Brn-3b enhances the pro-apoptotic effects of p53 but not its induction of cell cycle arrest by cooperating in trans-activation of bax expression

Vishwanie S. Budhram-Mahadeo*, Samantha Bowen, Sonia Lee, Christina Perez-Sanchez1, Elizabeth Ensor, Peter J. Morris and David S. Latchman

Medical Molecular Biology Unit, Institute of Child Health, University College London 30 Guilford Street, London WC1N 1EH, UK and 144 Lincoln’s Inns Field, London WC2A 3PX, UK

Received August 8, 2006; Revised October 6, 2006; Accepted October 11, 2006

ABSTRACT

The Brn-3a and Brn-3b transcription factor have opposite and antagonistic effects in neuroblastoma cells since Brn-3a is associated with differentiation whilst Brn-3b enhances proliferation in these cells. In this study, we demonstrate that like Brn-3a, Brn-3b physically interacts with p53. However, whereas Brn-3a repressed p53 mediated Bax expression but cooperated with p53 to increase p21cip1/waf1, this study demonstrated that co-expression of Brn-3b with p53 increases trans-activation of Bax promoter but not p21cip1/waf1. Consequently co-expression of Brn-3b with p53 resulted in enhanced apoptosis, which is in contrast to the increased survival and differentiation, when Brn-3a is co-expressed with p53. For Brn-3b to cooperate with p53 on the Bax promoter, it requires binding sites that flank p53 sites on this promoter. Furthermore, neurons from Brn-3b knock-out (KO) mice were resistant to apoptosis and this correlated with reduced Bax expression upon induction of p53 in neurons lacking Brn-3b compared with controls. Thus, the ability of Brn-3b to interact with p53 and modulate Bax expression may demonstrate an important mechanism that helps to determine the fate of cells when p53 is induced.

INTRODUCTION

The Brn-3b POU transcription factors was isolated from ND7 neuroblastoma cells by its homology to the highly conserved POU domain of Brn-3a. However, these distinct proteins show limited homology outside the POU domain and often display different functions in cells (1,2). For instance, in neuroblastoma cells, Brn-3a and Brn-3b demonstrate opposite expression and antagonistic functions since Brn-3a increases survival but induces differentiation of these cells whereas, Brn-3b is associated with increased growth and proliferation (3–5). In support of these observation, Brn-3b is elevated in many neuroblastoma tumours as well as in breast cancers (6,7). When increased in these cells, Brn-3b enhances cell proliferation in vitro and tumour growth in vivo in xenograft models (6,8) but also increases cell migration and confers resistance to growth arrest stimulus. In contrast, reducing Brn-3b levels in these cells is sufficient to slow many of these growth effects. However, Brn-3b appears to act in a complex manner since loss of this protein in knock-out (KO) mice result in significant loss of retinal ganglion cells and consequent blindness (9,10). The requirement for Brn-3b for specification, survival and differentiation in retinal ganglion cells, is in contrast to its growth promoting effects in cancer cells, suggesting distinct mechanisms by which this protein can act, depending on the specific cells that expresses it.

The distinct and often antagonistic effects of Brn-3b and Brn-3a in controlling growth of neuroblastoma cells, relates to their effects on specific target genes (1,11,12). Thus, whereas Brn-3a activates promoters of genes associated with differentiation in these cells (e.g. SNAP25; neurofilament; α-internexin), Brn-3b either represses these promoters or has no significant effects on their activity (12–17). In contrast, Brn-3b modulates expression of genes that are associated with proliferation e.g. it activates the cell cycle dependent kinase (cdk4) promoter and accordingly Brn-3b correlates well with cdk4 levels in tumours (18). Brn-3b also directly represses the BRCA1 tumour suppressor gene which would normally induce cell cycle arrest in breast cancer cells (7,19–21). However, in addition to its direct effects on gene transcription, Brn-3b also interacts with other cellular proteins to modulate transcription indirectly. For instance, Brn-3b physically associates with the estrogen receptor (ER) and enhances its effects on an ERE containing promoter (22). This was demonstrated on the HSP27 promoter which can be directly transactivated by Brn-3b but maximal stimulation of HSP27 expression in breast cancer cells requires cooperation between Brn-3b and the ER (23). Thus, the interaction of
Brn-3b with other transcriptional regulators, with which it is co-expressed, is likely to influence its regulation of target genes and hence cell fate.

Previously, we showed that Brn-3a (24) physically interacts with the p53 protein (25) and alters its effects on cell fate determination by differentially regulating the expression of different classes of p53 target genes (26–28). When expressed, p53 determines cell fate by either inducing apoptosis or cell cycle arrest/DNA repair, and this is achieved by its ability to regulate different subsets of genes (29). Thus the stimulation of factors such as p21\(^{CIP1/WAF1}\), GADD45 and 14-3-3\(\sigma\) are associated with cell cycle arrest/DNA repair, whereas other target genes such as Bax, Apaf-1, PUMA and Noxa that are increased in a p53 dependent manner are associated with apoptotic responses (30,31).

However, the effects of inducing p53 expression are dependent on cell type and growth conditions/stresses (e.g. DNA damage) and its responses are greatly influenced by other proteins that are co-expressed with it (28,32). Thus, co-expression of p53 with proteins such as ASPP1/2, enhances, expression of pro-apoptotic target genes (e.g. Bax; Apaf1) and increases cell death (32,33). In contrast, co-expression of Brn-3a with p53 antagonizes transcription of pro-apoptotic target genes such as Bax and Noxa, whilst enhancing p53 mediated transcription of p21\(^{CIP1/WAF1}\) gene associated with cell cycle arrest (26–28). Consequently, co-expression of Brn-3a with p53 results in increased survival and cell cycle arrest when compared with p53 alone.

The interaction of Brn-3a with p53 occurs via the POU domain of Brn-3a and the DNA binding domain of p53 (25). The POU domains of Brn-3b and Brn-3a share ~95% homology so we tested whether Brn-3b could also interact with p53. In this study, we demonstrate the physical interaction of proliferation associated Brn-3b transcription factor with the p53 protein and show the functional effects of this association on transcription of specific p53 target genes. Although high levels of Brn-3b acts to increase growth of some cancer cells we show that upon co-expression with p53, Brn-3b can cooperate with p53 to enhance the pro-apoptotic gene, Bax and so increase apoptosis in these cells.

