

An Organometallic Protein Kinase Inhibitor Pharmacologically Activates p53 and Induces Apoptosis in Human Melanoma Cells

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Abstract

Unlike other tumors, melanomas harbor wild-type (WT) p53 but exhibit impaired p53-dependent apoptosis. The mechanisms for the impaired p53 activation are poorly understood but may be linked to the high expression of the p53 suppressor Mdm2, which is found in >50% of melanoma lesions. Here, we describe an organometallic glycogen synthase kinase 3 β (GSK3 β) inhibitor (DW1/2) as a potent activator of p53 and inducer of cell death in otherwise highly chemoresistant melanoma cells. Using RNA interference and pharmacologic approaches, we show that p53 is required for the cytotoxic effects of this organometallic inhibitor. The DW1/2 compound was barely able to induce cell death in melanoma cells with p53 mutations, further confirming the requirement for p53-WT in the cytotoxic effects of the GSK3 β inhibition. Mechanistic analysis of the p53-dependent cell death indicated an apoptotic mechanism involving depolarization of mitochondrial membrane potential, caspase cleavage, and elevated NOXA expression. The effect of p53 was not simply due to passive up-regulation of protein expression as adenoviral-mediated overexpression of p53 was not able to induce cell death. Treatment of melanoma cells with DW1/2 was instead found to decrease levels of Mdm2 and Mdm4. The importance of Mdm2 down-regulation in DW1/2-induced apoptosis was confirmed by treating the p53-WT cells with the p53/Mdm2 antagonist Nutlin-3. Taken together, our data provide a new strategy for the pharmacologic activation of p53 in melanoma, which may be a viable approach for overcoming apoptotic resistance in melanoma and offer new hope for rational melanoma therapy. [Cancer Res 2007;67(1):209–17]

Introduction

New approaches to melanoma treatment are urgently needed (1) and the search is on to identify new targets for intervention. One target which has been little explored in melanoma therapy is the so-called “guardian of the genome,” the p53 tumor suppressor protein (2). Typically, p53 becomes activated in response to a range of stimuli, such as growth factor withdrawal, the acquisition of

oncogenes, and DNA damage. Increased p53 activity is associated with cell cycle arrest, through increased expression of the cyclin-dependent kinase inhibitor p21^{Waf-1}(3) and the induction of apoptosis, via the intrinsic mitochondrial pathway (4). Not surprisingly, the inactivation of the p53-dependent pathway is a key event in tumor initiation and progression. The loss of p53 activity is a major mechanism through which tumors become resistant to apoptosis, escape the control of their local microenvironment, and become insensitive to many types of therapeutic intervention. The majority of cancers harbor point mutations in p53, most of which are in the central region of the protein responsible for DNA binding (5).

Unlike other solid tumors, melanomas typically lack p53 mutations and retain expression of the wild-type (WT) protein, often at high levels (6–9). This is surprising, given the highly malignant nature of melanoma and its great resistance to therapeutic intervention. Evidence suggests that melanoma cells can tolerate high levels of transcriptionally active p53 (9) and have an abnormal p53 response following DNA damage (6). It is therefore likely that p53 is not a functional tumor suppressor in melanoma. One of the major mechanisms of p53 inactivation in melanoma is through the increased expression of Mdm2 (and its human homologue Hdm2), a nuclear protein that binds to, promotes the nuclear export of, and subsequently degrades p53 through its intrinsic E3 ubiquitin ligase activity (10). In addition to its role in proteasomal degradation, Mdm2 also inhibits the transcriptional activity of p53 activity through specific binding at key residues (11). More than 50% of primary invasive and metastatic melanoma samples overexpress Hdm2 at the protein level, and it is likely that this contributes to functional inactivation of p53 in melanoma (12). p53 function can also be negatively regulated by another protein, which is structurally related to Mdm2 called Mdm4 (and its human homologue HdmX; ref. 13). Although there is evidence that some melanoma cell lines overexpress Mdm4 (14), the exact relationship between p53 function and Mdm2/Mdm4 expression remains to be unraveled (15).

p53 can be pharmacologically activated in cancer cell lines through a variety of mechanisms, leading to the induction of apoptosis (16–19). Of these, two groups have identified glycogen synthase kinase (GSK) 3 β as a major regulator of p53 localization and expression (20, 21). Although GSK3 β has received little attention as a possible therapeutic target from the cancer research community, several very recent studies have suggested that inhibiting GSK3 β may induce apoptosis in colorectal carcinoma cells through a p53-dependent pathway (18, 19). Classically, GSK3 β was described as a key regulator of glycogen metabolism and is also known to regulate other processes, such as apoptosis in

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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neurons, protein synthesis, cell proliferation, microtubule dynamics, cell motility, and Wnt signaling (22–24).

In the current study, we explore whether tumors with p53-WT, such as melanoma, may benefit from strategies that pharmacologically activate the p53 pathway. Here, we describe for the first time the anticancer activity of organometallic GSK3 β inhibitors, represented by DW1/2 (25, 26). Treatment of melanoma cells with these compounds leads to the nuclear accumulation of p53, down-regulation of Mdm2 and Mdm4, increased NOXA expression, and apoptosis via the mitochondrial pathway. Similar results were seen when the melanoma cells were treated with Nutlin-3, an antagonist that disrupts the p53/Mdm2 binding interaction leading to enhanced p53 activation and apoptosis (27).

Materials and Methods

Synthesis of DW1 and DW2 and DW1/2. The synthesis of the compounds DW1 and DW2 and DW1/2 were described previously by Williams et al. (25), where DW1 corresponds to compound "1-OH." The racemic mixture DW1/2 was separated into the pure enantiomers DW1 and DW2 with a ChiralPak AD-H analytic high-pressure liquid chromatography column (Daicel/Chiral Technologies, West Chester, PA) using a gradient of hexanes and ethanol (7:3 to 1:4 in 20 min) with a flow rate of 0.95 mL/min. The compound structures are given in Fig. 1A.

In vitro kinase assays. *In vitro* kinase assays were done as described previously by Williams et al. (25).

Cell culture. Human melanocytes and melanoma cells were isolated and cultured as described by Smalley et al. (28). The lentiviral vector short hairpin RNA (shRNA) constructs for p53 were developed in the laboratory of Dr. Maria Soengas (University of Michigan, Ann Arbor, MI) based on published sequences (29, 30). The p53 adenovirus was from Dr. Wafik El-Deiry (University of Pennsylvania, Philadelphia, PA; ref. 6). Melanoma cells that were WT for p53 (WM793, 1205Lu, and WM115) or harbored p53 mutations (WM852, WM983A, WM983B, and WM164) have been sequenced previously (6, 7).

Adherent cell proliferation analysis. Cells were plated into a 96-well plate at a density of 2.5×10^4 per mL and left to grow overnight. Cells were treated with increasing concentrations of DW1, DW2, or DW1/2 in triplicate. In each instance, cells were left to grow for 72 h before being treated with 20 μ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for 3 h (Sigma, St Louis, MO). After this time, medium was rapidly removed and the resulting crystals were solubilized in DMSO. Absorbance was read in a plate reader at 540 nm. Absorbance readings were subtracted from the value of blank wells, and the reduction in cell growth was calculated as a percentage of control absorbance in the absence of any drug. Data show the mean of at least three independent experiments \pm SE.

