Targeting Wild-Type and Mutant p53 with Small Molecule 
CP-31398 Blocks the Growth of Rhabdomyosarcoma by 
Inducing Reactive Oxygen Species–Dependent Apoptosis

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Abstract

Rhabdomyosarcoma (RMS) is a common soft-tissue sarcoma of childhood in need of more effective therapeutic options. The expression of p53 in RMS is heterogeneous such that some tumors are wild-type whereas others are p53 mutant. The small molecule CP-31398 modulates both the wild-type and the mutant p53 proteins. Here, we show that CP-31398 blocks the growth of RMS cells that have either wild-type or mutant p53 status. In wild-type A204 cells, CP-31398 increased the expression of p53 and its downstream transcriptional targets, p21 and mdm2; enhanced the expression of apoptosis-related proteins; and reduced proliferation biomarkers. Flow profiling of CP-31398–treated cells indicated an enhancement in sub-G0 and G1 populations. CP-31398 inhibited proliferation in a manner associated with co-induction of SOX9 and p21. Apoptosis induced by CP-31398 occurred with translocation of p53 to mitochondria, leading to altered mitochondrial membrane potential, cytochrome c release, and reactive oxygen species release. In vivo, CP-31398 decreased the growth of tumor xenografts composed of wild-type or mutant p53 tumor cells, increasing tumor-free host survival. Our findings indicate that the ability of CP-31398 to modulate wild-type and mutant p53 results in the inhibition of RMS growth and invasiveness. Cancer Res; 70(16); 6566–76. ©2010 AACR.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in pediatrics, with an incidence of 4.6 cases per million children (1). Embryonal (ERMS) and alveolar (ARMS) RMS, the two major histologic subtypes of RMS, carry distinct clinical features (2). ERMS tumors are more common among young children, typically occurring in the head, neck, and genitourinary tract. ERMS tumors are generally more sensitive to chemotherapy and radiation. In contrast, ARMS tumors are more common in adolescents, often occurring in the extremities. Patients with ARMS and undifferentiated sarcoma carry a less favorable prognosis than patients with ERMS tumors (3). The overall 5-year survival rate for children with RMS is ∼64% for cases diagnosed from 1985 to 1994 (1, 2). The majority of RMS tumors occur sporadically, but a subset of tumors develop in patients with cancer predisposition syndromes such as Li-Fraumeni (4).

Using multiagent chemotherapy, surgery, and radiation, the outcome for patients with favorable features has steadily improved (3). However, the prognosis for metastatic and relapsed RMS tumors remains very poor (5). RMS therapies beyond cytotoxic chemotherapy are desperately needed. During the last decade, efforts were made to use tumor suppressor p53 as a major target of drug development for blocking the pathogenesis and progression of various cancers (6). It is well known that p53 is mutated in more than 50% of all human cancers including RMS (7). In the remaining 50% where p53 is not mutated and remains wild-type, the signaling downstream of p53 is frequently interrupted (8, 9). Wild-type p53 is usually not accumulated in the cells due to its short half life (<30 minutes). Therefore, attempts to increase transcriptionally active p53 either by enhancing the stability of wild-type p53 or by reverting mutant p53 to its wild-type conformation with its ability to block cell cycle progression and induce apoptosis has been considered as an important approach in cancer treatment. In this regard, CP-31398, a styrylquinazoline, can restore a wild-type associated epitope (monoclonal antibody 1620) on the DNA-binding domain of the mutant p53 protein (10–14). Furthermore, CP-31398 not only restores p53 functions in mutant p53-expressing cells but can also significantly increase the protein level and promote the activity of wild-type p53 in multiple human cancer cell lines, leading to cell cycle arrest or apoptosis (14). The putative mechanism by which CP-31398 enhances the protein levels of wild-type p53 includes blockade of ubiquitination and degradation of p53 without interrupting the physical association between p53 and MDM2 in vivo (14).
In this study, we investigated the chemotherapeutic effects of CP-31398 in a poorly differentiated RMS cell line, A204, which carries wild-type p53 (15, 16), and the ERMS cell line RD, which carries mutant p53. Our results show that CP-31398 induces p53-dependent cell cycle arrest and apoptosis in both A204 and RD cells. CP-31398–induced transcriptional activation of p53 is evident by the induction of its downstream targets, p21, mdm2, and puma, in both of these cells. The induction of apoptosis involved the mitochondrial translocation of p53 followed by the release of cytochrome c and activation of caspase-3. Parenteral administration of CP-31398 reduced the growth of xenograft tumors that developed

