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Targeting wild-type and mutant p53 with small molecule CP-31398 blocks the growth of rhabdomyosarcoma by inducing ROS-dependent apoptosis

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Abstract

Rhabdomyosarcoma (RMS) is a common soft-tissue sarcoma of childhood in need of more effective therapeutic options. 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 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 p53 expression and its downstream transcriptional targets, p21 and mdm2, enhanced expression of apoptosis-related proteins, and reduced proliferation biomarkers. Flow profiling of CP-31398-treated cells indicated an enhancement in sub-G0 and in 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 ROS release. In vivo, CP-31398 decreased the growth of tumor xenografts comprised 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.

Keywords

rhabdomyosarcoma; p53; CP-31398; mitochondria; apoptosis

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

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Précis: A novel small molecule-based strategy to modulate p53 function exerts antitumor activity in a common and aggressive type of childhood soft tissue cancer

Disclosure of Potential conflicts of Interest

No potential conflicts of interest were disclosed.

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 (UDS) 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 1985-94 (1,2). The majority of RMS tumors occur sporadically, but a subset of tumors develops in patients with cancer predisposition syndromes such as Li-Fraumeni (4).

Using multi-agent 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 utilize 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 min). Therefore, attempts to increase transcriptionally active p53 by either 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 protein levels of *wild-type* p53 includes the 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 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 following the subcutaneous 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 1, Santa Cruz Biotech.); HRP-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-1,1',3,3'-tetraethyl benzimidazolcarbocyanine iodide) staining kit (Molecular Probes Inc.); Cyclosporine A, N-Acetyl-cysteine (NAC), 2',7'-dichlorofluorescein diacetate (DCFDA) (Sigma) were purchased.

Cell culture

The ERMS A204 cells (*wild-type* p53) and the ERMS RD cells (mutant p53) were obtained from the ATCC. A204 cells were cultured in McCoy's 5A media whereas RD cells were cultured in DMEM media (Hyclone), supplemented with 10% fetal bovine serum, 100 U/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 analysis were performed as described previously (11).

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)

TUNEL staining was performed according to the vendor's guidelines.

Flow cytometric analysis of cell cycle

Flow cytometry was done using Becton Dickinson FACSCan and cell cycle was analyzed using FowJo (8.8.6) Watson pragmatic analysis software.

Mitochondrial membrane potential assay

Cells from various treatments were trypsinized, re-suspended in 200 nM JC-1 solution and incubated in a 5% CO₂ incubator at 37°C and then analyzed by flow cytometry. 5 µM of CCCP was used to completely disrupt mitochondrial potential as a positive control (11).

Measurement of intracellular ROS generation

The cells were incubated with 10 µM DCF-DA at 37°C for 15 min. The intracellular 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

6 to 8 weeks old female Nu/Nu mice from NCI were used in this study. Animals were divided into three groups of ten mice each. Each mouse from all three groups received subcutaneously 2×10^6 cells in 200 µL PBS in each flank. 24 hours after the inoculation of cells, group-1 mice received an i.p. injection of vehicle, whereas groups-2 and -3 received i.p. injections of CP-31398 (2mg/mouse) at 12hrs or at 24hrs intervals respectively for 5 consecutive days/week for one month. Tumors were measured by digital calipers and tumor volumes calculated using the formula volume = length × width × height/2 plotted as a function of days on test.

Statistics

Statistical analysis was performed using Microsoft Excel software. The significance between the 2 test groups was determined using Student's *t* test and *p* < 0.05 is considered as significant.

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 (Figure 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 (Figure 1A). This correlated with an increased percentage of cells stained with Annexin V and PI (Figure 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 that occurred in cells in all phases of the cell cycle equally, since no significant differences in the cell cycle distribution of the remaining viable cells as compared to control were discernable (Figure 1C, graph).

CP-31398 induces 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 Figure 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 following 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 pro-apoptotic and other cell-cycle regulatory proteins in these cells. A time- and dose-dependent (data not shown) increase in pro-apoptotic proteins such as Apaf-1, Bax, Bcl2, Bad, and caspase-3 were observed. Consistently, 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 was detected and often peaked at the earliest time points of 3 - 6 hours; while, increased levels of apoptotic pathway proteins Caspase 3, Bad, Puma, and PARP cleavage peaked at 12 hours. The enhanced CHK1/2 expression may in part be due to the induction of DNA damage-dependent ATM/ATR-regulated signaling pathway. However, this needs to be investigated in further detail, which is beyond the scope of this manuscript. Overall, these results are consistent with CP-31398-mediated stabilization of WT p53 protein conformation and activation of p53 downstream functions that block cell cycle and apoptosis in RMS A204 cells.