**MATERIALS AND METHODS**

**Expression vectors**

The p53 expression construct (p53 cDNA cloned into pcDNA3 expression vector) was a kind gift of Dr K. Vousden (Beaton Institute for Cancer Research, Glasgow). Brn-3b or Brn-3a cDNA cloned into pLTR expression vectors were described previously (60).

**Antibodies.** Primary Antibodies: Brn-3b (Goat pAb) (Santa Cruz, California, USA) or Rabbit pAb (BAbCo—now unavailable); α-Bax Ab (Pharminagen, BD Biosciences, 1:500 dilution used at 1:1000–1:1500; α-actin Ab (Calbiochem, I 19) used at 1:1500; α-p53 Ab (Abcam, ab4060) 1:1000 dilution; α-Brn-3a Ab (Chemicon, MAB1585) 1:500–1000. α-cleaved caspase-3 (Asp175—Cell Signaling Technology) used at 1:500 dilution. Phycoerythrin (PE) conjugated Annexin V R&D Systems (Germany), α-Bax (Ab6) and α-p21\(^{CIP1/WAF1}\) (Ab4) mAb were obtained from Oncogene Sciences.

Secondary antibodies: Peroxidase conjugated secondary antibodies (α-rat, anti-mouse, -rabbit and -goat) were obtained from DAKO (UK); used at dilutions of 1:3000–2000.

**Protein–protein interaction studies**

**Affinity Chromatography (Pull-down) assays.** ‘Pull down’ assays were carried out as described (25). Briefly, Brn-3b GST fusion proteins linked to glutathione Sepharose beads were equilibrated and stored in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris (pH8), 0.5% Nonidet P-40, 0.5% milk powder). For interaction studies, 1–2 μg of fusion protein were washed in transcription buffer (20 mM HEPES (pH 7.9), 60 mM NaCl, 1 mM DTT, 6 mM MgCl\(_2\), 8.2% glycerol and 0.1 mM EDTA) resuspended in 100 ul of the transcription buffer, then incubated for 1 h at room temperature with 3–5 ul of \(^{35}\)S-methionine labeled in vitro translated p53 proteins (FL or truncated proteins) prepared using the TNT reticulocyte lysate kit (Promega). The equivalent amount of the T7 luciferase protein provided with the TNT kit was used as a control. Following 5x washes in NETN buffer to removed non-interacting proteins in vitro translated proteins, the pellets of the beads with interacting proteins were boiled in Laemmli SDS loading buffer for 5 min then resolved on a 12% SDS–PAGE gel. The gel was vacuum dried then exposed to radiographic film.

**Immunoprecipitation (IP).** IP studies to demonstrate interaction of Brn-3b with p53 (with appropriate controls) was carried out as described (25). Briefly, 5 μg of primary anti-p53 antibody were used to immuno-precipitate p53 protein complexes from nuclear extracts prepared from ND7 cells either expressing endogenous proteins or transfected to over-express Brn-3b protein [Brn-3b(+)]. The ER antibody was used as a positive control to IP Brn-3b whilst secondary antibody only was used as negative control. Protein A or G Sepharose beads were used to immobilize the antibody bound proteins and washes were performed to remove unbound proteins. Following the IP, proteins were resolved on a 12% PAGE gel and subjected to western blot analysis using the Brn-3b antibodies.

In the reciprocal study, to test whether p53 could be immuno-precipitated in complex with Brn-3b, specific Brn-3b pAb were used for similar IP studies and studies with Brn-3a (mAb) was included as positive control. IP of Brn-3-containing complexes was undertaken using cellular extracts prepared from ND7 cells co-transfected to over-express both Brn-3a and p53 or Brn-3b and p53. Anti-actin antibody (goat pAb) was used as IP control to confirm specificity of interaction with Brn-3b. Proteins obtained following IP were resolved by PAGE and immunoblotted for either p53 or Brn-3b (25).

**Analysis of protein levels.** Total cellular proteins were prepared from either transiently or stably transfected cell lines by harvesting into 2x Laemmli Buffer (+5% β-mercaptoethanol or 1 mM DTT). Samples were heated to 95°C for 5 min then cell debris removed by centrifugation. Variation in protein concentration was assessed using Bradford assay or Coomassie blue stained PAGE gel, which allowed visualization of the protein integrity as well as
estimation of variation in protein concentration between different samples.

Western blot analysis was carried out as previously described using 50–100 μg of each protein sample, which were resolved by polyacrylamide gel electrophoresis on a 15% PAGE gel at a constant voltage of 150V (23). Proteins were transferred onto Hybond C membrane using a wet-transfer method as described by Maniatis et al. (61). Filters were incubated with the appropriate primary antibody while anti-actin antibody was used to equalize for protein loading. Peroxidase conjugated secondary antibodies (anti-rat and anti-goat) were used at dilutions of 1:3000 or 1:2000. Signals were developed using the enhanced chemiluminescence systems (Amersham PLC or Pierce, UK).

Cell culture, transfection and luciferase assays

The ND7 cell line, which was obtained by immortalization of primary sensory neurons from DRG (62), was grown in full growth medium (FGM) (L15 medium with 10% (v/v) foetal calf serum (FCS) supplemented with D-glucose and glutamine as described previously (63). Transient transfections were carried out using either the Fugene 6 Transfection Reagent (Roche) or GeneJuice™ Transfection Reagent (Merek) according to the manufacturer’s protocol. For transfections, ND7 cells were plated at a density of 5 × 10⁵ in 6 well plates and incubated overnight (5% CO₂, 37°C in a humidified incubator). Three hours prior to transfection, the medium was changed to DMEM + 10% FCS.

