Chemical Inhibition of Wild-Type p53-Induced Phosphatase 1 (WIP1/PPM1D) by GSK2830371 Potentiates the Sensitivity to MDM2 Inhibitors in a p53-Dependent Manner

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Abstract

Sensitivity to MDM2 inhibitors is widely different among responsive TP53 wild-type cell lines and tumors. Understanding the determinants of MDM2 inhibitor sensitivity is pertinent for their optimal clinical application. Wild-type p53-inducible phosphatase-1 (WIP1) encoded by PPM1D, is activated, gained/amplified in a range of TP53 wild-type malignancies, and is involved in p53 stress response homeostasis. We investigated cellular growth/proliferation of TP53 wild-type and matched mutant/null cell line pairs, differing in PPM1D genetic status, in response to Nutlin-3/RG7388 ± a highly selective WIP1 inhibitor, GSK2830371. We also assessed the effects of GSK2830371 on MDM2 inhibitor-induced p53Ser15 phosphorylation, p53-mediated global transcriptional activity, and apoptosis. The investigated cell line pairs were relatively insensitive to single-agent GSK2830371. However, a non–growth-inhibitory dose of GSK2830371 markedly potentiated the response to MDM2 inhibitors in TP53 wild-type cell lines, most notably in those harboring PPM1D-activating mutations or copy number gain (up to 5.8-fold decrease in GI50). Potentiation also correlated with significant increase in MDM2 inhibitor–induced cell death endpoints that were preceded by a marked increase in a WIP1 negatively regulated substrate, phosphorylated p53Ser15, known to increase p53 transcriptional activity. Microarray-based gene expression analysis showed that the combination treatment increases the subset of early RG7388-induced p53 transcriptional target genes. These findings demonstrate that potent and selective WIP1 inhibition potentiates the response to MDM2 inhibitors in TP53 wild-type cells, particularly those with PPM1D activation or gain, while highlighting the mechanistic importance of p53Ser15 and its potential use as a biomarker for response to this combination regimen. Mol Cancer Ther; 15(3): 379–91. ©2016 AACR.

Introduction

Mutational inactivation of the p53 tumor suppressor protein, encoded by the TP53 gene, occurs in approximately 50% of malignancies overall (1, 2). Nongenotoxic activation of p53 in the remaining TP53 wild-type malignancies has attracted attention as a therapeutic strategy (3–5). In unstressed cells, p53 is rapidly turned over by binding to one of its transcriptional target gene products, MDM2, which inhibits p53-mediated transcription, promotes p53 ubiquitin-mediated nuclear export and its proteasomal degradation (3). Cellular stress can activate effector molecules (e.g., DNA-PK, ATM, and ATR) that posttranslationally modify MDM2 and or p53, leading to their dissociation followed by p53-mediated reversible cell-cycle arrest, senescence, or programmed cell death (6). Proof-of-concept that pharmacologic inhibition of the MDM2-p53 interaction by small molecular weight MDM2 inhibitors can be successfully used for nongenotoxic activation of p53 has been established in preclinical and clinical settings with encouraging antitumor activity (4, 7). Although, it is firmly established that the most important determinant of response to MDM2 inhibitors is wild-type TP53 genetic status (Supplementary Fig. S1A and ref. 8), multiple independent studies using various classes of MDM2 inhibitors, and drug sensitivity data generated by the Sanger Institute, have shown that there is a wide range of sensitivity to MDM2 inhibitors among TP53 wild-type cell lines [Supplementary Fig. S1B (8–10)]. These highlight that the determinants of sensitivity to MDM2 inhibitors in a TP53 wild-type background are poorly understood. The use of combination regimens and patient stratification strategies are therefore being investigated to optimize tumor-specific response in TP53 wild-type malignancies (11–13).

