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Characterisation of the p53 pathway in cell lines established from TH-MYCN transgenic mouse tumours

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Abstract. Cell lines established from the TH-MYCN transgenic murine model of neuroblastoma are a valuable preclinical, immunocompetent, syngeneic model of neuroblastoma, for which knowledge of their p53 pathway status is important. In this study, the Trp53 status and functional response to Nutlin-3 and ionising radiation (IR) were determined in 6 adherent cell lines from TH-MYCN transgenic cell lines using Sanger sequencing, western blot analysis and flow cytometry. Sensitivity to structurally diverse MDM2 inhibitors (Nutlin-3, MI-63, RG7388 and NDD0005) was determined using XTT proliferation assays. In total, 2/6 cell lines were Trp53 homozygous mutant (NHO2A and 844MYCN+/+) and 1/6 (282MYCN+/−) was Trp53 heterozygous mutant. For 1/6 cell lines (NHO2A), DNA from the corresponding primary tumour was found to be Trp53 wt. In all cases, the presence of a mutation was consistent with aberrant p53 signalling in response to Nutlin-3 and IR. In comparison to TP53 wt human neuroblastoma cells, Trp53 wt murine control and TH-MYCN cell lines were significantly less sensitive to growth inhibition mediated by MI-63 and RG7388. These murine Trp53 wt and mutant TH-MYCN cell lines are useful syngeneic, immunocompetent neuroblastoma models, the former to test p53-dependent therapies in combination with immunotherapies, such as anti-GD₂, and the latter as models of chemoresistant relapsed neuroblastoma when aberrations in the p53 pathway are more common. The spontaneous development of Trp53 mutations in 3 cell lines from TH-MYCN mice may have arisen from MYCN oncogenic driven and/or ex vivo selection. The identified species-dependent selectivity of MI-63 and RG7388 should be considered when interpreting in vivo toxicity studies of MDM2 inhibitors.

Introduction

Neuroblastoma, an embryonal malignancy of the developing sympathetic nervous system, remains one of the most difficult paediatric cancers to cure with <50% of high-risk patients being long-term survivors despite intensive multi-modal therapy. MYCN amplification occurs in ~50% of high-risk cases, associated with rapid tumour progression and a poor prognosis (1). The role of MYCN in neuroblastoma tumourigenesis has been demonstrated by the generation of TH-MYCN transgenic mice in 1997 through the transfer of a construct incorporating human MYCN cDNA under the control of the rat tyrosine hydroxylase promoter into the nucleus of fertilised murine oocytes and subsequent integration into genomic DNA (2,3). The tyrosine hydroxylase promoter leads to tissue-targeted overexpression of human MYCN in neural crest cells, and mice develop spontaneous highly penetrant abdominal and para-spinal thoracic tumours consistent with sites of human neuroblastoma (2). Tumour penetrance and growth have been shown to be related to MYCN gene dosage where homozygotes develop tumours with increased incidence and decreased latency (2,4). Analysis of TH-MYCN transgenic mice has shown that they recapitulate many histological features of human neuroblastoma with varying degrees of neuronal differentiation and express synaptophysin and neuron-specific enolase (2). Moreover, tumours also exhibit chromosomal changes syntenic with those observed in human neuroblastoma tumours, such as gain of chromosome 17 (2,4). The TH-MYCN transgenic mouse is now a well-established model of neuroblastoma, and a panel of homozygous and hemizygous TH-MYCN cell lines have been derived from tumour resections from these mice, similarly reflecting both the genetic and biological features of human neuroblastoma (5). Although micrometastases can occur, one major limitation of the TH-MYCN transgenic model in preclinical drug development studies is the very low incidence of clinically relevant metastases to sites such as bone marrow, thus limiting its usefulness as a model for high-risk metastatic neuroblastoma (6). To overcome this, TH-MYCN cell lines

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Abbreviations: IR, ionising radiation; wt, wild-type; PBS, phosphate-buffered saline; FCS, fetal calf serum

Key words: neuroblastoma, TH-MYCN, p53 pathway, MDM2 inhibitors, Nutlin-3, RG7388, MI-63, NDD0005
have been used to generate highly valuable orthotopic and pseudometastatic syngeneic models of neuroblastoma in an immunocompetent background (7,8). This is of particular importance as immunotherapies, such as anti-GD2 antibody
are currently standard of care for the treatment of children
with high-risk neuroblastoma.

