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.

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, 17177 Stockholm, Sweden

E-mail: galina.selivanova@ki.se

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Abstract

Purpose: Restoration of the p53 function in tumours is a promising therapeutic strategy due to the high potential of p53 as tumour suppressor and the fact that established tumours depend on p53 inactivation for their survival. Here, we addressed the question whether small molecule RITA can reactivate p53 in neuroblastoma (NB) and suppress the growth of NB 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 NB, using cell viability assays, RNAi silencing, co-immunoprecipitation, qPCR and Western blotting analysis. In vivo experiments were performed to study the effect of RITA on human NB xenografts in mice.

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

Conclusions: Reactivation of wild type and mutant p53 resulting in the induction of pro-apoptotic factors along with ablation of key oncogenes by compounds such as RITA may be a highly effective strategy to treat NB.

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 NB survival, including N-Myc, the driving oncogene in NB. Inhibition of oncogenes by p53 may thus constitute a new therapeutic approach for high risk NBs. 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 NB, it would be highly relevant to implement this strategy in the clinic.
Introduction

Neuroblastoma 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 NB pathways and key molecules for its growth and progression.

Among the diversity of genetic variations in NB, 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 NB cells using different approaches showed encouraging results and provides a promising treatment strategy (3). In addition, several other oncogenes have been implicated in NB 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 anti-apoptotic factors Mcl-1 and Bcl-2 and resistance to therapy in NB (6). Mcl-1 depletion via RNA interference induced apoptosis in NB cell lines and sensitized them to cytotoxic chemotherapy, suggesting that Mcl-1, as well as Bcl-2, might be promising targets for NB treatment (6,7).

Notably, chemotherapy-resistant NB often express p53 inactivated by a point mutation (8,9,10). 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 tumours, either by point mutation of the gene, or via its inhibitors, mainly MDM2 and MDMX. MDM2 ubiquitinylates p53 and marks
it for destruction by the proteasome, thus keeping p53 at bay in the absence of stress (11). MDMX is a paralog of MDM2 required for the efficient inhibition of p53 by MDM2, but 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 tumour suppression, as well as for response to anticancer therapy in different types of cancer including high risk NB (13).

p53 dysfunction in NB 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 NB, 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 demonstrated that reinstatement of p53 has much more profound tumour suppressor effects in aggressive, metastatic tumours (19,20). These data greatly encouraged us to explore the effect of p53-reactivating molecules in NB.

Several p53 reactivating molecules have been developed and at least two 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<sup>MET</sup>/APR-246, identified by us (22). Nutlin3a has been shown to activate p53-dependent growth suppression in NB carrying wild type 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 NB (25).
However, recent studies demonstrate that treatment with nutlin3a creates a selective pressure for p53 mutations in NB 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 (28,29,30,31). Notably, RITA can also reactivate mutant p53, probably because RITA treatment impinges on p53 conformation (32). Further, reactivation of p53 by RITA leads to the ablation of survival signalling in cancer cells via downregulation of Myc, Bcl-2, Mcl-1, Wip-1, 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 NB.

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

**Materials and Methods**

**Cell lines.**

NB 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 medium, all other cell lines
were maintained in DMEM. Plasmid DNA and siRNA transfections were performed 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 short-term viability assay, 100000 cells were seeded in 12-well plates, treated with RITA for 48 hours and stained with crystal violet. For long-term viability assay, 3000 cells/well were plated in a 96-well plate, treated with RITA for 2 weeks, and cell viability was assessed using proliferation reagent WST-1 (Roche) according to the manufacturer’s instructions. TUNEL assay was performed as we previously described (34). FACS analysis of the PI stained cells was performed as in (29).

**Antibodies and Western blotting.**

The following primary antibodies were used: rabbit polyclonal anti-p53 CM1 was from Novocasta; antibodies for p53 (DO-1, FL393), PARP (H-250), Mcl-1 (S-19), N-Myc (C-19), MDM2 (SMP14), Bax (N-20), Bel-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 performed according to standard procedures.

**Chemicals.**

Pifithrin-α (PFTα) a kind gift from A. Gudkov (USA), was used at 10 μM two hours prior to RITA treatment. The proteasomal inhibitor MG132 was used at a concentration of 20 μM. RITA was obtained from National Cancer research Institute, USA.

