads and the serial protein dilutions of C-terminal truncated Bax and His-tagged nucleophosmin.

Results:

i) Verification of the initial experimental design was performed by confirming interaction of the specific antibodies in the right and left arms of the assay (Figure 12). Using an AlphaScreen™ mouse IgG detection kit, interaction of the biotinylated mouse secondary antibody with a monoclonal Bax antibody was demonstrated as a standard titration curve was observed.

ii) Increasing the detergent (CHAPS) concentration may enhance the interaction between the biotinylated mouse secondary antibody and the monoclonal Bax antibody. However there was no evidence to suggest that altering the CHAPS concentration affected the interaction (Figure 13). Verification of the interaction between the biotinylated mouse secondary antibody and the monoclonal nucleophosmin antibody conjugated acceptor beads was also demonstrated. Again, increasing CHAPS concentration did not affect the signal produced (Figure 14). These observations confirmed the successful conjugation of the monoclonal nucleophosmin antibody to the acceptor beads.

iii) Previous studies using Biotinylated C-terminal truncated Bax peptide identified nucleophosmin as a putative interacting protein in affinity chromatography studies (Example 2; Figure 2). Initial studies with the biotinylated C-terminal truncated Bax peptide and His-tagged nucleophosmin (Figure 15) revealed a titration curve with increasing protein concentration. Following optimisation of the interaction by reducing Tween concentration, this response could be more clearly observed (Figure 16). The typical curves observed in these assays demonstrate what is known as the "hooking effect", a signal increase is observed with increasing protein concentration, after which time the protein becomes inhibitory to the production of signal. This is due to saturation of the available binding sites on one or both of the AlphaScreen™ beads (PerkinElmer).

In conclusion, an interaction between C-terminal truncated Bax peptide and nucleophosmin has been demonstrated in a 384-well format assay.

REFERENCES

Antonawich FJ, Fiore-Marasa SM and Parker CP (2002) Modulation of apoptotic regulatory proteins and early activation of

cytochrome c following systemic 3-nitropropionic acid administration. Brain Res Bull **57**:647-649.

Antonsson B, Montessuit S, Sanchez B and Martinou JC (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem **276**:11615-11623.

Bonvini P, Gastaldi T, Falini B and Rosolen A (2002) Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), a novel Hsp90-client tyrosine kinase: down-regulation of NPM-ALK expression and tyrosine phosphorylation in ALK(+) CD30(+) lymphoma cells by the Hsp90 antagonist 17-allylamino,17-demethoxygeldanamycin. Cancer Research **62**:1559-1566.

Borer RA, Lehner CF, Eppenberger HM and Nigg EA (1989) Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell **56**:379-390.

Bykov VJN, Selivanova G and Wiman KG (2003) Small molecules that reactivate mutant p53. Eur J Cancer **39**:1828-1834.

Cao G, Minami M, Pei W, Yan C, Chen D, O'Horo C, Graham SH and Chen J (2001) Intracellular Bax translocation after transient cerebral ischemia: implications for a role of the mitochondrial apoptotic signaling pathway in ischemic neuronal death. J Cereb Blood Flow Metab **21**:321-333.

Chan PK, Aldrich M and Busch H (1985) Alterations in immunolocalization of the phosphoprotein B23 in HeLa cells during serum starvation. Exp Cell Res **161**:101-110.

Chan PK, Bloom DA, Hoang TT (1999) The N-terminal half of NPM dissociates from nucleoli of HeLa cells after anticancer drug treatments. Biochem Biophys Res Commun **264**:305-309.

Chou JJ, Honglin L, Salvesen GS, Yuan J and Wagner G (1999) Solution structure of Bid, an intracellular amplifier of apoptotic signalling. Cell **96**:615-624.

Colombo E, Marine JC, Danovi D, Falini B and Pelicci PG

(2002) Nucleophosmin regulates the stability and transcriptional activity of p53. Nature Cell Biol **4**:529-533.

Fiskum G, Starkov A, Polster BM, Chinopoulos C (2003) Mitochondrial mechanisms of neural cell death and neuroprotective Interventions in Parkinson's disease. Ann N Y Acad Sci **991**:111-119.

Giannakopoulos P, Kovari E, Savioz A, de Bilbao F, Dubois-Dauphin M, Hof PR, Bouras C (1999) Differential distribution of presenilin-1, Bax, and Bcl-X(L) in Alzheimer's disease and frontotemporal dementia. Acta Neuropathol (Berl) **98**:141-149.

Gonzalez de Aguilar JL, Gordon JW, Rene F, de Tapia M, Lutz-Bucher B, Gaiddon C, Loeffler JP (2000) Alteration of the Bcl-x/Bax ratio in a transgenic mouse model of amyotrophic lalteral sclerosis: evidence for the implication of the p53 signalling pathway. Neurobiol Dis **7**:406-415.