Bax-3b over-expressing or vector control MCF7 cells were maintained in DMEM + 10% FCS containing antibiotic G418 (800 μg/ml) to maintain selection of stably transfected cells. For reporter analysis, 0.5 μg of reporter plasmid (WT Bax or p21ΔN192 mutant Bax promoter with altered Brn-3 site) was co-transfected with 0.5 μg of each of p53 or Bax-3b or Bax-3a expression vector either alone or together as indicated. The total amount of DNA was kept constant in each experiment by using herring sperm DNA to adjust for different amounts of reporter or expression vector added to each experiment. The reporter construct with empty content was then subjected to IP overnight using the appropriate antibody [either Bax-3b goat polyclonal Ab (Santa Cruz) or the secondary anti-goat antibody (Dako, UK)]. Protein G Sepharose beads were used to immobilize protein-antibody complexes, which were washed thoroughly then eluted from the beads. Cross-linking was reversed by incubation at 65°C for 4 h and the proteins were digested using proteinase K. Following phenol/chloroform extraction, the DNA was precipitated with ethanol, then resuspended in water and used for PCR. The primers used in the PCR to amplify a 152 bp fragment of the promoter containing the Bax-3 site were BaxF:5'-CAAACAGAAAGAGCAGCTGG-3' - BaxR: 5'-GAGTTTGTGGTTGGTTGCAGAGA-3'. Standard conditions were used for PCR amplification and included the use of 2.5 mM MgCl₂ with the following cycling parameters, 1 cycle at 94°C for 15 min followed by 40 cycles of 95°C for 45 s; 60°C for 45 s and 72°C for 45 s. The reaction was completed by an incubation of 72°C for 5 min. PCR products were resolved on a 2.5% agarose/TAE gel.

Cell cycle analysis and measurement of Annexin V positive cells

For cell cycle analysis and viability assays, cells were transfected as described above together with an expression vector containing the cDNA encoding the green fluorescent protein (GFP). 30–40 h following transfection, cells were harvested. For the analysis of cell cycle, transfected cells were fixed in 80% ethanol, stained with propidium iodide (50 μg/ml with 500 μg/ml RNAse A added) and the DNA content was then measured by fluorescence-activated cell sorting (FACS) using the Beckman Coulter Epics XL and analyzed using Expo32 software as previously described (25).

For measurement of Annexin V positivity, cells were harvested by scraping into medium, pelleted by low speed centrifugation, washed in cold PBS followed by a wash with Annexin V buffer (Sigma). Cells were then incubated with PE conjugated Annexin V (10 μl of 30 μg/ml stock/1 × 10⁶ cells) in the dark for 15 min then analyzed by FACS immediately. GFP expressing Annexin V positive cells were counted and used as a measure of transfection cells undergoing early apoptosis.

Chromatin immuno-precipitation (ChIP) assay

To test whether Brn-3b can be immuno-precipitated when bound to p53 on the WT Bax promoter, ChIP analysis was undertaken. This technique was carried out as described by Lee et al. (23) using stably transfected MCF7 cells that over-expressed Bax-3b compared with vector only transfected cells. Cells plated at a confluence of 1 × 10⁶, in 10 cm² Petri dishes, were subjected to cisplatin treatment for 6 h after which they were processed for ChIP analysis. Following cross-linking, cells were harvested in cold phosphate buffered saline (PBS) washed, then lysed in lysis buffer (1% SDS, 0.01 M EDTA; 0.05 M Tris, pH 8.0 and 0.01% protease inhibitor cocktail). DNA was sheared by sonication and following centrifugation, a volume of supernatant retained for use as ‘input’ sample in subsequent PCR analysis whilst the remaining sample was divided and then subjected to IP overnight using the appropriate antibody [either Bax-3b goat polyclonal Ab (Santa Cruz) or the secondary anti-goat antibody (Dako, UK)]. Protein G Sepharose beads were used to immobilize protein-antibody complexes, which were washed thoroughly then eluted from the beads. Cross-linking was reversed by incubation at 65°C for 4 h and the proteins were digested using proteinase K. Following phenol/chloroform extraction, the DNA was precipitated with ethanol, then resuspended in water and used for PCR. The primers used in the PCR to amplify a 152 bp fragment of the promoter containing the Bax-3 site were BaxF:5'-CAAACAGAAAGAGCAGCTGG-3' - BaxR: 5'-GAGTTTGTGGTTGGTTGCAGAGA-3'. Standard conditions were used for PCR amplification and included the use of 2.5 mM MgCl₂ with the following cycling parameters, 1 cycle at 94°C for 15 min followed by 40 cycles of 95°C for 45 s; 60°C for 45 s and 72°C for 45 s. The reaction was completed by an incubation of 72°C for 5 min. PCR products were resolved on a 2.5% agarose/TAE gel.

DRG cultures

Bax-3b KO mice were described by Gan et al. (48) and were kindly provided by Mengqing Xiang (Center for Advanced Biotechnology and Medicine, Rutgers University, New Jersey). Heterozygote crosses were used to give rise to WT, heterozygote and homozygote offspring and maintenance of this strain was in compliance with the home office...
results. Genotyping was carried out using established PCR protocols with specific primers to detect Brn-3b and neomycin gene in genomic DNA prepared from tail tips. Cultures of neurons from DRG were prepared at postnatal day 1 from litters that contain Brn-3b heterozygous, Brn-3b heterozygous or WT offspring. The cultures were prepared using methods previously described (40). Briefly the mice were sacrificed and the tails removed for genotyping after the experiments to enable non bias blind counting. The ganglia were dissected out and then dissociated in 0.3% collagenase and 0.1% trypsin for 20 min. Excessive non-neuronal cells were removed by centrifuging the dissociated cells through a 6% metrizamide cushion (Sigma M3383). Pelleted neurons were resuspended in defined media with 10 ng/ml NGF and plated onto laminin coated coverslips. Neuronal death was induced after 24 h in culture by NGF withdrawal in some cultures whilst control cultures continued to be grown in medium containing NGF. Cell were either harvested for RNA in 100 ul of Trizol (Sigma) and processed according to the manufacturer’s protocol or observed for viability. This was assessed on the basis of morphology with live cells showing an intact nucleus and a rounded phase bright body whereas dead cells were identified by a dark, granular nucleus and an absence of phase bright ring around the neuron body. All scoring was done blind, prior to genotyping of stored tail DNA. Cell count after 48 h treatment is expressed as percentage of count at start of treatment. All data are represented as mean ± SEM. An asterisk indicates P < 0.001 versus control, according to a two-tailed Student t-test.

Quantitative RT–PCR (qRT–PCR)

To analyze for changes in mRNA encoding genes of interest, total cellular RNA was prepared from different cells following the treatments described using Trizol reagent (Sigma) and the protocol previously described (18,65). To analyze for changes in mRNA of specific target genes, qRT–PCR was undertaken using Taqman MGB probes. The Assay-on-Demand probe/primer mix Hs00180269-m1 was used for amplification and quantification of GAPDH (Applied Biosystems—http://www.appliedbiosystems.com/). The reaction was carried out according to the Assay-on-Demand protocol but using Quantitect Probe mastermix (Qiagen—http://www1.qiagen.com/).