Figure 1. CP-31398 (CP) treatment blocks cell cycle progression and induces apoptosis in RMS A204 cells carrying wild-type p53. Cells were incubated in the presence of CP at the indicated concentrations for 24 h and then stained with PI for immediate flow cytometric analysis. A, loss of viable cells. Two-parameter pseudocolor density plots of total un gated events for forward scatter and side scatter properties. Note the CP dose–dependent accumulation of dead cells and apoptotic debris to the left side of the panel. The large circular gate includes both live and dead single cells, excluding small debris. The percentage of gated total cellular events is shown in the top left corner of each ungated panel. The small circular gate encompasses viable cells. The percentage of cells is displayed in the bottom right corner of each un gated panel. A graph summarizing the percentage of viable cells within the single cell gate is shown at the bottom. B, increase in apoptotic cells. Two-parameter bitmap displays of gated cells (indicated by arrows in A) analyzed for Annexin V and PI staining (left) and histograms for PI alone (right). The percentages of Annexin V–positive cells within PI-negative (early apoptosis) and PI-positive (late apoptosis) subsets are shown in the top left and top right quadrants, respectively. A graph displaying total apoptotic and dead cells (% Annexin + PI) is shown at the bottom. The percentages of PI-stained cells are shown in the histograms, and a summary graph is shown at the bottom. Note the inverse correlation of viable cells (A), indicated by light scatter properties (small gate), and apoptotic cells (central and right graphs in B). C, G1–phase block of cell cycle. A204 cells were incubated with the indicated concentrations of CP-31398 for 24 h and DNA was stained with PI for cell cycle analysis using the Watson pragmatic model (results at the top of each panel). The summary graph below shows the mean percent of cells ± SEM in each phase (n = 3). *, P < 0.05, two-sided Student’s t test. D, kinetics of CP-mediated induction of p53-related, apoptosis-related, and cell cycle–related protein expression. Western blot analyses of A204 cell lysate proteins following exposure to 20 μg/mL CP for different times (as indicated).
following the s.c. inoculation of A204 or RD cells. Our data indicate that CP-31398 can be highly effective in promoting diminution of the growth and invasiveness of RMS tumors carrying wild-type or mutant p53.

Materials and Methods

Antibodies and reagents
Primary antibodies (Supplementary Table S1; Santa Cruz Biotechnology); horseradish peroxidase–secondary antibodies (Pierce) and Alexa Fluor 488 or 596 secondary antibodies (eBioscience); MitoTracker Red CMXRos (Invitrogen); Apoptosis Detection Kit (Roche Applied Science); JC-1 dye (5,5′,6,6′-tetrachloro-1′,3′,3′-tetraethyl benzimidazolocarbocyanine iodide) staining kit (Molecular Probes, Inc.); and cyclosporine A (CsA), N-acetyl-cysteine (NAC), and 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma) were purchased.

Cell culture
The ERMS A204 cells (wild-type p53) and the ERMS RD cells (mutant p53) were obtained from the American Type Culture Collection. A204 cells were cultured in McCoy’s 5A medium whereas RD cells were cultured in DMEM (Hyclone), supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Western blotting, immunofluorescence, and immunohistochemistry
Western blotting, immunofluorescence, and immunohistochemistry analyses were done as described previously (11).

Figure 2. CP-31398 induces apoptosis in A204 cells by translocating p53 to mitochondria and alters MP in A204 cells, which can be blocked by CsA pretreatment. A, immunofluorescence staining showing colocalization of p53 with MitoTracker, which stains mitochondria. B, immunofluorescence staining showing that CsA blocks CP-31398–induced p53 localization to mitochondria. C, CsA blocks CP-31398–induced cytochrome c release in the cytoplasm. D, CP-31398–mediated alterations in MP are attenuated by CsA treatment. For the experiments in A to C, A204 cells were treated with PBS (control) or CP-31398 (20 μg/mL) for various time intervals. However, for the experiments in D, cells were treated with PBS (control) or CP-31398 (20 and 40 μg/mL) for 15 min and then stained with JC-1 dye. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control. CsA (1 μmol/L) pretreatment was done for 15 min before CP treatment. Columns, mean of three independent experiments; bars, SD. a, P < 0.05, compared with control; b, P < 0.01, compared with control; c, P < 0.05, compared with 20 μg/mL CP-31398 treatment.
Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to the vendor's guidelines.

