(letters or pictures) they attended. This task was performed for epochs of 36.9 s with repetition targets occurring pseudorandomly every six stimuli on average. To ensure that attending pictures was demanding, pictures had different orientations within any repeated pair, rotated 30° clockwise or counterclockwise from their natural axis (Fig. 1). Repetitions also took place in the unattended stream (requiring no response), uncorrelated in time with repetition in the attended stream. A button press response was required with auditory feedback for hits and misses. Participants were instructed about which stream to attend by an auditory cue in the rest epoch preceding each task epoch. The number of targets per epoch was balanced across conditions. Task order followed a within-participant Latin-square design. During both attention to letter strings and attention to pictures, any words were drawn from one of two different lists balanced for word frequency, concreteness, and imageability and were fully counterbalanced across participants.

11. A Siemens VISION (Siemens, Erlangen) acquired blood oxygenation level–dependent (BOLD) contrast functional images at 2 T. Image volumes were acquired continuously every 4100 ms, each comprising 48 contiguous 3-mm-thick slices to give whole-brain coverage with an in-plane resolution of 3 mm by 3 mm. Functional imaging was performed in four scanning runs comprising 288 volumes in total. In each scanning run, after eight image volumes were discarded to allow for T1 equilibration effects, the experimental conditions were presented for 36.9 s (nine scans) alternating with fixation control (rest) for nine scans. Condition order was counterbalanced across participants.
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16. After scanning, a surprise recognition memory test was presented. Participants indicated by button press whether each word shown had been presented during the scanning experiment. Three randomly intermingled word lists were tested (attended words, unattended words, and never-seen foils), matched for word frequency, concreteness, and imageability, with list membership counterbalanced across participants. Memory for attended words was better than for unattended words ($t = 5.2$, $P < 0.05$) and the latter did not differ from the chance rate given by false-positive responses to never-seen foils ($t = 0.08$, $P > 0.5$).
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18. Statistical parametric mapping software (SPM 99, http://www.fil.ion.ucl.ac.uk/spm) was used. The imaging time series was realigned, spatially normalized to stereotactic Talairach space, and smoothed with a Gaussian kernel of 10 mm full-width at half-maximum. Voxels activated during the experimental conditions were identified by a statistical model containing four delayed boxcar waveforms that represented the mean activity evoked in the experimental conditions. High-pass filtering removed participant-specific low-frequency drifts in signal, and global changes were removed by proportional scaling. Each component of the model served as a regressor in a multiple regression analysis. Masking with the contrast between the four experimental conditions versus baseline fixation was used to restrict our analysis to areas activated by the experimental conditions. A statistical threshold of $P < 0.05$, corrected for multiple comparisons, was used except where specified. Further inspection of any simple effect of unattended words minus consonants lowered the threshold to uncorrected $P < 0.001$ but still found no activation.
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22. Spatial variability among activations across subjects might obscure an otherwise consistent neural response to ignored words. To test this, we repeated the analysis, but with the voxel of peak activation to attended words selected within individual subjects (for all the cortical areas in Table 1, within a 10-mm radius of the group activation coordinates) to identify any activation to unattended words. Again, we found no significant differential activation (all $P < 0.05$).
23. The numerically lower activity for unattended words versus unattended consonants in Fig. 3 raises the possibility that inhibitory mechanisms might suppress responses to unattended words [possibly related to the psychological phenomenon of negative priming (*6*)]. However, the apparent lowering of activity was not reliable; our data showed neither significant activation nor deactivation for unattended words in these cortical areas. Moreover, psychologically, negative priming is typically eliminated under conditions of high attentional load [N. Lavie and E. Fox, *J. Exp. Psychol. Hum. Percept. Perform.*, in press; A. Treisman and B. DeSchepper, in *Attention & Performance XVI*, T. Inui and J. L. McClelland, Eds. (MIT Press, Cambridge, MA, 1996), pp. 15–46] such as the present demanding picture task.
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Pharmacological Rescue of Mutant p53 Conformation and Function

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Compounds that stabilize the DNA binding domain of p53 in the active conformation were identified. These small synthetic molecules not only promoted the stability of wild-type p53 but also allowed mutant p53 to maintain an active conformation. A prototype compound caused the accumulation of conformationally active p53 in cells with mutant p53, enabling it to activate transcription and to slow tumor growth in mice. With further work aimed at improving potency, this class of compounds may be developed into anticancer drugs of broad utility.

