IGF-1R/MDM2 Relationship Confers Enhanced Sensitivity to RITA in Ewing Sarcoma Cells

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Abstract
Ewing sarcoma is one of the most frequent bone cancers in adolescence. Although multidisciplinary therapy has improved the survival rate for localized tumors, a critical step is the development of new drugs to improve the long-term outcome of recurrent and metastatic disease and to reduce side effects of conventional therapy. Here, we show that the small molecule reactivation of p53 and induction of tumor cell apoptosis (RITA, NSC652287) is highly effective in reducing growth and tumorigenic potential of Ewing sarcoma cell lines. These effects occur both in the presence of wt-p53 as well as of mutant or truncated forms of p53, or in its absence, suggesting the presence of additional targets in this tumor histotype. Further experiments provided evidence that RITA modulates an important oncogenic mark of these cell lines, insulin-like growth factor receptor 1 (IGF-1R). Particularly, RITA causes downregulation of IGF-1R protein levels. MDM2 degradative activity is involved in this phenomenon. Indeed, inhibition of MDM2 function by genetic or pharmacologic approaches reduces RITA sensitivity of Ewing sarcoma cell lines. Overall, these data suggest that in the cell context of Ewing sarcoma, RITA may adopt additional mechanism of action besides targeting p53, expanding its field of application. Noteworthy, these results envisage the promising utilization of RITA or its derivative as a potential treatment for Ewing sarcomas. Mol Cancer Ther; 11(6); 1247–56. ©2012 AACR.

Introduction
Ewing sarcoma is the second most common tumor of bone in children and young adults. Besides surgery, its treatment includes highly intensive chemo- and/or radiation therapies, which are associated with significant short- and long-term side effects, of particular relevance in consideration of the young age of patients (1, 2). The potential application of newly developed strategies or the identification of newly targeted therapies is a field of intense study in the treatment of Ewing sarcoma.

New anticancer therapies are mostly residing on the development of small chemical entities against specific oncogenic targets (3). Ewing sarcoma is molecularly characterized by the presence of a somatic translocation that involves chromosome 11, resulting in an oncogenic fusion protein that encompasses the EWS gene (the more abundant translocation gives rise to EWS-FLI-1 chimera ref. 4). Therefore, approaches have been developed to inhibit this oncogene, achieved mainly through the inhibition of its transcript product and have shown their efficacy to limit cell growth and tumorigenicity (5–8). Strictly related to EWS-FLI-1, a crucial oncogenic pathway in Ewing sarcoma is that one mediated by Insulin-like growth factor receptor 1 (IGF-1R; refs. 9–12). Indeed, activation of this receptor is necessary for EWS-FLI-1-induced transformation of human cells (9). As confirmation of the relevance of IGF-1R pathway, different therapeutic strategies are showing their efficacy in preclinical models of the disease and in Ewing sarcoma patients in I or II phase clinical trials (2, 12). At the same time, increasing evidence suggests that combination of IGF-1R inhibitory molecules with other treatments may further improve the efficacy of the first (13).

Reactivation of the oncosuppressor p53 function is a goal of different therapeutic approaches. These approaches can be grossly divided in 2 groups: (i) one aiming to correct the activity of mutant p53 in those tumors bearing a mutated gene; (ii) a second one aiming to reactivate p53 function in those tumors in which it is still preserved (14). TP53 gene is rarely altered in Ewing sarcoma (12) and therefore can be a potential target of the second type of approach. Among strategies for p53 reactivation, a promising molecule is the furanic compound reactivation of p53 and induction of tumor cell apoptosis (RITA, also NSC652287; refs. 15, 16). At its
In this work, we investigated the sensitivity to RITA of different cell lines derived from Ewing sarcoma and the biologic response caused by this molecule. To define the role of p53 in RITA sensitivity, we used cell lines characterized by a different status of p53. Our data show that RITA is highly effective in suppressing growth and tumorigenic properties of Ewing sarcoma cell lines independently of the status of p53. At the molecular levels, we observed a decrease of IGF-1R protein, at least in part, mediated by MDM2 degradative activity. Overall, these data indicate the ability of RITA to affect additional molecules other than p53 and its potential application in Ewing sarcoma.