RESULTS

Brn-3b protein associates with p53 via the POU domain

The high homology in POU domains of Brn-3b and Brn-3a protein lead us to test whether Brn-3b also interacted with the p53 protein. Affinity chromatography ‘pull-down’ assays were undertaken using Brn-3b GST fusion protein, immobilized on Sepharose beads and incubated with in vitro translated (IVT) wild type (WT) p53 protein. The ER was used as a positive control whilst the non-related IVT luciferase protein was used as a negative control. As shown in Figure 1A, incubation of Brn-3b GST with p53 IVT resulted in a significant percentage of the p53 protein being retained by Brn-3b (11.5% of input). This was comparable to the positive control ER protein that is known to interact with Brn-3b (15.2% of input). The associations of Brn-3b with p53 and ER IVT were specific as the IVT control (luciferase) protein was not retained by Brn-3b and neither p53 nor ER proteins were retained by the GST moiety alone.

The POU domain of Brn-3a interact with DBD of p53 and the high homology between the POU domain of Brn-3b and Brn-3a suggests that the POU domain of Brn-3b should interact with the DNA binding domain of p53. Therefore, similar studies were next undertaken to identify the domains of Brn-3b and p53 required for this interaction. Thus, the isolated Brn-3b POU domain linked to GST (Brn-3b POU–GST) was incubated with IVT full length (FL) p53 as well as truncated forms of the p53 protein [shown in Figure 1B(i)]. In our previous experiments to study Brn-3a/p53 interaction, two truncated proteins (1–106 and 1–228), which lacked the DNA binding domain (DBD) of p53, had failed to bind to Brn-3a but deletion construct, p53(44–393), lacking the N’ terminal transactivation domain but with the DBD intact, could still effectively interact with Brn-3a (25). Therefore, these constructs were used in this study to test whether the p53 DBD was required for interaction with Brn-3b POU domain. As expected, FL p53 protein interacted with Brn-3b POU domain with high relative affinity [Figure 1B(ii)]. However, the two constructs lacking the DBD (1–106 and 1–228) failed to be retained by Brn-3b POU domain. In contrast, p53 (44–393), lacking the N’ terminal trans-activation but with intact DBD could effectively bind to Brn-3b. Thus, as with Brn-3a (25), the Brn-3b POU domain is sufficient to interact with p53 and this requires the C’ terminal region of p53 protein that contains the DBD.

To determine whether the interaction of Brn-3b with p53 can also be observed in intact cells, we tested whether these proteins could be immuno-precipitated (IP) in complex from cellular extracts that co-express both proteins. Nuclear extracts were obtained from ND7 cells that either expressed endogenous Brn-3b or that were transfected with the expression vector encoding the 32 kDa Brn-3b(s) protein to increase the levels of this protein (+). Immuno-precipitation (IP) was carried out using either anti-p53 antibody or the positive control anti-ER, antibodies which was previously used to effectively immuno-precipitate Brn-3b. Secondary antibody alone was used as a negative control. The IP complexes were resolved by PAGE and the immunoblots were probed with Brn-3b antibody [shown in Figure 1C(i)]. As expected, Brn-3b was successfully immunoprecipitated with the ER antibody in cells with endogenous Brn-3b protein (E) and with transfected Brn-3b (+). Similarly, IP with anti-p53 antibody resulted in Brn-3b being retained in complex with p53. Quantification of the relative amount of Brn-3b that was IP with p53 was carried out by scanning densitometry. These results showed that IP using cellular extracts from cells transfected to over-express Brn-3b protein (+) resulted in a 2-fold increase of the Brn-3b protein (4.55 arbitrary units) when compared with cells expressing endogenous Brn-3b (E) (2.29 arbitrary units). Brn-3b did not immuno-precipitate with the negative control antibody confirming the specificity of the interaction with p53.

In the reciprocal study, we tested whether p53 could also be immunoprecipitated with Brn-3b by undertaking similar studies using α-Brn-3b antibodies and cell extracts obtained from ND7 cells transfected with Brn-3b (or Brn-3a for control). α-Brn-3a antibodies were used as positive control (+ve control).
and actin as negative control (−ve control). Complexes resolved by PAGE were immunoblotted with anti-p53 antibody to detect p53 protein. As shown in Figure 1C(ii), p53 was successfully isolated in complex with both Brn-3a and Brn-3b proteins but not using anti-actin antibody (−ve control). These results confirmed that Brn-3b does indeed interact with p53 in these neuroblastoma cells.

Brn-3b enhances cell death but not cell cycle arrest when co-expressed with p53

Since Brn-3b is associated with proliferation in ND7 cells (5), whilst high levels of p53 induces either cell cycle arrest or apoptosis (28), we next tested the effects of co-expressing Brn-3b with p53 on the fate of these cells. Cells were co-transfected with Brn-3b or p53 either alone or both together. GFP expressing vector was included in all experiments to mark transfected cells. Brn-3a + p53 was included as positive control whilst transfection of the empty expression vector was used to establish baseline activity. To measure transfected cells undergoing early apoptosis, FACS analysis was undertaken following annexin V staining of cells, 30 h after transfection. (see Material and Methods section). Changes in viable cells were expressed relative to vector control (value arbitrarily set at 1).

