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MYCN sensitizes neuroblastoma to the MDM2-p53 antagonists Nutlin-3 and MI-63

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Abstract

MYCN amplification is a major biomarker of poor prognosis, occurring in 25-30% of neuroblastomas. *MYCN* plays contradictory roles in promoting cell growth and sensitizing cells to apoptosis. We have recently shown that p53 is a direct transcriptional target of *MYCN* in neuroblastoma and that p53-mediated apoptosis may be an important mechanism of *MYCN*-induced apoptosis. Although p53 mutations are rare in neuroblastoma at diagnosis, the p53/MDM2/p14^{ARF} pathway is often inactivated through *MDM2* amplification or p14^{ARF} inactivation. We hypothesised that reactivation of p53 by inhibition of its negative regulator MDM2, using the MDM2-p53 antagonists Nutlin-3 and MI-63, will result in p53-mediated growth arrest and apoptosis especially in *MYCN* amplified cells. Using the SHEP Tet21N *MYCN* regulatable system, *MYCN*(-) cells were more resistant to both Nutlin-3 and MI-63 mediated growth inhibition and apoptosis compared to *MYCN*(+) cells and siRNA mediated knockdown of *MYCN* in 4 *MYCN* amplified cell lines resulted in decreased p53 expression and activation, as well as decreased levels of apoptosis following treatment with MDM2-p53 antagonists. In a panel of 18 neuroblastoma cell lines treated with Nutlin-3 and MI-63, the sub-set amplified for *MYCN* had a significantly lower mean GI₅₀ value and increased caspase 3/7 activity compared to the non *MYCN* amplified group of cell lines, but p53 mutant cell lines were resistant to the antagonists regardless of *MYCN* status. We conclude that amplification or overexpression of *MYCN* sensitizes neuroblastoma cell lines with wildtype p53 to MDM2-p53 antagonists and that these compounds may therefore be particularly effective in treating high risk *MYCN* amplified disease.

Keywords

MYCN; neuroblastoma; MDM2-p53 antagonists; apoptosis; growth inhibition

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Introduction

Neuroblastoma is an embryonal malignancy originating from developing neural crest cells which go on to form the sympathetic nervous system. It is the most common solid extracranial malignancy of childhood (Maris et al 2007). Over 50% of cases of neuroblastoma are high risk and difficult to cure, with long term survival rates remaining below 40% despite intense multimodal therapies. *MYCN* gene amplification is a major marker of adverse prognosis, occurring in 25-30% of neuroblastomas and is strongly associated with progressive disease and treatment failure (Cohn and Tweddle 2004). Infants under 18 months with *MYCN* amplified tumors have an event-free survival of 26% compared to 83% for infant stage 4 patients without *MYCN* amplification (Cohn et al 2009).

MYCN belongs to the *MYC* family of transcription factors that play roles in promoting cell proliferation, differentiation, oncogenesis and apoptosis (Kang et al 2006). These proteins transcriptionally activate target genes by forming heterodimers with *MAX*, which bind promoters of target genes, typically at E-box sequences (Wenzel et al 1991). *MYCN* expression alone, targeted to developing neural crest tissue, has been shown to directly result in neuroblastoma tumor formation in transgenic mice (Weiss et al 1997) and there is evidence that *MYCN* expression sensitizes neuroblastoma cells to apoptosis induced by cytotoxic drugs (Fulda et al 2000, Hogarty 2003). However, since patients with *MYCN* amplified tumors have such an inferior outcome, acquired aberrations in the apoptotic pathway are thought to be associated with *MYCN* amplification and to be essential for tumor progression.

p53 is a critical tumor suppressor gene that is mutated in over 50% of adult sporadic cancers. It plays a major role in protecting the cell from genomic instability and tumor development by inducing apoptosis and cell cycle arrest in response to cellular stresses and DNA damage (reviewed in ref. (Michalak et al 2005)). In neuroblastoma, p53 mutations are rare, occurring in <2% of cases at diagnosis and ~15% at relapse (Carr-Wilkinson et al 2010). However, in 1/3 cases in a study of relapsed tumors, p53 was found to be inactivated via other mechanisms that resulted in destabilisation of p53 or disruption of p53 activity (Carr-Wilkinson et al 2010). In neuroblastoma, other mechanisms of p53 inactivation include amplification of the E3 ubiquitin ligase gene *MDM2*, or impairment of *p14^{ARF}*, a negative regulator of *MDM2* (Carr-Wilkinson et al 2010, Carr et al 2006). *MDM2* is the major negative regulator of p53 activity and stability, promoting the ubiquitination and degradation of p53 but also blocking its transcriptional activity by binding to the transactivation domain within the N-terminal of p53. *MDM2* is also a transcriptional target of p53, forming a negative feedback loop that allows for tight regulation of p53 under normal circumstances. Non-syntenic co-amplification of *MDM2* and *MYCN* has been reported in neuroblastoma cell lines and tumors, resulting in constant negative regulation of p53 (Carr-Wilkinson et al 2010, Corvi et al 1995). More commonly in tumors, *p14^{ARF}* function is impaired through methylation or homozygous deletion of the gene (Carr-Wilkinson et al 2010). *p14^{ARF}* negatively regulates *MDM2* and therefore *p14^{ARF}* inactivation drives cell survival through increased *MDM2* activity. *MYCN* is a central modulator of the p53/*MDM2*/*p14^{ARF}* network. There is evidence that both p53 and *MDM2* are direct transcriptional targets of *MYCN* (Chen et al 2010, Slack et al 2005), and that p53 may be important for *MYCN* induced apoptosis (Chen et al 2010). It has also been reported that *p14^{ARF}* is activated by c-*MYC* (Zindy et al 1998), and due to the similarities between *MYCN* and c-*MYC*, *MYCN* may function in a similar way. *MDM2* haploinsufficiency in mice has been shown to suppress *MYCN*-driven neuroblastoma tumorigenesis (Chen et al 2009) and there is evidence that *MDM2* may be the critical oncogene by which *MYCN* amplified neuroblastomas acquire an aggressive phenotype (Slack and Shohet 2005). Since *MYCN*

amplification is thought to be associated with defects in activating or executing apoptotic pathways and that this may be related to overactive MDM2, we hypothesize that *MYCN* amplified tumors may be more susceptible to compounds that reactivate p53. Several studies have shown that the downstream apoptotic pathway of p53 is generally intact in neuroblastoma (Goldman et al 1996, Hogarty 2003).

The hydrophobic p53-binding pocket of MDM2 is ideal for developing low molecular weight compounds that occupy the pocket and prevent p53 from binding. Compounds have been developed that induce cell cycle arrest and apoptosis in cancer cells, but usually only produce reversible growth arrest in normal cells (Efeyan et al 2007, Vassilev et al 2004). Nutlin-3 is a *cis*-imidazoline analogue which binds MDM2 at the p53 binding site with a K_i of 36nM (Vassilev et al 2004) and MI-63, a spiro-oxindole, is more potent, binding MDM2 with a K_i of 3nM (Canner et al 2009). In wild-type p53 neuroblastoma cell lines, Nutlin-3 has been reported to induce cell cycle arrest and apoptosis, and surviving cells underwent senescence or neuronal differentiation in cell lines tested, however the relationship to *MYCN* status was not extensively explored (Van Maerken et al 2006). Although not previously investigated in neuroblastoma or in relation to *MYCN*, MI-63 has also been shown to induce apoptosis in cell lines with wildtype p53 (Canner et al 2009).

The current study provides evidence that *MYCN* expression sensitizes neuroblastoma cells to MDM2-p53 antagonists. We show that a) in a *MYCN* regulatable cell line (Tet21N), *MYCN*(+) cells are more sensitive to MDM2-p53 antagonist mediated growth inhibition and apoptosis than *MYCN*(-) cells, b) siRNA-mediated knockdown of *MYCN* results in a decreased p53 response and decreased induction of apoptosis by MDM2-p53 antagonist treatment, and c) in a panel of 18 cell lines, *MYCN* amplified cells are more sensitive to Nutlin-3 and MI-63 than non-*MYCN* amplified cell lines.