Materials and Methods

Cell cultures, transfections, and treatments

All ES cell lines were obtained by Orthopaedic Rizzoli Institute Bologna, Italy in 2006. Previously, SK-N-MC cell line was obtained from the American Type Culture Collection, TC-71 and 6647 cell lines were a generous gift from TJ. Triche (Childrens Hospital, Los Angeles, CA), WE68 cell line was established and kindly provided by F. van Valen (Laboratory for Experimental Orthopaedic Research, Department of Orthopaedic Surgery, University of Münster, Münster, Germany; ref. 20), LAP-35 and IOR-BRZ71 cell lines were established in Orthopaedic Rizzoli Institute (21). In all cell lines, but WE68, the presence of EWS-FLI1 has been verified by Western blot. All cell lines have been tested for mycoplasma contamination and verified to be Mycoplasma free (last control in 2011). SK-N-MC, TC-71, and WE68 have been further authenticated by DNA fingerprinting in 2008, using genes MPX-2 and MPX-3 kits (22). The status of TP53 gene in all cell lines, but SK-N-MC, was confirmed by sequencing in 2008. Genetic analysis of the TP53 gene was carried out by PCR amplification of exons 1–12 with flanking intronic primers, followed by sequencing. Genomic DNA was extracted from cell lines according to standard procedures. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco), supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FBS (Invitrogen) and maintained at 37°C in a humidified 5% CO2 atmosphere.

SK-N-MC cells were stably transfected with pCMV-MDM2, pCMV-MDM2C438L, or pCDNA3.1B using Lipo-fectamine Plus Reagent (Invitrogen) and were then maintained in medium supplemented with G418 700 μg/mL. Cells were treated with RITA 1 or 3 μmol/L, α-amanitin 10 μg/mL, MG132 10 or 15 μmol/L and with sempervirin 0.5 μg/mL.

Cell viability and TUNEL assay

Cell viability was assessed by collecting both floating and adherent cells counted in a hemocytometer after the addition of trypan blue. Alternatively, cell viability was determined using Cell Titer Blue colorimetric assay according to manufacturer’s instruction (Promega). Growth curves were previously set up with increasing cell number (4,000, 6,000, and 8,000 cells per well) for each cell line.

Apoptotic DNA fragmentation was carried out by terminal deoxynucleotidyl transferase–mediated DUTP nick end labeling (TUNEL) assays with fluorescent In Situ Cell Death Detection Kit (Roche) following the manufacturer’s instructions. Alternatively, apoptotic cells were determined using ApoAlert Annexin V Kit (Clontech) according to manufacturer’s instruction.

Immunoprecipitation and Western blot

For immunoprecipitation experiments, cells were lysed in Saito’s modified buffer (50 mmol/L Tris–HCl, pH 7.4, 0.15 mol/L NaCl, 0.5% Triton-X100, and 5 mmol/L EDTA) supplemented with a cocktail of protease inhibitors (Boehringer). Immunoprecipitations were done by preincubation lysates with protein G-sepharose (Pierce) and then with the indicated antibody, under gentle rocking at 4°C overnight. For Western blot, cells were lysed in radioimmunoprecipitation assay buffer (50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% Na desoxycholate, 0.1% SDS, and 1 mmol/L EDTA) supplemented with a cocktail of protease inhibitors (Boehringer). The following primary antibodies were used: rabbit anti-IGF-1R polyclonal antibody C-20 (Santa Cruz), goat anti-p53 polyclonal antibody FL393 (Santa Cruz), mouse anti-MDM2 monoclonal antibody (mAb) 2A10, and Ab1 (Calbiochem), mouse anti-α-tubulin mAb DM1A (Sigma), rabbit anti-FLI-1 polyclonal antibody C-19 (Santa Cruz), mouse anti-PARP1 monoclonal antibody (mAb) 2A10, and Ab1 (Calbiochem), mouse anti-α-tubulin mAb DM1A (Sigma), rabbit anti-FLI-1 polyclonal antibody C-19 (Santa Cruz), mouse anti-ParP1 mAb C2–10 (Pharmingen), mouse anti-Hsp70 mAb SPA-820 (StressGen), mouse anti-GFP mAb 7.1 and 13.1 (Roche), and mouse anti-actin mAb C-40 (Sigma).