CP-31398 induces mitochondrial translocation of mutant p53, resulting in cytochrome c release and apoptosis

Next, we investigated whether CP-31398 can enhance translocation of p53 to mitochondria. It is known that *wild-type* p53 migrates to mitochondria in response to genotoxic insult (17) and alters mitochondrial membrane potential (MP) (11). This leads to release of cytochrome *c* from mitochondria to cytoplasm followed by apoptosis induction. In this experiment, we employed MitoTracker red dye to stain mitochondria of vehicle- or CP-31398-treated A204 cells. The localization of p53 in mitochondria (stained in green) was confirmed by the orange to yellow color in the overlay. Thus CP-31398 enhanced p53 mitochondrial localization (Figure 2A), whereas no such effect was discernible in vehicle-treated control cells shown at 15 minutes.

Cyclosporine A (CsA) blocks CP-31398-mediated mitochondrial localization of p53 and consequent induction of apoptosis in A204 cells

CsA is known to be a potent and specific inhibitor of mitochondrial membrane permeability pore transition (MPT) (18,19). We employed this agent to determine whether MPT 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 Figure 2B, CsA pretreatment blocked mitochondrial translocation of p53. Concomitantly, mitochondrial cytochrome *c* release into the cytoplasm was also diminished (Figure 2C). In addition, CP-31398-mediated apoptosis was abrogated.

CP-31398 alters mitochondrial membrane potential

To confirm that CP-31398 alters MP, we employed 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 (Figure 2D). However, as expected, CsA pretreatment blocked changes in CP-31398-induced MP.

CP-31398 triggered reactive oxygen species generation which mediated apoptosis in A204 cells

Reactive Oxygen Species (ROS) are considered to play an important role in apoptosis in various cells 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 h and 24 h. The number of DCF-positive cells was 1.5%, 36.4%, and 52.3% at 0, 12, and 24 h, respectively (Fig. 3A). However, the ROS scavenger NAC (5 mM) markedly decreased the level of ROS to ~6% at 24 h. Furthermore, the enhanced ROS production was significantly reduced by pre-treating cells with CsA (Fig. 3B), suggesting that the major source of CP-31398-mediated ROS release may be mitochondria.

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

To investigate whether CP-31398 affects 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 following 4 weeks of treatment. We found that tumor growth in the CP-31398 treatment groups was reduced significantly as compared to vehicle-treated control group in a dose-dependent manner as shown in Fig 4A. The tumor volume in the control group was $277 \pm 47 \text{ mm}^3$, whereas it reduced to $127 \pm 41 \text{ mm}^3$ in the CP-31398 daily treatment group and to $68 \pm 24 \text{ mm}^3$ in the CP-31398 twice daily treatment group ($P < 0.05$ and 0.01 respectively). At early time-points (day 14 and 18), the differences between the two doses of CP-31398 were found to be significant ($P < 0.05$), while at later time-points (day 22 and 30), these differences became less significant. As compared to the vehicle-treated controls, the histology of tumors in CP-31398 treatment groups showed less mitotic figures (Fig 4B) and large necrotic areas. In addition, we observed a remarkably decreased 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 CP-31398-treated groups (Fig 4C).