The tumour suppressor gene, TP53, is critical in maintain-
ing genomic stability and is mutationally inactivated in >50% of all human malignancies (9). Abnormalities in containing genomic stability and is mutationally inactivated

In neuroblastoma tumours and cell lines established at

diagnosis, TP53 mutations are rare; however an increased frequency of mutations has been reported at relapse/post-
diagnosis, obtained from Dr Gilbert de Murcia (18) and NIH3T3 cells. The control human neuroblastoma cell lines used were TP53 mutant, MYCN-amplified SK-N-BE(2)-C, and TP53 wt non-MYCN-amplified SHSY5Y [obtained from Professor Penny Lovat (Newcastle University, Newcastle upon Tyne, UK) and Dr June Biedler (Memorial Sloan Kettering Cancer Center, New York, NY, USA) and authenticated by multiplex short tandem repeat profiling by NevGene Ltd. (Newcastle upon Tyne, UK) using the GenePrint® 10 system] and TP53 wt MYCN-amplified NGP cells (19). The NHO2A cells were cultured as previously described (5). The 844MYCN+/-, 282MYCN+/-, SHSY5Y, SK-N-BE(2)-C and NGP cells were cultured in RPMI-1640 (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal calf serum (FCS) (Gibco, Paisley, Scotland) and the 3261MYCN+/-, 3994MYCN+/- and 3399MYCN+/- cells were cultured in RPMI-1640 supplemented with 20% FCS. MEF^PARP-/- and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% FCS.

Trp53 sequencing. DNA was extracted from tumours and cell lines using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions. DNA from NHO2A, 844MYCN+/- and 282MYCN+/- cells was amplified for Trp53 exons 2-10 and sequenced in both directions by DBS genomics (Durham University, Durham, UK). The primer sequences are available upon request. The 3261MYCN+/-, 3994MYCN+/- and 3399MYCN+/- DNA samples were amplified and sequenced in both directions for Trp53 exons 5-10 by LG Genomics GmbH (Berlin, Germany).

MMD2 inhibitors, IR, western blot analysis and flow cytometry. In brief, RG7388 was provided by Hoffman-La Roche (Nutley, NJ, USA), Nutlin-3 was purchased from Cambridge Bioscience Ltd. (Cambridge, UK) and MI-63 and NDD0005 were synthesised as previously described (20,21). The cells were irradiated using a RS320 irradiator (Gulmay Medical, Surrey, UK). For protein analysis, whole cell lysates were harvested and proteins separated using 4-20% Mini-Protean TGX Precast Gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and transferred onto Hybond-C Extra membrane prior to incubation with antibodies and detection using enhanced chemiluminescence (both from GE Healthcare Life Sciences, Little Chalfont, UK) and X-ray film (Fujiﬁlm, Bedford, UK). The primary antibodies used were p53 (1:1,000; CM5; Leica Microsystems Ltd., Buckinghamshire, UK), MYCN (1:100; NCMI100; Merck Millipore, Billerica, MA, USA), p21^{WAF1} (1:1,000; SX118; BD Biosciences, San Jose, CA, USA), Mdm2 1:500 (2A10), p19^{ARF} 1:500 (ab80) (both from Abcam, Cambridge, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:500 (FL335; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Cell growth inhibition was determined using the XTT cell proliferation assay (Roche, Burgess Hill, UK). The cells were seeded in 96-well plates (Corning, VWR International Ltd., Lutterworth, UK), allowed to adhere overnight prior to treatment with MDM2-p53 antagonists for 48 h. For flow cytometry analysis, cells were fixed in ice-cold 70% (v/v) ethanol, and stained with