Cell-cycle analysis

Cell-cycle profiles were evaluated by fixing 5 × 105 cells in 70% ethanol for 1 hour on ice and staining DNA for 30 minutes at room temperature with 50 μg/mL propidium iodide in PBS containing 1 mg/mL RNase A. Cell percentages in the different phases of the cycle were measured by flow cytometric analysis of propidium iodide–stained nuclei using Multicycle Software (Phoenix Flow Systems) Epics XL (Coulter).

Soft-agar foci formation assay

The anchorage-independent growth of LAP-35, TC-71, and SK-N-MC cell lines was monitored by the soft-agar colony formation assay. In 60-mm dishes 5 mL of hard agar layer, 0.7% agar-noble (Difco), reconstituted in serum-containing medium, were used as substrate for the Ewing sarcoma cell lines that were plated on a 2.0-mL layer of 0.35% warm agar in serum-containing medium. Cells were then cultured at 37°C, 5% CO2 for 3 weeks and RITA treatment was renewed every 3 days.
SiRNA
MDM2 siRNA and control siRNA were generated by Invitrogen (Stealth RNAi) and consisted of a mix of 3 different siRNA. Cells were transfected using RNAiMAX reagent (Invitrogen) following the manufacturer’s instructions.

Real-time PCR
Total RNA extraction was carried out with TRIzol reagent (Invitrogen). For quantitative reverse transcription PCR (qRT-PCR), total RNA (1 μg) was reverse transcribed using Gene Amp Kit (Applied Biosystems) and subjected to PCR amplification using SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used:

- upper IGF-1R 5'-ATAGGGATTCCGGGAGTCAT-3'; lower IGF-1R 5'-GGTGGATGCTCTTCAGTTCGT-3';
- upper IGF-1 5'-CCACGATGCCTGTCTGAGG-3'; lower IGF-1 5'-TGCAAGTTCTGGTTGTCGAG-3';
- upper IGF-BP3 5'-TGTGGCCATGACTGAGGAAA-3'; lower IGF-BP3 5'-TGCGAGACCTTCTTGGTT-3';
- upper hTBP 5'-GAAACATCATGGATCAGAACA-3'; lower hTBP 5'-ATAGGGATTCCGGGAGTCAT-3'.

Each tissue sample mRNA was normalized relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA. Samples underwent 40 amplification cycles, monitored by the ABI Prism 7900 sequence detector (Applied Biosystems). The following primers were used:

- F: 5'-CTCTCTATGCCTTGTTCT-3';
- R: 5'-CCCTGATCCATGGTTCT-3'.