Another strategy for nongenotoxic activation of p53 currently in preclinical development is inhibition of wild-type p53-inducible phosphatase-1 (WIP1/PPM1D), which is involved in homeostatic regulation of p53 function and stability following cellular stress (14–16). PPM1D is a bona fide oncogene that is activated, gained, or amplified mostly in TP53 wild-type malignancies (17–19). Notably, PPM1D gain-of-function mutations (activation) and TP53-inactivating mutations are mutually exclusive in brainstem gliomas, consistent with the role of WIP1 (PPM1D gene product) in negative regulation of p53 (18). Following cellular
stress, p53 transcriptionally induces WIP1, which forms a negative autoregulatory loop with the p53 network by dephosphorylating p53 and other signaling components involved in p53 posttranslational regulation (15). In spite of selectivity and bioavailability challenges associated with pharmacological targeting of kinases (20), recently a highly selective allosteric WIP1 inhibitor, GSK2830371, which targets the unique flap subdomain on WIP1 was identified and characterized (16). Although, the response to GSK2830371 was contingent on wild-type TP53, some TP53 wild-type cell lines did not respond in the dose range associated with on-target activity (16). In a subsequent publication that highlighted WIP1 as a potential target in neuroblastoma, GSK2830371 was shown to effectively inhibit the growth of TP53 wild-type cell lines with PPM1D copy number gain. However, there was greater than 52-fold range in sensitivity, with NGP cells (PPM1D copy number gain) showing no response at all within the dose range tested (10 μmol/L cutoff; ref. 21).

MDM2 blocks the p53 transactivation domain by interacting with three key p53 amino acids (Phe19, Tyr23, and Leu26) that are proximal to a WIP1 substrate, phosphorylated p53Ser15 (pp53Ser15) (22, 23). Unlike the strong influence of p53Ser20 phosphorylation on binding to MDM2, the phosphorylation of p53Ser15 has been reported to either have no or a modest effect on binding of p53 to MDM2 (24, 25), it has nevertheless been shown to contribute to increased p53 proapoptotic transcriptional activity (25–27). After MDM2 inhibitor–mediated dissociation of p53 from MDM2, pp53Ser15 is generated by the basal unstimulated activity of effector kinases that normally phosphorylate p53Ser15 following genotoxic stress (26). This suggests that a dynamic equilibrium exists between kinases and phosphatases in regulating pp53Ser15 following MDM2 inhibitor–induced p53 stabilization, which can be tilted in favor of the p53-activating kinases by inhibiting WIP1. Therefore, in this study, we have investigated whether a selective WIP1 inhibitor GSK2830371 can potentiate the response to MDM2 inhibitors Nutlin-3 and RG7388 in a panel of cell line pairs differing in their TP53 and PPM1D genetic status. Our findings show that a nongrowth–inhibitory dose of GSK2830371 can substantially increase sensitivity to MDM2 inhibitors in TP53 wild-type cell lines, particularly in those with PPM1D copy number gain or gain-of-function mutation. Furthermore, global gene expression analysis showed that RG7388 in the presence of GSK2830371 induces additional early p53 transcriptional target genes involved in apoptosis in TP53 wild-type cell lines that are not responsive to the WIP1 inhibitor alone. We propose that the combination of WIP1 and MDM2 inhibitors can selectively accentuate the sensitivity to MDM2 inhibitors in TP53 wild-type tumors with increased WIP1 expression or activity, with elevated pp53Ser15 as a potential mechanistic biomarker for response to this combination.

### Materials and Methods

#### Cell lines and growth conditions

All cell lines used were obtained from Northern Institute for Cancer Research cell line bank that only includes cell lines that have been authenticated using short tandem repeat DNA profiling (LGC Standards). Postauthentication passages were limited to 30 for experimental procedures (<6 months) before replacing with lower passage number stocks. Cell line pairs used and their TP53 and PPM1D genetic status are described in Table 1. MCF-7 cells were used as a positive control for WIP1 protein expression and response to the WIP1 inhibitor GSK2830371. The S_N40R2 (SN40R2) and N_N20R1 (N20R1) cell lines were TP53 mutant, otherwise isogenic, Nutlin–3-resistant clones derived from SJA-1 osteosarcoma and NGP neuroblastoma cells, respectively, and have been cited in preclinical studies of MDM2 inhibitors (28; see Table 1 for mutation details). U2OS-DN cells overexpression the R175H variant of p53 that is reported to have a dominant negative effect (29).

#### Growth inhibition assay

Cells were seeded in 96-well plates 24 hours before treatment. Cells were then fixed with Carnoy fixative and Sulfurphoramide B assay was carried out as described in ref. 30. A spectrophotometer (Bio-Rad Model 680) was used for densitometry at 570 nm.