Gostissa M, Hofmann TG, Will H and Del Sal G (2003) Regulation of p53 functions: let's meet at the nuclear bodies. Curr Opin Cell Biol **15**:351-357.

Gross A, Jockel J, Wie MC and Korsmeyer SJ (1998) Enforced dimerization of Bax results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J **17**:3878-3885.

Guegan C and Przedborski S (2003) Programmed cell death in amytrophic lateral sclerosis. J Clin Invest **111**:153-161.

Hassouna I, Wickert H, Zimmermann M, Gillardon F (1996) Increase in bax expression in substantia nigra following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment of mice. Neurosci Lett **204**:85-88.

Hata R, Maeda K, Hermann D, Mies G and Hossmann KA (2000) Evolution of brain infarction after transient focal ischaemia in mice. J Cereb Blood Flow Metab **20**:937-946.

Hingorani K, Szebeni A and Olson MO (2000) Mapping the functional domains of nucleolar protein B23. Journal of Biological

Chemistry **275**:24451-24457.

Hsu YT, Wolter KG and Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-xL during apoptosis. Proc Nat Acad Sci USA **94**:3668-3672.

Hsu YT and Youle RJ (1998) Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem **273**:10777-10783.

Kirchoff SR, Gupta S and Knowlton AA (2002) Cytosolic heat shock protein 60, apoptosis, and myocardial injury. Circulation **105**:2899-2904.

Lev N, Melamed E and Offen D (2003) Apoptosis and Parkinson's disease. Prog Neuropsychopharmacol Biol Psychiatry **27**:245-250.

McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ and Cowburn D (1999) Solution structure of the proapoptotic molecule Bid: a structural basis for apoptotic agonists and antagonists. Cell **96**:625-634.

McGinnis KM, Gnegy ME and Wang KKW (1999) Endogenous Bax translocation in SH-SY5Y human neuroblastoma cells and cerebellar granule neurons undergoing apoptosis. J Neurochem **72**:1899-1906.

Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL and Look AT (1994) Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science **263**:1281-1284.

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL and Fesik SW (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature **381**:335-341.

Nechushtan A, Smith CL, Hsu YT and Youle RJ (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J **18**:2330-2341.

Okuwaki M, Matsumoto K, Tsujimoto M and Nagata K (2001) Function of nucleophosmin/B23, a nucleolar acidic protein as a histone chaperone. FEBS Lett **506**:272-276.

Oltvai ZN, Milliman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homologue, Bax, that accelerates programmed cell death. Cell **74**:609-619.

Paradis E, Douillard H, Koutroumanis M, Goodyer C and LeBlanc A (1996) Amyloid beta peptide of Alzheimer's disease downregulates Bcl-2 and upregulates bax expression in human neurons. J Neurosci **16**:7533-7539.

PerkinElmer: A practical guide to working with AlphaScreen™.

Perlaky L, Valdez BC, Busch H. (1997) Effects of cytotoxic drugs on translocation of nucleolar RNA helicase RH-II/Gu. Exp Cell Res **235**:413-420.

Redner RL, Rush EA, Faas S, Rudert WA and Corey SJ (1996) The t(5;17) variant of acute promyelocytic leukaemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood **87**:882-886.

Sathasivam S, Ince PG and Shaw PJ (2001) Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. Neuropathol Appl Neurobiol **27**:257-274.

Shackleford GM, Ganguly A and MacArthur CA (2001) Cloning, expression and nuclear localisation of human NPM3, a member of the nucleophosmin/nucleoplasmin family of nuclear chaperones. BMC Genomics **2**:8.

Sparagna GC, Hickson-Bick DL, Buja LM and McMillin JB (2000) A metabolic role for mitochondria in palmitate-induced cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol **279**:H2124-H2132;

Su JH, Deng G and Cotman CW (1997) Bax protein expression is increased in Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology. J Neuropathol Exp Neurol

56:86-93.

Suzuki M, Youle, RJ and Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell **103**:645-654.

Szebeni A and Olson MO (1999) Nucleolar protein B23 as molecular chaperone activities. Protein Sci **8**:905-912.

Tortosa A, Lopez E and Ferrer I (1998) Bcl-2 and Bax protein expression in Alzheimer's disease. Acta Neuropathol (Berl) **95**:407-412.

Trumper L, Daus H, Merz H, von Bonin F, Loftin U, Chochlovius C, Moller P, Feller AC and Pfreundschuh M (1997) NPM/ALK fusion mRNA expression in Hodgkin and Reed-Sternberg cells is rare but does occur: results from single cell cDNA analysis. Ann Oncol **8** Suppl 2:83-87.

Vis JC, Verbeek MM, de Waal RM, ten Donkelaar HJ and Kremer B (2001) The mitochondrial toxin 3-nitropropionic acid induces differential expression patterns of apoptosis-related markers rat striatum. Neuropathol Appl Neurobiol **27**:68-76.

Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG and Youle RJ (1997) Movement of Bax from cytosol to mitochondria during apoptosis. J Cell Biol **139**:1281-1292.

Wu MH, Lam CY and Yung BY (1995) Translocation of nucleophosmin from nucleoli to nucleoplasm requires ATP. Biochem J **305**:987-992.

Yang C, Maiguel DA and Carrier F (2002) Identification of nucleolin and nucleophosmin as genotoxic stress-responsive RNA-binding proteins. Nucleic Acids Research **30**:2251-2260.

Yung BY, Bor AM and Chan PK (1990) Short exposure to actinomycin D induces "reversible" translocation of protein B23 as well as "reversible" inhibition of cell growth and RNA synthesis in HeLa cells. Cancer Res **50**:5987-5991.

Yung BY, Busch H and Chan PK (1986) Effects of

luzopeptins on protein B23 translocation and ribosomal RNA synthesis in HeLa cells. Cancer Res **46**:922-925.

Yung BY, Busch RK, Busch H, Mauger AB and Chan PK (1985) Effects of actinomycin D analogs on nucleolar phosphoprotein B23 (37,000 daltons/pI 5.1). Biochem Pharmacol **34**:4059-4063.

CLAIMS

1. A method for identifying a compound that is effective in modulating the binding of nucleophosmin to Bax.

2. The use of a compound that is effective in modulating the binding of nucleophosmin to Bax for the manufacture of a medicament for treating cerebral (including spinal cord) and myocardial ischaemic injury, neonatal hypoxic brain injury, traumatic brain injury and other neurological disorders, including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and Parkinson's disease.

3. The method of claim 1, wherein the compound is identified by assessing its inhibitory effect on the interaction between Bax and nucleophosmin.

4. The method of claim 3, wherein the interaction between Bax and nucleophosmin is assessed by an amplified luminescent homogeneous proximity assay.

5. The method of claim 3, wherein the interaction between Bax and nucleophosmin is assessed by an amplified luminescent homogeneous proximity assay using the following components (5a) – (5f):

(5a) recombinant Bax protein, or fragment thereof, whether native protein or tagged with histidine, biotin or Gluthathione-S-Transferase (GST), and/or directly conjugated to acceptor or donor beads; and

(5b) recombinant nucleophosmin protein, or fragment thereof, whether native protein or tagged with histidine, biotin or Gluthathione-S-Transferase (GST), and/or directly conjugated to acceptor or donor beads; and/or:

(5c) either monoclonal or polyclonal nucleophosmin antibody conjugated to donor or acceptor beads; and/or

(5d) either monoclonal or polyclonal Bax antibody conjugated to donor or acceptor beads; and/or

(5f) streptavidin-coated donor or acceptor beads.

6. The method of claim 3, wherein the interaction between Bax and nucleophosmin is assessed by a method comprising the steps of:

(I) combining the following component (6a) with (6b):

(6a) recombinant Bax protein, or fragment thereof, directly or indirectly conjugated to donor beads

(6b) recombinant nucleophosmin protein, or fragment thereof, directly or indirectly conjugated to acceptor beads; or

(I') combining the following component (6c) with (6d):

(6c) recombinant Bax protein, or fragment thereof, directly or indirectly conjugated to acceptor beads

(6d) recombinant nucleophosmin protein, or fragment thereof, directly or indirectly conjugated to donor beads;

and the method further comprising the step of:

(II) determining whether the proximity-dependent chemical energy transfer occurs between the acceptor and donor beads.

7. A method for identifying a chemical substance which has an inhibitory effect on the interaction between Bax and nucleophosmin wherein the method comprises the steps of:

(7a) assaying the interaction between Bax and nucleophosmin, wherein the interaction is measured according to the method of claim 6 in the presence of a test compound; and

(7b) assaying the interaction between Bax and nucleophosmin, wherein the interaction is measured according to the method of claim 6 in the absence of any test compounds; and

(7c) selecting a test compound showing a lower affinity level of interaction in the step (7a) compared with that in the step (7b).

8. A method for identifying a chemical substance which has an inhibitory effect on the interaction between Bax and nucleophosmin,

wherein the interaction between Bax and nucleophosmin is detected with high sensitivity and specificity together with high signal/background ratios by the amplified luminescent homogeneous proximity assay using the multi-well plate comprising a 4-, 6-, 12-, 24-, 48-, 96-, 384-, or 1536-well design.