Results

***MYCN*(-) Tet21N SHEP cells are more resistant to Nutlin-3 and MI-63 mediated growth inhibition and apoptosis compared to *MYCN*(+) cells**

Tet21N *MYCN* regulatable cells were treated with the MDM2-p53 antagonists Nutlin-3 and MI-63 in the presence (*MYCN*-) or absence (*MYCN*+) of tetracycline. Growth inhibition assays were performed and as shown in Figure 1A and 1B *MYCN*(+) cells had significantly lower GI_{50} s compared to *MYCN*(-) cells ($p=0.02$ for Nutlin-3, and $p=0.0008$ for MI-63). GI_{50} values are shown in Table 1. As a control, growth inhibition assays were performed in Tet21 vector only cells and no difference in GI_{50} was observed.

As previously reported *MYCN*(+) cells have a doubling time of 78.5 hours compared to 90.4 hours for *MYCN*(-) cells (Bell et al 2006). This difference in growth rate is reflected in the higher proportion of *MYCN*(-) cells in G_1 compared to *MYCN*(+) cells, which have a greater proportion of cells in S and G_2 phases (Figure 1C). Upon 2.5 μ M Nutlin-3 and MI-63 treatment, both *MYCN*(+) and *MYCN*(-) cells G_1 arrest, but this effect is more pronounced in *MYCN*(+) cells due to the lower G_1 baseline proportions. Interestingly, there is an increase in the proportion of cells in G_2 at 10 μ M Nutlin-3 and MI-63 in both *MYCN*(+) and *MYCN*(-) cells.

Three techniques were used to investigate levels of apoptosis following Nutlin-3 or MI-63 treatment in Tet21N cells. As shown in Figure 2A, there was a significant increase in the percentage of sub G_1 DNA following Nutlin-3 treatment (0-20 μ M) in *MYCN*(+) cells compared to *MYCN*(-) cells ($p<0.0007$), and although not statistically significant ($p=0.058$), a similar effect was seen after MI-63 treatment. An increase in the induction of caspase 3/7 activity at increasing concentrations of Nutlin-3 ($p<0.0001$) and MI-63

($p < 0.0001$) was observed in MYCN(+) compared to MYCN(-) cells (Figure 2B) and Western blotting showed increased expression of the p53 target and apoptotic marker PUMA in MYCN(+) compared to MYCN(-) cells (Figure 2C). Induction of p53 and its transcriptional target gene products was observed in both MYCN(+) and MYCN(-) cells. Higher basal levels of p53 were detected in MYCN(+) cells and after Nutlin-3 treatment, increased p53 induction was observed in MYCN(+) cells compared to MYCN(-) cells. Both compounds induced greater levels of p53 phosphorylation at serine 15 in MYCN(+) cells. Together, this data provide evidence that MYCN(+) cells are more sensitive to MDM2-p53 antagonist mediated growth inhibition and apoptosis.

Knockdown of MYCN increases resistance of MYCN amplified neuroblastoma cell lines to Nutlin-3 and MI-63 mediated induction of p53 and apoptosis

To further investigate the role of MYCN on the sensitivity of neuroblastoma cell lines to MDM2-p53 antagonists, MYCN was knocked down by siRNA treatment in two *MYCN* and *MDM2* co-amplified cell lines TR14 and LS, and 2 *MYCN* amplified but non-*MDM2* amplified cell lines LAN5 and IMR-32. MYCN siRNA or SCR siRNA (30-50nmol/L) was added to cells for 24 hours then removed and replaced with 0, 5 or 10 μ M Nutlin-3 or MI-63 for a further 24 hours. In all four cell lines, TR14 (Figure 3A), LAN5 (Figure 4A), LS (Figure S1A) and IMR32 (Figure S2) treatment with Nutlin-3 or MI-63, resulted in a clear increase in p53 induction, p53 phosphorylation (serine 15), induction of p53 target genes (p21^{WAF1}, MDM2 and PUMA) and induction of the apoptotic markers PUMA, cleaved caspase 3 and cleaved PARP (Lanes 1-5). In all cell lines, high levels of MYCN knockdown were achieved as shown by comparison of DMSO controls with SCR and siMYCN (lane 1 vs. lane 6, Figures 3A, 4A, S1A and S2). MYCN knockdown alone had little effect on p53 or p53 targets but with MDM2-p53 antagonist treatment MYCN knockdown decreased the p53 and apoptotic response. p53 levels decreased in 3/4 cell lines, and levels of phosphorylated p53 decreased in all cell lines (Figures 3A, 4A, S1A and S2, lanes 7-10 compared to lanes 2-5) indicating reduced p53 activation. p21^{WAF1} or MDM2 levels either decreased or didn't change and in all cases, a decrease in at least 2 of the apoptotic markers PUMA, cleaved caspase 3 and cleaved PARP was observed.

Apoptosis following MYCN knockdown and MDM2-p53 antagonist treatment was further investigated in TR14, LAN5 and LS cells. Caspase 3/7 activity assays were performed for TR14 and LAN5 cells (Figure 3B and 4B). After Nutlin-3 or MI-63 treatment, a dose dependent increase in caspase 3/7 activity was seen in both cell lines with both compounds (Figure 3B and 4B), and an increase in sub G₁ DNA % in LS cells (Figure S1B). Following MYCN knockdown, there was a reduction in caspase 3/7 activity in TR14 and LAN5 cells (Figures 3B and 4B at 0 μ M) compared to SCR control, and a significant reduction in caspase activity was observed in both TR14 and LAN5 cell lines following MYCN knockdown and Nutlin-3 or MI-63 treatment, (TR14 $p=0.0015$ Nutlin-3, $p=0.0001$ MI63, and LAN5 $p=0.0026$ Nutlin-3 and $p=0.008$ MI-63). Levels of sub G₁ DNA in LS cells (Figure S1B) reduced after MYCN knockdown following Nutlin-3 ($p=0.08$) and MI-63 ($p<0.05$) treatment compared to SCR. These data are consistent with the reduction in levels of apoptotic markers shown by Western blot showing that removal of MYCN by siRNA knockdown results in a decreased apoptotic response to MDM2-p53 antagonists.

Knockdown of MYCN does not alter the cell cycle response to MDM2-p53 antagonists

The effect of MDM2-p53 antagonists on the cell cycle was investigated in TR14, LAN5 and LS cells. TR14 cells underwent a slight G₁ arrest upon Nutlin-3 or MI-63 treatment (Figure 3C), whereas LAN5 (Figure 4C) and LS (Figure S1C) cells did not arrest. Knockdown of MYCN induced a G₁ arrest in both TR14 and LS cells, but the effect of MDM2-p53 antagonists was not influenced by MYCN knockdown. This data suggests that upon MDM2-

p53 antagonist treatment, some neuroblastoma cell lines G₁ arrest and that the effect is cell line dependent, but the response is not altered upon MYCN knockdown.

