

Dual Targeting of Wild-Type and Mutant p53 by Small Molecule RITA Results in the Inhibition of N-Myc and Key Survival Oncogenes and Kills Neuroblastoma Cells *In Vivo* and *In Vitro*

Mikhail Burmakin¹, Yao Shi¹, Elisabeth Hedström¹, Per Kogner², and Galina Selivanova¹

Abstract

Purpose: Restoration of the p53 function in tumors is a promising therapeutic strategy due to the high potential of p53 as tumor suppressor and the fact that established tumors depend on p53 inactivation for their survival. Here, we addressed the question whether small molecule RITA can reactivate p53 in neuroblastoma and suppress the growth of neuroblastoma cells *in vitro* and *in vivo*.

Experimental Design: The ability of RITA to inhibit growth and to induce apoptosis was shown in seven neuroblastoma cell lines. Mechanistic studies were carried out to determine the p53 dependence and the molecular mechanism of RITA-induced apoptosis in neuroblastoma, using cell viability assays, RNAi silencing, co-immunoprecipitation, qPCR, and Western blotting analysis. *In vivo* experiments were conducted to study the effect of RITA on human neuroblastoma xenografts in mice.

Results: RITA induced p53-dependent apoptosis in a set of seven neuroblastoma cell lines, carrying wild-type or mutant p53; it activated p53 and triggered the expression of proapoptotic p53 target genes. Importantly, p53 activated by RITA inhibited several key oncogenes that are high-priority targets for pharmacologic anticancer strategies in neuroblastoma, including N-Myc, Aurora kinase, Mcl-1, Bcl-2, Wip-1, MDM2, and MDMX. Moreover, RITA had a strong antitumor effect *in vivo*.

Conclusions: Reactivation of wild-type and mutant p53 resulting in the induction of proapoptotic factors along with ablation of key oncogenes by compounds such as RITA may be a highly effective strategy to treat neuroblastoma. *Clin Cancer Res*; 19(18); 5092–103. ©2013 AACR.

Introduction

Neuroblastoma (NB) belongs to the most challenging oncologic diseases of childhood. Despite intensive multimodal therapy, often resulting in good immediate response in many children, high-risk neuroblastoma frequently acquires therapy resistance with fatal clinical outcome (1). There is a strong need to develop novel targeted strategies that inhibit specific neuroblastoma pathways and key molecules for its growth and progression.

Among the diversity of genetic variations in neuroblastoma, *MYCN* amplification, leading to overexpression of the transcription factor N-Myc, is a genetic hallmark of the disease and an independent marker of dismal prognosis (1, 2). Selective targeting of N-Myc in neuroblastoma cells

using different approaches showed encouraging results and provides a promising treatment strategy (3). In addition, several other oncogenes have been implicated in neuroblastoma tumorigenesis, invasion, and dissemination and are regarded as targets for therapy (4). Among others, these include *PPM1D*, which encodes oncogenic phosphatase Wip1 (wild-type p53 induced phosphatase 1), increased expression of which is likely to be associated with 17q gain, a predictor of poor prognosis (5). Recent studies have shown a correlation between high expression of antiapoptotic factors Mcl-1 and Bcl-2 and resistance to therapy in neuroblastoma (6). Mcl-1 depletion via RNA interference induced apoptosis in neuroblastoma cell lines and sensitized them to cytotoxic chemotherapy, suggesting that Mcl-1, as well as Bcl-2, might be promising targets for neuroblastoma treatment (6, 7).

Notably, chemotherapy-resistant neuroblastoma often express p53 inactivated by a point mutation (8–10). p53 is the potent tumor suppressor, which halts tumor progression by inducing apoptosis or cell-cycle arrest (11). p53 is inactivated in the majority of human tumors, either by point mutation of the gene or via its inhibitors, mainly MDM2 and MDMX. MDM2 ubiquitinates p53 and marks it for destruction by the proteasome, thus keeping p53 at bay

Authors' Affiliations: ¹Department of Microbiology, Tumour and Cell biology (MTC); and ²Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

Corresponding Author: Galina Selivanova, Department of Microbiology, Tumour and Cell biology (MTC), Nobels v.16, Karolinska Institutet, SE-17177, Stockholm, Sweden. Phone: 46-8-52486302; Fax: 46-8-330744; E-mail: galina.selivanova@ki.se

doi: 10.1158/1078-0432.CCR-12-2211

©2013 American Association for Cancer Research.

Translational Relevance

There is a strong need for novel target-specific therapeutic approaches to treat high-risk neuroblastoma. Restoration of p53 is a promising strategy to treat cancer. Several compounds reactivating p53 are currently being tested in clinical trials. Unlike chemotherapy regimens which kill healthy cells along with tumor cells, leading to severe side effects, target-specific drugs spare normal cells, and have the potential to be well-tolerated therapies, which will enable patients with cancer to live longer and have an improved quality of life. Here we report that reactivation of p53 by target-specific molecule RITA triggers ablation of key factors crucial for neuroblastoma survival, including N-Myc, the driving oncogene in neuroblastoma. Inhibition of oncogenes by p53 may thus constitute a new therapeutic approach for high-risk neuroblastomas. The capability of p53 to target several oncogenes might allow p53-based therapies to cope with the daunting challenge of therapy—multiple genetic abnormalities in individual cancers. With no current satisfactory strategy for treatment of high-risk neuroblastoma, it would be highly relevant to implement this strategy in the clinic.

in the absence of stress (11). MDMX is a paralog of MDM2 required for the efficient inhibition of p53 by MDM2, but it can also suppress p53 function independently of MDM2, therefore maximal activation of p53 requires inhibition of both MDM2 and MDMX (12). Multiple studies provided evidence of the crucial role of p53 for tumor suppression, as well as for response to anticancer therapy in different types of cancer including high-risk neuroblastoma (13).

p53 dysfunction in neuroblastoma has been linked to MDM2 amplification and Wip1 activation (5, 14), as well as to homozygous deletions of CDKN2A, encoding MDM2 inhibitor p14ARF (15). Moreover, N-Myc inactivates p53 by inducing the expression of MDM2 (16), which in turn upregulates N-Myc (17). p53 mutations occur very seldom in neuroblastoma, but in cell lines established at relapse p53 mutations are more frequent, implicating mutant p53 in the development of therapy-resistant phenotype (8, 9).

Albeit inactive, the p53 protein is expressed in cancers, leading to the idea of p53 reactivation to combat cancer (18). Moreover, *in vivo* studies in animal models showed that re-instatement of p53 has much more profound tumor suppressor effects in aggressive, metastatic tumors (19, 20). These data greatly encouraged us to explore the effect of p53-reactivating molecules in neuroblastoma.

Several p53-reactivating molecules have been developed and at least 2 of them are currently being tested in clinical trials: MDM2 inhibitor nutlin3a discovered by Hoffmann La Roche (21) and the mutant p53-reactivating compound PRIMA-1^{MET}/APR-246, identified by us (22). Nutlin3a has

been shown to activate p53-dependent growth suppression in neuroblastoma carrying wild-type (wt) p53 *in vitro* and *in vivo* (23, 24). Evidence that defects in effector molecules downstream of p53 are remarkably rare in neuroblastoma leads further support to the strategy to restore the function of p53 in neuroblastoma (25).

However, recent studies show that treatment with nutlin3a creates a selective pressure for p53 mutations in neuroblastoma and other types of cancer leading to nutlin3a resistance, which in some cases contributes to multidrug resistance (26, 27). Thus, it might be beneficial to develop therapies which will simultaneously reactivate wild-type and mutant p53.