Flow cytometric analysis of cell cycle

Flow cytometry was done using Becton Dickinson FACS-Can and cell cycle was analyzed using FlowJo (8.8.6) Watson pragmatic analysis software.

Mitochondrial membrane potential assay

Cells from various treatments were trypsinized, resuspended in 200 nmol/L JC-1 solution, and incubated in a 5% CO₂ incubator at 37°C and then analyzed by flow cytometry. Carbonyl cyanide 3-chlorophenylhydrazone (5 μmol/L) was used to completely disrupt mitochondrial potential as a positive control (11).

Measurement of intracellular reactive oxygen species generation

The cells were incubated with 10 μmol/L DCF-DA at 37°C for 15 minutes. The intracellular reactive oxygen species (ROS) mediates oxidation of DCF-DA to the fluorescent DCF. The cells were then harvested and suspended in PBS and analyzed by flow cytometry.

Tumor xenograft study

Six- to eight-week-old female Nu/Nu mice from the National Cancer Institute were used in this study. Animals were
tein (6). We further tested its effects on the expression of p53 in addition to the enhanced transcription of this pro-
of 24 hours, which may be due to the stabilization of enhanced p53 levels in A204 cells detectable over a period by CP-31398 was correlated with the results of Western downstream target proteins as compared with control were discernable (Fig. 1C, graph). 

Results

Treatment of A204 cells with CP-31398 induces G1 cell cycle arrest and apoptosis in RMS A204 cells

To characterize the effects of CP-31398 in A204 cells in vitro, cells were grown in 60-mm plates and treated with 0, 10, 20, and 40 μg/mL of CP-31398 for 24 hours, and then samples were analyzed by flow cytometry. We observed a dose-dependent increase in apoptosis of A204 cells following CP-31398 treatment (Fig. 1A–C). This was indicated by an accumulation of events with light scatter properties consistent with apoptotic cells and debris and a reduction in events with viable cell light scatter properties (Fig. 1A). This correlated with an increased percentage of cells stained with Annexin V and propidium iodide (PI; Fig. 1B). In the viable cell gate, we observed a dose-dependent accumulation of cells in the G1 phase (up to 38% more than control) following exposure to lower concentrations of CP-31398. At the highest concentration, CP-31398–induced apoptosis occurred in cells in all phases of the cell cycle equally, as no significant differences in the cell cycle distribution of the remaining viable cells as compared with control were discernable (Fig. 1C, graph).

CP-31398 induces the expression of p53 and its downstream target proteins

The induction of the cell cycle G1 block and apoptosis by CP-31398 was correlated with the results of Western blot analyses. As shown in Fig. 1D, CP-31398 slightly enhanced p53 levels in A204 cells detectable over a period of 24 hours, which may be due to the stabilization of p53 in addition to the enhanced transcription of this protein (6). We further tested its effects on the expression of p53-dependent downstream target genes p21 and mdm2. The expression of these proteins was upregulated at 3, 6, and 12 hours after treatment with CP-31398. It is known that p21 is required for G1 cell cycle arrest. Next, we assessed the effect of CP-31398 on the expression of proapoptotic and other cell cycle regulatory proteins in these cells. A time- and dose-dependent (data not shown) increase in proapoptotic proteins such as Apaf1, Bax, Bcl2, Bad, and caspase-3 was observed. Consistently, poly(ADP-ribose) polymerase (PARP) cleavage was also induced. CHK1/2 and Cdc2 levels were increased, whereas cyclin E expression was decreased. Accumulation of p53 and induction of p21, mdm2, and CHK1/2 proteins were detected and often peaked at the earliest time points of 3 to 6 hours, whereas increased levels of the apoptotic pathway proteins caspase-3, Bad and Puma and PARP cleavage peaked at 12 hours. The enhanced CHK1/2 expression may, in part, be due to the induction of the DNA damage–dependent ATM/ATR–regulated signaling pathway. However, this needs to be investigated in further detail, which is beyond the scope of this article. Overall, these results are consistent with CP-31398–mediated stabilization of wild-type p53 protein conformation and activation of p53 downstream functions that block cell cycle and apoptosis in RMS A204 cells.