Western blot analysis. Proteins were extracted and blotted as described by Smalley et al. (28). After analysis, Western blots were stripped once and reprobed for β -actin to show even protein loading. Antibodies to phosphorylated GSK3 β , phosphorylated p53, cleaved caspase-9, cleaved caspase-3, Bcl-2, BAD, Akt, extracellular signal-regulated kinase (ERK), and Bax were from Cell Signaling Technology (Beverly, MA). The polyclonal

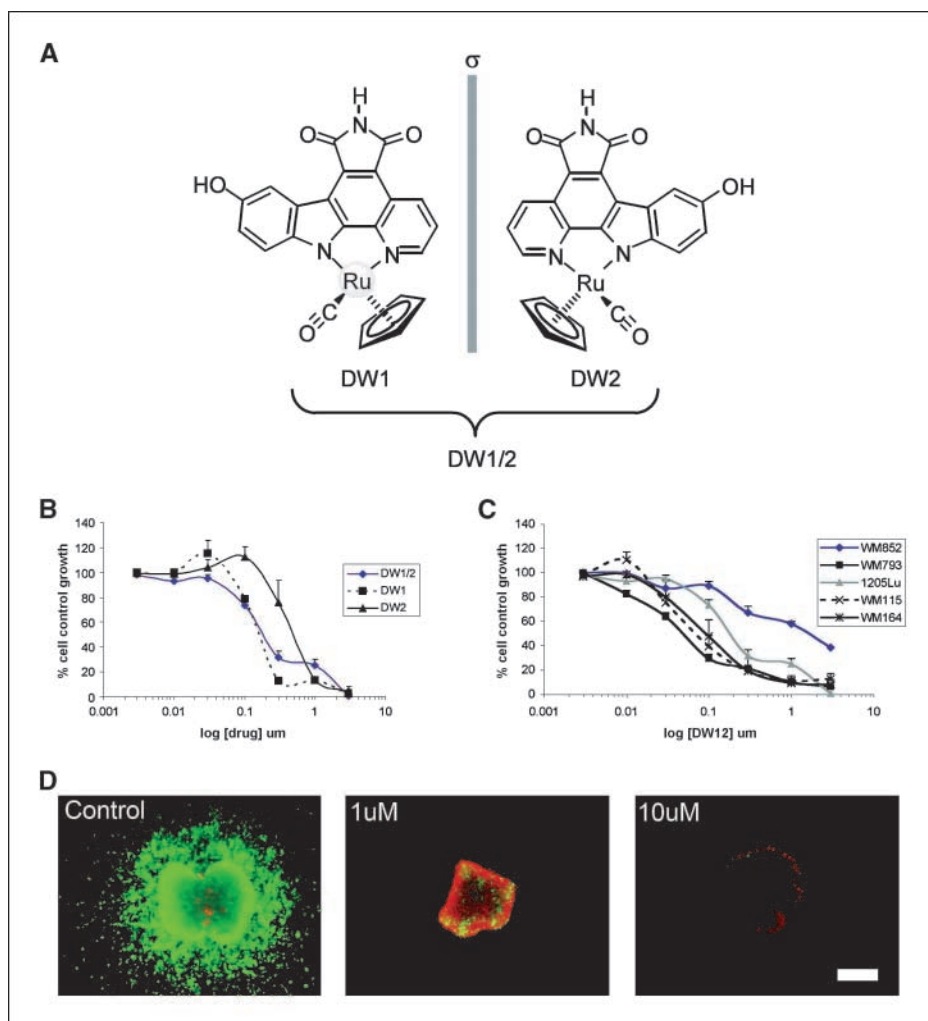


Figure 1. The novel GSK3 β inhibitors DW1, DW2, and DW1/2 inhibit the growth of human melanoma cell lines in both two-dimensional and three-dimensional cell culture models. **A**, the chemical structures of the enantiomers, DW1 and DW2. DW1/2 is a racemic mixture of DW1 and DW2. **B**, increasing concentrations of DW1, DW2, and DW1/2 reduce the growth of 1205Lu melanoma cells. Cells were treated with increasing concentrations (30 nmol–3 μ mol/L) of drug for 72 h, and cell numbers were quantified using the MTT assay. **Bars**, SD. **C**, DW1/2 reduces the growth of a panel of melanoma cell lines. A panel of five melanoma cell lines was treated with DW1/2 as described in (B). **Bars**, SD. **D**, DW1/2 reduces cell viability and invasion in 1205Lu cultured as collagen-implanted three-dimensional spheroids. Preformed 1205Lu spheroids were embedded into collagen and overlaid with medium. Cells were treated with DW1/2 (1 and 10 μ mol/L) for 72 h before being treated with calcein-AM and propidium iodide. **Green**, viable cells; **red**, dead cells. Lack of green staining also indicates loss of viability. **Bar**, 200 μ m.

antibody to Mdm4 (HdmX) was from Bethyl Laboratories (Montgomery, TX). The monoclonal antibody to β -actin was from Sigma and the monoclonal antibody for p53 (DO-1) was from Calbiochem (San Diego, CA). Monoclonal antibodies to GSK3 β , β -catenin, Mdm2, p21, p27, and NOXA were from BD Biosciences (Franklin Lakes, NJ).

Three-dimensional spheroid growth. Melanoma spheroids were prepared using the liquid overlay method and treated as described previously (31).

Cell cycle analysis. Cells were plated into 10-cm dishes at 60% confluency and left to grow overnight before being treated either with DW1/2 (100 nmol/L–1 μ mol/L) for 24 h or with Nutlin-3 (5–20 μ mol/L; Cayman Chemical Co. Ann Arbor, MI), LiCl (30 mmol/L), or SB216763 (20 μ mol/L) for 24 to 72 h. Cells were then treated and analyzed as described previously (31).

Flow cytometric analysis of apoptosis and mitochondrial membrane potential ($\Delta\psi_m$). After the treatment of the cells with DW1/2 (300 nmol/L or 1 μ mol/L, 24 h), cells were washed once with Annexin binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 5 mmol/L CaCl₂], resuspended in 100 μ L binding buffer containing 1 μ L FITC-conjugated Annexin V (R&D Systems, Minneapolis, MN) and 25 nmol/L tetramethylrhodamine, methyl ester, perchlorate (TMRM; Molecular Probes, Eugene, OR), and incubated at 37°C for 15 min. Cells were then analyzed for Annexin V fluorescence (green) and TMRM retention (red fluorescence) using flow cytometry.