CP-31398 treatment augments mesenchymal-epithelial transition (MET) in A204 xenograft tumors

To confirm whether CP-31398 alters the invasive growth of these tumors, we investigated its effects on the expression of MET pathway-related proteins (24) in A204 xenograft tumors employing immunofluorescence assay. In CP-31398-treated tumors, the expression of matrix metalloproteinases (MMP) 2/9 and 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 mice

CP-31398 induced the expression of SOX9 in xenograft tumors, which does not consistently co-localize 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 as these tumors are significantly smaller in size compared to 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 induces 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 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 subcutaneously to right and left flanks). Tumor volume was measured every 5 days. Animals were sacrificed following 11 weeks of treatment. We observed that tumor growth in the CP-31398 treatment groups was reduced significantly ($P < 0.05$) as compared to 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 $778 \pm 180 \text{ mm}^3$, whereas it reduced to $389 \pm 99 \text{ mm}^3$ in the CP-31398 single daily treatment group and to $240 \pm 109 \text{ mm}^3$ in the CP-31398 twice daily treatment group. At day 70, the differences between these two doses of CP-31398 were significant ($P = 0.0369$) while at the termination of experiment, these differences were not statistically significant. As compared to the vehicle-treated controls, the histology of tumors in CP-31398 treatment groups showed a larger area of necrosis. The tumor cells manifested less mitotic figures and a remarkable decrease in staining of 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 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 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 like Nutlins have been shown to be effective in blocking tumor progression (29). By disrupting mdm2 binding to p53, Nutlins enhance 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 employed CP-31398, a chemical agent that has both an ability to revoke wild-type functions of mutant p53 and potential to induce *wild-type* p53 (32,33). Earlier, we demonstrated that CP-31398 is highly efficacious against the induction of skin cancer carrying mutant p53 (11). Since 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 RD cells carrying mutant p53 (homozygous 742 C>T; R248W missense mutation) (34).

It is known that increase in *wild-type* p53 act by inducing 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 both 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 both the stabilization of p53 and enhanced transcriptional activity of p53. Similarly in CP-31398-treated nude mice, we observed smaller tumors which showed enhanced numbers of TUNEL-positive cells and reduced expression of Bcl2. These results suggest that these p53-dependant 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 a 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 observations that CP-31398 treatment induces mitochondrial localization of p53 in A204 cells suggest the involvement of mitochondria-regulated apoptosis as the underlying mechanism in the therapeutic response of CP-31398. This was verified employing CsA, a potent blocker of mitochondrial membrane pore transition. CsA blocked CP-31398-mediated p53 mitochondrial localization, alterations in MMPT 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 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 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/-9, and an increase in E-cadherin suggest that CP-31398-mediated *wild-type* p53 induction dampens invasiveness of A204 xenograft tumors and acts by altering the phenomenon of MET. This is confirmed by the observed decrease in the expression of proliferation biomarkers PCNA and cyclin E in these tumors. The mechanism(s) by which CP-31398 may reduce proliferation of RMS cells remain largely undefined. Recently, it has been demonstrated that SOX9 overexpression down-regulates melanoma cell proliferation through direct and indirect stimulation of the p21 promoter (25). However, our observation that SOX9 and p21 are co-expressed 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. 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 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 enhancing of tumor free survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ries, LAGSM.; Gurney, JG.; Linet, M.; Tamra, T.; Young, JL.; Bunin, GR., editors. Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975-1995, National Cancer Institute. NIH Pub; SEER Program Bethesda, MD: 1999.
2. Arndt CA, Crist WM. Common musculoskeletal tumors of childhood and adolescence. N Engl J Med. 1999; 341(5):342–52. [PubMed: 10423470]
3. Meza JL, Anderson J, Pappo AS, Meyer WH. Analysis of prognostic factors in patients with nonmetastatic rhabdomyosarcoma treated on intergroup rhabdomyosarcoma studies III and IV: the Children's Oncology Group. J Clin Oncol. 2006; 24(24):3844–51. [PubMed: 16921036]
4. Li FP, Fraumeni JF Jr. Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. J Natl Cancer Inst. 1969; 43(6):1365–73. [PubMed: 5396222]
5. Carli M, Colombatti R, Oberlin O, et al. European intergroup studies (MMT4-89 and MMT4-91) on childhood metastatic rhabdomyosarcoma: final results and analysis of prognostic factors. J Clin Oncol. 2004; 22(23):4787–94. [PubMed: 15570080]
6. Bassett EA, Wang W, Rastinejad F, El-Deiry WS. Structural and functional basis for therapeutic modulation of p53 signaling. Clin Cancer Res. 2008; 14(20):6376–86. [PubMed: 18927276]
7. Felix CA, Kappel CC, Mitsudomi T, et al. Frequency and diversity of p53 mutations in childhood rhabdomyosarcoma. Cancer Res. 1992; 52(8):2243–7. [PubMed: 1559227]
8. Hollstein M, Rice K, Greenblatt MS, et al. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res. 1994; 22(17):3551–5. [PubMed: 7937055]
9. Woods YL, Lane DP. Exploiting the p53 pathway for cancer diagnosis and therapy. Hematol J. 2003; 4(4):233–47. [PubMed: 12872148]
10. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Pharmacological rescue of mutant p53 conformation and function. Science. 1999; 286(5449):2507–10. [PubMed: 10617466]
11. Tang X, Zhu Y, Han L, et al. CP-31398 restores mutant p53 tumor suppressor function and inhibits UVB-induced skin carcinogenesis in mice. J Clin Invest. 2007; 117(12):3753–64. [PubMed: 18060030]
12. Marx J. Oncology. Recruiting the cell's own guardian for cancer therapy. Science. 2007; 315(5816):1211–3. [PubMed: 17332387]
13. Rippin TM, Bykov VJ, Freund SM, Selivanova G, Wiman KG, Fersht AR. Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. Oncogene. 2002; 21(14):2119–29. [PubMed: 11948395]
14. Wang W, Takimoto R, Rastinejad F, El-Deiry WS. Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. Mol Cell Biol. 2003; 23(6):2171–81. [PubMed: 12612087]