We have identified a small molecule RITA which binds to the N-terminus of p53 and induces a conformational change blocking its interaction with MDM2, leading to the robust induction of apoptosis in cancer cells of different origin *in vitro* and *in vivo*, without apparent toxic effects (28–32). Notably, RITA can also reactivate mutant p53, probably because RITA treatment impinges on p53 conformation (31). Furthermore, reactivation of p53 by RITA leads to the ablation of survival signaling in cancer cells via downregulation of Myc, Bcl-2, Mcl-1, Wip1, MDMX, and other oncogenes (30, 33). Taken together, these data inspired us to test whether RITA is capable of restoring wild-type and mutant p53 activity in neuroblastoma.

Here, we report that RITA triggers robust apoptosis in different neuroblastoma lines, including the ones with mutant p53. RITA-activated p53 induces the expression of its proapoptotic target genes such as PUMA and Noxa and also a rapid and substantial downregulation of several key survival factors in neuroblastoma, including N-Myc, Aurora kinase A, MDM2, MDMX, Wip1, and Mcl-1. Notably, RITA efficiently suppressed the growth of human neuroblastoma xenografts in mice.

Materials and Methods

Cell lines

Neuroblastoma cell lines used in this study and the status of p53 and N-Myc in these lines is indicated in Table 1. SKN-BE(2) and SHEP cells were maintained in RPMI 1640

Table 1. p53 status and N-Myc amplification status in the cell lines used in the study

Cell line	p53 status	N-Myc amp
SH-SY5Y	wt	–
SK-N-BE(2)	C135F	+
SK-N-AS	wt ^a	–
SKN-Fi	M246R	–
SK-N-DZ	wt	+
IMR-32	wt	+
SHEP	wt	–

p53 status is indicated according to ref. 35.

^aC-terminal homozygous deletion.

medium, all other cell lines were maintained in Dulbecco's Modified Eagle Medium. Plasmid DNA and siRNA transfections were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Plasmid encoding p53shRNA was kindly provided by A. Jochemsen (The Netherlands).

Growth suppression assays

For long-term viability assay, 100,000 cells were seeded in 12-well plates, treated with RITA for 2 weeks and stained with crystal violet. For short-term viability assay, 3,000 cells/well were plated in a 96-well plate, treated with RITA for 48 hours, and cell viability was assessed using proliferation reagent WST-1 (Roche) according to the manufacturer's instructions. TUNEL assay was conducted as we previously described (34). Fluorescence-activated cell sorting (FACS) analysis of the propidium iodide-stained cells was conducted as in ref. 29.

Antibodies and Western blotting

The following primary antibodies were used: rabbit polyclonal anti-p53 CM1 was from Novocastor; antibodies for p53 (DO-1, FL393), PARP (H-250), Mcl-1 (S-19), N-Myc (C-19), MDM2 (SMP14), Bax (N-20), Bcl-2 (C-2), from Santa Cruz. Anti-actin (AC15) from SIGMA and anti-p21 (Cip1/waf1) from Nordic Biosite. Antibodies for Noxa and PUMA (Ab-1) from Calbiochem, anti-MDMX (S403) and anti-Wip1 antibodies from Bethyl. Immunoblotting was conducted according to standard procedures.

Chemicals

Pifithrin- α (PFT α), a kind gift from A. Gudkov (USA), was used at 10 μ mol/L 2 hours before RITA treatment. The proteasomal inhibitor MG132 was used at a concentration of 20 μ mol/L. RITA was obtained from National Cancer research Institute (USA).

Quantitative real-time reverse transcriptase (RT)-PCR

Total RNA was extracted and purified with an RNeasy kit (QIAGEN) using the manufacturer's protocol. RNA (5 μ g) was reverse transcribed using a SuperScript First-Strand RT-PCR kit (Invitrogen). Real-time PCR was conducted with SYBR green reagent (Applied Biosystems) according to the manufacturer's protocol. Primers used for real-time RT-PCR were as we previously described (29, 30).

Co-immunoprecipitation

Neuroblastoma cells were treated with 1 μ mol/L RITA and harvested after 24 hours. Lysates (500 μ g) were pre-cleared with Protein A agarose beads and rabbit immunoglobulin G (Santa Cruz Biotechnologies) before immunoprecipitation with anti-p53 antibody FL-393 conjugated to agarose beads (Santa Cruz Biotechnologies). Beads were washed 5 times with IP buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP-40). Bound proteins were detected by Western blotting using MDM2 and MDMX antibodies.

In vivo experiments

The Northern Stockholm Animal Ethical Committee approved all animal studies and animal care was in accordance with the Karolinska Institutet guidelines. SKN-DZ cells (3×10^7) were injected subcutaneously on the left and right flanks of 6- to 8-week-old female severe combined immunodeficient (SCID) mice. Xenografts seemed palpable 7 days after inoculation, at which time the treatment was started. The mice were treated by intraperitoneal injections twice daily with injection of 200 μ L solution containing 10 mg/kg of RITA and 5% dimethyl sulfoxide (DMSO) in PBS or 5% DMSO in PBS for a period of 18 days. Xenograft volumes were measured every day. Animals were sacrificed on the last day of treatment; tumors were extracted, weighed, and photographed. Body weight of mice was measured before and after treatment.

Results

RITA inhibits the growth of neuroblastoma cells

We have previously shown that p53 reactivating compound RITA prevents p53/MDM2 interaction, induces p53 accumulation and activation, and triggers apoptosis in tumor cells of a different origin *in vitro* and *in vivo* (28–32). Here, we tested the effects of RITA in 7 neuroblastoma cell lines, differing in N-Myc and p53 status (Table 1).

Treatment with RITA efficiently suppressed the growth of neuroblastoma cell lines expressing wild-type p53 in a dose-dependent manner, as detected by cell-proliferation assay (Fig. 1A). These include 2 cell lines with amplified N-Myc, SKN-DZ, and IMR32. Furthermore, a long-term viability assay showed that treatment with RITA purged the entire population of neuroblastoma cells, leaving virtually no alive cells after several days of treatment (Fig. 1B).

In addition to the activation of the wtp53 activity, RITA can also restore the activity of mutant p53 in human tumor cells of different origin (31). In line with these results, we found that RITA efficiently inhibited the growth of SKN-BE (2) cells, which express C135F p53 mutant and SKN-FI, carrying M246R p53 mutant, as assessed in short- and long-term viability assays (Fig. 1A and B). In addition, the growth of SKN-AS cell line carrying p53 truncated at its very C-terminus, but retaining partial p53 activity (35), was also inhibited by RITA.

Thus, RITA efficiently suppressed the growth of neuroblastoma cells, carrying both wild-type and mutant p53, with or without N-Myc amplification.

RITA induces apoptosis in neuroblastoma cells

Nutlin3a, an inhibitor of p53/MDM2 interaction, induces a pronounced growth arrest and senescent phenotype in neuroblastoma cells (23). However, we did not observe senescent cells upon treatment with RITA. Microscopy analysis of cell morphology revealed the induction of cell death by RITA in all cell lines tested (Fig. 2A). Furthermore, we detected DNA fragmentation, the hallmark of apoptosis using TUNEL assay (Fig. 2B). Activation of caspases, manifested as induction of PARP cleavage, served as an additional proof of apoptosis. Using immunoblotting,