Materials and methods

Cell lines. The TH-MYCN transgenic murine cell lines used were MYCN homozygous NHO2A, 844^{MYCN+/-}, 3261^{MYCN+/-}, 3994^{MYCN+/-} and hemizygous 282^{MYCN+/-}. The NHO2A cell line has been previously described (5) and together with NHO2A mouse tumour DNA, was obtained from Professor Michelle Haber (Children’s Cancer Institute, Sydney, NSW, Australia). All other previously unre-
ported TH-MYCN cell lines were established from the TH-MYCN colony at the Children's Hospital of Philadelphia (Philadelphia, PA, USA) under an IACUC approved animal protocol. Genetically-engineered 129X1/Sv1 mice carrying the TH-MYCN transgene in one concatamer (TH-MYCN^+/mice) or two concatamers (TH-MYCN^+/mice) develop tumours closely resembling human neuroblastoma (2). The TH-MYCN^+/mice were bred and offspring genotyped as described previously (17). TH-MYCN mice were euthanised at the time of tumour progression according to humane approved guide-
lines using 3-5% isoflurane inhalation followed by cervical dislocation. The mice were then disinfected with 70% ethanol prior to resecting the tumour free. Tumours were fragmented and filtered through a 40 µm nylon mesh filter into a conical tube, spun-pelleted, and resuspended in sterile Tris Ammonia Chloride buffer, buffered to pH 7.65. Tumour cell pellets were harvested and proteins separated using 4-20% Mini-Protean TGX Precast Gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and transferred onto Hybond-C Extra membrane prior to incubation with antibodies and detection using enhanced chemiluminescence (both from GE Healthcare Life Sciences, Little Chalfont, UK) and X-ray film (Fujiﬁlm, Bedford, UK). The primary antibodies used were p53 (1:1,000; CM5; Leica Microsystems Ltd., Buckinghamshire, UK), MYCN (1:100; NCMI100; Merck Millipore, Billerica, MA, USA), p21^{WAF1} (1:1,000; SX118; BD Biosciences, San Jose, CA, USA), Mdm2 1:500 (2A10), p19^{ARF} 1:500 (ab80) (both from Abcam, Cambridge, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:500 (FL335; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Cell growth inhibition was determined using the XTT cell proliferation assay (Roche, Burgess Hill, UK). The cells were seeded in 96-well plates (Corning, VWR International Ltd., Lutterworth, UK), allowed to adhere overnight prior to treatment with MDM2-p53 antagonists for 48 h. For flow cytometry analysis, cells were fixed in ice-cold 70% (v/v) ethanol, and stained with

(>95% positive) and had surface expression of the GD, disi-
aloganglioside, as detected by flow cytometry. The control murine cell lines used were Trp53 wild-type (wt) MEF^PARP-/- and 844MYCN+/- obtained from Dr Gilbert de Murcia (18) and NIH3T3 cells. The control human neuroblastoma cell lines used were TP53 mutant, MYCN-amplified SK-N-BE(2)-C, and TP53 wt non-MYCN-amplified SHSY5Y [obtained from Professor Penny Lovat (Newcastle University, Newcastle upon Tyne, UK) and Dr June Biedler (Memorial Sloan Kettering Cancer Center, New York, NY, USA) and authenticated by multiplex short tandem repeat profiling by NevGene Ltd. (Newcastle upon Tyne, UK) using the GenePrint® 10 system] and TP53 wt MYCN-amplified NGP cells (19). The NHO2A cells were cultured as previously described (5). The 844MYCN+/-, 282MYCN+/-, SHSY5Y, SK-N-BE(2)-C and NGP cells were cultured in RPMI-1640 (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal calf serum (FCS) (Gibco, Paisley, Scotland) and the 3261MYCN+/-, 3994MYCN+/- and 3399MYCN+/- cells were cultured in RPMI-1640 supplemented with 20% FCS. MEF^PARP-/- and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% FCS.

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50 µg/ml propidium iodide and 50 µg/ml RNAse A (both from Sigma-Aldrich) at 37°C for 30 min prior to analysis on the FACSCalibur (Becton-Dickinson, Oxford, UK). Data were analysed using Cyflow (CyFlo Ltd., Turku, Finland).

Statistical analyses. Two-sided unpaired t-tests were performed using GraphPad Prism v6.0 software with a value of P<0.05 considered as the level of significance.