15. Barlow JW, Wiley JC, Mous M, et al. Differentiation of rhabdomyosarcoma cell lines using retinoic acid. *Pediatr Blood Cancer*. 2006; 47(6):773–84. [PubMed: 16283617]
16. Bache M, Dunst J, Wurl P, et al. G2/M checkpoint is p53-dependent and independent after irradiation in five human sarcoma cell lines. *Anticancer Res*. 1999; 19(3A):1827–32. [PubMed: 10470122]
17. Erster S, Mihara M, Kim RH, Petrenko O, Moll UM. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol*. 2004; 24(15):6728–41. [PubMed: 15254240]
18. Marques-Santos LF, Coqueiro VM, Rumjanek VM. Cyclosporin A does not protect the disruption of the inner mitochondrial membrane potential induced by potassium ionophores in intact K562 cells. *Cell Biol Int*. 2006; 30(3):197–204. [PubMed: 16376584]
19. Tay VK, Wang AS, Leow KY, Ong MM, Wong KP, Boelsterli UA. Mitochondrial permeability transition as a source of superoxide anion induced by the nitroaromatic drug nimesulide in vitro. *Free Radic Biol Med*. 2005; 39(7):949–59. [PubMed: 16140214]
20. Smiley ST, Reers M, Mottola-Hartshorn C, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci U S A*. 1991; 88(9):3671–5. [PubMed: 2023917]
21. Di Lisa F, Blank PS, Colonna R, et al. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. *J Physiol*. 1995; 486(Pt 1):1–13. [PubMed: 7562625]
22. Huang J, Wu L, Tashiro S, Onodera S, Ikejima T. Reactive oxygen species mediate oridonin-induced HepG2 apoptosis through p53, MAPK, and mitochondrial signaling pathways. *J Pharmacol Sci*. 2008; 107(4):370–9. [PubMed: 18719315]
23. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*. 2000; 5(5):415–8. [PubMed: 11256882]
24. Gentile A, Trusolino L, Comoglio PM. The Met tyrosine kinase receptor in development and cancer. *Cancer Metastasis Rev*. 2008; 27(1):85–94. [PubMed: 18175071]
25. Passeron T, Valencia JC, Namiki T, et al. Upregulation of SOX9 inhibits the growth of human and mouse melanomas and restores their sensitivity to retinoic acid. *J Clin Invest*. 2009; 119(4):954–63. [PubMed: 19273910]
26. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature*. 1991; 351(6326):453–6. [PubMed: 2046748]
27. Liang Y, Besch-Williford C, Hyder SM. PRIMA-1 inhibits growth of breast cancer cells by re-activating mutant p53 protein. *Int J Oncol*. 2009; 35(5):1015–23. [PubMed: 19787255]
28. Zache N, Lambert JM, Wiman KG, Bykov VJ. PRIMA-1MET inhibits growth of mouse tumors carrying mutant p53. *Cell Oncol*. 2008; 30(5):411–8. [PubMed: 18791272]
29. Miyachi M, Kakazu N, Yagyu S, et al. Restoration of p53 pathway by nutlin-3 induces cell cycle arrest and apoptosis in human rhabdomyosarcoma cells. *Clin Cancer Res*. 2009; 15(12):4077–84. [PubMed: 19509161]
30. Zhang L, Zhang J, Hu C, et al. Efficient activation of p53 pathway in A549 cells exposed to L2, a novel compound targeting p53-MDM2 interaction. *Anticancer Drugs*. 2009; 20(6):416–24. [PubMed: 19579266]
31. Coutts AS, Adams CJ, La Thangue NB. p53 ubiquitination by Mdm2: a never ending tail? *DNA Repair (Amst)*. 2009; 8(4):483–90. [PubMed: 19217357]
32. Tanner S, Barberis A. CP-31398, a putative p53-stabilizing molecule tested in mammalian cells and in yeast for its effects on p53 transcriptional activity. *J Negat Results Biomed*. 2004; 3:5. [PubMed: 15548325]
33. Bullock AN, Fersht AR. Rescuing the function of mutant p53. *Nat Rev Cancer*. 2001; 1(1):68–76. [PubMed: 11900253]
34. Taylor AC, Shu L, Danks MK, et al. P53 mutation and MDM2 amplification frequency in pediatric rhabdomyosarcoma tumors and cell lines. *Med Pediatr Oncol*. 2000; 35(2):96–103. [PubMed: 10918230]