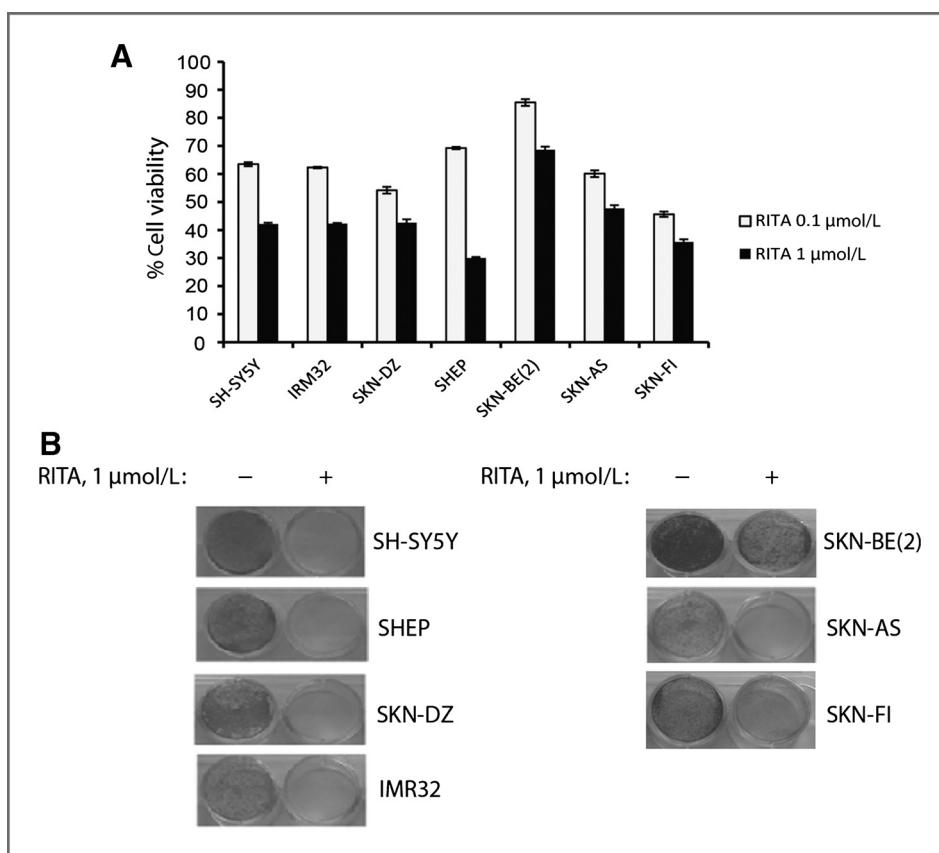


Figure 1. RITA inhibits the growth of neuroblastoma cells. A, B, inhibition of growth of 7 neuroblastoma lines by RITA, as assessed using short-term cell-proliferation assay (48 hr, A) and by long-term viability assay (2 weeks, B).

we showed the induction of PARP cleavage upon RITA in several neuroblastoma lines (Fig. 2C). Appearance of fragmented DNA, another indication of apoptosis, was observed upon FACS analysis of propidium iodide-stained SKN-BE(2) cells (Fig. 2D) and, as shown later, in SHEP and SKN-DZ cells. Taken together, our results strongly suggest that RITA induces neuroblastoma cell death via apoptosis.

RITA disrupts the interaction between p53 and MDM2/MDMX

We have previously shown that RITA induces apoptosis due to disruption of the p53/MDM2 complex (28), and also found similar inhibitory effect on the p53/MDMX complex. In line with these results, we found that RITA significantly decreased the complex formation between p53 and MDM2, as well as between p53 and MDMX, as assessed by coimmunoprecipitation assay (Fig. 3A). These data suggest that in wild type p53 cells the induction of apoptosis upon treatment with RITA is due to the inhibition of interaction between p53 and its negative p53 regulators MDM2 and MDMX.

Apoptosis induced by RITA is p53-dependent

To assess whether apoptosis induced by RITA is p53 dependent, we used 2 different approaches. First, we silenced p53 either by stably expressing p53shRNA in SHEP and SKN-DZ cells or by transient depletion of mutant p53 in SKN-BE(2) cells by pSUPER shp53 transfection. The silenc-

ing of p53 prevents apoptosis induction by RITA, as shown using a short-term viability assay (Fig. 3B, top), FACS analysis (Fig. 3C). Second, we assessed the p53 dependence by using chemical inhibitor of p53 transcriptional function, small molecule PFT α (36). Inhibition of p53 by PFT α before administration of RITA protects SHEP cells from apoptosis (Fig. 3B, bottom left). PFT α also rescued SKN-BE(2) cells carrying mutant p53 (Fig. 3B, bottom right). In addition, as shown below, PARP cleavage in SKN-BE(2) cells was rescued by p53 depletion. Taken together, our results show that apoptosis induced by RITA in neuroblastoma cell lines is triggered by p53. Thus, we set out to explore in more detail the mechanisms of p53-induced apoptosis.

p53 induced by RITA activates the expression of its proapoptotic targets

As expected, we observed the induction of p53 protein levels upon treatment with RITA in all neuroblastoma cell lines, except SKN-BE(2), carrying mutant p53 (Fig. 3D). Moreover, p53 accumulation upon RITA treatment resulted in the induction of p53 targets, the key proapoptotic factors PUMA, Noxa, and Bax, as well as CDK inhibitor p21 (Fig. 3D). These data are in line with the prevention of RITA-mediated apoptosis by RNAi-mediated silencing of p53 and the inhibitor of p53 transcriptional activity PFT α and suggest that p53 activated by RITA is transcriptionally active.

Furthermore, according to qPCR analysis, the expression of several p53 target genes was induced, including proapoptotic