Immunofluorescence microscopy. Cells were seeded thinly onto ethanol-sterilized glass coverslips in six-well plates and left to grow overnight. After which they were treated with either DW1/2 (1 μ mol/L), LiCl (30 mmol/L), SB216763 (20 μ mol/L), or Nutlin-3 (15 μ mol/L) for 24 h before being fixed in 4% paraformaldehyde and permeabilized with 0.2% (v/v) Triton X-100. Samples were then blocked in PBS containing 1% bovine serum albumin. Primary antibody incubations to p53, pH2AX, pATM (all at 1:100; Calbiochem), or cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology) were done at 37°C for 1 h under humidified conditions. Slides were prepared as described by Smalley et al. (28).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. Cells were thinly seeded onto coverslips in six-well plates and left to adhere overnight. Cells were treated with DW1/2 (100 nmol/L–3 μ mol/L) for 72 h before being fixed and permeabilized in 4% formaldehyde and 0.2% (w/v) Triton X-100. Cells were then treated using fluorescein/terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reagents from an *in situ* cell death detection kit (Roche Diagnostics, Basel, Switzerland) for 1 h before being washed and treated with antifade. Cells were analyzed as for immunofluorescence.

Statistical analysis. Unless otherwise stated, all experiments show the mean \pm SE of at least three independent experiments. Statistical significance was measured using the Student's *t* test, where *P* < 0.05 was judged to be significant.

Results

The novel organometallic kinase inhibitors block the growth of melanoma cells in both two-dimensional and three-dimensional culture models. *In vitro* kinase assays revealed the IC₅₀ values for DW1 to be 2 and 2.5 nmol/L for GSK3 α and GSK3 β , respectively, and the IC₅₀ values for DW2 to be 9 and 15 nmol/L for GSK3 α and GSK3 β , respectively, at an ATP concentration of 100 μ mol/L. Treatment of 1205Lu melanoma cells with increasing concentrations (3 nmol/L–3 μ mol/L) of DW1, DW2, and DW1/2 for 72 h (structures shown in Fig. 1A) led to concentration-dependent reduction in cell growth (Fig. 1B). In agreement with the *in vitro* kinase data, it was noted that DW2 was less potent than DW1. However, as the racemic mixture DW1/2 displayed similar potency to DW1 and did not require resolving, all subsequent experiments were done on this compound. DW1/2 had growth-inhibitory activity against a panel of five melanoma cell

lines (WM793, WM115, WM164, WM852, and 1205Lu; Fig. 1C). Often the pharmacologic profile of drugs in two-dimensional cell culture is not predictive of the response in three-dimensional melanoma spheroid culture (31). To investigate this further, spheroids formed from 1205Lu cells were implanted into a three-dimensional collagen I gel and treated with DW1/2 (1–10 μ mol/L) for 72 h. It was found that 1 μ mol/L DW1/2 markedly reduced cell viability of the spheroid, as shown by the loss of green staining and increased red staining and lack of invasion into the surrounding collagen (Fig. 1D).

Treatment with DW1/2 induces apoptosis through the intrinsic mitochondrial pathway. Cell cycle analysis showed that treatment of melanoma cells (WM793 and 1205Lu) with DW1/2 for 24 h led to a concentration-dependent increase in the number of cells in the sub-G₁ population, suggesting that DW1/2 was inducing apoptosis (Fig. 2A). At the highest concentration (1 μ mol/L), levels of apoptosis were 47% and 65% in the 1205Lu and WM793 cells, respectively. In other studies, cells were treated with drug for 24 h, fixed, stained, and labeled using a TUNEL kit. Again, like the cell cycle analysis, increasing concentrations of DW1/2 increased the level of TUNEL staining (data not shown). To investigate the mechanism of apoptosis, cells were treated with 1 μ mol/L DW1/2 for 0, 2, 6, and 24 h, and protein was extracted and probed for expression of the cleaved caspase-3 and caspase-9 (Fig. 2B). Caspase-9 was cleaved rapidly (<6 h) and caspase-3 was cleaved after 6 h of drug treatment. Following DW1/2 treatment, there was little change in levels of Bcl-2, Bax, or BAD (Fig. 2B). However, there was a marked reduction in levels of phosphorylated BAD after 2 h of DW1/2 treatment. A prolonged treatment of the cells with DW1/2 (24 h) led to increased expression of NOXA (Fig. 2B). Further evidence for DW1/2-induced apoptosis through the intrinsic pathway was shown by an increase in Annexin V positivity and loss of mitochondrial membrane potential ($\Delta\psi_m$) as measured by loss of TMRM staining (Fig. 2C). These processes started as early as 3 h, with rapid progression during subsequent time points (6, 12, 24, 48, and 72 h; Fig. 2C). To confirm whether the DW1/2 had any selectivity for tumor cells over normal cells, primary human skin melanocytes derived from foreskins were treated with increasing concentrations of DW1/2 (30 nmol/L–1 μ mol/L) for 24 h followed by Annexin V staining and flow cytometry (Fig. 2D). It was noted that only very little apoptosis was seen in the melanocytes (6%) following 1 μ mol/L DW1/2 treatment (24 h) compared with 31% in the 1205Lu melanoma cells at the same drug concentration. In other studies, we looked at the ability of DW1/2 to reduce the growth of primary human skin fibroblasts in MTT assays and found a markedly weaker antiproliferative effect relative to that seen for WM793 melanoma cells (Supplementary Fig. S1A). To determine the cross-reactivity of DW1/2 on other major melanoma survival pathways, we also looked at levels of phosphorylated Akt and phosphorylated ERK. It was noted that there was a substantial decrease in phosphorylated Akt activity after 2 h of DW1/2 treatment but little effect on phosphorylated ERK levels (Supplementary Fig. S1B).

GSK3 β as a potential pharmacologic target of DW1/2-induced apoptosis. It has been suggested that GSK3 β activity may be involved in the regulation of p53 expression. DW1/2 is a highly potent inhibitor of GSK3 β in *in vitro* kinase assays (25). Protein expression studies revealed that GSK3 β was highly expressed in all melanoma cell lines tested (Fig. 3A). Probing of the same protein extracts revealed that GSK3 β was constitutively phosphorylated at Ser⁹ (Fig. 3A). In spite of this, DW1/2 was still able to inhibit GSK3 β