MYCN amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonist mediated growth inhibition than non MYCN amplified neuroblastoma cell lines

Induction of a p53 response from MDM2-p53 antagonists can initiate a number of effects, including 2 important antitumor responses, cell cycle arrest and apoptosis. A panel of 12 MYCN amplified (including 4 MYCN and MDM2 co-amplified) and 8 non-MYCN amplified neuroblastoma cell lines were investigated for their sensitivity to Nutlin-3 and MI-63 mediated growth inhibition, which takes into account both growth arrest and cell death. GI₅₀ values for all p53 wildtype cell lines are shown in Table 1. In response to Nutlin-3 (Figure 5A, 5Bi) and MI-63 (Figure 5C, 5Di), MYCN amplified neuroblastoma cell lines underwent more growth inhibition compared to non-amplified cell lines with a significant difference in mean GI₅₀s (p<0.001 for Nutlin-3 and p<0.05 for MI-63). Overall a more varied response to the antagonists was seen in the non-amplified cell lines compared to MYCN amplified cell lines. Interestingly, the p53 wildtype SKNRA cell line was most resistant to both Nutlin-3 and MI-63. MYCN-only amplified cell lines were also more sensitive to Nutlin-3 mediated growth inhibition compared to MYCN and MDM2 co-amplified neuroblastoma cell lines (Figure 5Bii) (p<0.01) and although this difference did not reach statistical significance, the same trend was observed for MI-63 (Figure 5Dii). A number of cell lines in this panel had impaired p14^{ARF} function; PER-108 (methylated) and GIMEN (methylated), SHEP (homozygous deletion) and LAN-6 (homozygous deletion) (Carr et al 2006). There appeared to be a varied response to MDM2-p53 antagonist mediated growth inhibition with no evidence that p14^{ARF} status affects the response to these compounds. Two MYCN amplified p53 mutant cell lines (Be2C and IGRN91) and 1 non-MYCN amplified p53 mutant cell line (SKNAS) were investigated for their sensitivity to Nutlin-3 and MI-63. As shown in Figure 5E, p53 mutant cell lines were highly resistant to these compounds regardless of MYCN status. 50% growth inhibition was not achieved in these cell lines with the highest concentrations of Nutlin-3 (20μM) and MI-63 (10μM) used to generate GI₅₀ values in the other p53 wildtype cell lines tested.

MYCN amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonist mediated apoptosis compared to non MYCN amplified neuroblastoma cell lines

As a marker of apoptosis, caspase 3/7 activity following Nutlin-3 or MI-63 treatment was determined in the same panel of neuroblastoma cell lines that were assessed for growth inhibition. In response to 5μM Nutlin-3 (Figure 6A) and 2.5μM MI-63 (Figure 6B), MYCN amplified neuroblastoma cell lines showed higher mean caspase 3/7 activity compared to non MYCN amplified cell lines (p=0.0343 for Nutlin-3 and p=0.0085 for MI-63). Interestingly, despite no obvious resistance to growth inhibition, p14^{ARF} impaired cell lines, particularly those that are not MYCN amplified, appear to be especially resistant to MDM2-p53 antagonist mediated activation of caspase 3/7 activity, which was comparable to that of p53 mutant cell lines (Figure 6C). In addition the MYCN and MDM2 co-amplified cell lines that display increased caspase 3/7 activity have been previously reported to express high levels of p14^{ARF} mRNA and protein (Carr et al 2006).

Discussion

Neuroblastoma accounts for 15% of childhood cancer related deaths (Maris and Matthay 1999). MYCN amplification is a powerful and reliable biomarker of poor prognosis in neuroblastoma and is used to stratify patients into a high risk group requiring intensive treatment. However, current therapies are insufficient for these patients, resulting in high

mortality rates, a high incidence of relapse and treatment related toxicity (Matthay et al 1999).

MYCN plays roles in the contradictory pathways of promoting both cell survival (Lutz et al 1996) and sensitizing cells to apoptosis (Fulda et al 2000, van Noesel et al 2003), but in neuroblastomas abnormalities within the apoptotic pathways can occur in association with *MYCN* amplification (Hogarty 2003). p53, the major tumor suppressor in the cell, is generally wild-type and active at diagnosis and patients respond well to initial therapy. However, high risk patients often relapse and although p53 mutations are still relatively rare (~15% of cases), p53 function is often inactivated through disruption of the p53/MDM2/p14^{ARF} network, of which *MYCN* is a central modulator (Figure 7), resulting in chemoresistant disease (Carr-Wilkinson et al 2010). A number of studies have shown that pathways downstream of p53 are intact in neuroblastoma and that p53 can induce apoptotic responses (Hogarty 2003, Hosoi et al 1994, Tweddle et al 2003, Vogan et al 1993). However, in response to DNA damage, *MYCN* amplified cell lines fail to undergo G₁ arrest compared to non-amplified cell lines (Bell et al 2006). In response to Nutlin-3 and MI-63 we found that changes in cell cycle distribution were cell line dependent. It has been previously reported that the negative regulator of p53, MDM2, is the critical oncogene product by which *MYCN* amplified neuroblastomas acquire a more aggressive behaviour (Slack and Shohet 2005), and that *MYCN* and MDM2 work together to inhibit apoptosis (Alt et al 2003, Wang et al 2006). There are a number of mechanisms by which MDM2 might contribute to the aggressive phenotype of *MYCN* amplified neuroblastoma. MDM2 has been reported to be a direct transcriptional target of *MYCN* and therefore *MYCN*-driven expression of MDM2 may contribute to p53 inactivation in neuroblastoma (Slack et al 2005). *MDM2* is co-amplified with *MYCN* in about 6% of neuroblastomas but more frequently the negative regulator of MDM2, p14^{ARF} is inactivated through methylation or deletion, and has been reported to occur in 29% of tumors, resulting in hyperactive MDM2 (Carr-Wilkinson et al 2010, Corvi et al 1995). In addition, we have recently reported that p53 is a direct transcriptional target of *MYCN*, and that p53 induction by *MYCN* may be an important contributory mechanism by which *MYCN* sensitizes cells to apoptosis (Chen et al 2010).

Here we investigate the effect of *MYCN* on the response to the MDM2-p53 antagonist Nutlin-3 and the more potent MI-63, not previously investigated in neuroblastoma. We show by three independent methods that *MYCN* amplification and expression sensitizes neuroblastoma cell lines to MDM2-p53 antagonists. Firstly, using the SHEP Tet21N *MYCN* regulatable cell line, both Nutlin-3 and MI-63 are more effective at inducing growth inhibition and apoptosis in *MYCN*(+) compared to *MYCN*(-) cells, as shown by growth inhibition assays, caspase 3/7 activity, the sub G₁ DNA content on FACs analysis and induction of the p53 regulated apoptotic marker PUMA. Secondly, we show that following siRNA knockdown of *MYCN* in two *MYCN* amplified and two *MYCN* and *MDM2* co-amplified neuroblastoma cell lines, Nutlin-3 and MI-63 treatment resulted in a decreased p53 response and decreased levels of apoptosis. Thirdly, we assessed growth inhibition and caspase activity in a panel of 18 cell lines and found that both Nutlin-3 and MI-63 were more effective at inducing growth inhibition and apoptosis in *MYCN* amplified cells compared to non *MYCN* amplified cells. We also show that the effect is dependent on wildtype p53 in both *MYCN* amplified and non-amplified cell lines, consistent with a number of reports showing that these compounds require wild-type p53, as expected if their action is target-specific (Van Maerken et al 2006, Vassilev et al 2004). Whereas all *MYCN* amplified cell lines investigated were responsive to MDM2-p53 antagonists, the response of non-amplified cell lines was more variable. This is unsurprising as *MYCN* amplification is directly responsible for transformation; non amplified cases are likely to have a variety of

other genetic abnormalities or defects in apoptotic pathways that may not be present in *MYCN* amplified tumors.