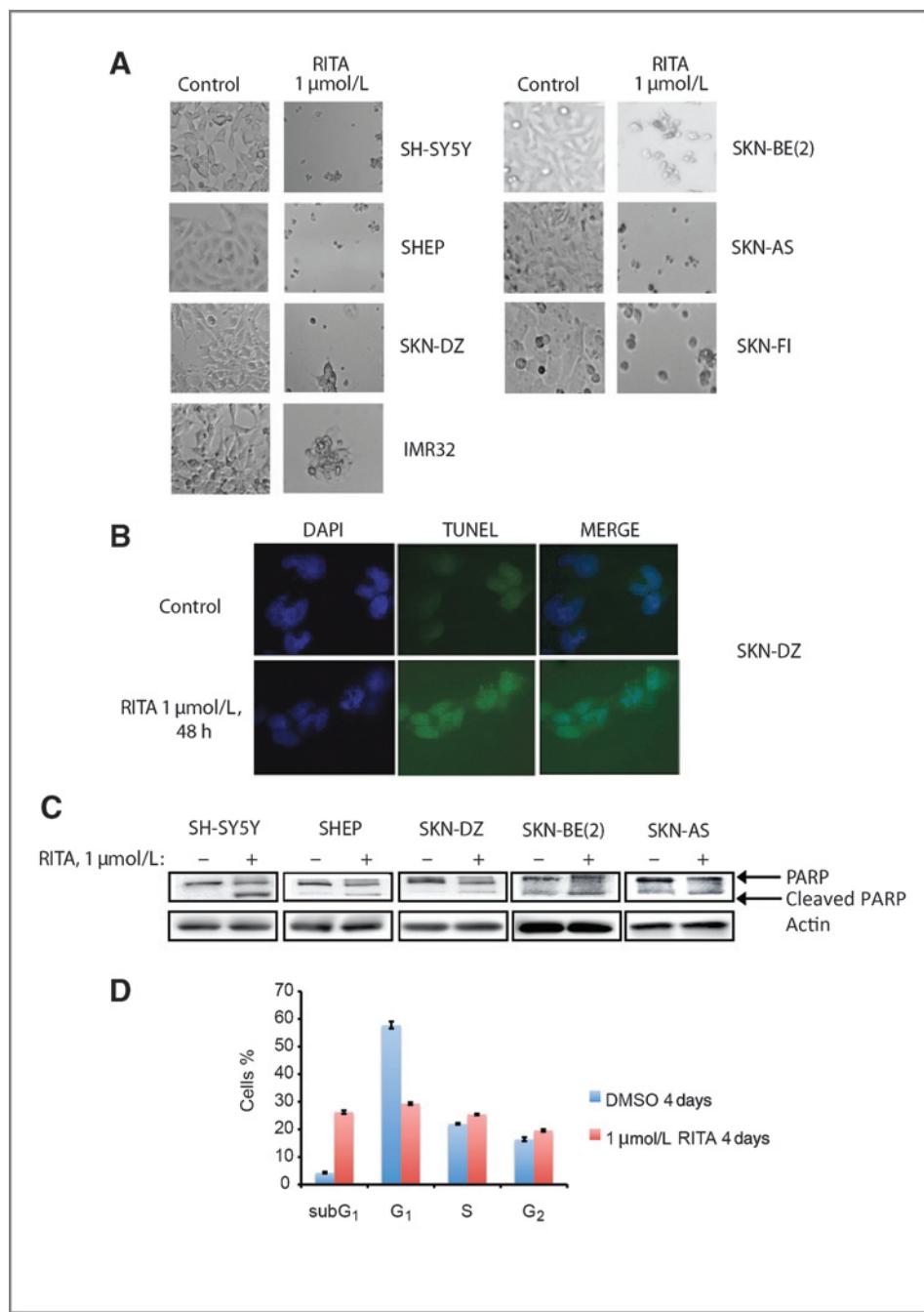


Figure 2. RITA induces apoptosis in neuroblastoma cells. A, induction of cell death in neuroblastoma cell lines was assessed by microscopy analysis. Cells were treated with RITA or DMSO as a control for 48 hours, except SKN-BE(2) cells, which were treated for 4 days, and images were taken under microscope. B, induction of DNA fragmentation by RITA was detected using TUNEL assay in SKN-DZ cells after 48 hours of RITA treatment. C, induction of PARP cleavage upon RITA treatment was assessed by immunoblotting. D, induction of apoptosis in SKN-BE(2) cells upon 4 days treatment with RITA as assessed by FACS analysis of propidium iodide-stained cell

Bax and *BBC3* (encoding PUMA), as well as *CDKN1A* gene encoding CDK inhibitor p21 (Fig. 3D, bottom).

p53 inhibits the expression of N-Myc and several other oncogenic factors important for neuroblastoma growth

Recently we reported a potent inhibition of crucial oncogenes by p53 *in vitro* and *in vivo* upon reactivation by RITA, which includes Mcl-1, Bcl-2, c-Myc, cyclin E, and β -catenin (30), as well as MDM2, MDMX, and Wip1 encoded by PPM1D (33). We found that the inhibition of oncogenes by

p53 reduces the cell's ability to buffer proapoptotic signals and elicits robust apoptosis (30). Thus, we decided to test whether p53 reactivation by RITA can inhibit oncogenes which play important role in neuroblastoma development, including N-Myc, Wip1, Mcl-1, and Bcl-2 (3, 5–7), as well as p53 inhibitors MDM2 and MDMX.

Analysis of protein levels of N-Myc in 3 cell lines carrying *MYCN* amplification, SKN-DZ, SKN-BE(2), and IMR32, revealed a strong downregulation of N-Myc upon RITA (Fig. 4A, top). Downregulation of N-Myc was p53-dependent, as evidenced by a rescue of N-Myc, albeit incomplete,

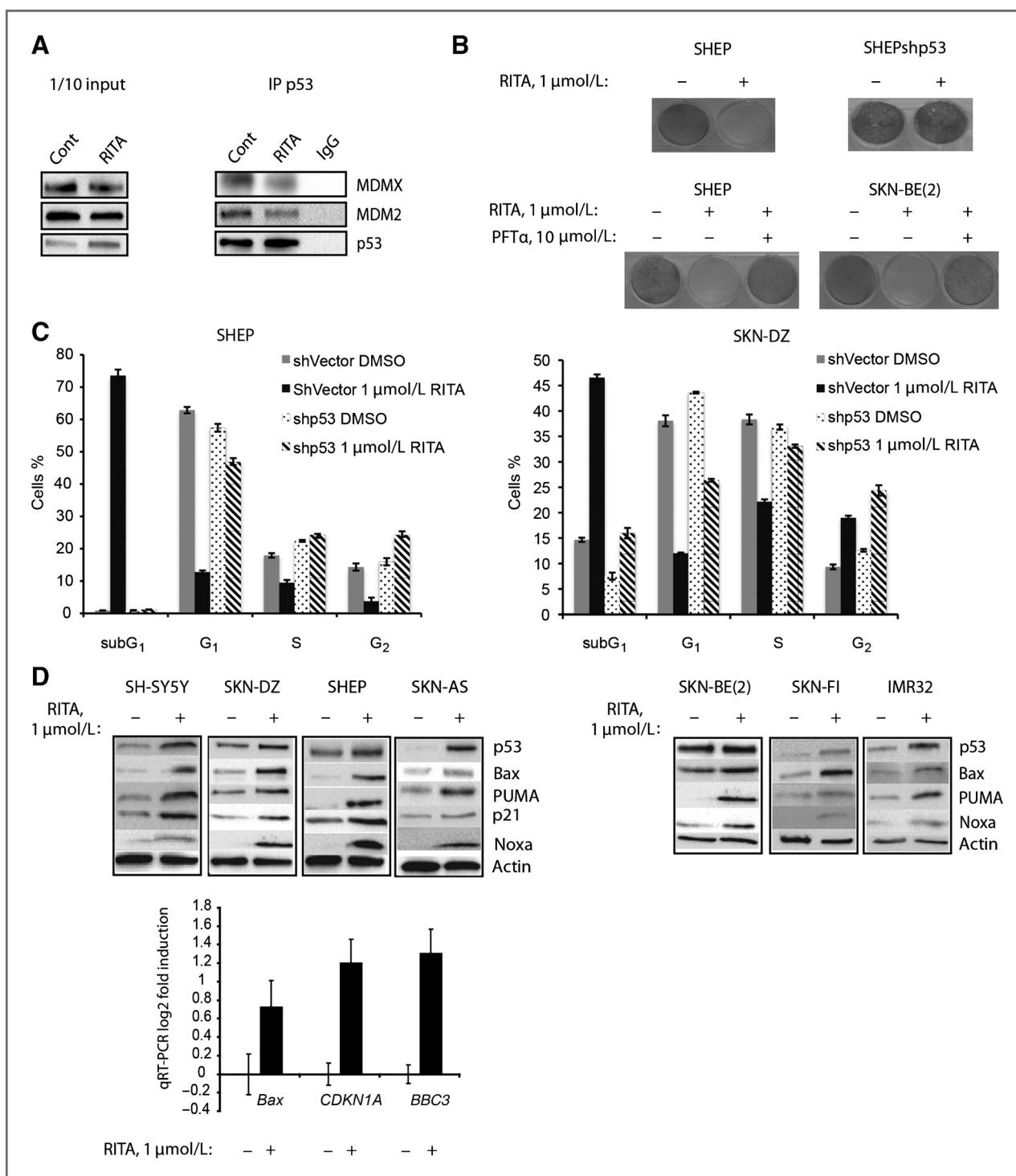


Figure 3. Induction of apoptosis in neuroblastoma cell by RITA is p53 dependent. A, RITA disrupts the interaction between p53 and MDM2/MDMX, as detected by co-immunoprecipitation in SKN-DZ cells followed by Western blotting. B, depletion of p53 by shRNA protects SHEP cells from RITA-induced cell death, as detected by short-term viability assay (top). Inhibition of p53 by pretreatment with PFT α prevents growth suppression by RITA in SHEP and SKN-BE(2) cells, as assessed using short-term viability assay (bottom). C, rescue of apoptosis induced by RITA upon p53 silencing in SHEP (left) and SKN-DZ (right) cell lines as analyzed by FACS of propidium iodide-stained cells. D, induction of p53 and its targets upon 24 hours of RITA treatment, as detected by immunoblotting (top). RITA induces the expression of p53 target genes encoding Bax, Puma (BBC3), and p21 (CDKN1A) in SKN-DZ cells, as detected by qPCR (bottom).