The p53 tumor suppressor gene is mutated with high frequency in human cancers, and reintroduction of wild-type p53 can suppress tumorigenicity (*1*). The transcription regulatory and tumor suppressor activity of p53 is absolutely dependent on the ability of the protein to maintain the DNA binding conformation (*2*). A large number of weakly interacting amino acids in the central DNA binding domain (DBD) of p53 contribute to the stability of a structured scaffold that orients the two loops and the loop-sheet-helix motif of the DNA binding surface (*3*). Recent evidence suggests that the most frequently encountered mutations in p53 reduce the thermodynamic stability of the DBD (*4*). Destabilization of the active conformation, which occurs under denaturing conditions or upon mutation of p53, reduces the binding of p53 to specific peptides, to cellular and viral proteins, and to the monoclonal antibody (mAb) mAb1620 (*5*). In contrast, the epitope for mAb240 is exposed when the active conformation is disrupted (*6*).

Ablation of a negative regulatory domain at the p53 COOH-terminus by antibodies and peptides has been used to promote the activity of certain mutant p53 forms (*7*). Here, we present an alternative approach to promoting p53 activity by stabilizing the active conformation of the DBD. We observed that purified wild-type p53 DBD is naturally temperature-sensitive for loss of the active conformation. The epitope for mAb1620 was lost in a temperature- and time-dependent manner when the protein was immobilized on microtiter plates and heated (Fig. 1A). An eight–amino acid epitope tag (FLAG) that was fused to the DBD remained fully stable under these conditions. Furthermore, loss of the 1620 epitope occurred in concert with the enhanced appearance of the 240 epitope, confirming the transition of the protein into a nonfunctional conformation. The half-life of

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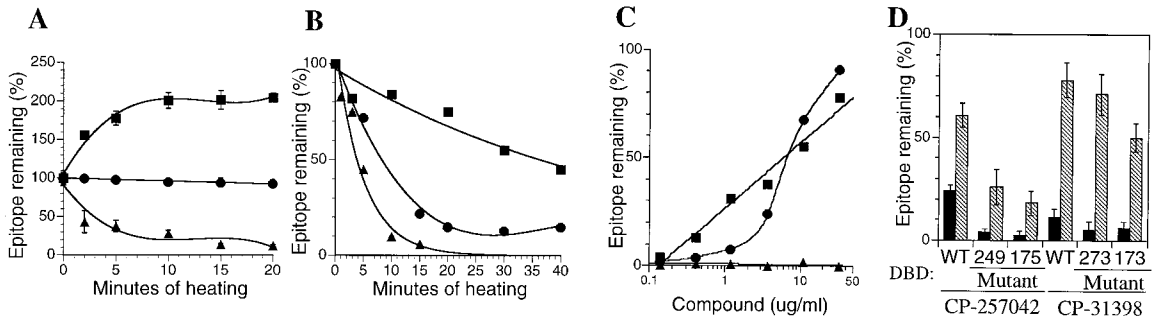

Fig. 1. Modulation of conformation-dependent epitopes on p53 DBD. (**A**) FLAG-tagged p53 DBD (1.25 ng) was immobilized on microtiter wells and incubated at 45°C, and the epitopes for mAb240 (■), antibody to FLAG (●), and mAb1620 (▲) were measured with each of the antibodies (*12*). Epitope remaining is shown as percentage of the control protein that was immobilized and maintained on ice. Error bars represent standard deviation. (**B**) Wild-type p53 DBD (0.5 ng) was immobilized and incubated at 23°C (■), 32°C (●), or 45°C (▲), and the epitope for mAb1620 was measured. Standard deviations were <10%. (**C**) Wild-type p53 DBD (1 ng) was immobilized and heated at 45°C for 30 min in the presence of CP-31398 (■), CP-257042 (●), or the equivalent concentration of the DMSO vehicle (▲). The remaining epitope for mAb1620 is shown as percentage of unheated control. (**D**) Wild-type (WT) and mutant p53 DBD preparations, with nearly equal amounts of epitope for mAb1620 (within 10%), were immobilized and heated at 37°C for 30 min in the presence of vehicle (solid bars) or compound (hatched bars). The remaining epitope for mAb1620 is shown as percentage of unheated controls. Error bars are the standard deviation for four replicates.