Our data differs from previous findings that report no correlation between *MYCN* status and response to Nutlin-3 (Barbieri et al 2006, Van Maerken et al 2006). Van Maerken *et al.* tested a panel of 7 p53 wildtype cell lines (3 *MYCN* amplified), and found no significant difference in the cell viability response or apoptotic response to Nutlin-3 in *MYCN* amplified compared to non-*MYCN* amplified cell lines (Van Maerken et al 2006). In another study IC₅₀ values for Nutlin-3 in 2 *MYCN* regulatable cell lines were determined and whilst in agreement with our extensive observations that there was increased sensitivity in *MYCN*(+) compared to *MYCN*(-) Tet21 cells, it was reported that there was no difference in IC₅₀s for *MYCN*(+/-) *MYCN*3 cells (Barbieri et al 2006). To test the effect of *MYCN* on sensitivity to MDM2-p53 antagonists we used several methods and a total of 22 cell lines to investigate the influence on cell growth and cell death, and found similar results using two structurally unrelated MDM2-p53 antagonists. Furthermore, previous studies with Tet21N cells have reported that *MYCN*(+) cells were more sensitive to apoptosis from cytotoxic drugs than *MYCN*(-) cells (Fulda et al 2000), and a recent study reports that *MYCN* sensitizes neuroblastoma cell lines to the apoptotic effects of bleomycin (Petroni et al 2011), in agreement with our data that *MYCN* is sensitizing cells to apoptosis. p14^{ARF} is often inactivated in neuroblastoma (Carr-Wilkinson et al 2010), is expressed at higher levels in *MYCN* and *MDM2* co-amplified neuroblastoma cell lines than *MYCN* amplified non-*MDM2* amplified cell lines (Carr et al 2006), and may inhibit *MYCN* transcriptional activity (Amente et al 2007). There is also evidence that p14^{ARF} is induced by c-MYC, and therefore *MYCN* may function in a similar way (Zindy et al 1998). Interestingly the 3 non *MYCN* amplified p14^{ARF} impaired cell lines were especially resistant to Nutlin-3 and MI-63 mediated apoptosis, supporting recent findings showing that silencing of p14^{ARF} results in decreased susceptibility to undergo apoptosis and overexpression of p14^{ARF} results in a stronger caspase 3/7 response (Van Maerken et al 2011).

We found that *MYCN* and *MDM2* co-amplified neuroblastomas had reduced sensitivity to Nutlin-3 mediated growth inhibition, and a similar trend with MI-63, compared to *MYCN* only amplified cell lines. However, we saw increased levels of caspase 3/7 activity in these cell lines compared to *MYCN* amplified and non-amplified cell lines, consistent with previous reports that increased levels or amplification of *MDM2* increases Nutlin-3 induced apoptosis as shown in liposarcomas and AML (Kojima et al 2005, Muller et al 2007). However, *MYCN* and *MDM2* co-amplified cell lines have previously been reported to have increased p14^{ARF} mRNA and protein expression (Carr et al 2006), and it may be this increase in p14^{ARF} that is sensitizing these cell lines to increased caspase activity. Our data indicates that *MDM2* co-amplification may increase the resistance of *MYCN* amplified neuroblastomas to Nutlin-3 mediated growth inhibition but may sensitise to apoptosis, and therefore the effect of MDM2 warrants further investigation.

Our observations support studies that both MDM2 and p53 are induced by *MYCN*, that MDM2 is a critical oncogene product by which *MYCN* amplified neuroblastomas acquire a more aggressive phenotype and that *MYCN* sensitizes cells to p53-mediated apoptosis. Under normal circumstances, both p53 and *MYCN* induce MDM2, but upon MDM2 inhibition *MYCN*-mediated transcription of p53 allows p53 to accumulate and increases activity. In addition there is evidence that both p53 and c-MYC inhibit anti-apoptotic factors such as the Bcl-2 and Bcl-x, another mechanism by which amplification of *MYCN* may promote apoptosis together with p53 if as is likely this is also true of *MYCN* (Chipuk and Green 2006, van Noesel and Versteeg 2004).

Interestingly, p53 has been shown to inhibit the mTOR pathway which is involved in senescence (Demidenko et al 2010), and there is evidence that p53 induced cell cycle arrest is reversible as long as mTOR is also inhibited, suggesting that induction of senescence, reversible cell cycle arrest and cell death in response to Nutlin-3 may be determined by the status of mTOR (Korotchkina et al 2010). The activation status of mTOR may therefore be important in determining the response to MDM2-p53 antagonists in neuroblastoma.

In conclusion, we have several lines of evidence that MYCN sensitizes neuroblastoma cell lines to MDM2-p53 antagonists through p53-dependent growth inhibition and apoptosis, and may provide a promising therapeutic approach for patients with high-risk MYCN amplified neuroblastoma with wild-type p53. Previous studies show that MYCN amplification sensitizes cells to chemotherapeutic drugs and that Nutlin-3 induces senescence in normal cells that might actually protect the cell against cytotoxic drugs (Efeyan et al 2007, Fulda et al 2000). Furthermore Nutlin-3 synergises with chemotherapy in neuroblastoma cells (Barbieri et al 2006) suggesting that patients with MYCN amplified tumors may be particularly responsive to MDM2- p53 antagonists in combination with chemotherapeutic drugs.

Materials and Methods

Cell Lines and reagents

MYCN amplified neuroblastoma cell lines used in this study were NGP, LS, TR14, NB1691, IMR-32, NBLW, SMSKCNr, LAN5, PER-108, CHLA136, and the p53 mutant cell lines SKNBe2C and IGRN91. Non-MYCN amplified cell lines used were SHSY5Y, GIMEN, SJNB1, NB69, LAN6, SKNRA, SHEP, NBL5 and the p53 mutant cell line SKNAS. The conditional MYCN-expressing SHEP Tet21N cell line was used and cells cultured for at least 48 hours in 1µg/ml of tetracycline (Sigma, Dorset, UK) to switch off MYCN. Tet21 vector only cells were used as a control (Lutz et al 1996). All cell lines were cultured in RPMI medium (Sigma, Dorset, UK) supplemented with 10% FCS, and 200µg/ml of G-418 antibiotic was added to Tet21N and Tet21 media. Cell lines were validated upon receipt using cytogenetic analysis courtesy of Nick Bown, Institute of Human Genetics, Newcastle University, U.K. Nutlin-3 was purchased from Enzo Life Sciences (Exeter, UK) and MI-63 was kindly provided by Siena Biotech (Siena, Italy) as part of a Framework Programme 6 DePPICT consortium collaboration.

Growth inhibition assays

Cells were plated in 96-well plates (Corning, UK) for 24 hours before treatment with 0-20µM Nutlin-3 or 0-10µM MI-63 for 72 hours. Cells were then fixed in 50% trichloroacetic acid and sulphorhodamine B assays performed as previously described (Skehan et al 1990). A SpectroMax 250 (Molecular Devices, Berkshire, UK) was used for densitometric scanning of the plates and GI₅₀ values calculated using Prism Version 4.0 software.

RNA interference

Cells were seeded at 4×10⁵ cells/well in 6-well plates (Corning, Amsterdam, Netherlands). At 30-50% confluency, 40nM (NGP, LS, IMR32) or 50nM (LAN5) of siRNA duplexes against MYCN were transfected into cells with Lipofectamine™ reagent (Invitrogen, Paisley, UK) using previously described sequences (Bell et al 2006), for 24 hours. All experiments were performed in triplicate.

Western analysis

Previously described methods were used with 25µg of protein (Tweddle et al 2001). Primary antibodies used were MYCN (Calbiochem, Nottingham, UK; OP13), MDM2 (Calbiochem;

OP40), p21^{WAF1} (Calbiochem; OP68), PARP-1/2 (Santa Cruz, CA, USA; H-250), at 1:100, p53 (Novocastra, Newcastle, UK; DO-7) at 1:200, Phospho-p53(Ser15) (Cell signalling, Danvers, MA, USA; 9284) and Cleaved Caspase-3 (Cell Signalling; Asp175) at 1:1000, and β -actin (Sigma-Aldrich, Dorset, UK; AC-40) at 1:500. Secondary goat anti-mouse/rabbit (Dako, Glostrup, Denmark) antibodies conjugated to horseradish peroxidase were used at 1:1000.

Flow cytometry

For cell cycle and sub-G₁ analysis, adherent and non-adherent cells were harvested, washed in PBS and fixed in 4:1 Methanol:Acetone. FACS analysis was performed using previously described methods (Bell et al 2006). The Becton Dickinson FACscan was used and data analysed using CellQuest software.

Caspase activity

5000 cells/well were seeded in 96 well plates. After treatment, a 1:1 volume of Caspase-Glo® 3/7 Reagent (Promega, Southhampton, UK) was added to wells for 1 hour, transferred to a white plate and then analysed on a microplate Luminometer (Berthold Technologies, Herefordshire, UK).