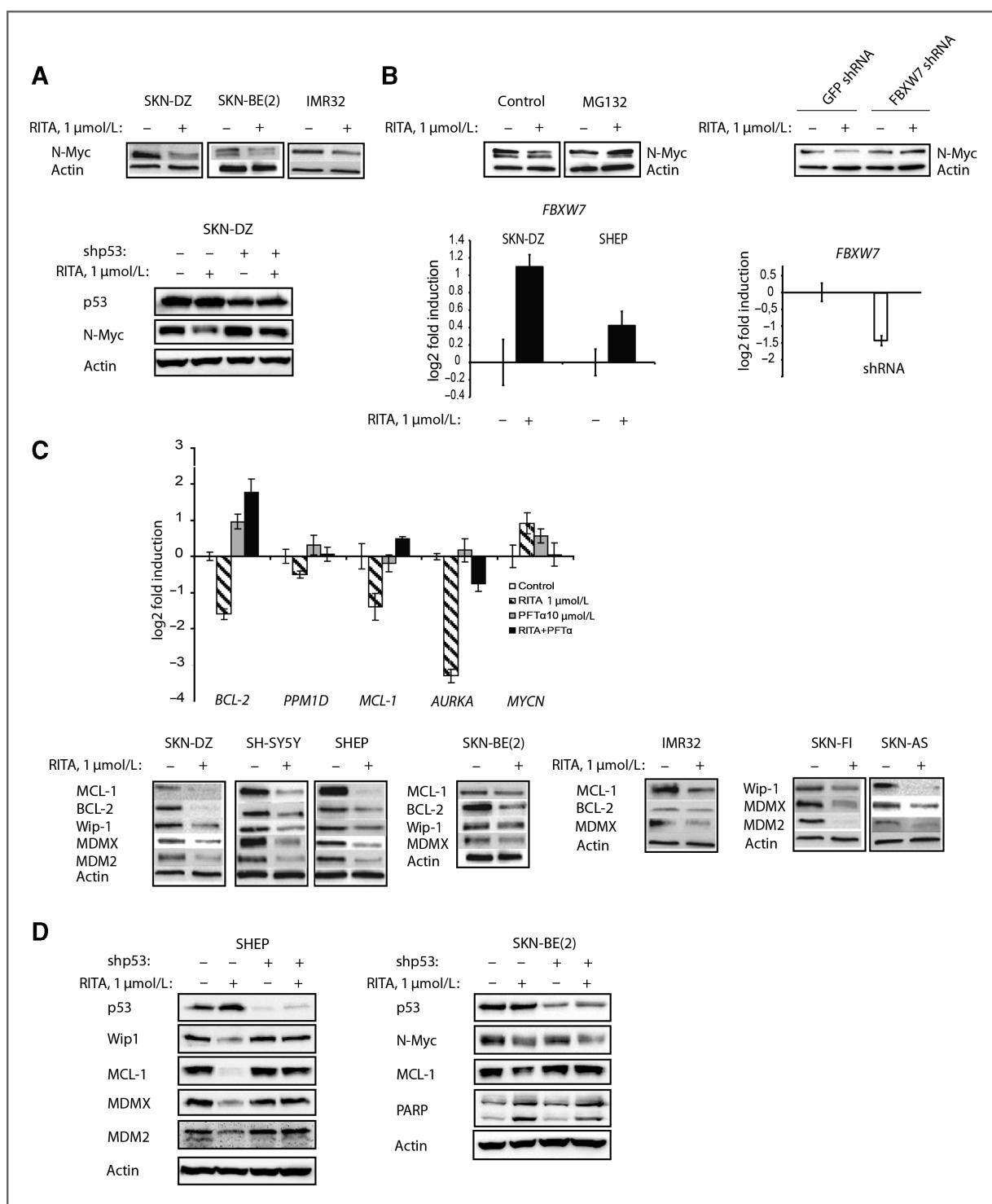


Figure 4. p53 reactivated by RITA inhibits crucial oncogenes in neuroblastoma cells. **A**, decrease of N-Myc protein level in SKN-DZ, SKN-BE(3), and IMR32 cells upon RITA treatment as detected by immunoblotting (top). Partial rescue of N-Myc in SKN-DZ cells upon inhibition of p53 by shRNA as assessed by Western blotting (bottom). **B** (top left), pretreatment with proteasome inhibitor MG132 rescues downregulation of N-Myc protein level by RITA; (top right) depletion of *FBXW7* by shRNA prevented downregulation of N-Myc by RITA, as assayed by immunoblotting; (bottom right) induction of *FBXW7* mRNA level upon RITA treatment, as detected by qPCR; (bottom left) shRNA decreased the level of *FBXW7* mRNA as detected by qPCR. **C**, downregulation of several oncogenes in neuroblastoma cells upon RITA treatment on mRNA and protein level; (top) transcriptional repression of *BCL-2*, *PPM1D*, *MCL-1*, and *AURKA*, but not *MYCN* upon RITA treatment, as assessed by qPCR. Downregulation of these genes was p53 dependent, because it was rescued by pretreatment with p53 inhibitor PFT- α ; (bottom) downregulation of survival oncogenes in neuroblastoma cells upon 24 hours of RITA treatment as detected by immunoblotting. **D**, effect of p53 silencing on downregulation of survival oncogenes in SHEP (left; 8 hours of RITA treatment) and in SKN-BE(2) cells (right; 3 days of RITA treatment).

upon partial silencing of p53 in SKN-DZ cells (Fig. 4A, bottom).

Pretreatment by MG132 rescued N-Myc level upon RITA, suggesting that the decline of N-Myc protein is proteasome dependent (Fig. 4B, top left). In addition, we did not detect a decrease of N-Myc mRNA levels by qPCR (Fig. 4C, top). It has been shown that Fbxw7 E3 ligase ubiquitinates N-Myc and triggers its proteasomal degradation (37). Therefore, we tested whether downregulation of N-Myc is dependent on Fbxw7. Indeed, silencing of the Fbxw7 expression by shRNA prevented N-Myc decline upon RITA (Fig. 4B, right). Moreover, qPCR analysis showed the induction of Fbxw7 mRNA upon RITA treatment (Fig. 4B, bottom left), in line with Fbxw7 being the p53 target gene (30).

Furthermore, we observed the p53-dependent transcriptional repression of AURKA gene, encoding Aurora kinase A (Fig. 4C, top), which we recently identified as a novel p53 target gene (38). It is possible that the transcriptional repression of AURKA encoding Aurora kinase, known to oppose Fbxw7-mediated degradation of N-Myc (37), might also contribute to the degradation of N-Myc upon RITA.

Moreover, in our set of neuroblastoma cell lines p53 activated by RITA triggered a potent decrease of protein levels of several oncogenes implicated in high-risk neuroblastoma, including Bcl-2, Mcl-1, and Wip-1 (Fig. 4C, bottom). In addition, we observed downregulation of the p53 inhibitor MDMX, which cooperates with MDM2 in p53 inhibition. Consistent with downregulation of MDM2 by RITA in other cell types (39), RITA treatment triggered a decline of MDM2 level (Fig. 4C, bottom).

Because p53 activated by RITA has been shown to be a potent transcriptional repressor of a number of genes, including p53 target genes *Bcl-2* and *Mcl-1* (30) and we have recently found that p53 can repress *PPM1D* encoding Wip1 (33), we addressed the question whether p53-mediated downregulation of these oncogenic factors in neuroblastoma is conferred on mRNA level. qPCR analysis showed that the treatment of cells with RITA lead to a decreased levels of *Bcl-2*, *Mcl-1*, and *PPM1D* mRNA (Fig. 4C, top). In contrast, *MDM4* and *MDM2* were not decreased (data not shown). This is in line with our published data that p53 activated by RITA induces degradation of MDMX in Wip1-dependent manner, along with decline of MDM2 (33). The transcriptional repression of oncogenes was p53 dependent, as it was rescued by the p53 inhibitor (Fig. 4C, top) and on protein level by RNAi-mediated silencing of p53 in wild-type and mutant p53 expressing cells SHEP and SKN-BE(2), respectively (Fig. 4D). However, in mutant p53 expressing SKN-BE(2) cells, N-Myc levels were not rescued by p53 silencing (Fig. 4D, right). It is possible that in SKN-BE(2) cells other mechanisms might contribute to N-Myc downregulation.