Results

Trp53 status of TH-MYCN cell lines. All TH-MYCN transgenic cell lines used in the present study were cultured as adherent monolayers (Fig. 1A). In 3/6 cell lines, the NHO2A, 844 MYCN+/+ and 282 MYCN+/+ cell lines, had missense coding region point mutations (Fig. 1B-D). The NHO2A cells were homozygous for a p.F106S (phenylalanine to serine; c.317 C>T) Trp53 mutation corresponding to the human p.F109S missense mutation (Fig. 1B). The 844 MYCN+/+ cells were Trp53 homozygous mutant for a p.C173W (cysteine to tryptophan; c.519 C>G) mutation corresponding to the human p.C176W missense mutation (Fig. 1C). Of note, the 282 MYCN+/+ cells were heterozygously mutated for the same Trp53 p.C173W mutant allele as the 844 MYCN+/+ cells (Fig. 1D). Both p.F106S and p.C173W mutations are within the DNA binding domain and were predicted to affect p53-mediated transactivation using the TP53Mutload database. The heterozygous mutations detected in NHO2A and 844 MYCN+/+ cells are most likely to be due to allele loss of one allele with mutation in the remaining allele.

To determine whether the mutations present in cell lines were also present in the original tumours or selected for during establishment of the cell line, tumour DNA was sequenced for Trp53 exons 2-10. Only 1/6 cell lines (NHO2A) had tumour DNA available and was found to be wt (Fig. 1E).

MYCN and the p53 pathway in TH-MYCN cell lines. Basal expression of MYCN and p53 pathway components, namely MYCN, P21 WAF1 and p19ARF were assessed in TH-MYCN cell lines by western blot analysis (Fig. 1F). The Trp53 wt murine MEF-PARP-/- and NIH3T3, and human MYCN amplified 3261 MYCN+/+ cells were included as controls. Overexpression of the MYCN transgene was observed in all TH-MYCN cell lines, with levels comparable to those of the MYCN-amplified SK-N-BE(2)-C cells. NHO2A and 844 MYCN+/+ cells had the highest MYCN levels compared to the other TH-MYCN cell lines. Accumulation of p53 was observed in NHO2A, 844 MYCN+/+, and 282 MYCN+/+ cells, consistent with their mutant Trp53 status. All mouse cell lines had very low or undetectable baseline Mdm2 expression (Fig. 1F). p21 WAF1 was expressed only in Trp53 wt 3399 MYCN+/+ cell line (Fig. 1F). p19ARF was expressed in NHO2A, 844 MYCN+/+, 3399 MYCN+/+ and 3399 MYCN+/+ cells (Fig. 1F).

The response of the p53 pathway to Nutlin-3 and IR. Using Nutlin-3 and IR as different methods to induce p53 activation, activation of the p53 signalling pathway was assessed by western blot analysis in the MYCN cell lines and control Trp53 wt MEF-PARP-/- cells (Figs. 2 and 3). Consistent with their Trp53 wt status, MEF-PARP-/- cells exhibited an intact p53 signalling pathway in response to both Nutlin-3 and IR, evident by p53 stabilisation and Mdm2 and p21 WAF1 upregulation (Figs. 2A and 3A). Of note, the p53 pathway response of MEF-PARP-/- cells to IR was slightly diminished and delayed in comparison to Nutlin-3 (Figs. 2A and 3A), and is most likely a consequence of PARP-1 knockout in this cell line (22). As expected, homozygously Trp53 mutant NHO2A and 844 MYCN+/+ cells failed to show p53 induction in response to Nutlin-3 (Fig. 2B and C) or IR (Fig. 3B and C). Consistent with this observation, no p21 WAF1 induction was observed in either cell line, and no Mdm2 induction was observed in 844 MYCN+/+ cells. Of note, despite a lack of p53 induction, weak induction of Mdm2 was observed in NHO2A cells following both treatment with Nutlin-3 and exposure to IR (Figs. 2B and 3B). The heterozygously Trp53 mutant 282 MYCN+/+ cells also failed to show p53 stabilisation in response to Nutlin-3 or IR; however in spite of this, an increase in both Mdm2 and p21 WAF1 expression was observed (Figs. 2D and 3D), most likely as a result of the presence of one remaining wt Trp53 allele. Finally, in line with their Trp53 wt status, the 3 remaining TH-MYCN cell lines, 3261 MYCN+/+, 3394 MYCN+/+ and 3399 MYCN+/+, all showed evidence of IR- and Nutlin-3-induced p53 pathway activation, with stabilisation of p53, and induction of p21 and Mdm2 (Figs. 2E-G and 3E-G).