Figure 1. Interaction of Brn-3b protein with p53, in vitro and in vivo. (A) Affinity chromatography (pull-down) analysis using full length Brn-3b GST fusion protein and 35S labelled IVT to show interaction of Brn-3b with p53. The p53 IVT protein was efficiently retained by Brn-3b (lane 2) showing similar affinity to positive control ER (lane 3) previously shown to interact with Brn-3b. The IVT luciferase protein (−ve control) was not retained by Brn-3b (lane 1). This interaction was specific to Brn-3b as none of the IVT proteins were retained by the GST moiety only. (B) (i) Schematic representation of the p53 deletion constructs used to identify the region of p53 required for interaction with Brn-3b POU domain. The positions of the amino acid shown represent those that are present with the deletions; AD, activation domain; DBD, DNA binding domain; OD, oligomerization domain (ii) Affinity chromatography analysis using Brn-3b POU–GST fusion protein showed that the isolated POU domain was sufficient to interact with WT FLp53 (lane 1). Use of the IVT truncated p53 constructs demonstrated that the amino acids between 1 and 106 (lane 2) or 1 and 228 (lane 3) of p53 were insufficient to mediate interaction with Brn-3b but the truncated protein, 228–393 lacking only the amino terminus (lane 4) could interact with affinity similar to the FLp53 protein. One-tenth of the input of IVT proteins used for the ‘pull-down’ assays are shown in the next panel (lanes 5–8) and * is used to indicate the size of expected polypeptide following IVT of the truncated constructs. (C) Co-immunoprecipitation assay demonstrating that Brn-3b can be isolated as a complex with p53 protein from cellular extracts. (i) IP undertaken using cellular extracts taken from neuroblastoma cells with endogenous Brn-3b (E) or with transiently transfected with Brn-3b (+), using antibodies to either ER (ER Ab) or p53 (p53 Ab) resulted in isolation of Brn-3b protein which was detected by western blotting. The control antibody (−ve) did not result in IP of Brn-3b. The isolated Brn-3b proteins are indicated by * and shows the shorter Brn-3b protein (~32 kDa) and longer 43 kDa Brn-3b isoforms (very weakly). The non-specific bands indicated by less than appear to be associated with use of the protein A/G Sepharose to immobilize the antibody bound proteins. (ii) Immunoprecipitation of p53 with either Brn-3a or Brn-3b from cellular extracts prepared from ND7 cells transfected with Brn-3a and p53 (lane 2) or Brn-3b and p53 (lane 3). The p53 protein was detected by western blotting. The p53 protein did not immuno-precipitate with the control actin antibody (−ve) (lane 1).
Figure 2A(i) shows the representative changes seen in the proportion of apoptotic (annexin V positive), transfected (GFP positive) ND7 neuroblastoma cells as measured by FACS analysis following transfection of cells with either p53, or Brn-3b or Brn-3a alone, or p53 co-transfected with either Brn-3b or Brn-3a control. Whereas p53 alone increased percentage of apoptotic cells; this was significantly increased upon co-expression with Brn-3b compared with p53 alone (denoted by **). In contrast, co-transfection of the positive control Brn-3a with p53 leads to protection from apoptosis. Data is expressed relative to LTR control (set at 1) and represents the mean ± standard error from three independent experiments. Statistical significance was determined using Students t-test. (ii) Similar effects were seen in MCF7 cells transfected with Brn-3b or p53 either alone or together. p53 alone resulted in increased cell death compared with control transfected cells (LTR) but upon co-expression of Brn-3b with p53, the percentage of apoptotic cells was increased. (B) Analysis of changes in the percentage of transfected cells undergoing cell cycle arrest (in G0/G1) following transfection with p53 and/or Brn-3b. Brn-3a+p53 was included as the positive control. DNA content was measured by FACS analysis following propidium iodide labeling. Although p53 alone resulted in a small increase in cells accumulating in G0/G1 phase of the cell cycle, co-expression Brn-3b did not alter its effects compared with the significant changes seen upon co-expression of p53 with Brn-3a. The results represent mean (± SE) of percentage of GFP positive cells in the G0/G1 phase of the cell cycle in four independent experiments.

Figure 2A(ii) shows that these effects were also observed in independent experiments using MCF7 cells which express Brn-3b alone did not increase apoptosis. However, co-expression of Brn-3b with p53 resulted in significantly enhanced apoptosis in these cells compared with p53 alone or controls (p=0.049). As expected from (previous studies), co-expression of p53 with Brn-3a was protective since cell death was decreased when compared with vector control, Brn-3a or p53 alone, Figure 2A(ii) shows that these effects were also observed in independent experiments using MCF7 cells which express Brn-3b and wt p53 (6,34). Thus in MCF7 cells, increasing p53 alone did induce apoptosis compared with vector control. However, although Brn-3b alone did not alter cell death, co-expression of p53 with Brn-3b resulted in further significant increases in the proportion of apoptotic cells.

Since p53 could also induce cell cycle arrest in ND7 cells, we next tested whether Brn-3b changes this function of p53 by measuring the percentage of transfected (GFP positive) cells that were in the G0/G1 phase of the cell cycle. Asynchronously growing ND7 cells were used for these studies and figure 2B shows that 20–30% of control (LTR) transfected cells were in G0/G1 phase of the cell cycle, in line with previous results seen with this cell line. Expression of Brn-3b alone did not alter the percentage of cells that were arrested in the G0/G1 phase of the cycle compared with control cells and its co-expression with p53 did not significantly affect cell cycle arrest induced by p53 alone. In contrast, as expected, Brn-3a enhanced the percentage of cells arrested in G0/G1, and this is markedly increased when Brn-3a was co-expressed with p53. Although p53 can induce arrest in G2 (35), these effects of Brn-3a were primarily observed in G1 phase of the cell cycle.

Therefore, co-expression of Brn-3b with p53 in ND7 cells appear to enhance death but did not alter cell cycle arrest function of p53, in contrast to the related Brn-3a protein, which enhanced cell survival and cell cycle arrest when co-expressed with p53 (28).

**Brn-3b enhances trans-activation of Bax promoter by p53**

Since the related Brn-3a protein could differentially regulate transcription by p53 on target promoters such as *bax* and *p21<sup>cip1/waf1</sup>* we next tested whether co-expressing of Brn-3b...
with p53 could alter transcription on these target promoters. Co-expression of Brn-3a with p53 was used as a control.

Figure 3A shows that p53 strongly transactivated the Bax promoter (lane 2), but Brn-3a and Brn-3b mildly inhibited its basal activity (lanes 3 and 5) compared with the control vector (lane 1). As expected, Brn-3a repressed p53 mediated activity of the promoter (lane 4), but co-transfection of Brn-3b with p53 (lane 6) resulted in a significant enhancement in promoter activity compared with p53 alone (lane 2) ($P < 0.05$). Values are expressed as a percentage of the empty LTR control vectors. All values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector. Values are the average of five experiments whose SD is shown by the bars. (B) Effect of Brn-3b and/or p53 expression vectors on the activity of the p21CIP1/Waf1 promoter driving a luciferase reporter gene in ND7 cells. As expected, p53 alone transactivated the promoter (lane 2) and Brn-3a mildly activated the promoter (lane 3) but cooperated with p53 to enhance the promoter activity (lane 4) compared with p53 alone only (lane 2). However, Brn-3b alone had no effect on promoter activity (lane 5) but it appears to reduce p53 mediated activation on this promoter (lane 6) compared with p53 alone. Values are expressed as a percentage of the activity obtained with the empty LTR expression vector alone (which was set at 100%). All values were equalised on the basis of the activity of a control renilla reporter plasmid which was co- transfected in all cells. Values are the average of five experiments ± SD (shown by error bars). (C) The ability of Brn-3b to increase Bax promoter activity is dependent on p53 since the p53 inhibitor, α-pifitrin, prevented the transactivation seen by p53 alone (set 3) or upon co-operation of Brn-3b and p53 (set 4). The changes in promoter activity following treatment with α-pifitrin are expressed as percentage of α-pifitrin treated LTR control (set at 100%) whereas induction in the absence of α-pifitrin is expressed as percentage of untreated LTR control (also set at 100%).