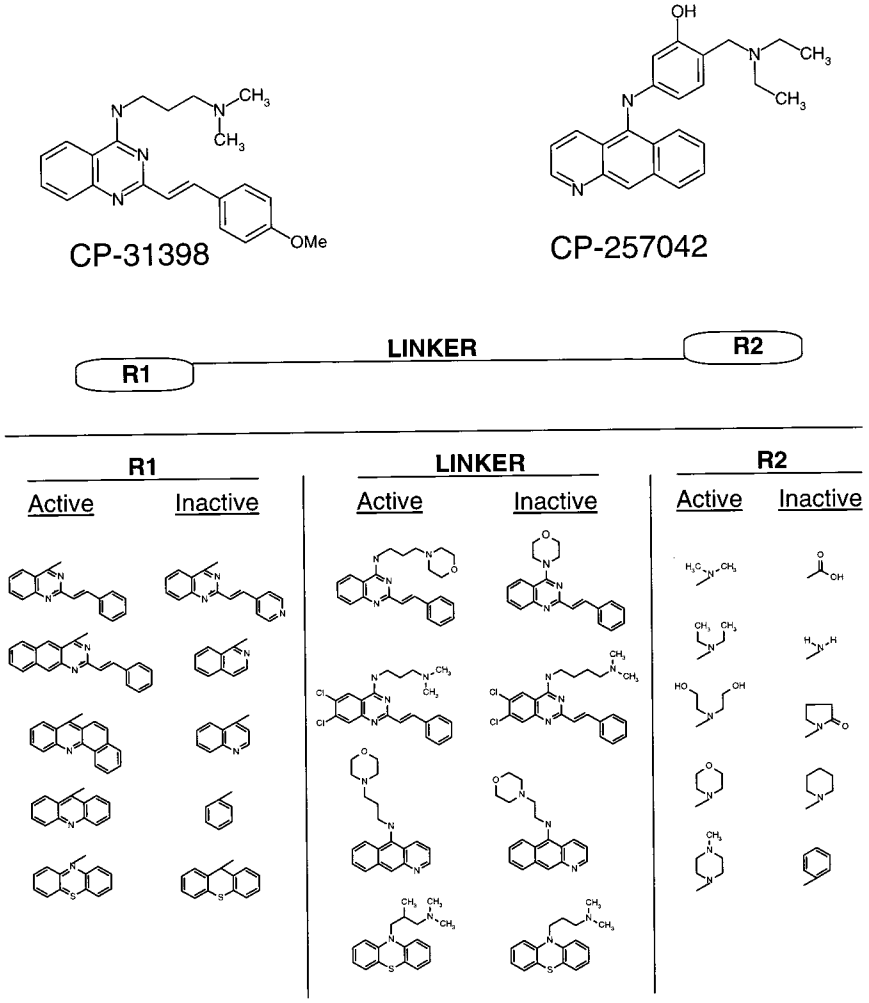

Fig. 2. Structural features of the active compounds. The relative activity of ~300 related compound analogs was assessed on the basis of the concentration of compound required to stabilize 50% of the mAb1620 epitope on wild-type p53 DBD. Active and inactive compounds denote >10-fold differences in potency of matched compound pairs.

the 1620 epitope on immobilized wild-type p53 DBD was ~35 min at 23°C and decreased to <5 min at 45°C (Fig. 1B). In parallel, the DNA binding capacity of p53 DBD in gel shift assays was reduced upon heating in solution (*8*), confirming the assertion that the 1620 epitope provides an accurate measure of the protein's DNA binding conformation (*2*, *5*).

Upon screening a library of >100,000 synthetic compounds and testing analogs of the active compounds, we identified multiple classes of small molecules (300 to 500 daltons) that promoted the conformational stability of wild-type p53 DBD as well as that of full-length p53 (*8*). Active compounds stabilized the epitope for mAb1620 in a dose-dependent manner when p53 was heated (Fig. 1C). The dimethyl sulfoxide (DMSO) solvent and several related analogs of the active compounds failed to stabilize.