9. The method of claim 3, wherein Bax is selected from the group consisting of any one of the following (9a) to (9h):

(9a) full-length native Bax protein; or

(9b) full-length recombinant Bax protein; or

(9c) an N-terminal or C-terminal truncated Bax peptide; or

(9d) a fragment derived from Bax protein; or

(9e) recombinant Bax protein or fragment thereof tagged with histidine; or

(9f) recombinant Bax protein or fragment thereof tagged with biotin; or

(9g) recombinant Bax protein or fragment thereof tagged with Glutathione-S-Transferase (GST); or

(9h) any of the above Bax peptides or fragments thereof containing point mutations.

10. The method of claim 9, wherein any one of (9b)-(9h) is prepared using any one of the following systems (10a) to (10c):

(10a) cell based expression systems; or

(10b) *in vitro* cell-free procaryotic expression systems based on coupled transcription and translation; or

(10c) *in vitro* cell-free eucaryotic expression systems based on coupled transcription and translation.

11. The method of claim 3, wherein nucleophosmin is selected from the group consisting of any one of the following (11a) to (11g):

(11a) full-length native nucleophosmin protein; or

(11b) full-length recombinant nucleophosmin protein; or

(11c) a fragment derived from nucleophosmin protein; or

(11d) recombinant nucleophosmin protein or fragment thereof tagged with histidine; or

(11e) recombinant nucleophosmin protein or fragment thereof tagged with biotin; or

(11f) recombinant nucleophosmin protein or fragment thereof tagged with Glutathione-S-Transferase (GST); or

(11g) any of the above nucleophosmin peptides or fragments thereof containing point mutations.

12. The method of claim 11, wherein any one of (11b)-(11g) is prepared using any one of the following systems (12a) to (12c):

(12a) cell based expression systems; or

(12b) *in vitro* cell-free procaryotic expression systems based on coupled transcription and translation; or

(12c) *in vitro* cell-free eucaryotic expression systems based on coupled transcription and translation.

13. The method of claim 3, wherein the interaction between Bax and nucleophosmin is evaluated by assaying the interaction between a C-terminal truncated Bax peptide and a histidine-tagged nucleophosmin protein using the following components (l) to (o) for an amplified luminescent homogeneous proximity assay:

(l) a biotinylated C-terminal truncated Bax peptide; and

(m) a histidine-tagged nucleophosmin protein; and

(n) nucleophosmin monoclonal antibody-conjugated acceptor beads; and

(o) streptavidin-coated donor beads.

14. The use of a compound identified by the method of any one of claims 3 to 13 for the manufacture of a medicament for treating cerebral (including spinal cord) and myocardial ischaemic injury, neonatal hypoxic brain injury, traumatic brain injury and other neurological disorders,

including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and Parkinson's disease.

15. A kit for assaying the interaction between Bax and nucleophosmin, wherein the interaction is detected with high sensitivity and specificity together with high signal/background ratios by the amplified luminescent homogeneous proximity assay using the multi-well plate comprising a 4-, 6-, 12-, 24-, 48-, 96-, 384-, or 1536-well design.