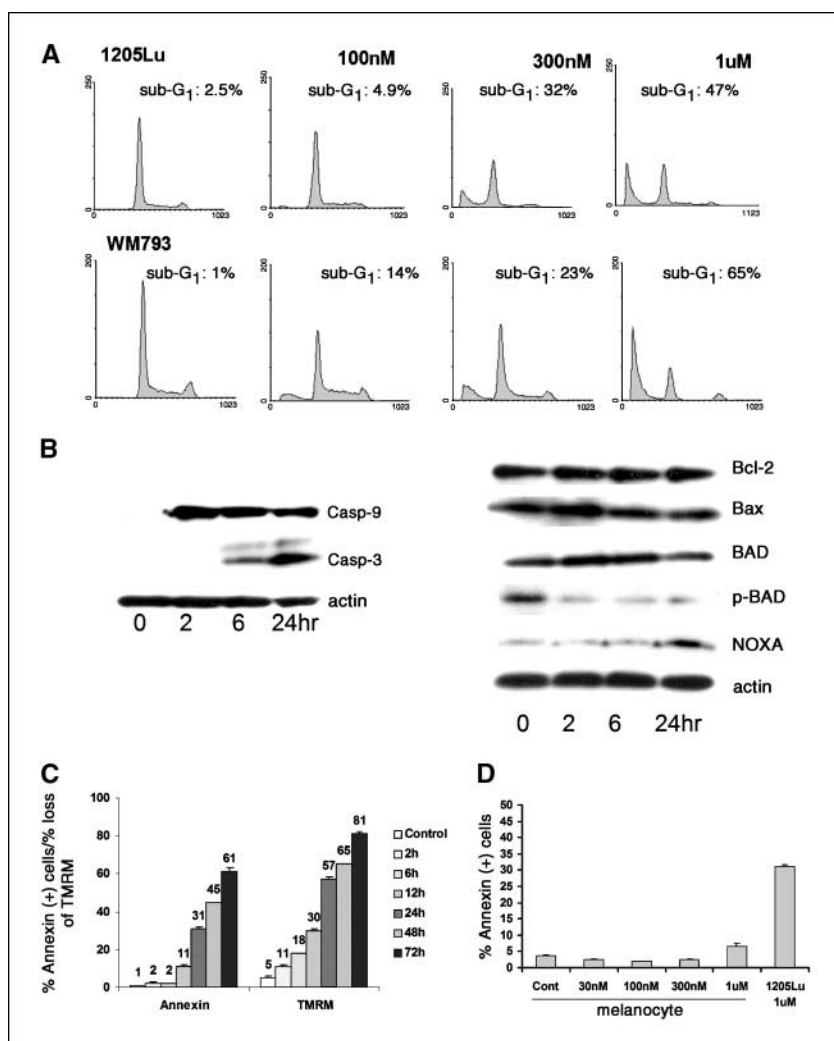


Figure 2. DW1/2 induces apoptosis in human melanoma cells via the intrinsic mitochondrial pathway. **A**, DW1/2 induces concentration-dependent apoptosis in human melanoma cells. 1205Lu and WM793 cells were treated with increasing (100 nmol–1 μ mol/L) concentrations of DW1/2 for 24 h. Cells were then harvested and ethanol-fixed before being stained with propidium iodide and analyzed using flow cytometry. **B**, DW1/2 induces cleavage of caspase-3 (*Casp-3*) and caspase-9 (*Casp-9*) and reduces levels of phosphorylated BAD (*p-BAD*) and increases NOXA expression in melanoma cells. 1205Lu cells were treated with DW1/2 (1 μ mol/L) for 6, 16, and 24 h, after which proteins were extracted. Resulting proteins were resolved by Western blotting and probed for expression of cleaved caspase-3, caspase-9, Bcl-2, Bax, BAD, phosphorylated BAD, and NOXA. Equal protein loading was confirmed by stripping the blots and reprobing for actin expression. **C**, phosphatidylserine externalization in 1205Lu cells was determined using Annexin V staining and reduction in mitochondrial membrane potential ($\Delta\psi_m$) by decrease of TMRM staining. *Columns*, mean of three replicate experiments; *bars*, SE. Percentages are given above the columns. **D**, DW1/2 induces little apoptosis in FOM139-1 human melanocytes. FOM139-1 cells were treated with increasing concentrations of DW1/2 (30 nmol/L–1 μ mol/L) for 24 h before being stained with Annexin V. *Columns*, mean of three experiments; *bars*, SE. Control levels of apoptosis induced in 1205Lu melanoma cells are also shown.

activity in 1205Lu cells and increase levels of β -catenin expression (Fig. 3B). Similar results were also seen when the cells were treated for 24 h with the unrelated GSK3 β inhibitor LiCl (30 mmol/L; Fig. 3B). In other studies, we treated melanoma cells with two other GSK3 β inhibitors, LiCl and SB216763, and found that both were able to increase nuclear p53 accumulation (Fig. 3C) and induce substantial levels of apoptosis (Fig. 3D).

DW1/2 up-regulates nuclear p53 expression and induces apoptosis in melanoma cells with p53-WT. Nonspecific GSK3 β inhibitors, such as LiCl, up-regulate p53 expression (Fig. 3C). To investigate the possible role of p53 in the proapoptotic activity of DW1/2, melanoma cells that were p53-WT (1205Lu) and p53-mut (WM852) were treated with DW1/2 (1 μ mol/L) for 24 h and stained for p53 expression. It was found that DW1/2 up-regulated nuclear levels of p53 in the 1205Lu cells (Fig. 4A and B) but not the WM852 cells (data not shown). Interestingly, nuclear staining for p53 was seen in the WM852 in the absence of drug treatment, but this did not alter following administration of DW1/2.

Treatment of WM852 cells with DW1/2 (1 μ mol/L) for 24 h did not increase the proportion of cells in the sub-G₁ apoptotic phase of the cell cycle and was instead associated with increased G₂-M phase arrest (Fig. 4C). Similar results (<10% apoptosis) were also seen with other p53-mut cell lines (WM983A and

WM983B; Supplementary Fig. S1C). Further evidence for the p53-dependent effects of DW1/2 came from apoptosis studies where either 1205Lu or WM852 cells were drug treated for 24 h followed by staining for either TUNEL or cleaved PARP (Fig. 4D). In each case, DW1/2 treatment increased levels of both TUNEL and cleaved PARP staining in the 1205Lu cells but not the WM852 cells.

p53-dependent apoptosis can also arise indirectly, as a consequence of DNA damage and through the generation of reactive oxygen species (ROS). As some metal ions, such as platinum, can induce p53 expression following DNA damage, we investigated whether the ruthenium-based DW1/2 compound also induced a DNA damage response. Treatment of 1205Lu cells for 0, 2, 6, or 24 h (1 μ mol/L) did not lead to an increase in the nuclear staining of phosphorylated H2AX or phosphorylated ataxia-telangiectasia mutated (ATM), which are both markers of DNA damage (Supplementary Fig. S2A). In contrast, treatment of the same cells with cisplatin led to increased phosphorylation of both H2AX and ATM after 24 h of treatment. As further evidence of DW1/2 working through a non-DNA damage-mediated p53 induction mechanism, we showed the rapid up-regulation of the p53 target gene p21 after 6 h of DW1/2 treatment (Supplementary Fig. S2B). Finally, we investigated whether DW1/2 induced

apoptosis through a ROS-dependent mechanism by pretreating the cells with the antioxidants TROLOX (200 $\mu\text{mol/L}$; Supplementary Fig. S3) or *N*-acetylcysteine (5 $\mu\text{mol/L}$) and found that there was no protection from DW1/2-induced apoptosis.

Knockdown of p53 reduces DW1/2-induced apoptosis in p53-WT melanoma cells. As melanoma cells have abnormalities in many apoptotic pathways, it is often difficult to compare between cell lines. To overcome this, we investigated the role of p53 in DW1/2-induced apoptosis within a defined, isogenic system where p53 was knocked down in WM793 cells using a stable shRNA lentiviral vector system (29, 30). After green fluorescent protein (GFP)-based selection studies, two p53 shRNA clones (p53.1 and p53.2) gave significant protein knockdown (>90%; Fig. 5A). Control vectors with mutated shRNA sequences were identical to uninfected controls (data not shown). The functional knockdown of p53 protein in the WM793 cells was shown following treatment of the cells with Nutlin-3 (15 $\mu\text{mol/L}$, 48 h; Fig. 5A). In the WM793-p53 cells, there was little apoptosis following Nutlin-3 treatment and the cells continued to proliferate (sub-G₁, 4%), whereas the control cells underwent apoptosis (sub-G₁, 27%).