**Quantitative real-time RT-PCR.**
Total RNA was extracted and purified with an RNeasy kit (QIAGEN) using the manufacturer's protocol. RNA (5 μg) was reverse transcribed using a SuperScript First-Strand RT-PCR kit (Invitrogen). Real-time PCR was performed 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.**

NB cells were treated with 1 μM RITA and harvested after 24 h. Lysates (500 μg) were pre-cleared with Protein A agarose beads and rabbit IgG (Santa Cruz Biotechnologies) prior to immunoprecipitation with anti-p53 antibody FL-393 conjugated to agarose beads (Santa Cruz Biotechnologies). Beads were washed five times with IP Buffer (50 mM Tris, pH7.5, 5 mM EDTA, 150 mM 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 × 10⁷) were injected subcutaneously on the left and right flanks of 6- to 8-week-old female severe combined immunodeficient (SCID) mice. Xenografts appeared palpable 7 days after inoculation, at which time the treatment was started. The mice were treated by intra-peritoneal injections twice daily with injection of 200 μL solution containing 10 mg/kg of RITA and 5%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; tumours were extracted, weighted and photographed. Body weight of mice was measured before and after treatment.

**Results**
RITA inhibits the growth of NB cells.

We have previously shown that p53 reactivating compound RITA prevents p53/MDM2 interaction, induces p53 accumulation and activation and triggers apoptosis in tumour cells of a different origin in vitro and in vivo (28,29,30,31). Here, we tested the effects of RITA in seven NB cell lines, differing in N-Myc and p53 status (Table 1).

Treatment with RITA efficiently suppressed the growth of NB cell lines expressing wild type (wt) p53 in a dose-dependent manner, as detected by cell proliferation assay (Fig. 1A). These include two 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 NB 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 tumour 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 NB cells, carrying both wt and mutant p53, with or without N-Myc amplification.

RITA induces apoptosis in NB cells.
Nutlin3a, an inhibitor of p53/MDM2 interaction, induces a pronounced growth arrest and senescent phenotype in NB 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). Further, 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, we showed the induction of PARP cleavage upon RITA in several NB lines (Fig. 2C). Appearance of fragmented DNA, another indication of apoptosis, was observed upon FACS analysis of PI stained SKN-BE(2) cells (Fig. 2D) and, as shown below, in SHEP and SKN-DZ cells. Taken together, our results strongly suggest that RITA induces NB 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 co-immunoprecipitation assay (Fig. 3A). These data suggest that 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.*

In order to assess whether apoptosis induced by RITA is p53-dependent, we used two 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 silencing of p53 prevents apoptosis induction by RITA, as shown using a short-term
viability assay (Fig. 3B, upper panels), FACS analysis (Fig. 3C) and, as shown below, also PARP cleavage. Second, we assessed the p53-dependence by using chemical inhibitor of p53 transcriptional function, small molecule Pifithrin-α (36). Inhibition of p53 by Pifithrin-α prior to administration of RITA protects SHEP cells from apoptosis (Fig. 3B, lower left panel). Pifithrin-α also rescued SKN-BE(2) cells carrying mutant p53 (Fig. 3B, lower right panel). In addition, as shown below, PARP cleavage in SKN-BE(2) cells was rescued by p53 depletion. Taken together, our results demonstrate that apoptosis induced by RITA in NB 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 pro-apoptotic targets*

As expected, we observed the induction of p53 protein levels upon treatment with RITA in all NB cell lines, except in SKN-BE(2), carrying mutant p53 (Fig. 3D). Moreover, p53 accumulation upon RITA treatment resulted in the induction of p53 targets, the key pro-apoptotic 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 Pifithrin-α and suggest that p53 activated by RITA is transcriptionally active.

Further, according to qPCR analysis, the expression of several p53 target genes was induced, including pro-apoptotic Bax and BBC3 (encoding PUMA), as well as CDKN1A gene encoding CDK inhibitor p21 (Fig. 3D, lower panel).

*p53 inhibits the expression of N-Myc and several other oncogenic factors important for NB 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 PPMID (33). We found that the inhibition of oncogenes by p53 reduces the cell’s ability to buffer pro-apoptotic signals and elicits robust apoptosis (30). Thus we decided to test whether p53 reactivation by RITA can inhibit oncogenes which play important role in NB development, including N-Myc, Wip1, Mcl-1 and Bcl-2 (3,5,6,7), as well as p53 inhibitors MDM2 and MDMX.

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

Pretreatment by MG132 rescued N-Myc level upon RITA, suggesting that the decline of N-Myc protein is proteasome-dependent (Fig. 4B, upper left panel). In addition, we did not detect a decrease of N-Myc mRNA levels by qPCR (Fig. 4C, upper panel). 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 panels). Moreover, qPCR analysis demonstrated the induction of Fbxw7 mRNA upon RITA treatment (Fig. 4B, lower left panel), in line with Fbxw7 being the p53 target gene (30).