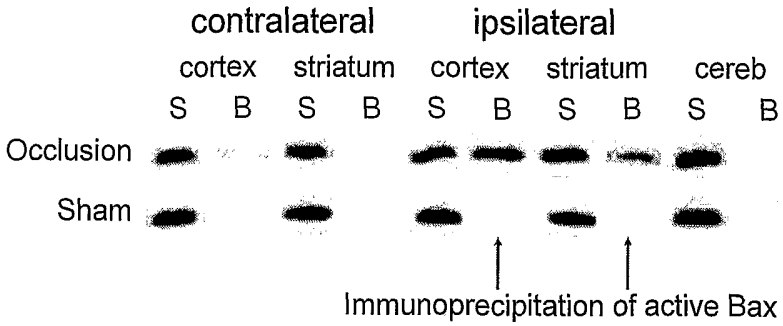


Figure 1

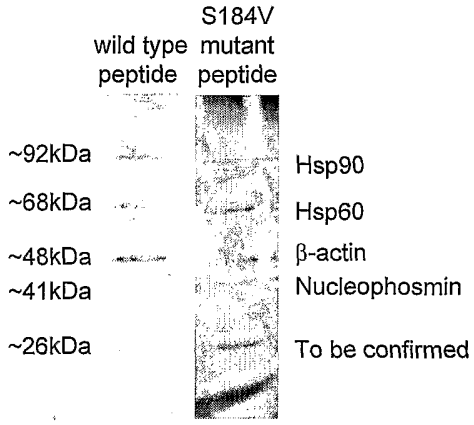


Figure 2

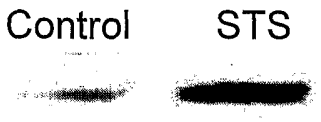


Figure 3

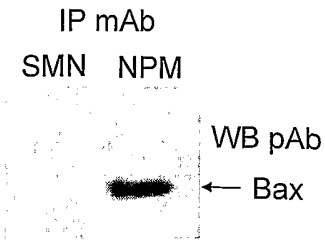


Figure 4

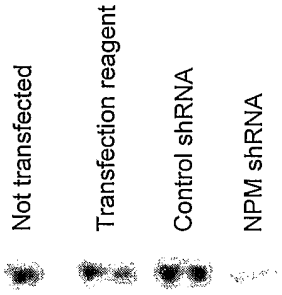


Figure 5

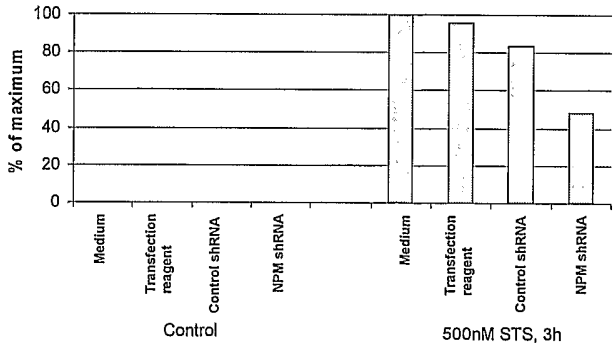


Figure 7

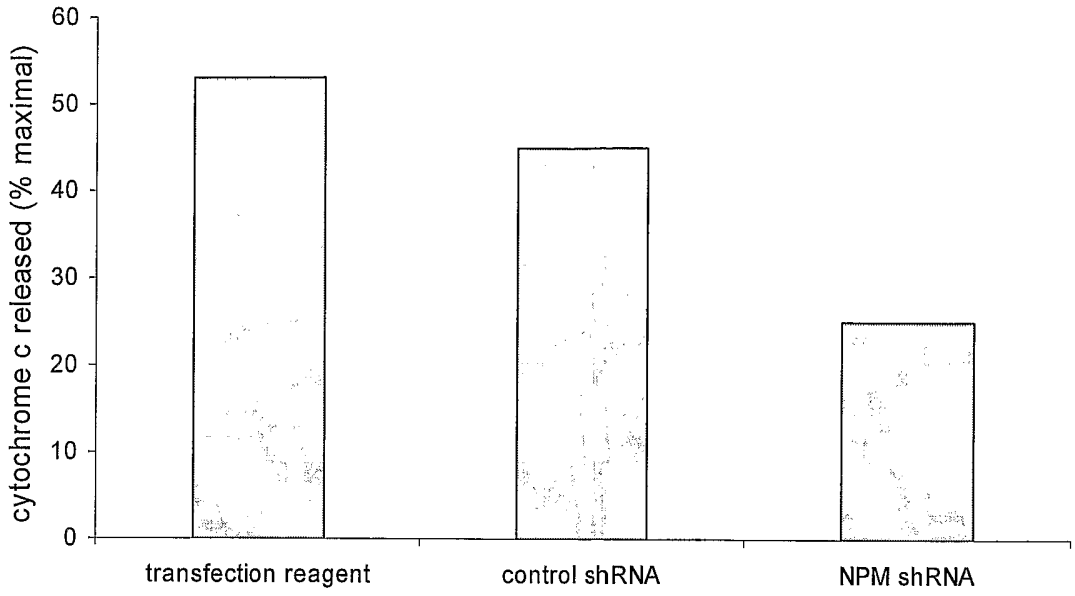

cytochrome c released (% maximal)
60
50
40
30
20
10
0
transfection reagent
control shRNA
NPM shRNA