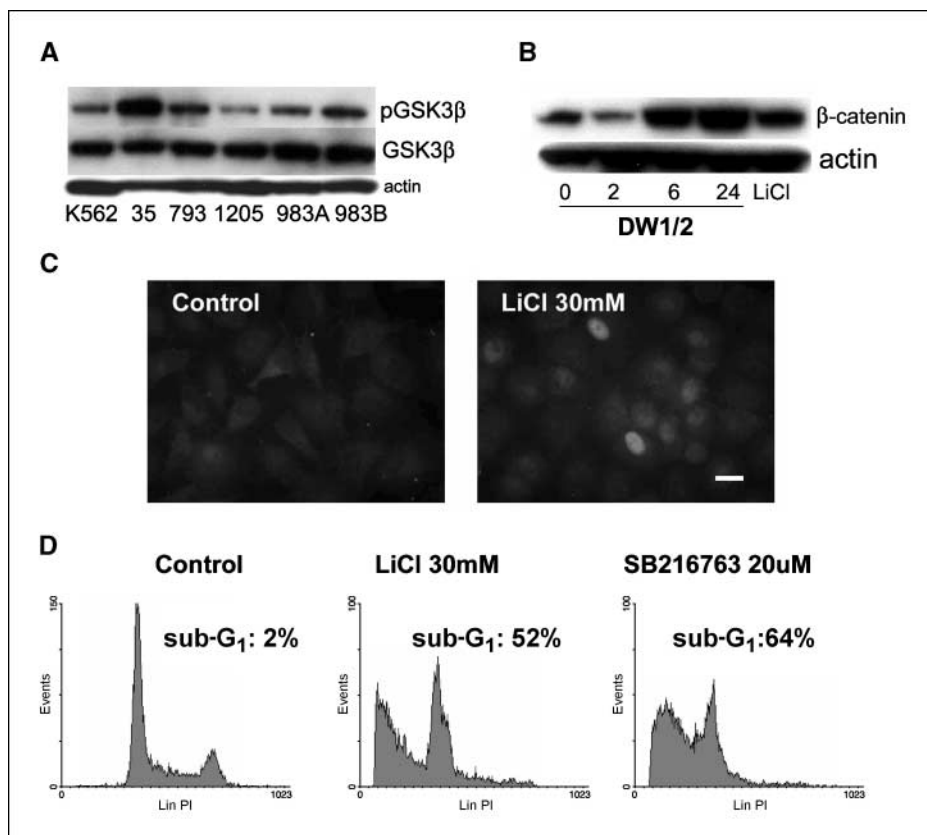
Treatment of the WM793-p53 RNA interference cells with increasing concentrations of DW1/2 was associated with significantly less apoptosis ($P < 0.05$) than seen in the WT cells (Fig. 5B and C), with $54.2 \pm 3.3\%$ and $26.1 \pm 2.7\%$ apoptosis being induced following DW1/2 treatment (1 $\mu\text{mol/L}$) in the WM793 and WM793-p53 cells, respectively.

To investigate the effects of DW1/2 on p53 regulation, cells were treated with drug for increasing periods before being probed for expression of p53, Mdm2, and Mdm4. It was noted that there was a rapid increase in p53 expression (<2 h) with a slower but marked

reduction of Mdm2 and Mdm4 expression (>2 h; Fig. 5D). In contrast, cisplatin (40 $\mu\text{mol/L}$) induced a much slower up-regulation of p53 expression, which was seen only after 24 h of treatment (data not shown).

Pharmacologic activation of p53, but not p53 overexpression, induces apoptosis in p53-WT melanoma cells. Nutlin-3 activates p53 through the disruption of the p53/Mdm2 interaction (27). Here, we show that 24-h treatment of the p53-WT 1205Lu melanoma cells with Nutlin-3 leads to nuclear accumulation of p53 (Fig. 6A). In contrast, treatment of p53-mut melanoma cells with Nutlin-3 had little effect on either p53 expression or nuclear localization (data not shown). Treatment of the p53-WT 1205Lu cell line with Nutlin-3 (10 $\mu\text{mol/L}$) was associated with up-regulation of the p53 transcriptional target p21 as well as p27 (Fig. 6B). In contrast, no up-regulation of either p21 or p27 was seen following Nutlin-3 treatment in the p53-mut cell line WM852 (Fig. 6B). Cell cycle analysis of p53-WT 1205Lu cells showed that Nutlin-3 induced a progressive accumulation of cells in the sub-G₁ phase, indicating that this compound was inducing apoptosis (Fig. 6C). Similar experiments in the p53-mut cell line WM852 revealed little apoptosis following Nutlin-3 treatment (Fig. 6C). It was important to ascertain whether p53-dependent apoptosis in melanoma cells was the result of inhibiting the p53/Mdm2 complex or only increased p53 expression. To investigate this point, we infected p53-WT and p53-mut cells with an adenovirus for p53. It was noted that overexpression of p53 induced apoptosis in the p53-mut WM852 cells but not the p53-WT 1205Lu cells (Fig. 6D). Western blot analysis confirmed that the adenoviral infection led to increased p53 expression in both melanoma cell lines (data for 1205Lu is shown in Fig. 6D).

Figure 3. Evidence that GSK3 β inhibition induces p53 up-regulation and apoptosis in human melanoma cells. **A**, GSK3 β is expressed in human melanoma cell lines. Protein was harvested from a panel of human melanoma cell lines (WM35, WM793, 1205Lu, WM983A, and WM983B) as well as one leukemic cell line (K562). Proteins were resolved by Western blotting and probed for expression of total GSK3 β and Ser⁹ phosphorylated GSK3 β . **B**, DW1/2 and LiCl stabilize β -catenin in human melanoma cells. 1205Lu cells were treated with DW1/2 (1 $\mu\text{mol/L}$) for increasing periods (0, 2, 6, and 24 h) or LiCl (30 mmol/L) for 24 h. Proteins were extracted and probed for expression of β -catenin. Equal protein loading was confirmed by stripping the blot and probing for actin. **C**, LiCl up-regulates nuclear p53 expression in p53-WT melanoma cells. 1205Lu cells were treated with LiCl (30 mmol/L) for 24 h before being fixed, permeabilized, and stained for p53. Bar, 20 μm . **D**, LiCl and SB216763 induce apoptosis in 1205Lu cells. Cells were treated with LiCl (30 mmol/L) or SB216763 (20 $\mu\text{mol/L}$) for 48 h before being harvested and ethanol-fixed. Cells were then stained with propidium iodide and analyzed using flow cytometry.