Results
RITA inhibits growth and tumorigenic potential of Ewing sarcoma cell lines independently of p53 status
To ascertain RITA efficacy in Ewing sarcoma, we used cell lines characterized by the same chromosomal translocation, t(11;22)(q24;q12), that generates the oncogenic protein EWS/FLI-1. To verify the relevance of p53 in RITA sensitivity, we used cell lines characterized by a different p53 status: LAP-35 and WE68 with wild-type p53, TC-71 and IOR-BRZ71 with a truncated p53 lacking the tetramerization and DNA-binding domains (p53-213STOP), 6647 with a point mutant p53 (p53-S241F) and SK-N-MC lacking p53 expression (23). The status of TP53 gene in all cell lines, but SK-N-MC, was confirmed by sequencing (data not shown). At first, we analyzed cell growth of all cell lines in the presence or absence of RITA. Because analysis of NCI-60 cell panel has evidenced a mean IC_{50} > 3.5 μmol/L for RITA in mutant p53 carrying cancer cell lines (24), we treated Ewing sarcoma cell lines with 1 and 3 μmol/L RITA. Unexpectedly, analysis of cell viability evidenced the ability of RITA to reduce this parameter in all tested cell lines, independently of the presence and of the status of p53 (Fig. 1A–F). In wt-p53 cell lines, cell viability was strongly reduced in WE68 cells, whereas in LAP-35 it was similar to that observed in mutant p53 cell lines or even lower in comparison with null p53 SK-N-MC. Cell growth reduction was enhanced by increased doses (1 or 3 μmol/L) and treatment times of RITA, although with a different kinetics among cell lines. To confirm these data and ascertain the specificity of the observed effects, we measured viability by analyzing cell metabolic activity (by reduction of resazurin) and compared the dose of RITA able to reduce this parameter to 50% in Ewing cell lines and in H1299, a lung tumor cell line lacking p53. Although not overlapping with the results obtained by Trypan blue uptake, RITA confirmed its efficacy in all Ewing cells in comparison with H1299 cells that resulted unaffected by the used doses of RITA (Fig. 1G). Particularly, the p53-null SK-N-MC confirmed the highest sensitivity whereas wt-p53 LAP35 showed a sensitivity similar to that of truncated-p53 IOR-BRZ71. HCT116 /−/, a colon cancer cell line lacking p53 too, and insensitive to the drug (16), showed results similar to those observed in H1299 (data not shown). These data overall confirmed the specific sensitivity of Ewing sarcoma cell lines to RITA.

Fluorescence-activated cell sorting (FACS) analysis evidenced a strong alteration of cell cycle caused by RITA as early as 16 hours after drug treatment in 5 representative cell lines (Fig. 2A–C) and in the other cell lines (Supplementary Fig. S1A–C). In all cell lines it was evident that a progressive accumulation of cells in the S–G2 phases of cell cycle, in agreement to what previously reported (15, 18); in TC-71 and especially in SK-N-MC, it was also evident a sub-G1 population indicative of cell death (Fig. 2A–C). LAP-35, although containing wt-p53, showed a modest increase of sub-G1 at the latest time point (36 hours). To further characterize the loss of viability evidenced by FACS analysis, a TUNEL assay was done. The experiments confirmed a strong increase of apoptotic cells in TC-71 and SK-N-MC cells upon RITA 3 μmol/L treatment, whereas only a mild increase was observed in LAP-35 in agreement to FACS data (Fig. 2D).

Overall, these data indicated that RITA is effective in suppressing cell growth of Ewing sarcoma cell lines and that its effects are mediated by a strong growth suppressive and/or apoptotic activity. Noteworthy, these effects seem to be not related to the status of p53.

RITA exerts a strong antitumor activity in tumor cell lines (16, 25). To verify whether RITA is able to affect tumorigenic properties of Ewing sarcoma cells too, we analyzed their anchorage-independent growth by a soft-agar assay using 1 and 3 μmol/L RITA. Different amounts of cells were plated (20,000 or 100,000 cells/60 mm dish) and after 48 hours treated with vehicle (CTR) or RITA, repeating the administration every 3 days. After 3 weeks, all CTR cells have formed marked colonies, more evident in TC-71 and SK-N-MC, less evident in LAP-35 in agreement with the reduced tumorigenicity of this cell line.
Strikingly, both doses of RITA were able to completely abolish anchorage-independent growth independently of the number of seeded cells (Fig. 3A and data not shown), indicating that RITA is highly effective in reducing in vitro tumorigenic potential of these cells. Again these effects were irrespective of p53 status, suggesting that RITA may affect additional pathways in this tumor histotype.