Figure 6

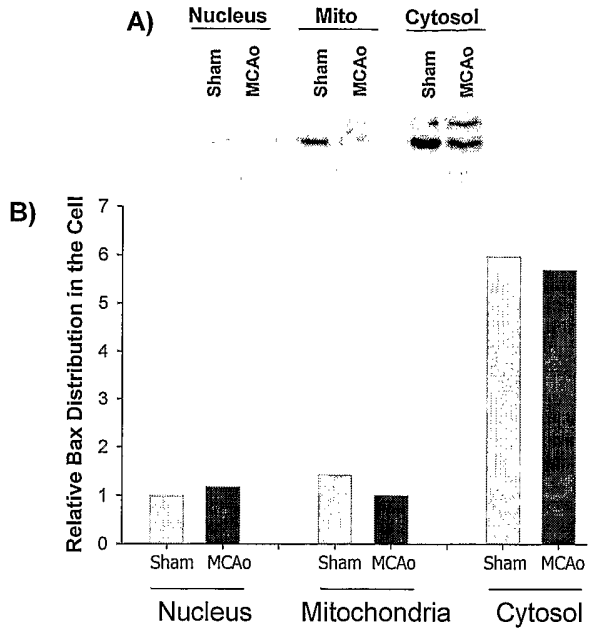


Figure 8

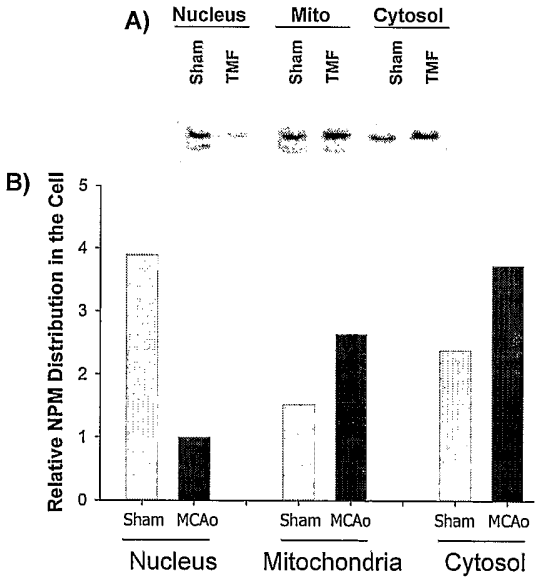


Figure 9

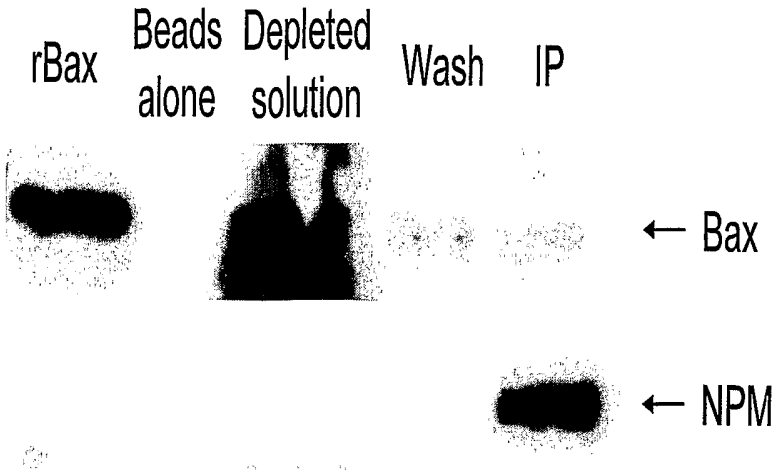


Figure 10

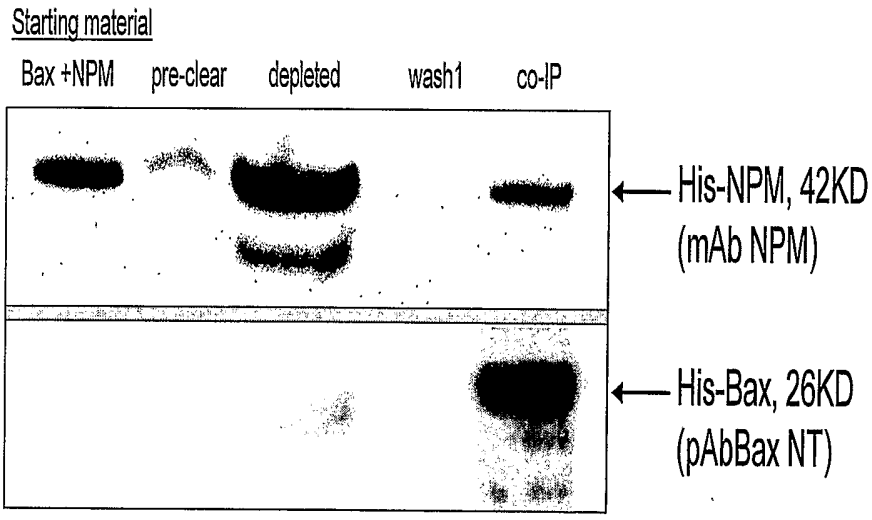
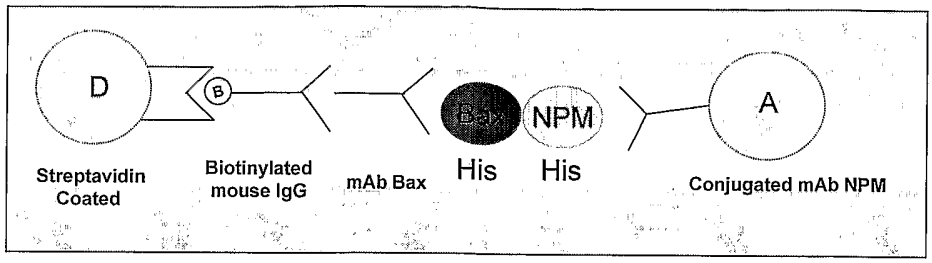
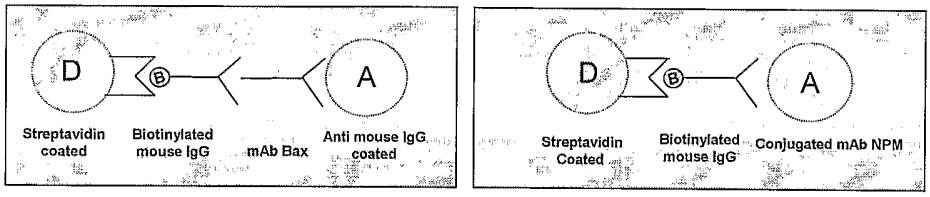