These results confirm that Brn-3a and Brn-3b POU proteins mediate different effects on gene expression when co-expressed with p53 with Brn-3b and p53 cooperating on the Bax but not p21CIP1/Waf1 promoter.

We next tested whether the cooperative effect seen upon co-expression of Brn-3b with p53 on the bax promoter specifically required the p53 protein by undertaking similar experiments in the presence (+) or absence (−) of the p53 inhibitor, pifitrin-α-hydrobromide. As shown in Figure 3C, the addition of pifitrin-α-hydrobromide resulted in a significant loss of p53 mediated transcription of this promoter. Furthermore, co-expression of Brn-3b with p53 failed to give rise to the increased activation previously seen on this promoter. These results therefore suggest that Brn-3b requires expression of functional p53 in order to stimulate bax promoter activity.
Brn-3 binding site is required for the cooperative effects on the bax promoter

Previous studies using in vitro site-directed mutagenesis showed that two binding sites which flanked the p53 consensus site in the bax promoter [shown in Figure 4A(i)] were essential for Brn-3a to repress p53 mediated transactivation of this promoter (27). We therefore tested whether loss of these Brn-3 binding sites could also alter the ability of Brn-3b to cooperate with p53 on this promoter. Thus, similar co-transfection studies were undertaken using mutant bax promoter that lacked both Brn-3 sites [Figure 4A(i)]. Results of reporter assays following co-transfection of Brn-3b and p53 with mutant Bax promoter (mutated Brn-3 sites but intact p53 binding site) into ND7 cells showed that the Brn-3 sites within the Bax promoter are required for Brn-3b to repress p53 mediated transcription of this promoter (Figure 4A(ii)). Results of reporter assays using the mutated bax promoter are shown in Figure 4A(ii) and demonstrated that Brn-3b, on its own, fails to repress the mutated bax promoter (111% ± 60 of basal activity seen in control LTR transfected cells, set at 100%). However, more interestingly, whilst p53 alone continued to transactivate this promoter, co-expression of Brn-3b with p53 failed to cooperate and enhance the promoter activity as seen with the WT promoter (Figure 3A). Therefore, the intact Brn-3 binding sites in the bax promoter are required for Brn-3b to cooperate with p53 to enhance transcription of this gene.

In order to determine whether Brn-3b is bound to the bax promoter in vivo in intact cells, under appropriate conditions, we undertook Chromatin Immuno-precipitation (ChIP) assays using Brn-3b over-expressing cells treated with cisplatin to stimulate p53 expression. Figure 4B(i) shows that p53 mRNA is increased ~2-fold following cisplatin treatment compared with levels in untreated cells [quantified using quantitative (q)RT–PCR (see Material and Methods)]. Similarly, Bax mRNA showed a corresponding increase following treatment. These results confirm that p53 mRNA is induced by cisplatin treatment and is accompanied by elevation in bax mRNA expression.

ChIP assays were undertaken using Brn-3b antibody to immunoprecipitate this protein bound to DNA of target promoters in cisplatin treated Brn-3b over-expressing cells. Figure 4B(ii) shows the PCR products obtained following the ChIP analysis and demonstrates that IP of Brn-3b protein cross-linked to DNA under these conditions resulted in successful amplification of a region in the Bax promoter that contains the Brn-3 site. This effect was only observed following IP with anti-Brn-3b antibody but not with the control actin antibody. These results therefore confirm that Brn-3b is indeed bound to the bax promoter in intact cells and may thus act by associating with p53 once it is bound to the promoter.

Co-expression of Brn-3b with p53 increases endogenous levels of Bax protein

Since co-expression of Brn-3b with p53 could significantly increase bax promoter activity, we next tested whether
increasing the expression of both these factors in ND7 cells would enhance endogenous Bax expression. Western blot analysis was undertaken to analyze Bax protein levels using cellular extracts prepared from cells transfected with Brn-3b and p53, either alone or both together. This was compared with control cells transfected either with the empty vector, LTR or co-transfected positive control, Brn-3a + p53. As shown in figure 5A(i), p53 alone increased the expression of Bax protein when compared with LTR whereas co-expression of p53 with control protein Brn-3a resulted in decreased Bax expression compared with p53 alone. Although Brn-3b alone did not increase Bax levels, co-transfecting Brn-3b and p53 together resulted in significantly increased levels of Bax protein compared with p53 alone. Figure 5A(ii) shows
the increased levels of Brn-3b or p53 in cells transfected with the appropriate expression constructs. Figure 5A(iii) shows that these effects were specific to Bax as co-expression of Brn-3b and p53 did not change p21cip1/waf1 protein levels but co-expression of Brn-3a with p53 resulted in significant increases, as expected. These results therefore confirm that co-expression of Brn-3b with p53 increases endogenous Bax protein levels in ND7 cells.

To test whether Brn-3b could cooperate with p53 to increase Bax expression in other relevant cell types also, we utilized Brn-3b over-expressing MCF7 breast cancer cells (known to express WT p53) or control MCF7 cells (LTR) which were either treated with cisplatin, to induce p53 expression or left untreated. RNA prepared from treated cells and untreated controls were used for cDNA synthesis and real-time PCR (qRT–PCR) was undertaken to quantify Bax mRNA levels. As shown in Figure 5B, treatment of control cells (LTR) with cisplatin, resulted in nearly 2-fold increased bax mRNA in control cells which is expected when p53 expression is induced (see Figure 4B). However, there was significant increase in the levels of bax mRNA in Brn-3b over-expressing cells treated with cisplatin compared with untreated Brn-3b over-expressing cells or in control cells. These results therefore confirm that induction of p53 expression in cells expressing high levels of Brn-3b results in a significant increase in bax mRNA in these cells also.