35. Conzen SD, Gottlob K, Kandel ES, et al. Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-Myc: transrepression correlates with acceleration of apoptosis. *Mol Cell Biol.* 2000; 20(16):6008–18. [PubMed: 10913183]
36. Chen F, Chang D, Goh M, Klibanov SA, Ljungman M. Role of p53 in cell cycle regulation and apoptosis following exposure to proteasome inhibitors. *Cell Growth Differ.* 2000; 11(5):239–46. [PubMed: 10845424]
37. Takimoto R, Wang W, Dicker DT, Rastinejad F, Lyssikatos J, el-Deiry WS. The mutant p53-conformation modifying drug, CP-31398, can induce apoptosis of human cancer cells and can stabilize wild-type p53 protein. *Cancer Biol Ther.* 2002; 1(1):47–55. [PubMed: 12174820]
38. Luu Y, Bush J, Cheung KJ Jr, Li G. The p53 stabilizing compound CP-31398 induces apoptosis by activating the intrinsic Bax/mitochondrial/caspase-9 pathway. *Exp Cell Res.* 2002; 276(2):214–22. [PubMed: 12027451]
39. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell.* 2003; 11(3):577–90. [PubMed: 12667443]
40. Kurrey NK, Jalgaonkar SP, Joglekar AV, et al. Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. *Stem Cells.* 2009; 27(9):2059–68. [PubMed: 19544473]
41. Wang SP, Wang WL, Chang YL, et al. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. *Nat Cell Biol.* 2009; 11(6):694–704. [PubMed: 19448627]
42. Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J Cell Sci.* 2003; 116(Pt 3):499–511. [PubMed: 12508111]

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 hours then stained with propidium iodide (PI) for immediate flow cytometric analysis. A, Loss of viable cells. Two parameter pseudocolor density plots of total un-gated events for forward 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 upper left corner of each un-gated panel. The small circular gate encompasses viable cells. The percentage of cells is displayed in the lower right corner of each un-gated panel. A graph summarizing the % of viable cells within the single cell gate is shown at the bottom. B, Increased apoptotic cells. Two parameter bitmaps displays of gated cells (indicated by arrows in A.) analyzed for Annexin V and PI staining (left) and histograms for PI alone (right). The percentage of Annexin V positive cells within PI negative (early apoptosis) and PI positive (late apoptosis) subsets are shown in the upper left and right quadrants, respectively. A graph displaying total apoptotic and dead cells (% Annexin + PI) is shown at the bottom. The percentage of PI stained cells is shown in histogram, and a summary graph is shown at the bottom. Note the inverse correlation of viable cells (panel for A), indicated by light scatter properties (small gate) and apoptotic cells (central and right panel graphs in B). C, G1 phase block of cell cycle. A204 cells were incubated with the indicated concentration of CP-31398 for 24 hrs and DNA 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 $\pm$ SEM (n=3) for the percent of cells in each phase. *Student's t-Test (2-sided) $P < 0.05$. 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 μ g/ml CP for different times (as indicated).