Strong antitumor effect of RITA in SKN-DZ xenografts in mice

The most rigorous test for the antitumor effect of novel compounds which could predict their potency as possible anticancer drugs is the assessment of their effects *in vivo*. To

study the effects of RITA *in vivo*, we used SKN-DZ xenografts grown in SCID mice. Upon formation of palpable tumors, we injected intraperitoneally 10 mg/kg of RITA or vehicle twice daily. RITA treatment significantly suppressed the growth of neuroblastoma *in vivo*, resulting in a 2-fold decrease in the volume of SKN-DZ xenografts and decrease of the weight of tumors (Fig. 5A–C, left). The substantial reduction of tumor volume caused by RITA was not followed by body weight loss (Fig. 5C, right), suggesting the absence of systemic toxicity. Notably, treatment with RITA decreased microvascular density in some tumors, probably due to the downregulation of N-Myc, known to have strong proangiogenic function (ref. 3; Fig. 5B). Indeed, we observed downregulation of N-Myc, along with the p53 target antiapoptotic factor Mcl-1, in xenograft tumors treated with RITA (Fig. 5D).

Discussion

The relapse and chemoresistance in cancers, including neuroblastoma, is often associated with inactivation of the p53 tumor suppressor. Elegant studies in mice show that reinstatement of p53 causes regression of aggressive metastatic tumors (19, 20). This makes pharmacologic rescue of p53 an attractive strategy to combat cancer. Several compounds are currently undergoing clinical trials: JNJ-26854165 (Johnson & Johnson), PXn727 and PXn822 (Priaxon), RG7112/nutlin3a (F. Hoffmann-la Roche), and PRIMA-1^{MET}/Apr-246 identified by us (22). High attrition rate of novel drugs observed during later stages of clinical trials due to unfavorable pharmacokinetics or toxicity demand the search for novel compounds targeting p53.

Rescue of wild-type p53 in neuroblastoma by nutlin3a has been reported (23, 24), supporting the idea that reactivation of p53 by small molecules could be a good strategy to combat neuroblastoma. Nutlin3a is highly selective: sensitivity to nutlin-3a was highly predictive of absence of p53 mutation (25). However, recent study shows that continuous treatment with nutlin-3a confers selective pressure for p53 mutations, resulting in resistance (27). Moreover, p53-mutated nutlin-3a-resistant neuroblastoma cells display an MDR phenotype (26). Emergence of nutlin3a-resistant clones via *de novo* p53 mutations was observed also in osteosarcoma and colon carcinoma (27). Expression of mutant p53 in neuroblastoma is known to result in establishment of a MDR phenotype (10), thus it is imperative that anticancer drugs and/or their combinations be developed that target both wild-type and mutant p53.

In this study, we report that the small molecule RITA causes disruption of p53/MDM2 and MDMX complex and induces apoptosis in a set of neuroblastoma cell lines. However, in contrast with nutlin-3a, which does not inhibit the growth of mutant p53-expressing neuroblastoma (23), RITA can reactivate mutant p53 in neuroblastoma cell lines.

In our previous study we have shown that RITA binds to the N-terminal domain of p53 and induces a conformational change which propagates from the N-terminus to the core and C-terminal domain. This prevents the binding to

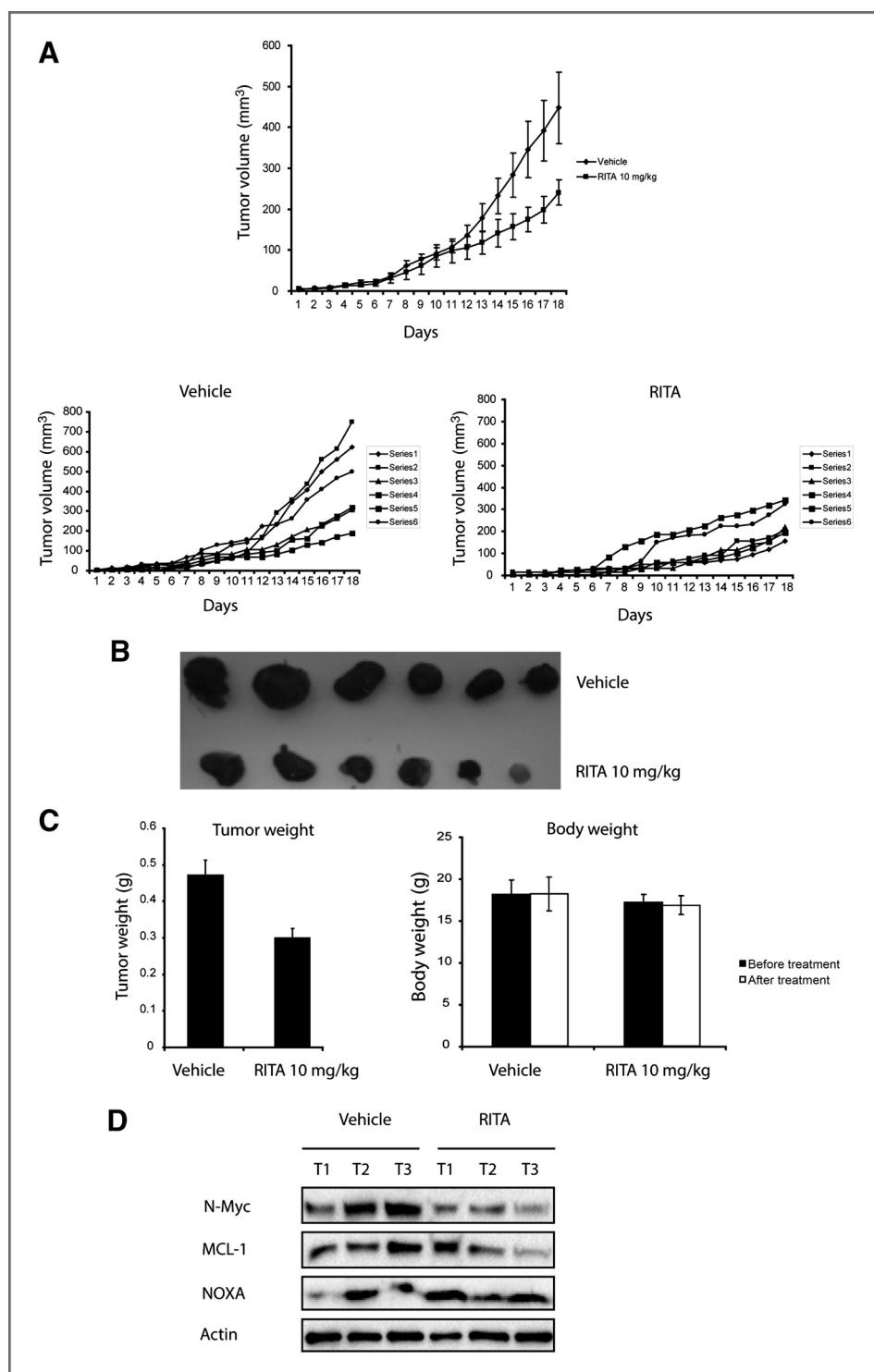


Figure 5. Antitumor effect of RITA in SKN-DZ xenografts in mice. **A** (top), growth of SKN-DZ tumor xenografts *in vivo* upon injection of 10 mg/kg RITA twice daily in comparison to vehicle treatment; (bottom) growth curves of individual tumors upon RITA or vehicle treatment. **B**, pictures taken from excised SKN-DZ tumors treated or nontreated with RITA. **C** (left), comparison of the weight of SKN-DZ tumors treated and nontreated with RITA; (right), body weight of mice before and after treatment with RITA. **D**, treatment with RITA decreased the protein level of N-Myc and MCL-1 *in vivo*, as assessed by immunoblotting.

p53 of several inhibitors, including MDM2, iASPP, Parc, and E6-AP (28, 32). These observations imply that RITA treatment may affect the global folding of the p53 protein and thus might also affect the folding of mutant p53. Indeed, we have found that a broad range of p53 mutants were reactivated by RITA, including several hot spot

mutants (31). Taken together with this study, our results promote the idea of developing compounds capable of simultaneously targeting wild type and mutant p53. This type of compounds should reduce the chance of emergence of *de novo* resistance and enhance clinical success. Indeed, in line with our data on the ability of RITA to reactivate mutant

p53 in neuroblastoma, recent study using UKF-NB-3 neuroblastoma cells as a model does not suggest p53 mutations being the mechanism of acquired resistance to RITA, in contrast to nutlin3a (41). Interestingly, several p53-binding molecules that rescue mutant p53 have been shown to activate the function of wild-type p53 as well. These include CDB3 (42), SCH529074 (43), CP-3139 (44), and PRIMA-1^{MET}/Apr-246 (45). At least some of them seem to inhibit the p53/MDM2 interaction via induction of a conformational change (43), although in most cases the mechanism remains elusive and awaits a detailed investigation.