Figure 11

Amplified Luminescent Proximity Homogeneous Assay



Verification of Interaction



mAb Bax/ Biotinylated mouse IgG **mAb NPM conjugated acceptor beads**

Figure 12

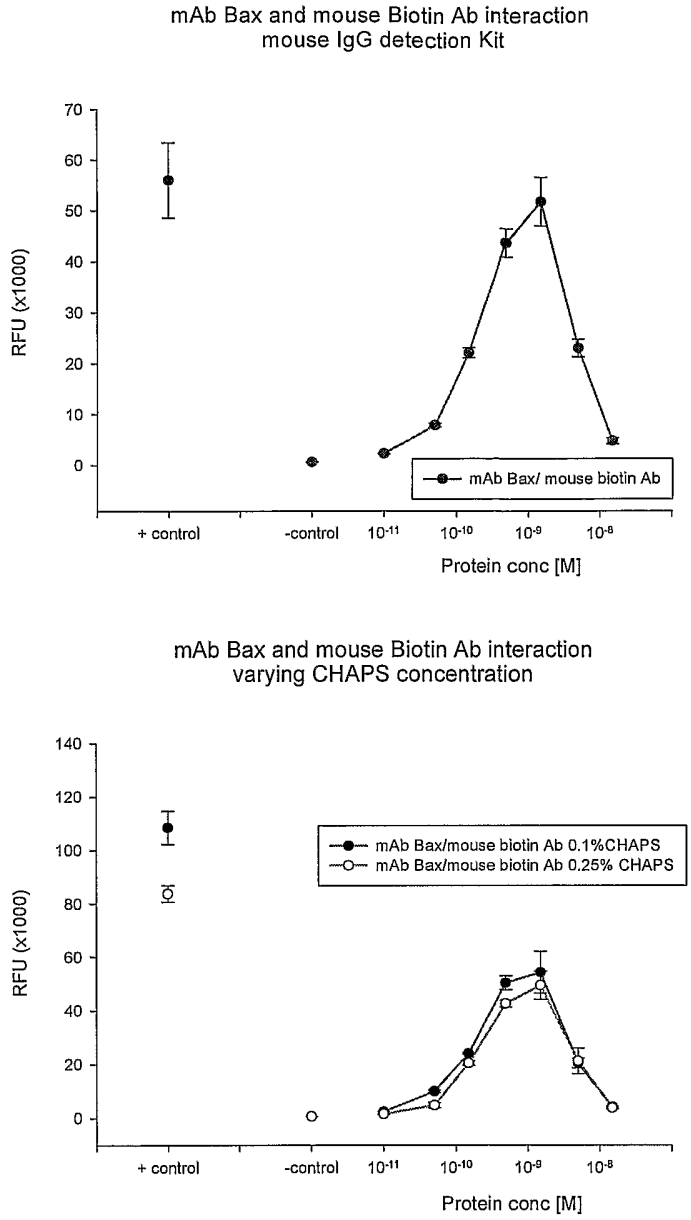

mAb Bax and mouse Biotin Ab interaction
mouse IgG detection Kit
RFU (x1000)
70
60
50
40
30
20
10
0
mAb Bax/ mouse biotin Ab
+ control
-control
10-11
10-10
10-9
10-8
Protein conc [M]
mAb Bax and mouse Biotin Ab interaction
varying CHAPS concentration
RFU (x1000)
140
120
100
80
60
40
20
0
mAb Bax/mouse biotin Ab 0.1%CHAPS
mAb Bax/mouse biotin Ab 0.25% CHAPS
+ control
-control
10-11
10-10
10-9
10-8
Protein conc [M]