Nutlin-3-induced cell cycle distribution. To further characterise the p53 functional response in the TH-MYCN cell lines to Nutlin-3, the sub-G1 and cell cycle distribution of all the 6 TH-MYCN cell lines following 24 h treatment with 20 µM Nutlin-3 were analysed by propidium iodide-based flow cytometry. Sub-G1 events was used as a surrogate marker of apoptosis and the G1:S ratio calculated as an indicator of G1 cell cycle arrest. Trp53 wt NIH3T3 and MEF-PARP-/- cells were included as positive controls. The functional assessment of Nutlin-3-mediated cell cycle arrest and apoptosis of human neuroblastoma cell lines have previously been reported (23,24). In response to Nutlin-3 treatment, NIH3T3 cells underwent G1 arrest, evident by a 4.6-fold increase in their G1/S ratio [16.58±4.13 (Nutlin-3) vs. 3.16±0.17 (DMSO)] (Table I and Fig. 4A). Consistent with the observed activation of the p53 pathway in Fig. 2A, the MEF-PARP-/- cells demonstrated a 3.2-fold increase in their G1:S ratio [7.19±3.32 (Nutlin-3) vs. 2.23±0.23 (DMSO)], and a significant increase in the percentage of cells in G1/M phase [53.32±4.19 (Nutlin-3) vs. 34.75±1.63 (DMSO); P<0.005, paired t-test] (Table I and Fig. 4A), indicative of a Nutlin-3-induced G1 arrest in this cell line. In contrast to control Trp53 wt murine cell lines, and as expected, no noticeable changes in the G1/S ratio or percentage of cells in G1/M phase were observed in any Trp53 mutant TH-MYCN NIH2A, 844 MYCN+/+ or 282 MYCN+/+ cells (Table I and Fig. 4A). Of note, compared to the other murine cell lines, NIH2A and 282 MYCN+/+ cells had a high proportion of sub-G1 basal events (Table I and Fig. 4A), a surrogate marker of apoptosis, which following Nutlin-3 treatment, increased slightly and remained unaltered, respectively. Consistent with their Trp53 wt status and observed p53 pathway activation, as shown in Fig. 2E-G, the 3 Trp53 wt TH-MYCN cell lines, 3261 MYCN+/+, 3394 MYCN+/+ and 3399 MYCN+/+, all underwent a G1 arrest, as evident by a 5.2-, 5- and 3.4-fold increase in G1/S ratio compared to control cells,
respectively (Table I and Fig. 4A). In addition, the 3399^{MYCN+/-} cells also exhibited an increased sub-G1 population (Table I and Fig. 4A).

Species-dependent MDM2 inhibitor selectivity. MDM2 inhibitors are currently under preclinical and clinical evaluation as a novel therapeutic for neuroblastoma. To further evaluate
the p53 pathway status of cell lines studied and establish their response to MDM2 inhibitors, sensitivity to Nutlin-3 and additional structurally unrelated MDM2 inhibitors, NDD0005, MI-63 and RG7388, and their effects on growth inhibition were assessed (Table II). Human TP53 wt non-MYCN amplified SHSY5Y and MYCN amplified NGP cells, which have previously been shown to be sensitive to the tested MDM2 inhibitors (20,21,23), were included as positive controls. The concentrations of Nutlin-3, MI63, NDD0005 and RG7388, which led to a 50% growth inhibition (GI<sub>50</sub>) after 48 h of treatment, are shown in Table II. In comparison to TP53 wt human neuroblastoma cells, the Trp53 wt control and TH-MYCN murine cell lines were less sensitive to Nutlin-3-mediated growth inhibition, as evidenced by higher GI<sub>50</sub> concentrations.