We next examined whether the compounds could stabilize the active conformation of mutant p53. DBDs from several mutant p53 proteins were isolated from bacteria grown at 17°C where a substantial fraction of the protein retained the epitope for mAb1620 (*4*, *9*). The mutant DBDs were less stable than wild-type DBD when heated, but the stability of the mutants in the presence of compound equaled or exceeded that of the wild-type p53 in the absence of compound (Fig. 1D). These compounds did not rescue p53 that had already lost the active conformation. Indeed, there was no increase in mAb1620 reactivity when p53 DBD was heated before compound addition. Also, the compounds reduced the rate of epitope loss, but prolonged heating resulted in loss of the active conformation. Compound wash-out before incubation at 37°C did not prevent epitope loss, suggesting that the effect may be reversible (*8*).

All of our active compounds join together a hydrophobic group (R1, polycyclic) and an ionizable group (R2, often an amine) by a linker of a specific length (Fig. 2). Certain substitutions at R1 and R2 positions maintained activity, whereas even subtle changes in these groups rendered the compounds in-

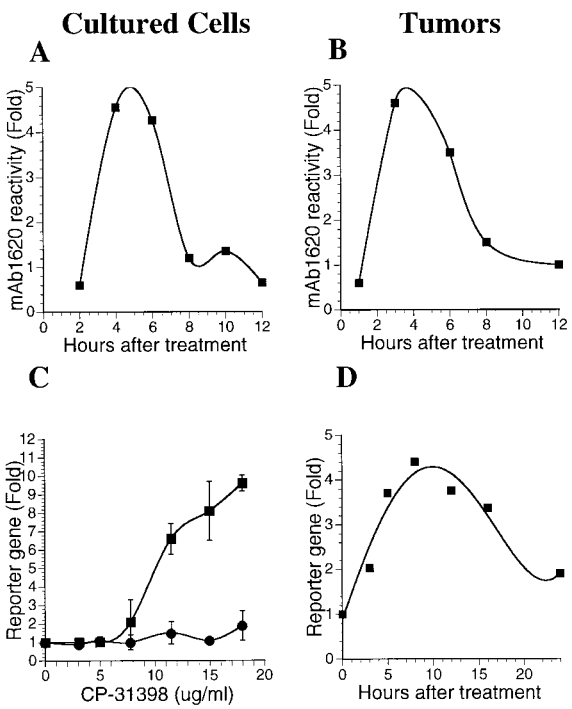

Fig. 3. Modulation of p53 conformation and transcription activity in cultured cells (A and C) and in tumors (B and D). (**A**) Cultured H1299 cells transfected with 173A mutant p53 were treated with CP-31398 (15 μg ml^{-1}) (*13*). Total levels of p53 were unchanged as assessed with mAbDO-1. Cell lysates representing equal amounts of p53 were added to microtiter plates coated with mAb1620, and the amount of p53 that displayed the epitope for mAb1620 was measured (*14*). Values represent the average of three replicates. Reactivity with mAb1620 did not change in vehicle-treated cells. (**B**) Mice with subcutaneous tumors derived from H1299 transfectants were given a single intraperitoneal injection of CP-31398 (100 mg kg^{-1}), and tumor lysates from two mice at each time point were prepared. Lysates representing equal amounts of total p53 were assayed as above to determine the relative fraction of p53 that displayed the epitope for mAb1620. Each sample deviated by <15% from the average (shown), and similar results were obtained when the experiment was repeated. (**C**) Matched H1299 transfectants with a luciferase reporter gene (●) or with the reporter gene and the 173A mutant p53 (■) were treated in microtiter wells for 16 hours (*15*). As a measure of p53 transcription activation function, the expression of luciferase reporter gene was corrected for the basal level of expression in the absence of compound. Shown are results of a representative experiment with standard deviation for four replicate wells. (**D**) Tumor lysates from animals treated as in (B) were normalized for protein content and analyzed for luciferase expression. Luciferase expression was unchanged in lysates from vehicle-treated tumors.