Statistical Analyses

All statistical tests were performed in GraphPad Prism (GraphPad Software, Inc.). Tests were performed using log values and all t-tests were two-sided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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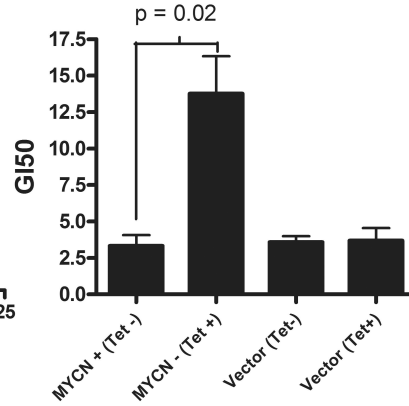
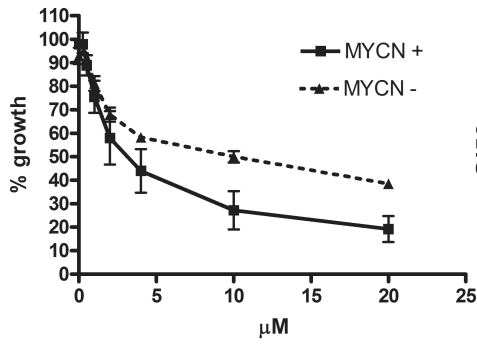
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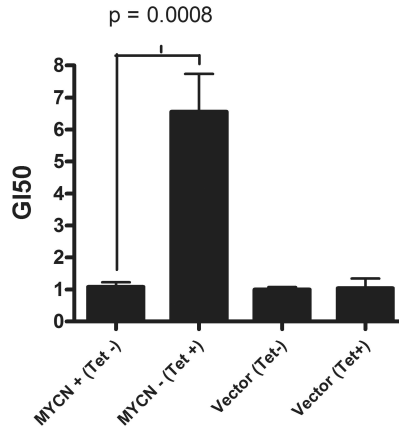
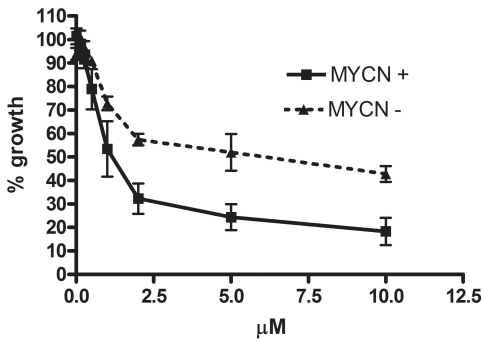
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A Nutlin-3



B MI-63



C

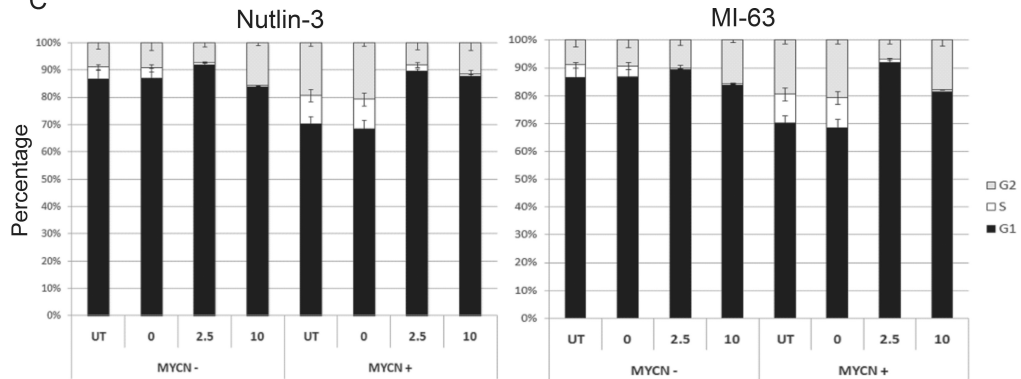


Figure 1. MYCN(+) Tet21N cells are more sensitive to MDM2-p53 antagonist mediated growth inhibition than MYCN(-) Tet21N cells

Growth inhibition assays for **A)** Nutlin-3 and **B)** MI-63 were performed after 72 hour drug exposure. MYCN(+) cells have a significantly lower mean GI₅₀ compared to MYCN(-) cells (p=0.02 for Nutlin-3 and p=0.0008 for MI-63, paired t-test). No difference in GI₅₀s was observed between the control Tet21 cells in the presence and absence of tetracycline. **C)** Cell cycle analysis after 24 hour drug exposure shows that both MYCN(-) and MYCN(+) cells G₁ arrest in response to 2.5µM and 10µM Nutlin-3 or MI-63, and show that MYCN(-) cells have an increased proportion of cells in G₁ in control samples compared to MYCN(+) cells.

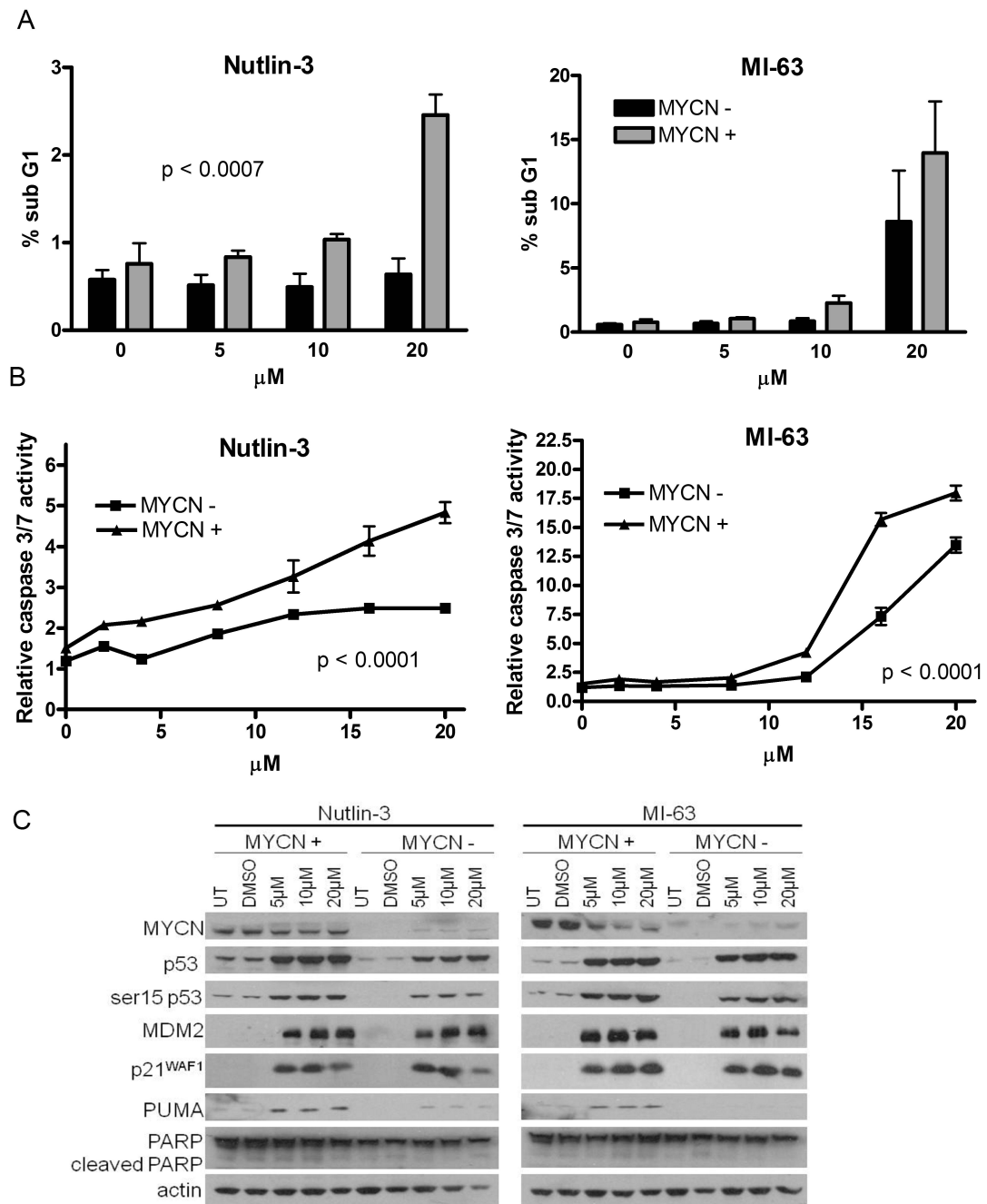


Figure 2. MYCN(+) Tet21N cells are more sensitive to MDM2-p53 antagonist mediated apoptosis than MYCN(-) Tet21N cells, following 24 hours treatment