Figure 4. CP-31398 treatment reduces the growth xenograft tumors and augments MET in nude mice developed by inoculating A204 cells carrying wild-type p53. A, CP-31398 reduces the volume of xenograft tumors. B, histology of tumors developed in vehicle- or CP-31398–treated mice. Inset shows mitotic figures (red arrow) and apoptosis (green arrow). C, TUNEL, immunofluorescence, and immunohistochemical staining for apoptosis, Bcl2, PCNA (arrow indicates positive nuclear staining), cyclin E (arrow indicates positive nuclear staining), and E-cadherin expression (data showing that CP-31398 treatment reduces proliferation and increases E-cadherin expression in xenograft tumors). D, CP-31398 reduces the expression of MMP-2/MMP-9, snai, slug, twist, and fibronectin in xenograft tumors. D1 represents CP-31398 daily treatments (24-h intervals) whereas D2 represents twice-daily (12-h interval) treatments. Mice were treated over 4 wk, beginning treatment at the time of tumor cell inoculation. Each value represents the mean ± SE of 10 mice (*, P < 0.05; **, P < 0.01).
blockade in A204 cells inhibits CP-31398–mediated p53 mitochondrial translocation and the associated alterations such as cytochrome c release and induction of apoptosis. As shown in Fig. 2B, CsA pretreatment blocked mitochondrial translocation of p53. Concomitantly, mitochondrial cytochrome c release into the cytoplasm was also diminished (Fig. 2C). In addition, CP-31398–mediated apoptosis was abrogated.

**CP-31398 alters mitochondrial membrane potential**

To confirm that CP-31398 alters MP, we used JC-1 dye, which accumulates in mitochondria and forms aggregates that emit red-orange fluorescence when exposed to light at 590 nm. Formation of these aggregates requires normal MP. With altered MP following the membrane depolarization, the dye remains as a monomer in the cytoplasm and shows green fluorescence (20, 21). The ratio of red/green fluorescence is a function of MP. CP-31398 decreased dose-dependently the MP in A204 cells (Fig. 2D). However, as expected, CsA pretreatment blocked changes in MP.

**CP-31398 triggers ROS generation, which mediates apoptosis in A204 cells**

ROS are considered to play an important role in apoptosis in various cell types (22, 23). To investigate whether CP-31398 stimulated ROS generation in A204 cells, we measured intracellular ROS levels using a ROS-detecting fluorescence dye, DCF-DA. Generation of ROS was evidenced by the increased intensity of DCF fluorescence. Following treatment of A204 cells with CP-31398, an increased generation of ROS could be observed at 12 and 24 hours. The percentage of DCF-positive cells was 1.5%, 36.4%, and 52.3% at 0, 12, and 24 hours, respectively (Fig. 3A). However, the ROS scavenger NAC (5 mmol/L) markedly decreased the level of ROS to ∼6% at 24 hours. Furthermore, the enhanced ROS production was significantly reduced by pretreating cells with CsA (Fig. 3B), suggesting that the major source of CP-31398–mediated ROS may be the mitochondria.

**CP-31398 reduces the growth of xenograft tumors developed by A204 RMS cells in nude mice**

To investigate whether CP-31398 affects the growth of A204 RMS xenograft tumors in nude mice, we injected CP-31398 i.p. (2 mg/mouse daily or twice daily, every 12 hours) to nude mice over a period of 4 weeks after inoculating them with A204 cells. Tumor volume was measured every other day. Animals were sacrificed after 4 weeks of treatment. We found that tumor growth in the CP-31398 treatment groups was reduced significantly in a dose-dependent manner as compared with the vehicle-treated control group as shown in Fig. 4A. The tumor volume in the control group was 277 ± 47 mm³, whereas it was reduced to 127 ± 41 mm³ in the CP-31398 daily treatment group and to 68 ± 24 mm³ in the CP-31398 twice-daily treatment group (P < 0.05 and P < 0.01, respectively). At early time points (day 14 and day 18), the differences between the two doses of CP-31398 were found to be significant (P < 0.05), but at later time points (day 22 and day 30), these differences became less significant. As compared with the vehicle-treated controls, the histology of tumors in the CP-31398 treatment groups showed less mitotic figures (Fig. 4B) and large necrotic areas. In addition, we observed remarkably decreased proliferating cell nuclear antigen (PCNA) and cyclin E staining with a dramatic increase in the number of TUNEL-positive cells. We also found a concomitant reduction in Bcl2 expression in tumors excised from the CP-31398–treated groups (Fig. 4C).

**CP-31398 treatment augments mesenchymal-epithelial transition in A204 xenograft tumors**

To confirm whether CP-31398 alters the invasive growth of these tumors, we investigated its effects on the expression of mesenchymal-epithelial transition (MET) pathway–related proteins (24) in A204 xenograft tumors using immunofluorescence assay. In CP-31398–treated tumors, the expression of matrix metalloproteinase (MMP)-2/MMP-9, snail, slug, and twist was found to be reduced (Fig. 4D). Parallel to these observations, we noticed a concomitant decrease in the expression of fibronectin with an increase in E-cadherin expression (Fig. 4C).