Further, we observed the p53-dependent transcriptional repression of AURKA gene, encoding Aurora kinase A (Fig. 4C, upper panel), 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 NB cell lines p53 activated by RITA triggered a potent decrease of protein levels of several oncogenes implicated in high risk NB, including Bcl-2, Mcl-1, and Wip-1 (Fig. 4C, lower panels). 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, lower panels).

Since 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 NB is conferred on mRNA level. qPCR analysis demonstrated that treatment of cells with RITA lead to decreased levels of Bcl-2, Mcl-1 and PPM1D mRNA (Fig. 4C, upper panel). In contrast, mRNA levels of MDMX 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 Wip-1-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, upper panel) 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 panel). It is possible, that in SKN-BE(2) cells other mechanisms might contribute to N-Myc downregulation.

*Strong anti-tumour effect of RITA in SKN-DZ xenografts in mice.*
The most rigorous test for the anti-tumour effect of novel compounds which could predict their potency as possible anti-cancer 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 tumours we injected intra-peritonealy (i.p.) 10 mg/kg of RITA or vehicle twice daily. RITA treatment significantly suppressed the growth of NB in vivo, resulting in a two-fold decrease in the volume of SKN-DZ xenografts and decrease of the weight of tumours (Fig. 5A, 5B and 5C, left panel). The substantial reduction of tumour volume caused by RITA was not followed by body weight loss (Fig. 5C, right panel), suggesting the absence of systemic toxicity. Notably, treatment with RITA decreased microvascular density in some tumours, probably due to the downregulation of N-Myc, known to have strong pro-angiogenic function (3) (Fig. 5B). Indeed, we observed downregulation of N-Myc, along with the p53 target anti-apoptotic factor Mcl-1, in xenograft tumours treated with RITA (Fig. 5D).

Discussion

The relapse and chemoresistance in cancers, including NB, is often associated with inactivation of the p53 tumor suppressor. Elegant studies in mice demonstrate that re-instatement of p53 causes regression of aggressive metastatic tumours (19,20). This makes pharmacological rescue of p53 an attractive strategy to combat cancer. Several compounds are currently undergoing clinical trials: JnJ-26854165 (Johnson & Johnson, USA), PXn727 and PXn822 (Priaxon, Munich, Germany), RG7112/nutlin3a (F.Hoffmann–la Roche, Basel, Switzerland) and PRIMA-1MET/Apr-246 identified by us (22). High attrition rate of novel drugs observed during later stages of clinical trials due to unfavourable pharmacokinetics or toxicity demand the search for novel compounds targeting p53.
Rescue of wild type p53 in NB by nutlin3a has been reported (23,24), supporting the idea that reactivation of p53 by small molecules could be a good strategy to combat NB. 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 NB cells display a multi-drug-resistant (MDR) phenotype (26). Emergence of nutlin3a-resistant clones via de novo p53 mutations was observed also in osteosarcoma and colon carcinoma (27,40). Expression of mutant p53 in NB 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 the current study, we report that the small molecule RITA causes disruption of p53/MDM2 and MDMX complex and induces apoptosis in a set of NB cell lines. However, in contrast with nutlin-3a, which does not inhibit the growth of mutant p53-expressing NB (23), RITA can reactivate mutant p53 in NB 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 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 NB, recent study using UKF-NB-3 NB 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\textsuperscript{MET}/Apr-246 (45). At least some of them appear 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 \textit{MYCN} gene predicts poor prognosis and resistance of NB 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 NB. 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 NB (46), which, according to our recent study, is a \textit{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 four 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 NB. Our data suggest that the repression of Bcl-2 and Mcl-1, reducing the cancer cell's ability to buffer pro-apoptotic signal, might contribute to the robust induction of apoptosis in NB by pharmacologically reactivated p53.

Another factor downregulated in NB cells by RITA-reactivated p53 is Wip1, encoded by the PPM1D gene at 17q, whose gain is associated with poor prognosis in NB (5). Wip1 interferes with the DNA damage response and p53 activation by de-phosphorylating crucial effectors, thus conferring resistance to standard treatments. It is overexpressed in different cancers and is important for the survival of tumour 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 anti-cancer therapy – multiple genetic abnormalities in individual cancers. Since 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 anti-cancer therapies targeting p53. Thus, the ability of reactivated p53 to inhibit several key oncogenes in NB adds a new dimension to the mechanism of tumor suppression upon p53 activation by small molecules.