Brn-3b(−/−) neuronal cultures that are resistant to apoptosis express less Bax protein

Brn-3b is expressed in the dorsal root ganglion (DRG) and neurons of the spinal cord where bax expression is essential for apoptosis during neurogenesis (36–38). Previous studies by Ensor et al. (39) showed that primary cultures of DRG prepared from mice lacking Brn-3b [Brn-3b(−/−)] are highly resistant to death-inducing stimuli, e.g. Nerve Growth Factor (NGF) withdrawal which requires bax expression (37,38). Since Brn-3b cooperates with p53 to enhance Bax expression and hence increase apoptosis under certain conditions, we tested whether the resistance to apoptotic stimuli in Brn-3b(−/−) neurons might be associated with reduced expression of pro-apoptotic Bax. Therefore, primary DRG cultures prepared from Brn-3b KO mice and WT littermates (39,40) were either grown in defined medium that contained NGF at concentration of 10 ng/μl (+) or were deprived of NGF (−) which results in increased apoptosis in WT DRG cultures. Analysis of mRNA encoding the Bax protein was determined by RT–PCR (see Materials and Methods section).

Figure 5C shows that bax transcripts were increased in WT cultures grown in medium lacking NGF compared with matched control cultures grown in the presence of NGF. In contrast, cultures prepared from Brn-3b KO mice and grown in full growth medium containing NGF showed reduction in bax mRNA. Furthermore, there was no induction in bax expression even when the cultures from Brn-3b KO were grown in medium lacking NGF. Although Bax levels were reduced in the KO cultures compared with WT controls, the structural protein GAP43 remained unchanged in WT and KO cultures under these conditions.

Given the critical role for bax in controlling apoptosis in these cells during development (36–38), these results suggest that although Brn-3b on its own might influence survival of neurons during development (10,41), if it is co-expressed when p53 is induced in these cells, cooperation between these two factors will result in maximal induction of Bax and hence apoptosis.

DISCUSSION

Fate decision made by cells, in terms of whether they continue to proliferate, undergo cell cycle arrest or die, is crucial during development as well as for normal tissue homeostasis / functions. When induced, the p53 transcription factor, either leads to cell cycle arrest or apoptosis and its ability to control such events depends on which of its target genes are induced under different cellular conditions (42–44). While many p53 dependent target genes have been identified, it is now clear that transcriptional regulation of these target genes by p53 is highly dependent not only on the levels and localization of p53, but also on other cellular proteins that are co-expressed and physically associate with p53. For instance, whilst ASPP1/2 proteins can enhance the expression of pro-apoptotic p53 target genes such as Bax and Apaf1 (32,33), co-expression of the differentiation-associated Brn-3a POU protein with p53 results in antagonism on pro-apoptotic Bax and Noxa promoters, but cooperation on p21cip1/waf1 promoter with consequent increased survival and cell cycle arrest in neuronal cells (22–25).

In this study, we have shown that the related Brn-3b POU protein also physically interacts with p53 via the POU domain, a region with very high homology to Brn-3a. Deletion constructs that lacked the DBD and C' terminus of p53 failed to associate with Brn-3b, suggesting that this region of the p53 protein is required for effective interaction with Brn-3b. Because of the significant similarity (95%) between the POU domains of Brn-3a and Brn-3b (45), it is likely that Brn-3b will bind p53 via the DBD. Moreover, like Brn-3a, Brn-3b can be immunoprecipitated in complex with p53 from cellular extracts, demonstrating a physiologically relevant association of the Brn-3 proteins with p53 in intact cells. However, analysis of the functional effects of co-expressing Brn-3b with p53 showed that in contrast to Brn-3a (which prevented p53 mediated cell death but supported cell cycle arrest when co-expressed with p53 in these cells), increasing both Brn-3b and wt-p53 expression, enhanced cell death in ND7 neuroblastoma cells compared with either factor alone but did not significantly alter cell cycle arrest caused by p53. At the molecular level, Brn-3b can cooperate with p53 to enhance the expression of the pro-apoptotic Bax promoter but not the p21cip1/waf1 promoter. This is in contrast to Brn-3a which antagonized p53 mediated bax expression but cooperated to enhance p21cip1/waf1 expression. Consequently, co-expression of Brn-3b with p53 resulted in increased endogenous Bax protein expression compared with p53 alone or with controls.

The stimulation of Bax expression in the presence of Brn-3b requires wt-p53, since on its own, Brn-3b represses bax promoter activity but when it is co-expressed with p53, bax promoter activity is significantly enhanced. Although the experiments in ND7 cells relied on over-expression of exogenous Brn-3b and p53 and has to be interpreted with
caution, we observed that induction of wt p53 in MCF7 cells which express high levels of Brn-3b resulted in higher levels of bax and accompanying apoptosis. This was also supported by findings that the p53 inhibitor, pifithrin-α-hydrobromide, prevented p53 mediated trans-activation of the Bax promoter and abolished the cooperative effects of Brn-3b when it is co-expressed with p53. Moreover the distinct effects of Brn-3a and Brn-3b upon co-expression with p53 suggest specific and significant effects that are dependent on which POU protein is co-expressed with p53.