Figure 2. The response of the p53 pathway of the MEF<sub>PARP/-</sub> and TH-MYCN transgenic murine cell lines to the MDM2 inhibitor, Nutlin-3. Western blot analysis showing levels of p53 and p53 target genes, Mdm2 and p21<sup>WAF1</sup>, in (A) MEF<sub>PARP/-</sub>, (B) NHO2A, (C) 844<sup>MYCN</sup>+, (D) 282<sup>MYCN</sup>−, (E) 3261<sup>MYCN</sup>+, (F) 394<sup>MYCN</sup>− and (G) 3399<sup>MYCN</sup>− cells after 6, 24 and 48 h of treatment with 20 µM Nutlin-3. Control cells were treated with either media alone (M) or media containing an equal volume of DMSO (DMSO).
As expected, Trp53 mutant TH-MYCN cell lines NHO2A, 844 MYCN+/+ and 282 MYCN+/- had the highest GI50 values (14.1-21.8-fold less sensitive) (Table II).

Further evaluation and comparison of Trp53 wt TH-MYCN and MEF PARP−/− cells to NDD0005, MI-63 and RG7388, demonstrated that although the MEF PARP−/− cells were significantly (2.5-3.6-fold) less sensitive to NDD0005, there was no difference in the sensitivity of Trp53 wt TH-MYCN cell lines to NDD0005 compared with human SHSY5Y and NGP neuroblastoma cells (Table II and Fig. 4C). Of the tested MDM2 inhibitors, MI-63 and RG7388 were the most potent against human neuroblastoma cell lines, consistent with the findings of our previous studies (20,23). The data also revealed that compared with SHSY5Y and NGP cells, Trp53 wt MEF PARP−/− cells were significantly (11.3-15.4- and 11.9-15.0-fold) less sensitive to NDD0005.

Figure 3. The p53 pathway response of MEF PARP−/− and TH-MYCN transgenic murine cell lines to ionising radiation (IR)-induced DNA damage. Western blot analysis showing the levels of p53 and p53 target genes, Mdm2 in (A) MEF PARP−/−, (B) NHO2A, (C) 844 MYCN+/+, (D) 282 MYCN+/-, (E) 3261 MYCN+/-, (F) 3394 MYCN+/- and (G) 3399 MYCN+/- cells at 6, 24 and 48 h following exposure to 10 Gy IR. Control cells received no IR (M).
sensitive to both MI-63 and RG7388, respectively (Table II and Fig. 4D and E). Furthermore, the data also indicated that Trp53 wt TH-MYCN cell lines were significantly (3.5-5.1-fold) less sensitive to MI-63 (Table II and Fig. 4D), and even less so (13.6-59.1-fold) to RG7388 (Table II and Fig. 4E). This highlights an inverse association between potency in TP53 wt human neuroblastoma cells and potency in Trp53 wt murine cells.

Discussion

Cell lines established from primary tumours resected from TH-MYCN mice have been used to develop valuable preclinical immunocompetent, syngeneic models of neuroblastoma (7,8), for which knowledge of their p53 pathway status is important. In this study, we demonstrated that 2/6 TH-MYCN cell lines were Trp53 homozygous mutant (NHO2A and 844 MYCN+/+) and 1/6 was heterozygous mutant (282 MYCN+/-). In the only case where DNA from the original tumour was available (NHO2A), the original tumour was Trp53 wt. This is consistent with our previous analysis of 13 primary TH-MYCN tumours, which did not show any Trp53 mutations (exons 4-8) (25). It is possible that Trp53 mutant subpopulations existed within the primary tumour, but were below the level of detection of Sanger sequencing, or that the mutation spontaneously developed and was selected for during ex vivo culturing pressures and cell line establishment. A MYCN oncogenic drive may be a strong contributing factor for the positive selection of Trp53 mutations.
and may account for why 3/6 TH-MYCNU cells tested in the present study were either homozygous or heterozygous Trp53 mutant. Certainly, MYCN is known to play a dual role in driving both proliferation and apoptosis, and there are several lines of evidence, including studies using TH-MYCNU models, which suggest that MYCN driven p53-dependent apoptosis is an important mechanism for tumour suppression in neuroblastoma and that MYCN amplified neuroblastoma cells may circumvent MYCN driven p53-dependent apoptosis by selecting for cells with aberrations in the p53/MDM2/p14ARF pathway, as has been observed for MYCC-driven lymphoma (3,19,26-29).