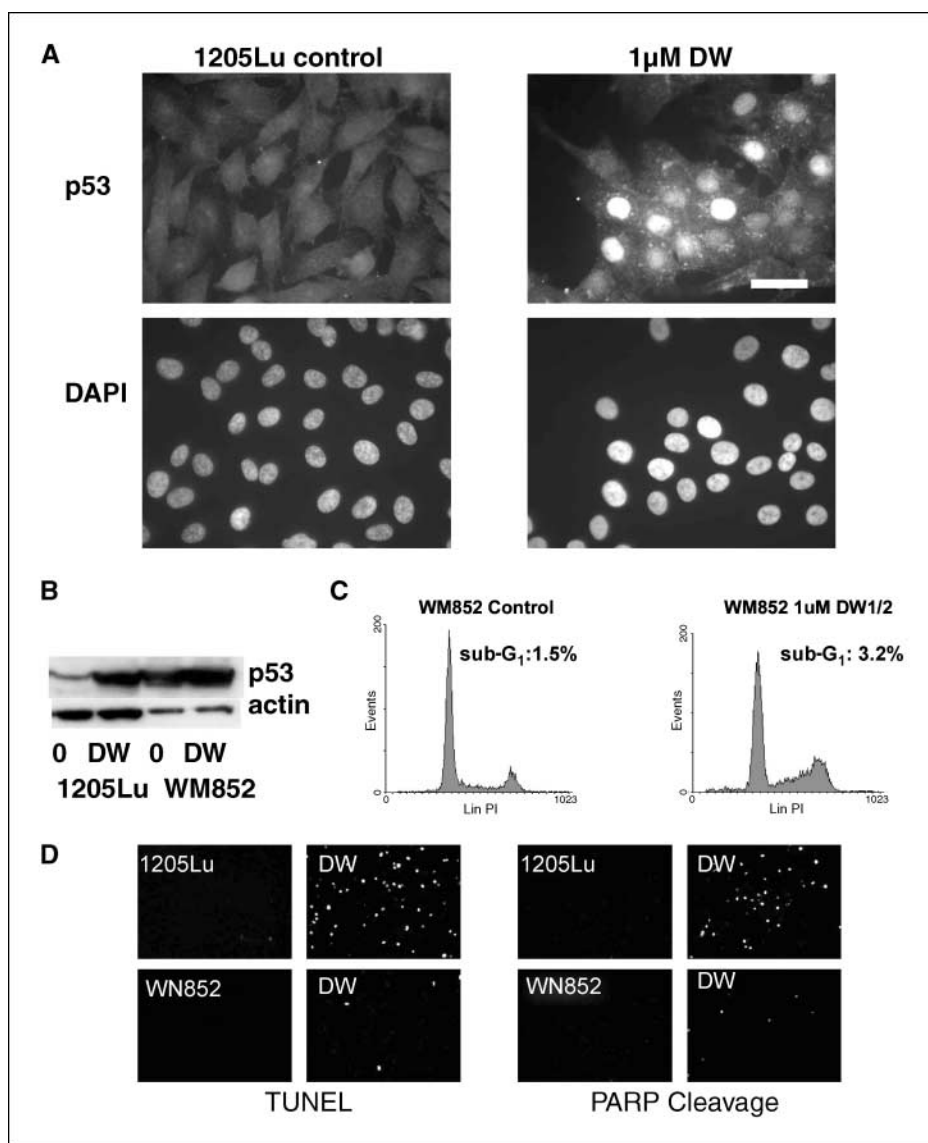


Figure 4. DW1/2 induces apoptosis in a p53-dependent manner. *A*, DW1/2 up-regulates nuclear p53 expression in p53-WT melanoma cells. 1205Lu (p53-WT) melanoma cells were treated with DW1/2 (1 µmol/L) for 24 h before being fixed, permeabilized, and stained for p53 or 4',6-diamidino-2-phenylindole (DAPI). Bar, 20 µm. *B*, DW1/2 up-regulates p53 expression in p53-WT but not p53-mut melanoma cells. 1205Lu (p53-WT) and WM852 (p53-mut) melanoma cells were treated with DW1/2 (1 µmol/L) for 24 h, and proteins were extracted and probed for p53. Equal protein loading was shown by stripping the blot and probing for β-actin. *C*, DW1/2 induces little apoptosis in p53-mut melanoma cells. WM852 cells were treated with DW1/2 (1 µmol/L) for 24 h before being harvested and ethanol-fixed. Cells were then stained with propidium iodide and analyzed using flow cytometry. *D*, DW1/2 (DW) induces little TUNEL or cleaved PARP staining in p53-mut melanoma cells. p53-WT cells (1205Lu) and p53-mut cells (WM852) were treated for 24 h with DW1/2 (1 µmol/L) before being fixed and stained using a TUNEL kit or with antibody for cleaved PARP. Stained nuclei indicate apoptosis.

Discussion

Unlike many other tumors, melanomas express p53-WT (6, 7) but exhibit impaired p53-dependent apoptosis. In the current study, we investigated two classes of drugs that activate p53 through different mechanisms. First, we characterized an organometallic kinase inhibitor with high affinity for GSK3β and show that it induces p53-dependent apoptosis in human melanoma cells. Second, we showed that Nutlin-3, a p53/Mdm2 antagonist, induces a similar p53-dependent apoptosis. It is therefore suggested that the pharmacologic manipulation of p53 in melanoma could represent a novel approach to therapy.

Here, we report for the first time the remarkable anticancer activity of DW1/2 on human melanoma cells. In most of the melanoma cell lines tested, 300 nmol/L of the DW1/2 were able to maximally inhibit cell growth. DW1/2 represents a member of a series of organometallic kinase inhibitors derived from the class of indolocarbazole alkaloids (e.g., staurosporine) with very high selectivity for GSK3β (25, 26, 32).

All of the melanoma cells tested had high levels of the Ser⁹ phosphorylated form of GSK3β, suggesting that this kinase was at

least partly inactivated in melanoma cells, which is most likely a consequence of constitutive high Akt activity (33, 34). Although GSK3β was Ser⁹ phosphorylated in the melanoma cells, DW1/2 treatment was still able to up-regulate β-catenin expression in the 1205Lu melanoma cells, showing that the melanoma cells still possessed some GSK3β activity that could be blocked by these compounds. The lower selectivity of DW2 over DW1 in the MTT assays shows that growth inhibition was directly correlated with the ability of these compounds to inhibit GSK3 in the *in vitro* kinase assay. Similar β-catenin-stabilizing and growth-inhibitory results were also seen following treatment with two other GSK3β inhibitors, SB216763 and LiCl.