RITA efficiently inhibited the growth of NB tumour xenografts, without the apparent toxicity. Notably, the morphology of tumours suggest that reactivation of p53 by RITA is able to inhibit the growth of tumours’ blood vessels, in line with inhibition of potent pro-angiogenic 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 tumour 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 (50).

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

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References


Figure legends

Figure 1. RITA inhibits the growth of NB cells. (A,B) Inhibition of growth of seven NB lines by RITA, as assessed using short-term cell proliferation assay (48h, A) and by long-term viability assay (2 weeks, B).

Figure 2. RITA induces apoptosis in NB cells. (A) Induction of cell death in NB 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 48h 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 fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI)-stained cell.

**Figure 3.** Induction of apoptosis in NB 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 (upper panel). Inhibition of p53 by pre-treatment with Pifithrin-α prevents growth suppression by RITA in SHEP and SKN-BE(2) cells, as assessed using short-term viability assay (lower panels). (C) Rescue of apoptosis induced by RITA upon p53 silencing in SHEP (left panel) and SKN-DZ (right panel) cell lines as analysed by FACS of PI-stained cells. (D) Induction of p53 and its targets upon 24 hr of RITA treatment, as detected by immunoblotting (upper panels). RITA induces the expression of p53 target genes encoding Bax, Puma (*BBC3*) and p21 (*CDKN1A*) in SKN-DZ cells, as detected by qPCR (lower panel).

**Figure 4.** p53 reactivated by RITA inhibits crucial oncogenes in NB cells. (A) Decrease of N-Myc protein level in SKN-DZ, SKN-BE(3) and IMR32 cells upon RITA treatment as detected by immunoblotting (upper panel). Partial rescue of N-Myc in SKN-DZ cells upon inhibition of p53 by shRNA as assessed by Western blotting (lower panel). (B) Upper left panel: Pre-treatment with proteasome inhibitor MG132 rescues downregulation of N-Myc protein level by RITA. Upper right panel: Depletion of Fbxw7 by shRNA prevented downregulation of N-Myc by RITA, as assayed by immunoblotting. Lower left panel: Induction of *FBXW7* mRNA level upon RITA treatment, as detected by qPCR. Lower right panel: shRNA decreased the level of *FBXW7* mRNA as detected by qPCR. (C) Downregulation of several oncogenes in NB cells upon RITA treatment on mRNA and protein level. Upper panel: 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, since it was rescued by pre-treatment.
with p53 inhibitor PFT-α. Lower panels: Downregulation of survival oncogenes in NB cells upon 24 h of RITA treatment as detected by immunoblotting. (D) Effect of p53 silencing on downregulation of survival oncogenes in SHEP (left panel, 8 h of RITA treatment) and in SKN-BE(2) cells (right panel, 3 days of RITA treatment).

**Figure 5.** Anti-tumour effect of RITA in SKN-DZ xenografts in mice. (A) Upper panel: Growth of SKN-DZ tumour xenografts *in vivo* upon injection of 10 mg/kg RITA twice daily in comparison to vehicle treatment. Lower panels: Growth curves of individual tumours upon RITA or vehicle treatment. (B) Pictures taken from excised SKN-DZ tumours treated or non-treated with RITA. (C) Left panel: Comparison of the weight of SKN-DZ tumours treated and non-treated with RITA. Right panel: 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.

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

<table>
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<th>Cell line</th>
<th>p53 status</th>
<th>N-myc amp</th>
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<tr>
<td>SH-SY5Y</td>
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<tr>
<td>SK-N-BE(2)</td>
<td>C135F</td>
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<tr>
<td>SK-N-AS</td>
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<td>SKN-FI</td>
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<tr>
<td>SK-N-DZ</td>
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<td>+</td>
</tr>
<tr>
<td>IMR-32</td>
<td>wt</td>
<td>+</td>
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<tr>
<td>SHEP</td>
<td>wt</td>
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p53 status is indicated according to ref 35
*- C-terminal homozygous deletion
Figure 2

A

<table>
<thead>
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<td>IMR32</td>
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B

- DAPI
- TUNEL
- MERGE

Control

RITA 1 µM, 48h

SKN-DZ

C

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<tbody>
<tr>
<td>RITA 1 µM:</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PARP cleaved PARP actin

D

Graph showing cell percentage in subG1, G1, S, and G2 phases for DMSO 4d and 1 µM RITA 4d.
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 Burmakin, Yao Shi, Elisabeth Hedström, et al.

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