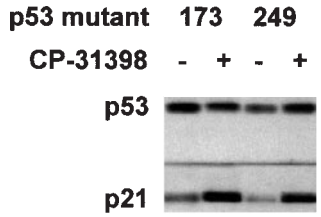

Fig. 4. Induction of p21 in cells expressing only mutant p53. Saos-2 cells expressing transfected mutant p53 were treated with CP-31398 (16 μg ml^{-1}) for 16 hours. Cell lysates were normalized for total protein and analyzed on protein immunoblots for the expression of p21 and total p53 (*16*).

active. Negatively charged or uncharged groups at R2 were always inactive. The spacing between R1 and R2 was also critical because a propyl length linker was optimal for activity. Branched linkers often improved activity. Although the exact nature of the molecular interaction remains to be elucidated, these observations suggest a bivalent contact between p53 and the compounds through the R1 and the R2 groups. The optimal length of the linker may reflect the necessity of precisely distancing or orienting these two sites on the protein while providing a tether that enhances the stability of the active conformation.

To examine the effect of compounds on the conformational stability of p53 in living cells, we used the p53-null H1299 lung carcinoma cells that were transfected with mutant p53. The mAbDO-1, which does not discriminate between the active and the inactive conformations of p53, was used in protein immunoblots to select clones expressing mutant p53. Immunoprecipitation of the transfected cell extracts with mAb1620 confirmed that only a small fraction of mutant p53 in cells retains the active conformation (*10*). Low micromolar concentrations of a prototype compound, CP-31398, increased the steady-state fraction of 1620-positive p53 in cells by fivefold at 4 to 6 hours after treatment (Fig. 3A). CP-31398 did not alter the total amount of p53 as measured with mAbDO-1. Despite the large pool of inactive p53 in these cells, the compounds are likely to stabilize only the newly synthesized p53 that is in the active conformation and thus allow for time-dependent accumulation of this fraction.

CP-31398 also enhanced the steady-state levels of the p53 fraction that displays the epitope for mAb1620 in tumors derived from transfected H1299 cells. Tumor-bearing mice were killed after a single dose of 100 mg kg^{-1}, and the conformational status of p53 was quantified in the tumor lysates. Total p53 levels were unchanged as measured on protein immunoblots with mAbDO-1. The relative amount of the mAb1620 epitope was increased by ~fivefold at 3 hours after treatment (Fig. 3B).

We next examined the effect of the compound on the sequence-specific transcription activity of p53. H1299 cells were transfected with a p53-inducible luciferase reporter gene, and a stable clone (H1299/Reporter) was retransfected to express mutant p53. In the presence of mutant p53, CP-31398 increased reporter gene expression by ~10-fold (Fig. 3C). This induction of the reporter gene was dependent on the presence of mutant p53 because H1299/Reporter cells did not activate the reporter gene.

To determine if mutant p53 could be functionally restored in vivo, we measured the reporter gene in tumors. A maximum 4.5-fold induction was observed at 8 hours after a single intraperitoneal dose of CP-31398 (Fig. 3D). No induction was observed in mice treated with the vehicle. The peak plasma concentration of compound in mice was ~10 μg ml^{-1} (*8*). This may account for the lower levels of reporter gene induction in tumors as compared with cultured cells, where a 10-fold induction was observed at 18 μg ml^{-1} (Fig. 3C).

We also examined the ability of CP-31398 to induce the cellular p21 gene in the absence of wild-type p53. As compared with vehicle treatment, CP-31398 elevated p21 expression by ~threefold in Saos-2 osteosarcoma cells that express either position 173 or position 249 mutant p53 (Fig. 4). The total amount of mutant p53 protein in these cells was unchanged, suggesting that conformationally stabilized p53 can activate a relevant downstream gene.

We next examined whether CP-31398 could inhibit the growth of small human tumor xenografts with naturally mutated p53. The compound appeared safe, and no mortality was observed when mice were dosed at 200 mg kg day^{-1} (100 mg kg^{-1}, every 12 hours) for 14 consecutive days (*8*). Doses above 100 mg kg^{-1} did not appreciably increase plasma concentrations, suggesting that absorption may be limited. The A375.S2 melanoma cell line (mutated at p53 position 249) and the DLD-1 colon carcinoma cell line (mutated at p53 position 241) rapidly formed tumors in nude mice (Fig. 5). Seven daily injections of CP-31398 (l00 mg kg^{-1}) suppressed A375.S2 tumor growth by ~50%, and twice daily administrations inhibited tumor growth by 75% (Fig. 5). Twice daily treatments completely inhibited DLD-1 tumor growth. The growth of both tumor types resumed upon cessation of treatment, confirming the inhibitory activity of the com-