A) The sub G₁ DNA fraction was significantly lower in MYCN(-) compared to MYCN(+) cells following Nutlin-3 treatment ($p=0.0007$, 2-way ANOVA) and although of borderline significance, a decrease in the sub G₁ fraction is observed in MYCN(-) cells compared to MYCN(+) cells following MI-63 treatment. ($p=0.058$ for MI-63, 2-Way ANOVA), **B)** Caspase 3/7 activity was significantly reduced in MYCN(-) compared to MYCN(+) cells following Nutlin-3 and MI-63 treatment ($p<0.0001$ for both Nutlin-3, and MI-63, 2-way ANOVA). **C)** Western blots showed no difference in induction of p53, MDM2 and p21^{WAF1} in MYCN(-) and MYCN(+) cells following Nutlin-3 and MI-63 treatment but there were

increased levels of phosphorylated p53 and PUMA in MYCN(+) compared to MYCN(-) cells. Actin was used as a loading control.

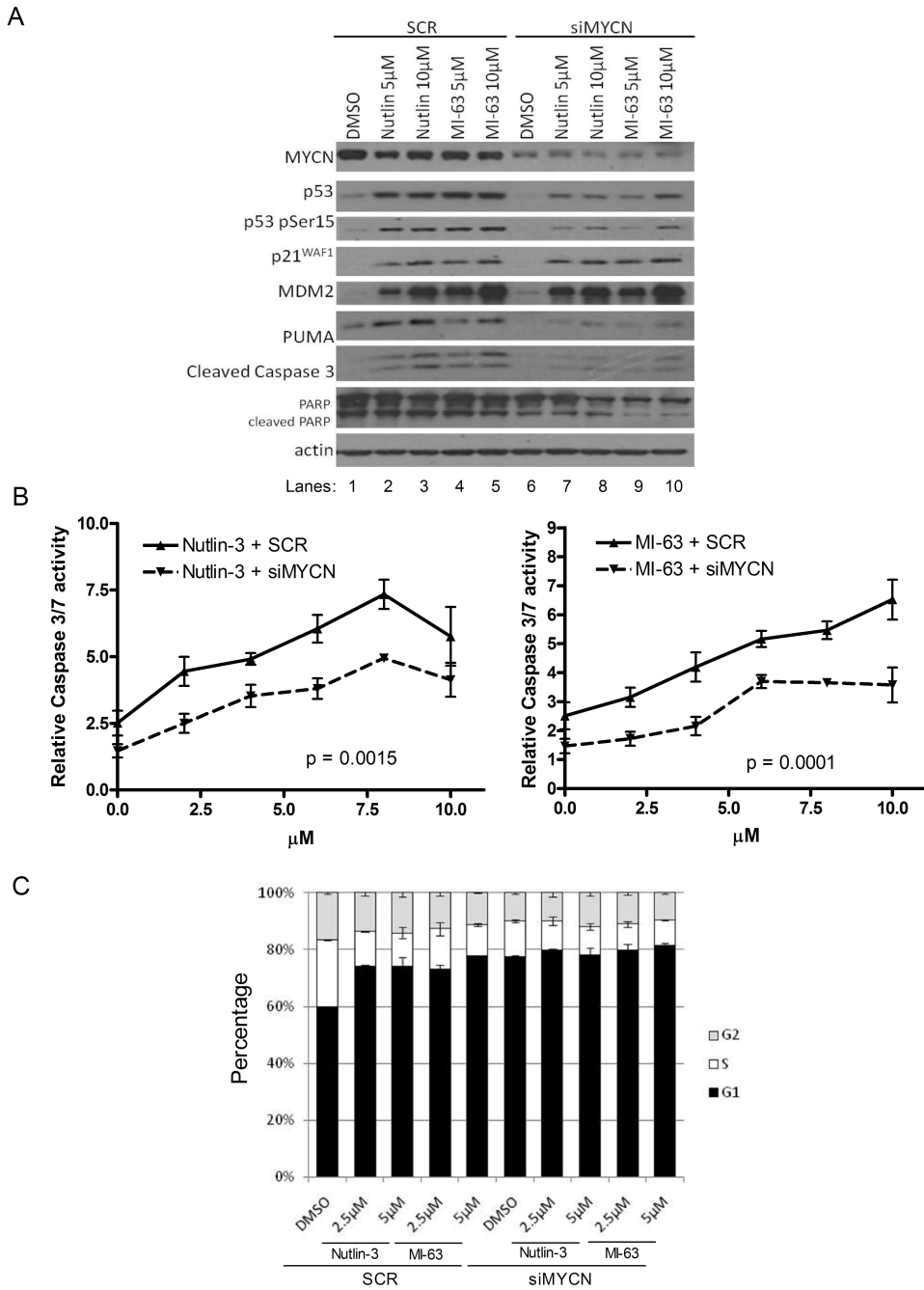


Figure 3. siRNA knockdown of MYCN (30nmol/L for 24 hours) decreases the sensitivity of MYCN and MDM2 co-amplified TR14 cells to Nutlin-3 and MI-63 treatment (24 hours)
A) Western blot showing induction of p53 responsive genes and levels of apoptotic markers. Actin was used as a loading control. **B)** Caspase 3/7 activity was significantly reduced compared to SCR siRNA following MYCN knockdown ($p=0.0015$ for Nutlin-3, $p=0.0001$ for MI-63, 2-way ANOVA). **C)** Cell cycle analysis showed a slight G₁ arrest following Nutlin-3 or MI-63 treatment and no further effect upon MYCN knockdown despite MYCN knockdown alone inducing a slight G₁ arrest.