Figure 13

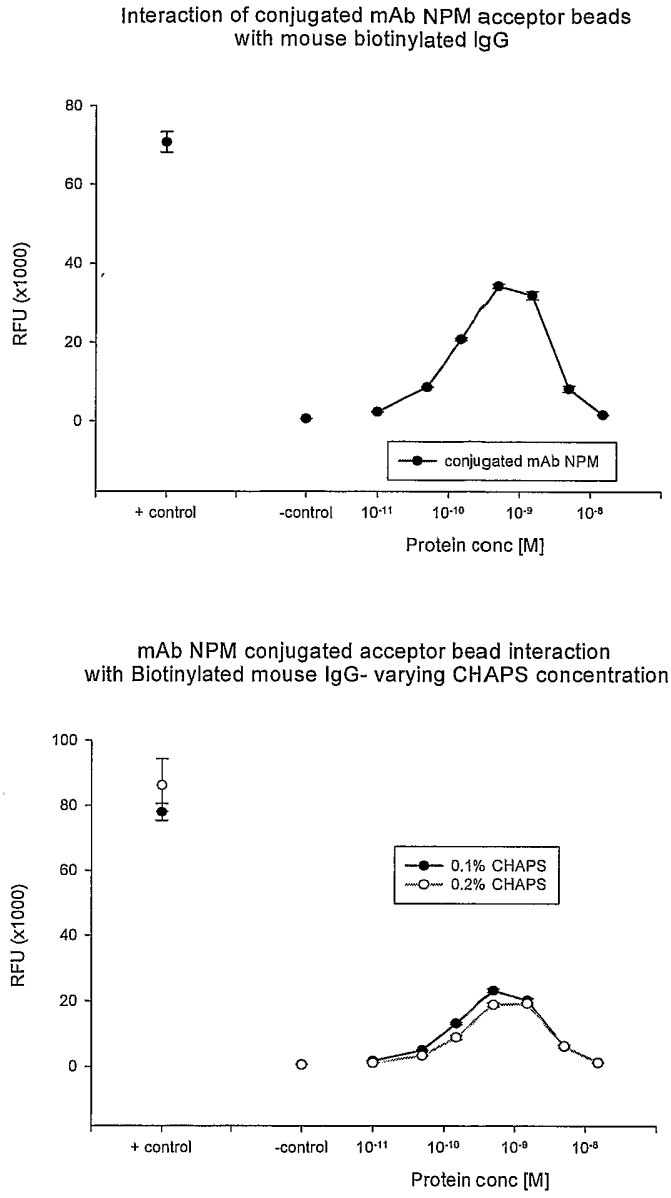

Interaction of conjugated mAb NPM acceptor beads
with mouse biotinylated IgG
RFU (x1000)
80
60
40
20
0
conjugated mAb NPM
+ control
-control
10-11
10-10
10-9
10-8
Protein conc [M]
mAb NPM conjugated acceptor bead interaction
with Biotinylated mouse IgG- varying CHAPS concentration
RFU (x1000)
100
80
60
40
20
0
0.1% CHAPS
0.2% CHAPS
+ control
-control
10-11
10-10
10-9
10-8
Protein conc [M]

Figure 14

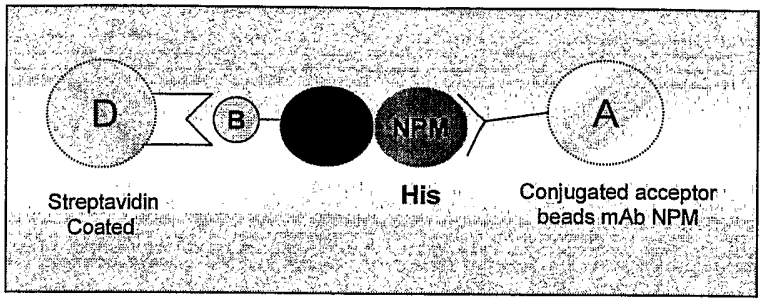

D
B
NPM
A
Streptavidin
Coated
His
Conjugated acceptor
beads mAb NPM

Figure 15

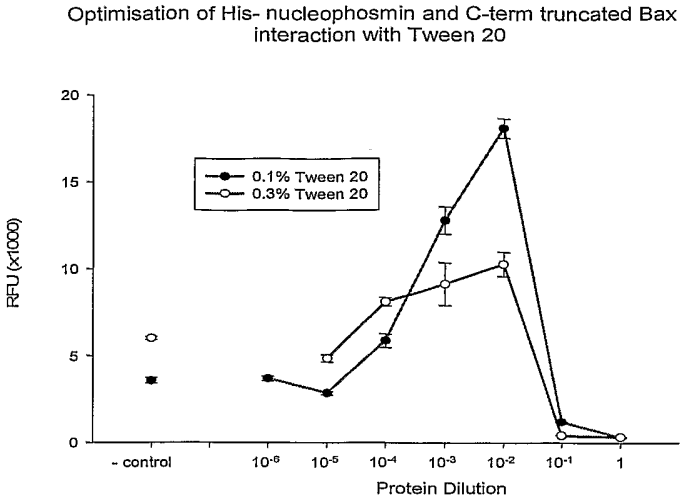

Optimisation of His- nucleophosmin and C-term truncated Bax interaction with Tween 20
RFU (x1000)
20
15
10
5
0
0.1% Tween 20
0.3% Tween 20
- control
10-6
10-5
10-4
10-3
10-2
10-1
1
Protein Dilution

Figure 16