Figure 2. CP-31398 induces apoptosis in A204 cells by translocating p53 to mitochondria, and alters mitochondrial membrane potential (MP) in A204 cells which can be blocked by Cyclosporin A (CsA) pretreatment

A, IF staining showing co-localization of p53 with Mitotracker, which stains mitochondria; B, IF staining showing that CsA blocks CP-31398-induced p53 localization to mitochondria; C, CsA blocked CP-31398-induced cytochrome *c* release in the cytoplasm; and D, CP-31398-mediated alterations in MP are attenuated by CsA treatment. For experiments described in A to C, A204 cells were treated with PBS (control) or CP-31398 (20 μ g/ml) for various time intervals. However, for experiments described in D, cells were treated with PBS (control) or CP-31398 (20 and 40 μ g/ml) for 15 minutes, and then stained with JC-1 dye. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control. CsA (1 μ M) pretreatment was done for 15 min prior to CP treatment. Each value represents mean $\pm$ S.D. of three independent experiments. A $P < 0.05$ when compared to control; b $P < 0.01$ when compared to control; c $P < 0.05$ when compared to 20 μ g/ml CP-31398 treatment.

Figure 3. CP-31398 induces ROS and ROS-dependent cell death in A204 cells

A, CP-31398 stimulated the generation of ROS, which was blocked by pre-treating cells with NAC. Cells were cultured in medium containing CP-31398 (20 μ g/ml) for 0, 12 and 24 h in the absence or presence of 5 mM NAC; B, Pretreatment of CsA blocked CP-31398-induced generation of ROS. ROS generation was measured by using the ROS-detecting fluorescent dye, DCF-DA on a flow cytometry. The corresponding linear diagram of FACSscan is also shown ($n=3$, mean $\pm$ S.D; $**P < 0.01$). Cells were treated with CP-31398 (20 μ g/ml) for 15min. Each value represents mean $\pm$ S.D. of three independent experiments ($n=3$, $*P < 0.01$).

Figure 4. CP-31398 treatment reduces the growth xenograft tumors and augments mesenchymal-epithelial transition 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 indicated by red arrow and apoptosis indicated by green arrow); C, TUNEL, IF and IHC 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 MMP2/9, snai, slug, twist and fibronectin in xenograft tumors. D1 represents CP-31398 daily treatments (24h intervals) whereas D2 represents twice daily (12h intervals) treatments. Mice were treated over 4 weeks beginning treatment at the time of tumor cell inoculation. Each value represents mean $\pm$ S.E. of 10 mice (* P < 0.05, ** P < 0.01).

Figure 5. CP-31398 treatment induces SOX9 expression which co-localizes in cells showing induction of p21 but does not co-localize with TUNEL-positive cells in A204 xenograft tumors
A, CP-31398-induced expression of SOX9 (red arrows) which does not co-localize with TUNEL-positive (green arrows) cells; and B, CP-31398-induced expression of SOX9 that co-localizes 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. Insets show a 40X magnification.

Figure 6. CP-31398 treatment reduces the growth of xenograft tumors developed by RD cells carrying mutant p53 in nude mice

A, CP-31398 reduces the volume of xenograft tumors ($P < 0.05$). D1 represents CP-31398 daily treatments at 24h interval whereas D2 represents twice daily treatments at 12h interval. B, Histology of tumors developed in vehicle or CP-31398-treated mice (insets show mitotic figures with red arrows and apoptosis with green arrows). IHC and TUNEL staining showing that CP-31398-treatment reduces proliferation as assessed by PCNA, cyclin E expression (arrow indicates positive nuclear staining) and induces apoptosis (as assessed by an increase in TUNEL positive cells) in xenograft tumors. C, Western blot analysis showing that CP-31398 induces stabilization of p53, enhances the expression of its downstream target genes. In addition, it induces apoptosis related genes and reduces the expression of proliferation marker proteins. Mice were treated over 11 weeks starting at the time of tumor inoculation. Each value represents mean $\pm$ S.E. of 5 mice.