**RITA downregulates IGF-1R levels**

Anchorage-independent growth requires a combination of proliferation and survival signaling strongly affected by the presence of a functional IGF-1R pathway (13, 27). To investigate RITA activity in Ewing sarcoma cells, the levels of IGF-1R were analyzed in 2 cell lines characterized by a null and a truncated p53, SK-N-MC, and TC-71, respectively. RITA treatment caused a progressive down-regulation of IGF-1R levels in both cell lines (Fig. 4A and B). IGF-1R decrease appeared earlier and more evident in cells that undergo apoptotic response evidenced also by the progressive appearance of PARP cleavage product (Fig. 4A and B). Under the same conditions, MDM2 levels decreased at the later time points as previously reported (Fig. 4A and B; refs. 28, 29). Similar results were observed also in the other cell lines (Supplementary Figs. S2 and S3). P53, wt as well as mutant S241F forms, were not substantially altered or slightly increased in LAP-35 and 6647 (Supplementary Fig. S3). Conversely, a strong decrease of IGF-1R levels was observed, paralleling cell viability.

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**Figure 1. RITA effects on cell growth.** RITA reduces cell viability in LAP-35 (A), WE68 (B), TC-71 (C), IOR-BRZ71 (D), 6647 (E), and SK-N-MC (F). Cells were treated with RITA at the indicated concentrations for 24 or 48 hours and then stained with trypan blue for immediate count analysis. Cell growth was calculated as number of trypan blue negative cells. Percentage of cell viability was obtained by setting 100% the cell growth of control vehicle-treated cells (CTR) at each time point and calculating the relative cell growth of treated cells. Data are representative of at least 3 different experiments. G, cells were plated in 96-well plate and treated in octuplicate with increasing concentration of RITA (0.5-1-2-3-6 μmol/L) for 24 or 48 hours (*). Cell viability was assessed by Cell Titer Blue. The dose reported in the graph is the dose able to reduce cell viability to 50% in comparison with untreated cells.
wt-p53 coexistent with IGF-1R was observed in WE68 (Supplementary Fig. S2), suggesting a specific susceptibility of this cell line to RITA. IGF-1R has been previously described as a transcriptional target of RITA-mediated p53 transcriptional activity (30, 31). Although, in the cell lines here used, but LAP-35 and WE68, p53 is transcriptionally inactive or absent, we tested whether IGF-1R downregulation is correlated to a decrease of its mRNA. Indeed, the levels of IGF-1R mRNA were not significantly altered in both cell lines (Fig. 4C and D). We therefore tested whether the downregulation of IGF-1R was mediated by increased protein turnover. IGF-1R degradation is mediated by proteasome and lysosome (32). To analyze the involvement of proteasome in RITA-mediated IGF-1R downregulation, TC-71 and SK-NM-C cell lines were treated with the proteasome inhibitor, MG132, or RITA or both drugs. MG132 treatment per se increased the levels of IGF-1R, confirming the proteasome-mediated degradation of the receptor in these cell lines. Noteworthy, addition of MG132 to RITA-treated cells was able to recover the decrease of IGF-1R levels (Fig. 4E and F), indicating that RITA-induced downregulation was indeed caused by degradation of the receptor.

It has been reported that IGF-1R levels are downregulated by IGF binding protein 3 (IGF-BP3; ref. 33), a protein acting as inhibitor of IGF-1R activity by sequestering its ligand IGF-1. IGF-BP3 is a transcriptional target of EWS-FLI-1, the oncogenic mark of Ewing sarcoma (34).