Specifically, as previously demonstrated, tumours formed with greater penetrance and reduced latency in TH-MYCNU mice heterozygous for an inactivated germline p53 allele (27). The analysis of human neuroblastoma cell lines reported to date with aberrations in the p53/MDM2/p14ARF pathway as has been observed for MYCC-driven lymphoma (3,19,26-29). Specifically, as previously demonstrated, tumours formed with greater penetrance and reduced latency in TH-MYCNU mice heterozygous for an inactivated germline p53 allele (27).

The analysis of human neuroblastoma cell lines reported to date with aberrations in the p53/MDM2/p14ARF pathway demonstrates that 31/40 (78%) are MYCN-amplified (19). More recently, a study of the role of p53 function in neuroblastoma pathogenesis using TH-MYCNU murine models observed that loss of p53 function led to reduced survival (30).

Nutm-3 is a potent selective inhibitor of the MDM2-p53 interaction (31), previously shown to be highly effective against TP53 wt neuroblastoma cell lines, inducing cell cycle arrest and/or apoptosis, and used to functionally screen large neuroblastoma cell line panels for p53 pathway aberrations (23,24,32). In this study, we found that in all cell lines tested, the presence of a mutation was consistent with high basal levels of p53 and aberrant p53 signalling in response to Nutlin-3 and IR, and failure to growth arrest in response to Nutlin-3. Consistent with the mechanisms of action of MDM2 inhibitors and existing data from human neuroblastoma cell lines, overall, Nutlin-3 was found to induce cell cycle arrest and/or apoptosis of TP53 wt control and TH-MYCNU murine cell lines (23,24). Of note, in the current study, in response to Nutlin-3 and IR, although there was no induction of p53, an increase in both Mdm2 and p21WAF1 expression was observed in heterozygously mutant 282 MYCN+/– cells, suggesting there is some residual p53 function from the remaining wt allele (33); however this did not lead to growth arrest or apoptosis in response to Nutlin-3.

MDM2 inhibitors are currently under preclinical and clinical development as a novel therapeutic, both alone and in combination, for human malignancies including neuroblastoma. Of particular interest in view of the latter, data from the present study demonstrated that in comparison to human TP53 wt neuroblastoma cells, murine control (MEPARP/-) and TH-MYCNU cell lines were significantly less sensitive to MI-63 and RG7388 induced growth inhibition. Although human and murine MDM2 show a high degree of amino acid sequence homology, with only 2 non-identical amino acids within the p53 binding domain (34), these subtle differences could account for a weaker binding affinity to murine Mdm2 which is believed to contribute to the increased resistance and higher GI50 concentrations of murine cells to some MDM2 inhibitors that have been designed with high potency against human MDM2 (35). In support of this, the present

Table I. Flow cytometric analysis of the proportion of cells in Sub-G1, G1, S, and G2/M phase, and the G1/S ratios in the panel cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell line and Trp53 status</th>
<th>Treatment condition</th>
<th>Sub-G1</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
<th>G1/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF3261 MYCN+/+</td>
<td>DMSO</td>
<td>18.60±7.73</td>
<td>49.16±6.4</td>
<td>20.95±1.81</td>
<td>11.28±2.12</td>
<td>2.4±0.41</td>
</tr>
<tr>
<td>(wt) Nutlin-3</td>
<td>17.37±9.66</td>
<td>51.58±5.73</td>
<td>19.69±2.29</td>
<td>11.37±2.22</td>
<td>2.68±0.41</td>
<td></td>
</tr>
<tr>
<td>NHO2A (mutant)</td>
<td>DMSO</td>
<td>12.29±3.2</td>
<td>53.35±1.98</td>
<td>23.28±0.79</td>
<td>11.07±1.33</td>
<td>2.29±0.01</td>
</tr>
<tr>
<td>844 MYCN+/+</td>
<td>(wt) Nutlin-3</td>
<td>0.86±0.09</td>
<td>69.45±0.65</td>
<td>18.28±0.32</td>
<td>11.40±0.87</td>
<td>3.8±0.03</td>
</tr>
<tr>
<td>(mutant)</td>
<td>DMSO</td>
<td>1.58±0.05</td>
<td>88.61±0.28</td>
<td>4.57±0.41</td>
<td>5.24±0.41</td>
<td>19.67±1.68</td>
</tr>
</tbody>
</table>