Amplification of the *MYCN* gene predicts poor prognosis and resistance of neuroblastoma to therapy. Inhibition of N-Myc is therefore regarded as a promising approach for the development of targeted therapies (3). Here, we have identified p53 as a potent inhibitor of N-Myc expression in neuroblastoma. We found that p53 activated by RITA induced the expression of its target Fbxw7, which has a critical function in proteasomal degradation of the N-Myc protein (37). Moreover, we showed that p53 represses the transcription of the antagonist of Fbxw7-mediated degradation of N-Myc, Aurora A (37). Aurora A is a negative prognostic factor and a potential therapeutic target in neuroblastoma (46), which, according to our recent study, is a *bona fide* p53 target (38). In addition, RITA treatment leads to the decrease of MDM2, which upregulates N-Myc (17). Taken together, our data suggest that reactivation of p53 by RITA causes inhibition of N-Myc via induction of its E3 ligase Fbxw7. This might be further facilitated by transcriptional repression of Aurora A and inhibition of MDM2.

It is possible that additional mechanisms of N-Myc inhibition by RITA might exist, as we did not detect N-Myc rescue upon mutant p53 silencing in SKN-BE(2) cells. For example, inhibition of TrxR1 by RITA might play a role (47). We would like to note, however, that the mutant p53 silencing by 4 different RNAi constructs caused SKN-BE(2) cell death, limiting our analysis. We speculate that the survival of SKN-BE(2) cells might depend on mutant p53 expression, due to gain-of-function of mutant p53. This limitation precludes a more vigorous analysis of N-Myc regulation by p53 in SKN-BE(2) cells.

Our study reveals the ability of p53 to unleash the transcriptional repression of several major survival factors in neuroblastoma. Our data suggest that the repression of Bcl-2 and Mcl-1, reducing the cancer cell's ability to buffer proapoptotic signal, might contribute to the robust induction of apoptosis in neuroblastoma by pharmacologically reactivated p53.

Another factor downregulated in neuroblastoma cells by RITA-reactivated p53 is Wip1, encoded by the *PPM1D* gene at 17q, whose gain is associated with poor prognosis in neuroblastoma (5). Wip1 interferes with the DNA damage response and p53 activation by dephosphorylating crucial effectors, thus conferring resistance to standard treatments. It is overexpressed in different cancers and is important for the survival of tumor stem cells, which makes the development of Wip1 inhibitors an attractive strategy for therapy (48). The multitude of oncogenes, inhibited by

RITA-reactivated p53 creates a robust p53 response. It might allow p53 to cope with the daunting challenge of anticancer therapy—multiple genetic abnormalities in individual cancers. Because tumors are often "addicted" to the oncogenes, such as increased expression of N-Myc, Wip1, Aurora A, Bcl-2, or Mcl-1, their inhibition might be an essential component of anticancer therapies targeting p53. Thus, the ability of reactivated p53 to inhibit several key oncogenes in neuroblastoma adds a new dimension to the mechanism of tumor suppression upon p53 activation by small molecules.

RITA efficiently inhibited the growth of neuroblastoma tumor xenografts without the apparent toxicity. Notably, the morphology of tumors suggests that reactivation of p53 by RITA is able to inhibit the growth of tumors' blood vessels, in line with inhibition of potent proangiogenic factor N-Myc and previous studies suggesting that p53 can affect the transcription of several genes involved in angiogenesis (49). The effect of RITA on tumor blood vessels is very interesting and will be investigated further. Although we did not attempt to maximize the therapeutic response *in vivo*, it is conceivable that the dosing regimen and the schedule of treatment could be improved, for example, by the administration of higher dose (50–100 mg/kg, shown previously to be safe in mice; ref. 50).

In conclusion, we showed that RITA is efficient and potent activator of both wild-type and mutant p53 and inducer of p53-dependent apoptosis in neuroblastoma *in vitro* and *in vivo*. Ablation of oncogenes driving neuroblastoma, in particular, N-Myc, by pharmacologically reactivated p53 might be a very important factor for future application of p53-based therapy in neuroblastoma. Our study provides further support for the notion of using molecules reactivating p53 to combat neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Burmakin, Y. Shi, P. Kogner, G. Selivanova
Development of methodology: M. Burmakin, Y. Shi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Burmakin, Y. Shi, E. Hedström
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Burmakin, Y. Shi, E. Hedström, G. Selivanova
Writing, review, and/or revision of the manuscript: M. Burmakin, Y. Shi, P. Kogner, G. Selivanova
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Selivanova
Study supervision: G. Selivanova

Grant Support

This study was funded by the Swedish Cancer Foundation, Swedish Childhood Cancer Foundation, the Swedish Research Council, the Ragnar Söderberg's Foundation, and the Karolinska Institutet (ACT! Medical Theme Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 11, 2012; revised June 4, 2013; accepted June 28, 2013; published OnlineFirst July 3, 2013.