Figure 2. RITA causes G2 arrest and cell apoptosis. FACS analysis of LAP-35 (A), TC-71 (B), and SK-N-MC (C) carried out at 12, 24, or 30 hours after treatment with 3 μmol/L RITA. Cells were grown in the presence or absence of RITA and then stained with PI for the immediate cell-cycle analysis. Each panel indicates the percentage of cells in each cell-cycle phase. Sub-G1 region (as indicated) has been independently evaluated relative to the total population. These analyses are representative of at least 2 different experiments. D, TUNEL analysis of indicated cells at 16 or 24 hours after treatment with 3 μmol/L RITA. The percentage of apoptotic cells was measured as TUNEL-positive cells relative to total cells. The data report the mean of 3 independent counts.
Particularly, IGF-BP3 is repressed by EWS-FLI-1. Because it has been shown that RITA downregulates various key oncogenic pathways (30), we investigated whether IGF-1R decrease was related to modification of EWS-FLI-1 levels and/or activity. Nor substantial modification of EWS-FLI-1 protein levels (Supplementary Fig. S4A–C) or of its transcripational targets IGF-1 and IGF-BP3 mRNA (Supplementary Fig. S4D) was observed upon RITA treatment in SK-N-MC and TC-71, suggesting that downregulation of IGF-1R by RITA is not mediated by alteration of EWS-FLI-1. Conversely, in LAP-35 RITA caused a decrease of EWS-FLI-1 (Supplementary Fig. S4C), supporting the p53-mediated downregulation of this oncogenic pathway too (30).

**MDM2 mediates RITA-induced IGF-1R degradation**

Degradation of IGF-1R is mediated by 2 RING ubiquitin ligases, c-Cbl and MDM2 (35, 36). Although p53 is the only known target of RITA, MDM2 has been reported as an important mediator of RITA activity (28, 37). We therefore investigated whether MDM2 may be involved in IGF-1R degradation. We interfered MDM2 expression by siRNA and analyzed IGF-1R levels of siMDM2 interfered cells in comparison with control cells interfered with scramble siRNA (siCTR), both in the absence and in the presence of RITA. Interference of MDM2 expression was indeed able to counteract RITA-mediated IGF-1R downregulation (Fig. 5A), supporting the potential involvement of MDM2 in RITA-mediated IGF-1R degradation. To further ascertain whether MDM2 activity is involved in RITA...
sensitivity, cell viability was analyzed by Annexin staining. Indeed, cell apoptosis induced by RITA was reduced in siMDM2 in comparison with siCTR cells, paralleling IGF-1R recovery. These effects were more pronounced in SK-N-MC cells in agreement with the increased apoptosis observed in these cells.

To ascertain whether MDM2-induced degradation of IGF-1R is mediated by transcriptional activation of other cofactors by RITA, we induced transcriptional block by pretreating cells with the inhibitor of RNA polymerase II, α-amanitin (38, 39). In the absence of RITA, α-amanitin does not affect IGF-1R levels, in agreement with the long half-life of the receptor (24 hours, as reported in 40). Importantly, the presence of α-amanitin does not alter RITA-induced decrease of IGF-1R levels (Fig. 5C and D), suggesting that transcription of other factors is not required for RITA-mediated IGF-1R downregulation. Functionality of α-amanitin was evidenced by the downregulation of Pol II levels, as previously reported (39).

MDM2-mediated degradation of IGF-1R is mediated by direct association between the 2 proteins (35). To support the activity of MDM2 toward IGF-1R, we analyzed the binding between IGF-1R and MDM2 in the presence or absence of RITA after few hours of RITA treatment. In the absence of RITA, association between the 2 proteins was barely detectable. Conversely, after 4 hours of RITA treatment, an evident increase in the association between the 2 proteins was observed in both SK-N-MC and TC-71 (Fig. 5E and F). This early association is in agreement with the observation that MDM2-mediated ubiquitination of IGF-1R precedes subsequent internalization and degradation of the receptor (36) and supports the hypothesis that RITA treatment increases the association between MDM2 and IGF-1R, leading to IGF-1R degradation.