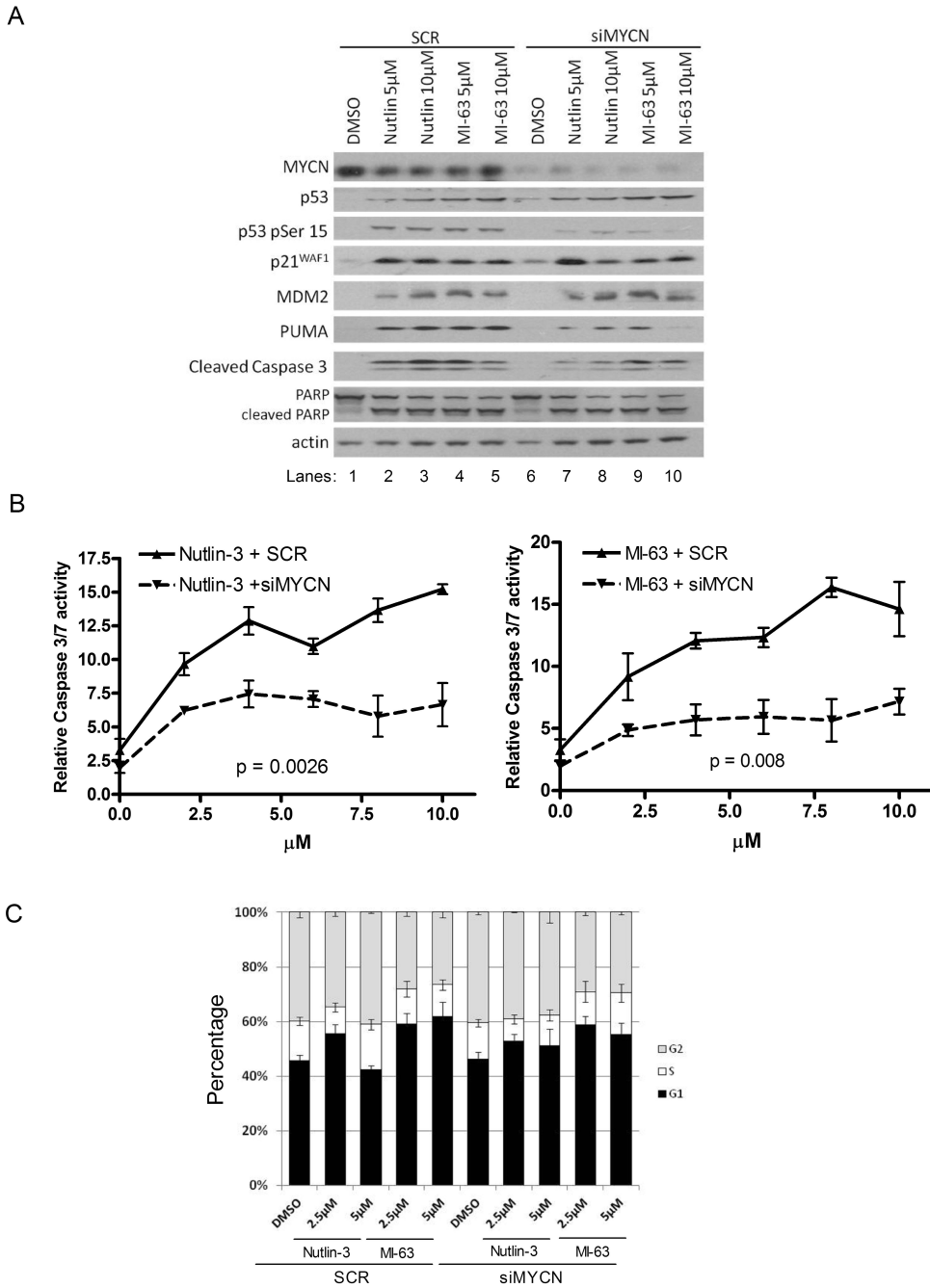


Figure 4. siRNA knockdown of MYCN (50nmol/L for 24 hours) decreases the sensitivity of MYCN amplified LANS5 cells to MDM2-p53 antagonist treatment (24 hours)

A) Western blot showing the effect on the p53 response and levels of apoptotic markers. Actin was used as a loading control. **B)** Caspase 3/7 activity was significantly reduced following MYCN knockdown ($p < 0.0026$ for Nutlin-3, $p < 0.008$ for MI-63, 2-way ANOVA). **C)** Cell cycle analysis showed no G₁ arrest following Nutlin-3 or MI-63 treatment and no further effect upon MYCN knockdown.

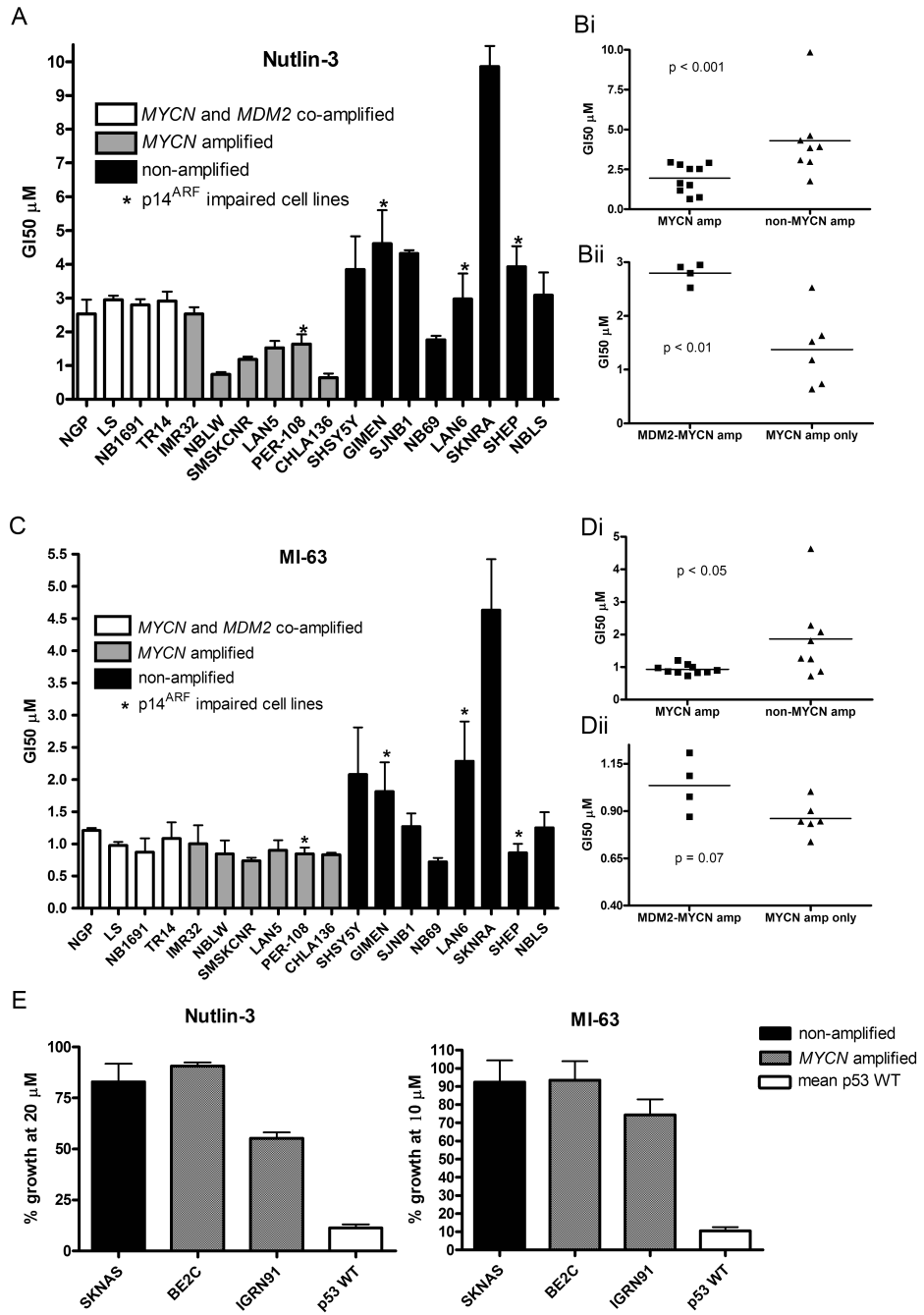
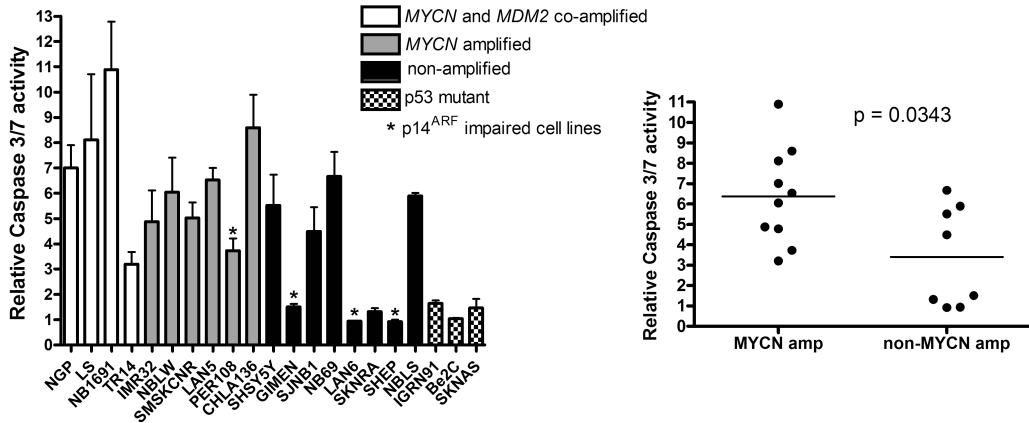