References

- Tonini GP, Boni L, Pession A, Rogers D, Iolascon A, Basso G, et al. MYCN oncogene amplification in neuroblastoma is associated with worse prognosis, except in stage 4s: the Italian experience with 295 children. *J Clin Oncol* 1997;15:85–93.
- Canete A, Gerrard M, Rubie H, Castel V, Di Cataldo A, Munzer C, et al. Poor survival for infants with MYCN-amplified metastatic neuroblastoma despite intensified treatment: the International Society of Paediatric Oncology European Neuroblastoma Experience. *J Clin Oncol* 2009;27:1014–9.
- Johnsen JL, Kogner P, Albihn A, Henriksson MA. Embryonal neural tumours and cell death. *Apoptosis* 2009;14:424–38.
- Wagner LM, Danks MK. New therapeutic targets for the treatment of high-risk neuroblastoma. *J Cell Biochem* 2009;107:46–57.
- Saito-Ohara F, Imoto I, Inoue J, Hosoi H, Nakagawara A, Sugimoto T, et al. PPM1D is a potential target for 17q gain in neuroblastoma. *Cancer Res* 2003;63:1876–83.
- Lestini BJ, Goldsmith KC, Fluchel MN, Liu X, Chen NL, Goyal B, et al. Mcl1 downregulation sensitizes neuroblastoma to cytotoxic chemotherapy and small molecule Bcl2-family antagonists. *Cancer Biol Ther* 2009;8:1587–95.
- Bettayeb K, Baunbaek D, Delehouze C, Loaec N, Hole AJ, Baumli S, et al. CDK inhibitors roscovitine and CR8 trigger Mcl-1 down-regulation and apoptotic cell death in neuroblastoma cells. *Genes Cancer* 2010;1:369–80.
- Keshelava N, Zuo JJ, Chen P, Waidyaratne SN, Luna MC, Gomer CJ, et al. Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. *Cancer Res* 2001;61:6185–93.
- Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J. Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. *Cancer Res* 2001;61:8–13.
- Xue C, Haber M, Flemming C, Marshall GM, Lock RB, MacKenzie KL, et al. p53 determines multidrug sensitivity of childhood neuroblastoma. *Cancer Res* 2007;67:10351–60.
- Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell* 2009;137:413–31.
- Bernal F, Wade M, Godes M, Davis TN, Whitehead DG, Kung AL, et al. A stapled p53 helix overcomes HDMX-mediated suppression of p53. *Cancer Cell* 2010;18:411–22.
- Wolter J, Angelini P, Irwin M. p53 family: therapeutic targets in neuroblastoma. *Future Oncol* 2010;6:429–44.
- Corvi R, Savelyeva L, Breit S, Wenzel A, Handgretinger R, Barak J, et al. Non-syntenic amplification of MDM2 and MYCN in human neuroblastoma. *Oncogene* 1995;10:1081–6.
- Caren H, Erichsen J, Olsson L, Enerback C, Sjoberg RM, Abrahamsson J, et al. High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene. *BMC Genom* 2008;9:353.
- Slack A, Chen Z, Tonelli R, Pule M, Hunt L, Pession A, et al. The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. *Proc Natl Acad Sci U S A* 2005;102:731–6.
- Gu L, Zhang H, He J, Li J, Huang M, Zhou M. MDM2 regulates MYCN mRNA stabilization and translation in human neuroblastoma cells. *Oncogene* 2012;31:1342–53.
- Selivanova G. Therapeutic targeting of p53 by small molecules. *Semin Cancer Biol* 2010;20:46–56.
- Feldser DM, Kostova KK, Winslow MM, Taylor SE, Cashman C, Whitaker CA, et al. Stage-specific sensitivity to p53 restoration during lung cancer progression. *Nature* 2010;468:572–5.
- Junttila MR, Karnezis AN, Garcia D, Madriles F, Kortlever RM, Rostker F, et al. Selective activation of p53-mediated tumour suppression in high-grade tumours. *Nature* 2010;468:567–71.
- Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8.
- Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 2002;8:282–8.
- Van Maerken T, Speleman F, Vermeulen J, Lambertz I, De Clercq S, De Smet E, et al. Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma. *Cancer Res* 2006;66:9646–55.
- Van Maerken T, Ferdinand L, Taildean J, Lambertz I, Yigit N, Vercruyse L, et al. Antitumor activity of the selective MDM2 antagonist nutlin-3 against chemoresistant neuroblastoma with wild-type p53. *J Natl Cancer Inst* 2009;101:1562–74.
- Van Maerken T, Rihani A, Dreida D, De Clercq S, Yigit N, Marine JC, et al. Functional analysis of the p53 pathway in neuroblastoma cells using the small-molecule MDM2 antagonist nutlin-3. *Mol Cancer Ther* 2011;10:983–93.
- Michaelis M, Rothweiler F, Barth S, Cinatl J, van Rikxoort M, Loschmann N, et al. Adaptation of cancer cells from different entities to the MDM2 inhibitor nutlin-3 results in the emergence of p53-mutated multi-drug-resistant cancer cells. *Cell Death Dis* 2011;2:e243.
- Aziz MH, Shen H, Maki CG. Acquisition of p53 mutations in response to the non-genotoxic p53 activator Nutlin-3. *Oncogene* 2011;30: 4678–86.
- Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M, et al. Small molecule RITA binds to p53, blocks p53-HDM2 interaction and activates p53 function in tumors. *Nat Med* 2004;10: 1321–8.
- Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A, Selivanova G. MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer Cell* 2009;15:171–83.
- Grinkevich VV, Nikulenkov F, Shi Y, Enge M, Bao W, Maljukova A, et al. Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis. *Cancer Cell* 2009;15:441–53.
- Zhao CY, Grinkevich VV, Nikulenkov F, Bao W, Selivanova G. Rescue of the apoptotic-inducing function of mutant p53 by small molecule RITA. *Cell Cycle* 2010;9:1847–55.
- Zhao CY, Szekely L, Bao W, Selivanova G. Rescue of p53 function by small-molecule RITA in cervical carcinoma by blocking E6-mediated degradation. *Cancer Res* 2010;70:3372–81.
- Spinnler C, Hedstrom E, Li H, de Lange J, Nikulenkov F, Teunisse AF, et al. Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction via activation of ATM and inhibition of HdmX. *Cell Death Differ* 2011;18:1736–45.
- Selivanova G, Lotsova V, Okan I, Fritzsche M, Strom M, Groner B, et al. Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. *Nat Med* 1997;3:632–8.
- Nakamura Y, Ozaki T, Niizuma H, Ohira M, Kamijo T, Nakagawara A. Functional characterization of a new p53 mutant generated by homozygous deletion in a neuroblastoma cell line. *Biochem Biophys Res Commun* 2007;354:892–8.
- Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 1999;285:1733–7.
- Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N, et al. Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma. *Cancer Cell* 2009;15:67–78.
- Nikulenkov F, Spinnler C, Li H, Tonelli C, Shi Y, Turunen M, et al. Insights into p53 transcriptional function via genome-wide chromatin occupancy and gene expression analysis. *Cell Death Differ* 2012;19: 1992–2002.
- Rinaldo C, Prodromo A, Siepi F, Moncada A, Sacchi A, Selivanova G, et al. HIPK2 regulation by MDM2 determines tumor cell response to the p53-reactivating drugs nutlin-3 and RITA. *Cancer Res* 2009;69: 6241–8.
- Shen H, Moran DM, Maki CG. Transient nutlin-3a treatment promotes 723 endoreduplication and the generation of therapy-resistant tetraploid 724 cells. *Cancer Res* 2008;68:8260–8.
- Michaelis M, Rothweiler F, Agha B, Barth S, Voges Y, Loschmann N, et al. Human neuroblastoma cells with acquired resistance to the p53 720 activator RITA retain functional p53 and sensitivity to other p53 721 activating agents. *Cell Death Dis* 2012;3:e294.

42. Issaeva N, Friedler A, Bozko P, Wiman KG, Fersht AR, Selivanova G. Rescue of mutants of the tumor suppressor p53 in cancer cells by a designed peptide. *Proc Natl Acad Sci U S A* 2003;100:13303–7.
43. Demma M, Maxwell E, Ramos R, Liang L, Li C, Hesk D, et al. SCH529074, a small molecule activator of mutant p53, which binds p53 DNA binding domain (DBD), restores growth-suppressive function to mutant p53 and interrupts HDM2-mediated ubiquitination of wild type p53. *J Biol Chem* 2010;285:10198–212.
44. Wang W, Takimoto R, Rastinejad F, El-Deiry WS. Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. *Mol Cell Biol* 2003;23:2171–81.
45. Bao W, Chen M, Zhao X, Kumar R, Spinnler C, Thullberg M, et al. PRIMA-1 (Met)APR-246 induces wild-type p53-dependent suppression of malignant melanoma tumor growth in 3D culture and *in vivo*. *Cell Cycle* 2011;10:301–7.
46. Shang X, Burlingame SM, Okcu MF, Ge N, Russell HV, Egler RA, et al. Aurora A is a negative prognostic factor and a new therapeutic target in human neuroblastoma. *Mol Cancer Ther* 2009;8:2461–9.
47. Hedstrom E, Eriksson S, Zawacka-Pankau J, Arner ES, Selivanova G. p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA. *Cell Cycle* 2009;8:3576–83.
48. Le Guezenec X, Bulavin DV. WIP1 phosphatase at the crossroads of cancer and aging. *Trends Biochem Sci* 2010;35:109–14.
49. Teodoro JG, Evans SK, Green MR. Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med (Berl)* 2007; 85:1175–86.
50. Nieves-Neira W, Rivera MI, Kohlhagen G, Hursey ML, Pourquier P, Sausville EA, et al. DNA protein cross-links produced by NSC 652287, a novel thiophene derivative active against human renal cancer cells. *Mol Pharmacol* 1999;56:478–84.