### MDM2-degradation of IGF-1R mediates RITA sensitivity

To confirm the role of MDM2 activity and of MDM2/IGF-1R relationship in RITA sensitivity, we interfered with MDM2 ubiquitination function. It has been recently described the ability of sempervirine, a plant alkaloid with well-known anticancer activities (41), to inhibit ubiquitination and degradative activity of MDM2 (42). We therefore impaired MDM2 function by sempervirine and analyzed RITA effects. A dose of 0.5 mg/mL of sempervirine showed its efficacy to inhibit MDM2 degradative function both in TC-71 and in SK-N-MC cells, after 8 or 4 hours of treatment, respectively (data not shown). Simultaneous administration of RITA and sempervirine was able to antagonize downregulation of IGF-1R levels in both TC-71 and SK-N-MC (Fig. 6A and C). Most importantly, these effects correlate with an impairment of cell death induced by RITA (Fig. 6B and D), confirming that MDM2-mediated IGF-1R degradation is an important step of...
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MC. Indeed, the expression of wt-MDM2 potentiated the molecular data correlate with altered survival of SK-N-MC. This mutation disrupts the RING region where wt-MDM2 and a mutant MDM2, MDM2-C438L, in SK-N-MC. The canonical target of RITA is wt-p53, although additional molecules have been hypothesized (16–18). Here, we compared the effects of RITA among different cell lines characterized by wt, mutant, truncated, and null p53. The first result was that RITA strongly affects the growth and tumorigenic properties of these cell lines independently of p53, suggesting a specific sensitivity of this tumor type to RITA. Particularly, RITA reduced to a similar extent cell viability of wt-p53 LAP-35, null p53 SK-N-MC, and IOR-BRZ71 cells expressing a truncated form p53-213STOP that resembles a null condition. In addition, a significant cell death was observed in wt-p53 WE68 cells as well as in p53-213STOP TC-71 and null-p53 SK-N-MC cells. Conversely, a reduced cell death was evident in the wt-p53 LAP-35 cell line. Although this may be attributed to the reduced tumorigenicity and growth rate of this cell line, the presence of additional factors affecting RITA sensitivity could contribute as well (28).

RITA has been identified through a cell-based drug screening strategy (16) based on its biologic effects on target function, specifically p53. The possibility that it can affect additional pathways cannot be excluded. Indeed, RITA activates a DNA damage response (44) and affects wt p53-related off-targets (28). In addition, cell lines carrying mutant p53 have been reported to be sensitive to RITA, although at higher doses in comparison with cell lines carrying wt-p53 (24, 45).

In fact, accumulation of cells in the S-G2 phases of cell cycle, as observed in Ewing sarcoma cell lines, has been previously reported both in wild-type as well as in mutant and null p53-carrying cancer cell lines (16, 18), suggesting that RITA-mediated alteration of the cell cycle may depend on additional factors besides p53.

The hallmarks of Ewing sarcoma are the presence of oncogenic protein EWS-FLI-1 and the autocrine-activation of IGF-1R. We did not observe substantial modification of EWS-FLI-1 levels. Conversely, we observed a consistent decrease of IGF-1R levels upon RITA treatment. IGF-1R is a crucial pathway for Ewing sarcoma survival. Molecular and translational studies have shown that inhibition of this pathway suppresses tumor growth (reviewed in ref 12) and in fact clinical trials with IGF-1R inhibitors are under evaluation (2). The data here presented indicate that IGF-1R may be indirectly affected by a targeted therapy too, opening a new potential field of anti-IGF-1R therapies.

Discussion

The aim of this study was to evaluate the efficacy of the furanic compound RITA in Ewing sarcoma. Indeed, the requirement of new therapeutic tools for this type of tumor is a field of intense study (2).

![Image](88x428 to 313x735)

Figure 6. RITA sensitivity is affected by MDM2 function. A, Western blot analysis of SKNMC cells treated with vehicle, 3 μmol/L RITA for 22 hours, or 3 μmol/L RITA plus 0.5 μg/mL sempervirine added 4 hours before cell lysate collection. B, percentage of cell death after treatment of indicated cells as in A. C, Western blot analysis of TC-71 cells treated with vehicle, 3 μmol/L RITA for 24 hours, or 3 μmol/L RITA plus 0.5 μg/mL sempervirine added 8 hours before cell lysate collection. D, percentage of cell death after treatment of indicated cells as in C. The percentage of cell death was calculated as fold of increase of cell death of RITA treated cells/untreated cells. Columns, average of 3 independent experiments. Bars, SD.