Figure 5. MYCN amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonists than non-MYCN amplified neuroblastoma cell lines

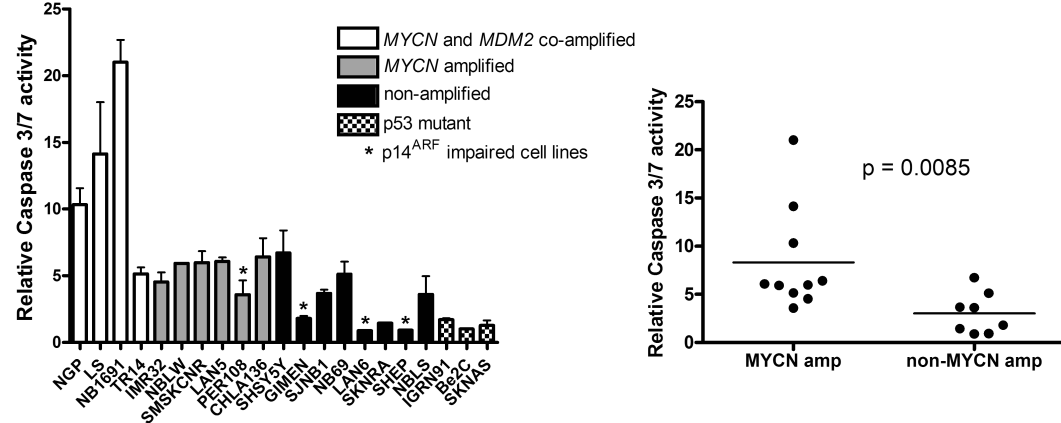
A panel of neuroblastoma cell lines were treated with **A**), **B**) Nutlin-3 and **C**), **D**) MI-63 with doses ranging from 0-20 μ M and 0-10 μ M respectively to obtain GI₅₀s. A significant difference was observed between GI₅₀s for both **Bi**) Nutlin-3 (p<0.001) and **Di**) MI-63 (p<0.05). There was an increase in GI₅₀s in MYCN and MDM2 co-amplified cell lines compared to MYCN only amplified cell lines for **Bii**) Nutlin-3 (p<0.01) but not **Dii**) MI-63 (p=0.07). Mann-Whitney tests were used to calculate p values. **E**) p53 mutant cell lines including one non-amplified and 2 MYCN amplified were treated with up to 20 μ M Nutlin-3 and 10 μ M MI-63. All three cell lines were resistant to Nutlin-3 and MI-63 compared to

wildtype p53 cell lines. Cell lines marked * are $p14^{ARF}$ impaired (methylation or homozygous deletion).

A. Nutlin-3



B. MI-63



C.

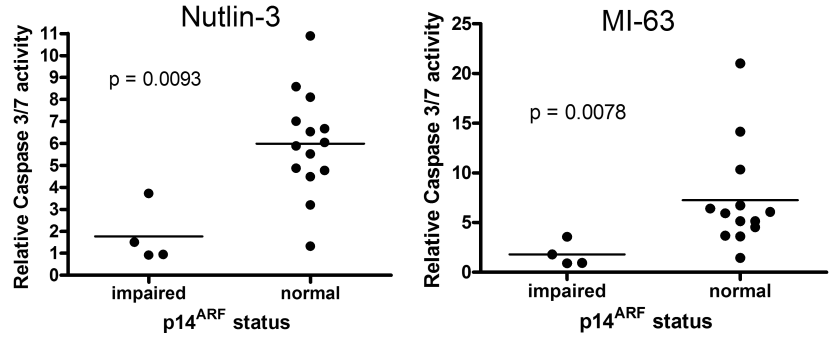


Figure 6. MYCN amplified neuroblastoma cell lines are more sensitive to MDM2-p53 antagonists mediated apoptosis than non-MYCN amplified neuroblastoma cell lines

A panel of neuroblastoma cell lines were treated with A) 5µM Nutlin-3 and B) 2.5µM MI-63 and relative caspase 3/7 activity determined. The difference in caspase 3/7 activity was significantly higher in MYCN amplified compared to non-MYCN amplified cell lines (p=0.0343 for Nutlin-3, and p=0.0085 for MI-63). p53 mutant cell lines had low levels of caspase activity. Cell lines marked * are p14^{ARF} impaired (methylation or homozygous deletion). C) p14^{ARF} impaired cell lines have significantly lower levels of caspase 3/7 activity compared to cell lines with normal p14^{ARF} (p=0.0093 for Nutlin-3, p=0.0078 for MI-63, Mann-Whitney test).

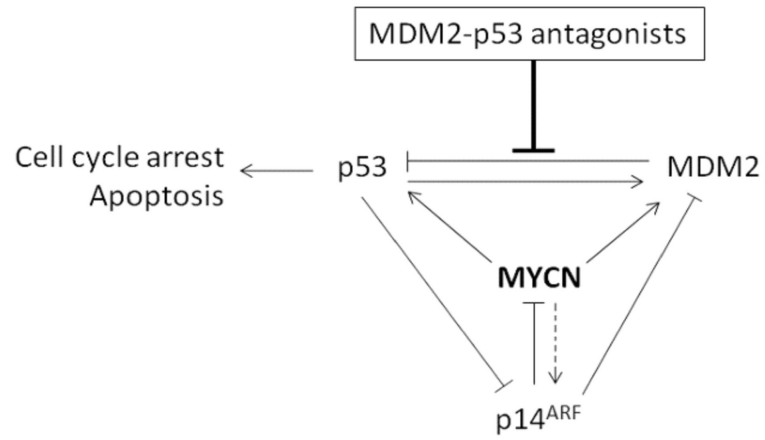


Figure 7. MYCN is a central modulator of the p53/MDM2/p14^{ARF} negative feedback loop
MDM2-p53 antagonists block the MDM2-p53 interaction.

Table 1

Summary of GI₅₀ values for Nutlin-3 and MI-63 in 18 neuroblastoma cell lines of varying *MYCN* and *MDM2* amplification status, and the Tet21N conditional *MYCN* expression system. amp - amplified, non-amp – non-amplified.

Cell Line	<i>MYCN</i> status	<i>MDM2</i> status	GI ₅₀ (μM)	
			Nutlin-3	MI-63
NGP	amp	amp	2.53 ± 0.43	1.21 ± 0.04
LS	amp	amp	2.95 ± 0.12	0.98 ± 0.06
NB1691	amp	amp	2.80 ± 0.17	0.87 ± 0.22
TR14	amp	amp	2.91 ± 0.28	1.09 ± 0.25
IMR32	amp	non-amp	2.53 ± 0.20	1.00 ± 0.29
NBLW	amp	non-amp	0.74 ± 0.07	0.85 ± 0.21
SMSKCNR	amp	non-amp	1.18 ± 0.08	0.74 ± 0.05
LAN5	amp	non-amp	1.52 ± 0.21	0.90 ± 0.16
PER108	amp	non-amp	1.64 ± 0.29	0.85 ± 0.09
CHLA136	amp	non-amp	0.64 ± 0.12	0.83 ± 0.03
SHSY5Y	non-amp	non-amp	3.85 ± 0.98	2.01 ± 0.73
GIMEN	non-amp	non-amp	4.61 ± 0.99	1.81 ± 0.45
SJNB1	non-amp	non-amp	4.32 ± 0.10	1.27 ± 0.20
NB69	non-amp	non-amp	1.77 ± 0.12	0.72 ± 0.06
LAN6	non-amp	non-amp	2.97 ± 0.75	2.28 ± 0.62
SKNRA	non-amp	non-amp	9.85 ± 0.61	4.63 ± 0.79
SHEP	non-amp	non-amp	3.92 ± 0.61	0.86 ± 0.14
NBLS	non-amp	non-amp	3.08 ± 0.68	1.25 ± 0.24
MYCN + (Tet21N)		non-amp	3.33 ± 0.74	1.08 ± 0.14
MYCN – (Tet21N)		non-amp	13.76 ± 2.57	6.56 ± 1.18