**CP-31398 treatment induces SOX9 expression in xenograft tumors developed by A204 cells in nude mouse**

CP-31398 induced the expression of SOX9 in xenograft tumors, which does not consistently colocalize with TUNEL–positive cells (Fig. 5A). These results suggest that SOX9 does not target the cells destined to die. However, the observed increase of SOX9 and p21 expression in CP-31398–treated A204 xenograft tumors (Fig. 5B) indicates their role in the blockade of tumor growth because these tumors are significantly smaller in size compared with vehicle-treated controls. These results are also supported by Western blot analysis of CP-31398–treated A204 cells showing an identical pattern of SOX9 and p21 induction kinetics (Fig. 5C). This is consistent with previous reports where SOX9 was shown to bind with the promoters of p21 and induce its expression, thereby reducing tumor growth (25).

**CP-31398 reduces the growth of xenograft tumors developed by RD RMS cells in nude mice**

To determine whether CP-31398 affects the growth of mutant p53–expressing RMS, we examined the growth of RD RMS xenograft tumors in nude mice. We administered CP-31398 i.p. (2 mg/mouse daily or twice daily, every 12 hours) over a period of 11 weeks after inoculating these animals with RD cells (injected s.c. into right and left flanks). Tumor volume was measured every 5 days. Animals were sacrificed after 11 weeks of treatment. We observed that tumor growth in the CP-31398 treatment groups was reduced significantly (P < 0.05) as compared with the vehicle-treated control group in a dose-dependent manner, as shown in Fig. 6A. The tumor volume in the control group was found to be 778 ± 180 mm³, whereas it was reduced to 389 ± 99 mm³ in the CP-31398 once-daily treatment group and to 240 ± 109 mm³ in the CP-31398 twice-daily treatment group. At day 70, the differences between these two doses of CP-31398 were significant.
(P = 0.0369), but at the termination of experiment, these differences were no longer statistically significant. As compared with the vehicle-treated controls, the histology of tumors in CP-31398 treatment groups showed a larger area of necrosis. The tumor cells manifested less mitotie figures and a remarkable decrease in staining of the proliferation biomarkers PCNA and cyclin E (Fig. 6B). The CP-31398–treated tumors also showed a substantially increased number of TUNEL-positive cells. To show that these effects of CP-31398 are specifically dependent on p53, we treated RD cells with various concentrations of CP-31398 and assessed the levels of p53 and its downstream transcription target genes, p21, mdm2, and Apaf1. We observed that CP-31398 treatment stabilizes the levels of p53 and enhances the expression of p21, mdm2, and Apaf1 (Fig. 6C). In addition, consistent with immunohistochemistry and TUNEL data in xenograft tumors, CP-31398 treatment reduces the expression of cyclin E and enhances PARP cleavage.

Discussion

p53 is known to be a potent tumor suppressor and functions as a tetrameric transcription factor by binding to specific DNA sequences and transactivating or repressing a large number of target genes involved in cell cycle regulation and apoptosis (26). Therefore, it is considered to be an important drug target for blocking cancer growth. In tumors carrying mutant p53, use of small molecules such as PRIMA-1, capable of refolding mutant p53 to its wild-type conformation, may be effective in tumor regression (27, 28). In tumors where p53 is not mutated, agents such as Nutlins have been shown to be effective in blocking tumor progression (29). By disrupting mdm2 binding to p53, Nutlins enhance the levels of p53 protein in tumors carrying wild-type p53 (30). Mdm2 is a ubiquitin ligase that degrades wild-type p53 by its polyubiquitination (31). In this study, we used CP-31398, a chemical agent that has both an ability to revoke wild-type functions of mutant p53 and a potential to induce wild-type p53 (32, 33). Earlier, we showed that CP-31398 is highly efficacious against the induction of skin cancer carrying mutant p53 (11). Because the expression of p53 in RMS is heterogeneous, we investigated the chemotherapeutic effects of CP-31398 on the growth of human xenograft tumors developed by A204 cells carrying wild-type p53 as well as tumors developed by RD cells carrying mutant p53 (homzygous 742 C>T; R248W missense mutation; ref. 34).