RITA activity and supporting the role of MDM2/IGF-1R relationship in RITA sensitivity of these tumor cells.

To confirm the role of MDM2 and of its ubiquitinating activity in RITA sensitivity, we stably overexpressed wt-MDM2 and a mutant MDM2, MDM2-C438L, in SK-N-MC. This mutation disrupts the RING region where ubiquitin ligase activity of MDM2 resides and therefore abolishes it (43). Upon 15 hours of RITA treatment, the overexpression of wt-MDM2 accelerated IGF-1R downregulation, whereas this was poorly observed in the pcDNA3.1 transfected- (CTR) and the MDM2-C438L-expressing cell populations (Fig. 6E). Of note, these molecular data correlate with altered survival of SK-N-MC. Indeed, the expression of wt-MDM2 potentiated the effects of RITA, whereas the mutant MDM2-C438L did not exhibit such activity and rather slightly reduced cell sensitivity to the small molecule (Fig. 6F), supporting the role of MDM2 degradative activity in RITA sensitivity of Ewing sarcoma cells.
Our experiments indicate that downregulation of IGF-1R protein levels is mediated, at least in part, by the E3 ubiquitin ligase, MDM2. Various studies have shown the ability of MDM2 to control IGF-1R levels (35, 46), although it is not completely clear what activates and controls this MDM2 function. Moreover, it has been shown that MDM2 counteracts the IGF-1R survival function, independently of p53 (47). Our data indicate that in Ewing sarcoma cell lines, RITA activates MDM2 function. How this happens, it is not clear. Cell fractionation experiments did not show any difference in MDM2 localization in the presence or absence of RITA, excluding a delocalization of MDM2 by RITA treatment (data not shown). A study of docking simulation indicated that RITA can bind MDM2 (17), although with an affinity ~15,000 less than p53. The possibility that MDM2 modifications alter RITA affinity to MDM2 has not been tested. However, the selective sensitivity of Ewing sarcoma to RITA suggests that additional specific factors mediate and direct MDM2 activity toward IGF-1R in this tumor type. Interestingly, a recent study reported the overexpression in Ewing sarcoma of a protein involved in ubiquitin ligase activity, CDT2, raising the hypothesis that deregulation of protein ubiquitin machinery may be of relevance in this tumor type (48). Finally, downregulation of MDM2 upon RITA treatment, as observed in this work and reported by other authors (28, 29), but yet its role in RITA function may seem to counteract its role in RITA sensitivity. However, the crucial role of MDM2-degradative activity in RITA sensitivity has been already reported by other authors (28, 37), indicating that MDM2 is important at least for the early response to RITA.

It remains to be ascertained whether MDM2-mediated IGF-1R downregulation is the main determinant of RITA activity. Unfortunately, experiments of rescue of RITA effects by overexpression of IGF-1R failed because of the inability of 2 different IGF-1R coding plasmids to substantially overexpress IGF-1R levels in the Ewing sarcoma cell lines here studied (data not shown). Of note, MDM2 is able to alter cell-cycle progression and different studies have reported the ability of MDM2 to induce a G0/G1 arrest in specific cell lines, independently of p53 (49). Therefore, growth control activities of MDM2 too might be involved in RITA activity in Ewing sarcoma. However, the complete understanding of the mechanism by which RITA is effective in this tumor type is a point of relevance before any consideration of this drug as therapeutic agent in Ewing sarcoma.

Overall, these data expand the spectrum of antitumor RITA activity showing its efficacy in Ewing sarcoma. Interestingly, p53 mutation, rare in Ewing sarcoma (12), is increased in a subset characterized by high aggressive behavior and poor chemoresponse (50), suggesting the useful application of RITA in those cases escaping standard therapies. Given the low toxicity of this molecule in normal cells (16), these results pave the way to the potential application of a new therapeutic tool to Ewing sarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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