Several studies have suggested that intracellular signaling is modified by the tumor microenvironment (31, 35, 36). Our previous studies have shown that melanoma cells derived from metastases become completely resistant to the growth-arresting activity of inhibitors of the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein/ERK pathways when grown as three-dimensional collagen-implanted spheroid (31). Here, we show that the 1205Lu cells still respond well to the DW1/2 when grown under these

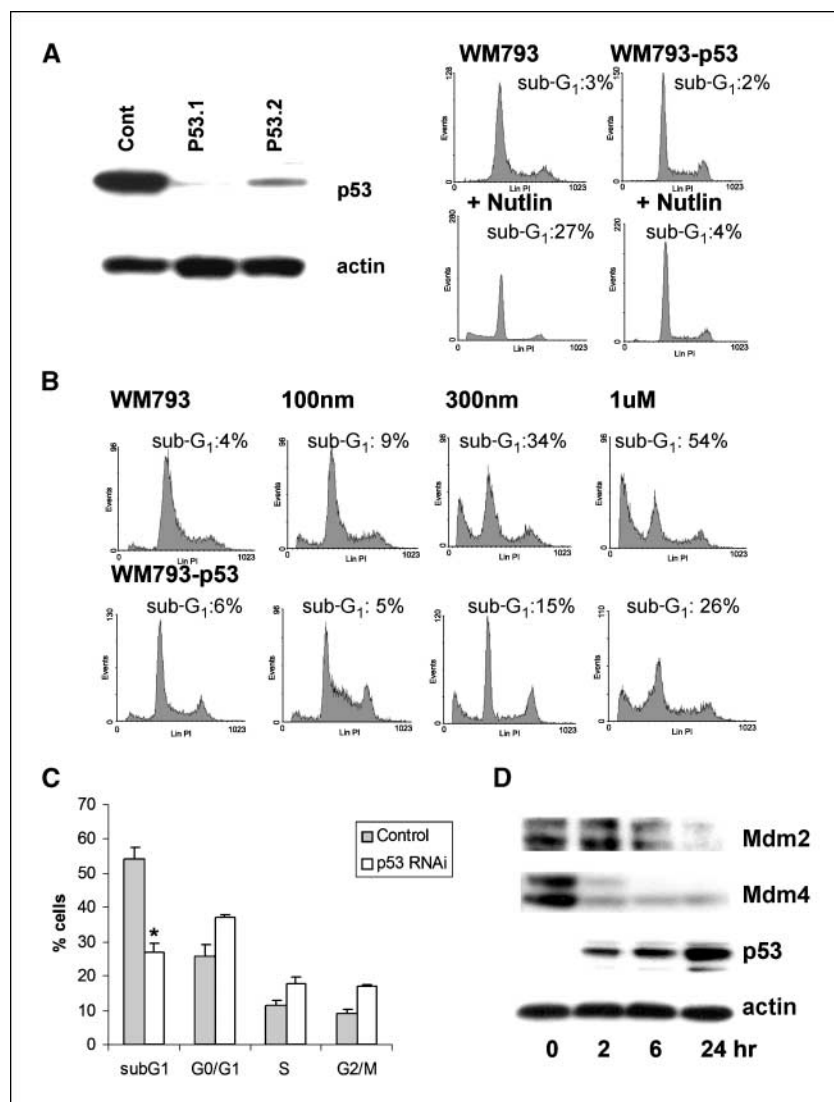
conditions, suggesting that there is no acquisition of drug resistance to DW1/2 under three-dimensional culture conditions.

Treatment of melanoma cells that were WT for p53 (p53-WT) with DW1/2 led to the rapid induction apoptosis via the intrinsic caspase-9/caspase-3 mitochondrial pathway. Treatment of the melanoma cells with other GSK3 β inhibitors, such as LiCl and SB216763, were also found to induce apoptosis. The p53 pathway is a major activator of mitochondrial-dependent apoptosis. Consistent with this, treatment of p53-WT cells with DW1/2 led to the up-regulation of nuclear p53 expression. Interestingly, in p53-mut cells, the levels of p53 were high in the absence of the DW1/2 and were not altered following treatment, suggesting that mutations in p53 rendered the cells unresponsive to DW1/2. This was further shown by the fact that melanoma cells with mutated p53 (WM852, WM983A, and WM983B) did not undergo any (WM852) or very little (<10%; WM983A and WM983B) apoptosis following administration of DW1/2. The possible role of GSK3 β in the observed up-regulation of p53 expression in melanoma cells was also confirmed when cells were treated with LiCl and SB216763.

Tumor cells are genetically heterogeneous and harbor many mutations that work together to suppress apoptosis. To account for this, we further explored the role of p53 in DW1/2-induced

apoptosis in a defined isogenic system where melanoma cells with p53-WT (WM793) had the p53 stably knocked down using a lentiviral shRNA (WM793-p53). Knockdown of p53 in WM793 cell line was found to significantly reduce the levels DW1/2-induced apoptosis by 48%, suggesting that p53 played an important role in DW1/2-mediated apoptosis. The fact that total suppression of apoptosis was not achieved can be either explained by the fact that the levels of p53 knockdown were not sufficient to completely abolish all DW1/2-mediated apoptosis or that there are other p53-independent apoptosis mechanisms involved in the activity of DW1/2. The fact that the WM793-p53 cells also display a blunted response to the p53/Mdm2 antagonist Nutlin-3 shows that levels of p53 knockdown achieved are sufficient to block p53-induced apoptosis, suggesting that DW1/2 may have additional mechanisms of apoptosis induction. The p53 dependency of GSK3 β inhibitor/chemotherapy-mediated apoptosis has been shown previously in two separate studies using the HCT116 colorectal carcinoma cell lines, which either express or were null for p53 (18, 19). In these studies, it was shown that a panel of GSK3 β inhibitors (SB 216763, purvalanol A, and LiCl) only induced p21 expression (indicative of p53 activity) and sensitized the cells to chemotherapy-induced apoptosis in the p53-expressing cells and not in the p53-null cells (18, 19).

Figure 5. Knockdown of p53 reduces DW1/2-induced apoptosis in human melanoma cells. **A**, infection of WM793 melanoma cells with shRNA to p53 gives near-complete protein knockdown and reduces Nutlin-3-induced apoptosis. Cells were infected with a shRNA lentivirus selective for p53. Cells were sorted according to their expression of GFP, after which protein was harvested and levels of p53 expression were measured by Western blotting. Even protein loading was confirmed by stripping and reprobing the blot for actin expression. WM793 cells and WM793 p53 knockdown cells (WM793-p53) were treated with 15 μ mol/L Nutlin-3 for 48 h, after which cells were harvested, fixed, stained with propidium iodide and analyzed using flow cytometry. Percentages of cells undergoing apoptosis (*sub-G*₁) are given. **B**, knockdown of p53 significantly reduces apoptosis to DW1/2. WM793 cells and WM793 p53 knockdown cells (WM793-p53) were treated with 1 μ mol/L DW1/2 for 24 h. Cells were processed as in (A). **C**, shRNA to p53 reduces levels of apoptosis to DW1/2 in human melanoma cells. Mean cell cycle data from three independent experiments as done in (B). Columns, mean; bars, SE. *, $P < 0.05$, statistically significant difference between WM793 and WM793-p53 cells. **D**, DW1/2 treatment induces p53 expression and reduces Mdm2 and Mdm4 expression. 1205Lu cells were treated with DW1/2 (1 μ mol/L) for 0, 2, 6, and 24 h, after which proteins were harvested and probed for expression of p53, Mdm2, and Mdm4. Equal protein loading was confirmed using actin.