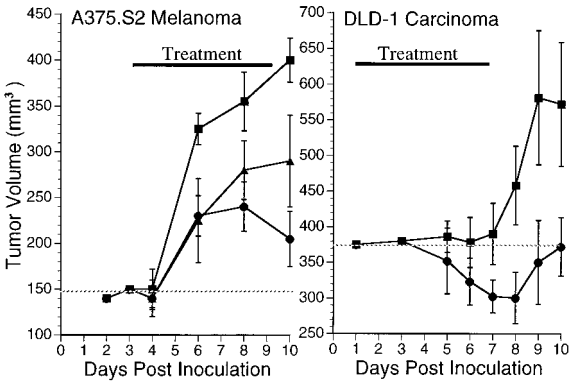

Fig. 5. Growth inhibition of tumors with naturally mutated p53. Mice were inoculated with human tumor cells in Matrigel, a gel matrix with growth factors, and treated by intraperitoneal injections of CP-31398 or vehicle (*17*). Groups of mice were randomized for equal inoculum size on the basis of measurement of the Matrigel plug and treated for 7 days at 24-hour (▲) or 12-hour (●) intervals. Vehicle-treated mice received injections at 12-hour intervals (■). Dotted lines represent initial volume, which includes Matrigel. Tumor volume was determined by standard methods (*18*). Error bars represent standard error for 5 to 10 mice in each group. Results were reproducible in repeated experiments.

pound. Pharmacokinetic data indicated that even twice daily dosing of CP-31398 did not maintain concentrations of the compound that were sufficient to continuously sustain p53 activity in tumors of treated animals. This suggests that intermittent threshold levels of p53 activity may be sufficient to suppress tumor growth.

Pharmacological agents that specifically reduce the free energy of a protein's active conformation may have utility in cancer, cystic fibrosis, and prion-mediated neurodegeneration, where protein conformation, folding, and aggregation contribute to the disease (*11*). Other benefits of specific conformation stabilizing agents may be envisioned whereby naturally unstable proteins are maintained in their active conformation, enabling them to compensate for disease-related deficiencies. Our findings support the notion that broadly effective anticancer therapies may one day be developed from compounds that conformationally stabilize p53. We have shown here that prototype compounds can conformationally modulate all of the four randomly chosen p53 mutants in vitro and functionally activate three mutants in vivo. Evaluating the full potential of these compounds will ultimately require improvements in their potency.