The role for Brn-3b in regulating p53 mediated Bax expression was also demonstrated in primary cultures of DRG neurons prepared from Brn-3b KO mice. Thus, cultures prepared from WT littermates underwent apoptosis upon NGF withdrawal which is associated with increased bax mRNA levels (39). However, this response was attenuated in Brn-3b(-/-) cultures, which showed significantly reduced death when grown under similar conditions (39), and this correlated with decreased Bax expression. This observation highlights an important mechanism by which Brn-3b may act to control cell fate during development. Brn-3b and the related Brn-3a proteins are both expressed during development in neuronal precursors and in specific neurons but evidence so far suggest that they give rise to distinct effects. The role for Brn-3a during neurogenesis in the PNS has been widely studied and this transcription factor was shown to be essential for survival and differentiation of specific neurons since Brn-3a(-/-) mutants suffer loss of somatotheral neurons during development with resultant early lethality (46,47). Although Brn-3b has been identified as being critical for survival and normal function of retinal ganglion cells (41,48), there is still much to be understood about its expression and function during neurogenesis in the PNS. Our findings showing that neurons cultured from Brn-3b KO are resistant to apoptosis is very interesting particularly in view of the decreased bax mRNA expression found in these cells. It is known that p53 levels are increased during neuronal development, when it is associated with either cell cycle arrest or apoptosis (26,49–52). Furthermore, Bax expression in DRG appears to peak between E13.5–15.5 coincident with increased death in Brn-3a KO embryos, which suffer significant neuronal apoptosis during development (36). A number of studies have shown that Bax plays a critical role in controlling apoptosis in sensory neurons of the DRG. For instance, DRG neurons fail to undergo apoptosis in bax KO mice (38,53) and in vitro cultures of DRG neurons prepared from bax KO mice survived in the absence of the neurotrophic support, NGF which induces widespread apoptosis in WT cultures (37). Furthermore, loss of bax prevented death in bcl-x deficient mice (54) whereas over-expression of bax in chick embryos increases the susceptibility of sensory neurons to apoptosis (55). Given the critical role for bax in controlling death in these neurons, we hypothesize that if p53 is induced in neuronal progenitor cells or neurons when Brn-3b is elevated there will be increased apoptosis as a consequence of elevated Bax. However, if Brn-3a is expressed at high levels, it will repress pro-apoptotic p53 target genes but cooperate to increase differentiation-associated genes to enhance cell cycle arrest and survival. Thus when p53 is induced in these neurons, the relative ratio of Brn-3a to Brn-3b will be critical in determining cell fate. The resistance of Brn-3b KO neurons to apoptosis could result either because Brn-3b is required for maximal induction of bax in these cells or in the absence of Brn-3b, the related Brn-3a factor (which may be increased in a compensatory manner) is likely to associate with p53 and repress Bax expression, even at baseline, with consequent enhanced survival.

Although these hypotheses will need to be tested further, the opposite expression and antagonistic effects of Brn-3b and related Brn-3a has previously been observed in neuroblastoma cells. Thus, Brn-3b but not Brn-3a is expressed in proliferating neuroblastoma cells. Upon induction of differentiation by growth of cells in serum free medium, Brn-3b levels are significantly decreased whilst there is a concomitant increase in Brn-3a expression (4). Moreover, changing the relative levels of Brn-3a and Brn-3b can alter the growth and behavior of neuroblastoma cells since forced over-expression of Brn-3b prevents differentiation of ND7 cells, whereas increasing Brn-3a induces cell cycle arrest and neurite outgrowth (associated with differentiation) even under growth conditions that should maintain active proliferation (11,16).

Because of the high homology in the conserved DNA binding POU domain, Brn-3a and Brn-3b recognize similar DNA sequences on promoters of target genes. However, there is very limited similarity outside this region in the full length protein. These differences contribute to distinct transcriptional effects by these two POU proteins that are dependent on many factors, including the target gene and cell types. For instance, whereas Brn-3a can stimulate the expression of neuronal target genes such as neurofilament, α-internexin, SNAP 25 and synaptophysin, Brn-3b either represses these genes and antagonizes the effects of Brn-3a or has no effect on the promoter activity (15). Thus, although these two transcription factors appear to bind to p53 via their POU domains and are thus likely to make similar contacts on the DBD of p53, it is unlikely that their expression would overlap, at least in neuroblastoma cells, where Brn-3b is expressed in actively proliferating cells and will be decreased when Brn-3a is induced upon differentiation. However, it is possible that in other cell types or during development, there may be co-expression of Brn-3a and Brn-3b and thus competition for binding to p53 with consequent changes in cell fate. Under such circumstances, the relative ratio of Brn-3a to Brn-3b factors, which depends on the cellular context, will determine outcome on cellular fate, given the distinct and opposite effects of Brn-3a and Brn-3b on specific p53 mediated target gene expression.

Brn-3b but not Brn-3a protein is elevated in both breast cancers and in neuroblastoma tumours (4,6–8). When elevated in these tumours, Brn-3b transcription factor can alter the growth and behavior of cells by modulating expression of target genes that help to confer growth advantages to the cells, e.g. increasing CDK4 (associated cell cycle progression) and HSP27 (involved in migration and drug resistance) but represses the tumour suppressor protein, BRCA1 (7,8,18,23,57). However, its ability to alter p53 mediated effects becomes quite important in these cell types. Although on its own, Brn-3b represses Bax, when co-expressed with wt p53 there is cooperative activation to increase bax expression. This observation is very interesting as it provides a distinct mechanism by which Brn-3b can act in cancer cells in response to different conditions. For instance, many
neuroblastomas express wt p53 that remains inactive (e.g., by cytoplasmic sequestration) so does not prevent the initiation or progression of tumourigenesis (56). Such tumours are often responsive to chemotherapy that relies on induction and activation of wt p53 to give rise to apoptosis. Our results lead us to hypothesize that increasing Brn-3b in cancer cells promotes growth changes whereas induction of wt p53 (e.g., by irradiation or chemotherapy) will induce cell cycle arrest and inhibit growth. When such opposing signals arise in the cells, they triggers a crisis and cells will undergo apoptosis instead of proliferation. Such an effect was previously observed when the co-expression of the oncogenic Myc protein with p53 in quiescent cells lead to apoptosis rather than proliferation or cell cycle arrest (58,59). Thus, the ability of Brn-3b to enhance p53 mediated bax transcription may help to explain the molecular mechanism by which cells that express wt p53 and Brn-3b, respond to such conflicting signals.

Although p53 can transactivate the bax promoter in the absence of Brn-3b, it is clear that high levels of both Brn-3b and p53 can significantly enhance the expression of this pro-apoptotic protein in the cells used in this study. The ability of Brn-3b proteins to associate with and enhance p53 mediated apoptosis in contrast to the related Brn-3a protein, which prevents apoptosis by p53 and promotes cell cycle arrest, may prove to be important in understanding how the molecular responses of p53 can be modified in different cell types depending on particular proteins that are co-expressed with it.

ACKNOWLEDGEMENTS

We thank the following for kind gifts of reagents: Dr W. El Deiry (p21CIP1/Waf1 promoter constructs), Dr K. Vosden (p53 expression vector) and Dr J. Reed (Bax promoter construct). We also thank J. Sinclair for assistance with FACS analysis. This work was supported by the following UK funding agencies Breast Cancer Campaign, Association for International Cancer Research, Child Health Research Action Trust (CHRAT), ICH and Great Ormond Street Children’s Charity. Funding to pay the Open Access publication charges for this article was provided by Medical Molecular Biology Unit, ICH.

Conflict of interest statement. None declared.

REFERENCES