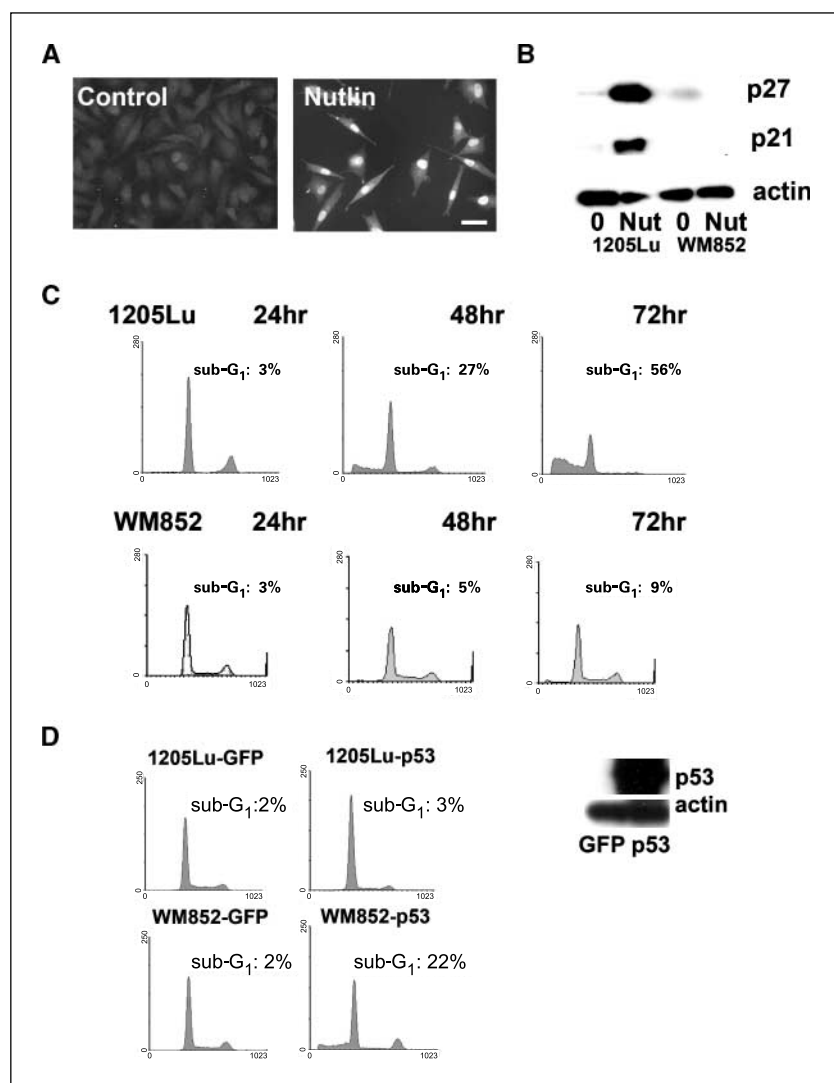


Figure 6. Pharmacologic activation but not overexpression of p53 induces apoptosis in melanoma cells. **A**, Nutlin-3 induces nuclear p53 accumulation in 1205Lu cells. 1205Lu (p53-WT) cells were treated with Nutlin-3 (15 $\mu\text{mol/L}$) for 48 h before being fixed, permeabilized, and stained for p53. Bar, 20 μm . **B**, Nutlin-3 (Nut) induces p21 and p27 expression in p53-WT but not p53-mut cells. 1205Lu cells (p53-WT) and WM852 (p53-mut) cells were treated with Nutlin-3 (15 $\mu\text{mol/L}$) for 24 h, after which proteins were extracted and probed for expression of p27 and p21. Equal protein loading was confirmed by stripping the original blots and probing for total actin. **C**, induction of apoptosis in p53-WT cells but not p53-mut melanoma cells. 1205Lu or WM852 cells were treated with Nutlin-3 (15 $\mu\text{mol/L}$) for 24, 48, and 72 h. Cells were processed as described previously. Sub-G₁ cells given are indicative of apoptosis. **D**, overexpression of p53 induces apoptosis in p53-mut cells but not p53-WT cells. 1205Lu cells (p53-WT) and WM852 cells (p53-mut) were infected with adenovirus encoding for p53-WT for 72 h (right, protein expression), after which cells were harvested and processed for flow cytometry as described previously. Cells undergoing apoptosis are within the sub-G₁ fraction. Control cells were infected with GFP adenovirus and found to be identical to untreated controls.

To further investigate whether p53 activation was a bona fide approach for the induction of apoptosis in melanoma, we next tried Nutlin-3, another drug known to increase p53 activity, through disruption of the p53/Mdm2 interaction (16, 27). Like DW1/2, Nutlin-3 also increased nuclear accumulation of p53 in p53-WT melanoma cells. Like DW1/2, Nutlin-3 also increases the transcriptional activity of p53, as shown by the increased expression of p21^{Waf-1} in p53-WT cells but not in p53-mut cells. In a similar manner, Nutlin-3 also induced apoptosis in p53-WT melanoma cells but not p53-mut cells. In this instance, onset of apoptosis was slower (>48 h) than that seen to DW1/2, which induced significant apoptosis after 24 h of treatment.

To gain further insight into the mechanisms behind the proapoptotic activity of DW1/2 and Nutlin-3, we overexpressed p53 in both p53-WT and p53-mut melanoma cells using an adenoviral vector. It was found that p53 overexpression only induced apoptosis in the p53-mut cells and not in the p53-WT cells. This suggested that mere overexpression of p53 was not sufficient to induce apoptosis and that the p53-WT cells possessed other mechanisms to suppress p53-induced apoptosis. Indeed, others have shown the poor transcriptional activation of p53 target genes in p53-WT melanoma cells following DNA damage (37). It has also

been shown that melanoma cells with p53-WT can tolerate high levels of exogenous p53 (9). It therefore seems likely that DW1/2 may function in a similar manner to Nutlin-3 in freeing the p53 from its suppressors rather than only increasing p53 expression. To investigate this further, we treated the 1205Lu cells with DW1/2 and looked for up-regulation of p53 and down-regulation of Mdm2 and Mdm4. Treatment with DW1/2 induced a rapid increase in p53 expression, a rapid down-regulation of Mdm4 expression (<2 h), and a slower down-regulation of Mdm2 (6 h). Recent studies have shown that both Mdm4 and Mdm2 play important roles in regulating p53 function, with Mdm4 regulating p53 activity and Mdm2 being more responsible for p53 stability (15). To our knowledge, this is the first report showing the pharmacologic down-regulation of Mdm4 expression.

Several recent studies have begun to shed light on the GSK3 β -mediated regulation of p53 expression. It has been suggested that GSK3 β cooperates with Mdm2 to directly regulate the export of p53 from the nucleus via phosphorylation of p53 at Ser³¹⁵ and Ser³⁷⁶ (20). Our own studies revealed a transient increase in Ser⁶ but not Ser³¹⁵ phosphorylation of p53 following DW1/2 treatment (data not shown). Others have shown that overexpression of GSK3 β directly reduces levels of p53 expression through the direct phosphorylation of Mdm2 (21).

Here, we have shown for the first time that activation of p53, using either the novel organometallic kinase inhibitor DW1/2 or the Mdm2/p53 antagonist Nutlin-3, led to the efficient induction of apoptosis in human melanoma cells. At present, GSK3 β inhibitors are being explored in both preclinical and clinical settings for a wide range of disease states from diabetes to neurodegeneration (38). The fact that melanomas harbor p53-WT, which can be activated pharmacologically, makes GSK3 β inhibitors and Nutlin-3 attractive novel therapeutic approaches.

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