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12. Recombinant DBD (residues 94 to 312) from wild-type and mutant p53 proteins (173A, 175S, 249S, and 273H) and FLAG-tagged wild-type p53 DBD were diluted in buffer containing 25 mM Hepes (pH 6.8), 150 mM KCl, and 10 mM dithiothreitol (DTT), and 0.25 to 1.0 ng of the protein was immobilized onto Reactibind microtiter wells (Pierce, Rockford, IL) by incubating on ice for 30 min. Compounds were dissolved in DMSO at 10 mg ml^{-1} and diluted before use. The wells were rinsed with 25 mM Hepes (pH 6.8) and 150 mM KCl, compound or diluted DMSO vehicle was added, and the plates were incubated at the indicated temperatures. Incubation was terminated by placing the wells on ice, and the enzyme-linked immunosorbent assays were performed on ice to avoid further alterations of the epitopes. Wells were blocked for 1 hour with cold 5% skim milk in Hepes-KCl buffer before addition of the primary antibodies. Monoclonal antibodies mAb1620 and mAb240 (Calbiochem, San Diego, CA) and antibody to FLAG M2 (Sigma, St. Louis) were diluted at 1:100 to 1:250 in Hepes-KCl and added at 100 μl per well for 30 min. The plates were rinsed twice with cold Hepes-KCl buffer and incubated with horseradish peroxidase (HRP)–conjugated antibody to mouse immunoglobulin G (IgG; Roche, Indianapolis) for another 30 min. The HRP signal was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) developer (Pierce), and the optical density of the signal was read on a Bio-Rad microplate reader set at 450 nm.
13. Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in the recommended media with 10% fetal bovine serum. Cells were transfected with expression plasmids encoding the 173A mutant p53 and a neomycin selectable marker with *N*-[2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) transfection reagent (Roche). Transfected clones were selected for growth in media containing G418.
14. Cells (1×10^7) were treated overnight, rinsed three times with cold tris-buffered saline, and lysed in 1.5 ml of hypotonic lysis buffer [20 mM Hepes (pH 7.4), 10 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, and 10 mM DTT with protease inhibitors]. Cells were pelleted in microfuge tubes at 2000 rpm for 5 min at 4°C, and nuclear extracts were prepared by resuspending the pellets in the same buffer with 0.5 M NaCl. Tumor samples were homogenized in a Dounce homogenizer in three volumes of the above buffer with 0.5 M NaCl. The lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4°C. Nuclear extracts were normalized for p53 content with protein immunoblots with mAbDO-1 antibody, and p53 was captured onto wells of MaxiSorp F96 plates (Nunc, Naperville, IL) that had been coated overnight at 4°C with mAbDO-1 at 1 μg ml^{-1} in 0.05 M carbonate buffer (pH 9.6). The wells were washed with cold phosphate-buffered saline (PBS), blocked for 3 hours at 4°C with 4% skim milk in PBS, and probed with HRP-conjugated mAb1620 antibody in skim milk. The antibody incubation was for 1 hour on ice, after which wells were washed three times in PBS with 0.05% Tween 20, and TMB substrate was used to develop the signal. A standard curve was established with the lysate from temperature-shifted (32°C) cells that expressed large quantities of 1620-positive p53. Quantitation of the samples was within the linear range of the standard curve and was corrected for total p53 in each sample as well as for the 1620-positive p53 fraction in untreated cell lysates.
15. Cells were transfected with a plasmid encoding the hygromycin resistance marker and a p53 reporter gene made up of four copies of a p53-binding sequence (GCCTTGCCTGGACTTGCCTGGCCTTGCCTTTTC) placed upstream of the simian virus 40 basal promoter driving the luciferase gene. Transfected clones were selected for growth in Hygromycin and subsequently transfected with the mutant p53 as above. Monolayers of cells in 96-well tissue culture plates were treated with compound, and luciferase activity was determined with a substrate conversion assay (Promega, Madison, WI) and quantified with a Dynatech microplate luminometer.
16. Cultured cells were treated for 21 hours, rinsed three times with cold tris-buffered saline, scraped, and pelleted at 10,000 rpm for 30 s before being resuspended in 50 mM Hepes (pH 7.5), 0.1% NP-40, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM DTT, aprotinin (50 μg ml^{-1}), and Pefabloc (1 mg ml^{-1}; Roche). Protein concentrations were determined with Bradford reagent (Bio-Rad, Hercules, CA), and 10 μg of cell lysate was loaded onto 8 to 16% gradient polyacrylamide-SDS gels (Novex, San Diego, CA). Proteins were transferred onto Immobilon P membrane (Millipore, Marlborough, MA). Membranes were bisected between the 32.5- and 47.5-kD molecular mass markers and blocked for 1 hour at room temperature in SuperBlock (Pierce) plus 3% skim milk. The bottom half of the blot was probed for p21 expression with monoclonal antibody clone EA10 (Calbiochem), and the top half of the blot was probed for total p53 expression with mAbDO-1 (Calbiochem). The blots were washed for 1 hour in three changes of tris-buffered saline with 0.1% Tween 20, before the addition of a secondary antibody, HRP-conjugated antibody to mouse IgG. The bands were visualized with Renaissance ECL (DuPont, Boston) and exposure to Hyperfilm ECL (Amersham, Arlington Heights, IL).
17. Cultured cells were rinsed with PBS, and 1×10^6 A375.S2 or 5×10^6 DLD1 cells were inoculated in 90% Matrigel (Becton Dickinson, Franklin Lakes, NJ) unilaterally into the right flanks of ~20-g female NU/NU-nuBR mice (Charles River Laboratories, Wilmington, MA). CP-31398 was administered intraperitoneally in a saline solution with 0.1% Pluronic P-105 (BASF, Parsippany, NJ) as the vehicle. Tumor diameter was measured in two dimensions with calipers and converted to tumor volume. The care of animals was in accordance with institutional guidelines.
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