Cell cycle analysis was performed using Trp53 wild-type (wt) and mutant murine cell lines after 24 h of treatment with 20 µM Nutlin-3 or an equal volume of DMSO. Values represent the mean of n=3 ± SEM.
data showed that the more potent the MDM2 inhibitor was against human neuroblastoma cells, the less sensitive the murine cells were, and the greater the fold difference between GI50 values of Trp53wt murine cells vs. TP53wt human cells. These observations are consistent with the inter-species selectivity of spiro-oxindole-based MDM2 inhibitors (36) and dihydroisoquinolinone NVP-CGM097 (37), but not pyrazolo-pyrrolidinone NVP-HDM201 (38,39), and should be taken into account when designing studies of MDM2 inhibitors either alone or in combination using either preclinical transgenic or human tumour xenograft models as p53-dependent normal tissue toxicity will not be adequately modelled.

Currently, murine neuroblastoma models include genetically engineered mouse models, syngeneic models, and subcutaneous, orthotopic, pseudometastatic and patient-derived xenografts (3,40-43). All models have associated advantages and disadvantages, and it is likely that the most comprehensive preclinical assessment of efficacy will include a combination of existing models. Several of the models predominantly use immunocompromised mice and thus are unsuitable for assessment of immunotherapies, which are emerging as effective targeted therapies in patients with neuroblastoma. To overcome this, cell lines established from the TH-MYCNtransgenic model/or other murine neuroblastoma cell lines can be used to generate orthotopic or pseudometastatic syngeneic models of neuroblastoma in an immunocompetent background (7,8). For this, the genetic and functional characterisation of murine cell lines, including Trp53 status and pathway function, are very important and highly warranted, as existing data are limited.

In conclusion, the Trp53wt and mutant TH-MYCN cell lines characterised in this study can be used in syngeneic models of neuroblastoma, the former to test p53-dependent therapies in combination with immunotherapies, such as anti-GD2 antibody, and the latter as models of immunocompetent, chemoresistant relapsed neuroblastoma in which aberrations in the p53 pathway are more common (14-16).

Table II. Summary of 48-h GI50 values for MDM2 antagonists in human and murine cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>48 h</th>
<th>Nutlin-3</th>
<th>NDD0005</th>
<th>MI-63</th>
<th>RG7388</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHSY5Y</td>
<td></td>
<td>1.95±0.56</td>
<td>1.93±0.69</td>
<td>0.78±0.16</td>
<td>0.11±0.05</td>
</tr>
<tr>
<td>NGP</td>
<td></td>
<td>2.51±0.3</td>
<td>2.78±0.02</td>
<td>1.06±0.12</td>
<td>0.14±0.03</td>
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<tr>
<td>MEFPPRTP</td>
<td></td>
<td>7.10±0.38</td>
<td>6.98±0.06</td>
<td>12.05±0.63</td>
<td>1.61±0.11</td>
</tr>
<tr>
<td>NIH3T3</td>
<td></td>
<td>14.1±2.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NHO2A</td>
<td></td>
<td>41.4±2.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>282MYCN+/-</td>
<td></td>
<td>35.3±1.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>844MYCN+/-</td>
<td></td>
<td>36.5±1.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3261MYCN+/-</td>
<td></td>
<td>3.53±0.54</td>
<td>2.38±0.39</td>
<td>3.71±0.3</td>
<td>6.36±1.65</td>
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<tr>
<td>3394MYCN+/-</td>
<td></td>
<td>3.09±0.19</td>
<td>1.57±0.63</td>
<td>4.0±0.41</td>
<td>1.84±0.50</td>
</tr>
<tr>
<td>3399MYCN+/-</td>
<td></td>
<td>4.3±1.2</td>
<td>1.58±0.65</td>
<td>3.94±0.58</td>
<td>3.49±0.37</td>
</tr>
</tbody>
</table>

Values represent the means ± SEM. P-values were determined using unpaired t-tests vs. human neuroblastoma SHSY5Y or NGP cells, *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001; ns, not statistically significant. Rel Fold, fold change relative to the GI50 value of SHSY5Y cells and the GI50 value of NGP cells; vs., versus; ND, not determined.
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Competing interests

The authors declare that they have no competing interests.

References