Figure 5. CP-31398 treatment induces SOX9 expression, which colocalizes with cells showing induction of p21 but does not colocalize with TUNEL-positive cells in A204 xenograft tumors. A, CP-31398–induced expression of SOX9 (red arrows) that does not colocalize with TUNEL-positive (green arrows) cells. B, CP-31398–induced expression of SOX9 that colocalizes with p21 expression (yellow arrow). C, Western blot showing the kinetics of SOX9 and p21 induction in A204 cells treated with CP-31398 (20 μg/mL) for various time intervals. Magnification, ×40 (insets).
It is known that increase in wild-type p53 induces proteins that block cell cycle progression and subsequently induce apoptosis (35, 36). Consistent with previous observations, we observed increased p53 levels and the induction of downstream transcriptional target genes of p53, such as p21, mdm2, puma, etc., in CP-31398–treated A204 and RD cells. This is further confirmed by the observed cell cycle arrest and apoptosis in RMS cells that manifest augmented PARP cleavage, reduced Bcl2, and enhanced Bax expression. The effects may be due to the stabilization of p53 and enhanced transcriptional activity of p53. Similarly in CP-31398–treated nude mice, we observed smaller tumors that showed enhanced numbers of TUNEL-positive cells and reduced expression of Bcl2. These results suggest that these p53-dependent effects of CP-31398 on cells in culture and in xenograft tumors are almost identical (37, 38). Interestingly, we also observed enhanced tumor-free survival of these animals, suggesting the potential of CP-31398 in the treatment of RMS.

To probe the molecular mechanism by which CP-31398 invokes apoptosis in A204 cells, we investigated its effects on the migration of p53 to the mitochondria. It is known that wild-type p53 migrates to mitochondria where it disrupts permeability pore potential and activates mitochondria-regulated intrinsic apoptotic pathways characterized by the release of mitochondrial proteins into the cytoplasm (39). Our observation that CP-31398 treatment induces mitochondrial localization of p53 in A204 cells suggests mitochondria-regulated apoptosis as the underlying mechanism in the therapeutic response of CP-31398. This was verified using CsA, a potent blocker of mitochondrial membrane pore transition. CsA blocked CP-31398–mediated p53 mitochondrial localization, alterations in MPT, and apoptosis induction in these cells. Similar results were obtained for RD cells. The involvement of mitochondria in CP-31398–mediated killing of RMS cells was further confirmed by observations in this study that CP-31398 treatment enhances ROS production;
that NAC, a cell-permeable antioxidant, affords protection against CP-31398–mediated cell death; and that NAC and CsA manifest similar protective effects in CP-31398–treated A204 cells. These data suggest the possibility that CP-31398 may induce ROS production through mitochondrial membrane disruption and that ROS play a crucial role in the induction of CP-31398–mediated apoptosis.

p53 is known to play a role in altering the expression of proteins that regulate the balance between epithelial and mesenchymal phenotypes and thus determine the invasiveness and metastatic potential of cancer cells (40). It is known that wild-type p53 suppresses cancer cell invasion by inducing MDM2-mediated slug degradation (41). Slug is a member of the Snail family of transcription repressors and is capable of repressing E-cadherin expression thereby triggering EMT (42). The observations in this study that CP-31398 reduces the expression of mesenchymal markers such as fibronectin, slug, snai, and twist with a concomitant decrease in the expression of matrix-degrading enzymes, MMP-2/MMP-9, and an increase in E-cadherin suggest that CP-31398–mediated wild-type p53 induction dampens the invasiveness of A204 xenograft tumors and acts by altering the MET. This is confirmed by the observed decrease in the expression of proliferation markers PCNA and cyclin E in these tumors. The mechanism(s) by which CP-31398 may reduce proliferation of RMS cells remains largely undefined. Recently, it has been shown that SOX9 overexpression downregulates melanoma cell proliferation through direct and indirect stimulation of the p21 promoter (25). However, our observation that SOX9 and p21 are coexpressed and that SOX9 induction does not occur in cells undergoing apoptosis is consistent with a novel mechanism by which CP-31398 may inhibit proliferation in wild-type p53–positive RMS cells. A similar efficacy of CP-31398 in abrogating the growth of xenograft RMS tumors developed in nude mice by inoculating A204 or RD cells suggests that restoring wild-type functions of mutant p53 is equally efficacious in tumor regression as activating wild-type p53. Together, these data suggest that CP-31398 has potential to block the growth of human RMS irrespective of the mutational status of p53. In summary, our data indicate that small molecular weight compounds such as CP-31398 can be highly effective in diminishing the growth and invasiveness of RMS tumors and in enhancing tumor-free survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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