#### Immunoblotting

Western blotting was carried out as described in ref. 31. Antibodies used were MDM2 (Ab-1) 1:300 (Cat No.: OP46-100UG, Merck Millipore), MDMX (Cat No.: A300287A-2 Bethyl Laboratories), WIP1 (F-10) 1:200 (Cat No.: sc-376257, Santa Cruz Biotechnology), p53 1:500 (Cat No.: NCL-L-p53-D07, Leica Microsystems Ltd.), phospho-p53Ser15 1:1,000 (Cat No.: 9284 Cell Signaling Technology), p21 WAF1 1:100 (Cat No.: OP64, Calbiochem). BAX 1:1,000 (Cat No.: 27725, Cell Signaling Technology), cleaved caspase-3 1:1,000 (Cat No.: 9664S, New England Biolabs Ltd.), and actin 1:3,000 (Cat No.: A4700, Sigma-Aldrich).

#### Denaturing immunoprecipitation

Treated cells were lysed [50 mmol/L Tris, 150 mmol/L NaCl, 0.2 mmol/L NaVO₄, 1% NP40 v/v, 1 mmol/L phenylmethylsulfonylfluoride, Roche Complete protease inhibitor tablet, 1 mmol/L dithiothreitol (DTT), 2% SDS] then aliquots were conserved as 10 μmol/L cutoff (ref. 21).

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**Table 1. TP53 wild-type and mutant/null cell line pairs with different PPM1D genetic status**

<table>
<thead>
<tr>
<th>TP53 wild-type parental cell lines</th>
<th>PPM1D-mutant/null daughter lines</th>
<th>Tumor of origin</th>
<th>PPM1D genetic alteration (citation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJSA-1</td>
<td>SN40R2 (E285K)</td>
<td>Osteosarcoma</td>
<td>Wild-type (51)</td>
</tr>
<tr>
<td>HCT116</td>
<td>HCT116 −/− (Null)</td>
<td>Colorectal cancer</td>
<td>c.1544delT:WT (L450X) gain-of-function (59)</td>
</tr>
<tr>
<td>U2OS</td>
<td>U2OS-DN (R175H)</td>
<td>Osteosarcoma</td>
<td>c.1572C&gt;T:WT (R458X) gain-of-function (59)</td>
</tr>
<tr>
<td>NGP</td>
<td>N20R1 (P52T and P98H)</td>
<td>Neuroblastoma</td>
<td>Copy number gain (27)</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td>Breast adenocarcinoma</td>
<td>Amplified (38)</td>
</tr>
</tbody>
</table>

**NOTE:** PPM1D status of SJSA-1 cells were obtained from sanger.ac.uk (51). TP53-mutant daughter cell lines of NGP and SJSA-1 cell lines were derived as described in Materials and Methods.

**Abbreviations:** Mt, mutant; Wt, wild-type.
remainder of the lysates (>0.1% SDS). A total of 1 to 2 μg of rabbit antiubiquitin antibody (Cat No.: FL-76, Santa Cruz Biotechnology) or rabbit IgG control (Cat No.: X0903, Dako) was added to each appropriate vessel and incubated overnight at 4°C. Sepharose beads (Cat No.: 17-0618-01, GE Healthcare) were then added and incubated for a further 4 hours at 4°C. Beads were washed first with 0.5 mol/L KCl then with 0.1 mol/L KCl, then treated as lysates in the immunoblotting protocol above.

Caspase-3/7 assay
A total of 2 × 10^4 cells/well (>60%–70% confluence) were seeded in white 96-well plates (CELLSTAR, Greiner Bio-One international) and treated after 24 hours. Caspase-3/7 enzymatic activities were quantified by adding a 1:1 ratio of CaspaseGlo 3/7 reagent (Promega) to growth media 30 minutes before measuring the luminescence signal using a FLUOstar Omega plate reader (Bio-Tek) and all values were expressed as a ratio of signal relative to solvent control.

Expression array
NGC cells were seeded at 6 × 10^5 cells per well of a 6-well plate and treated with either DMSO or 75 nmol/L of RG7388 (≥ GI50) for 4 hours before RNA extraction using RNeasy Plus Mini Kit (Qiagen). Concentration and quality of mRNA were determined using Agilent RNA 6000 nano kit on an Agilent 2100 Bioanalyzer (RNA integrity numbers > 9). RNA samples were sent to AROS Applied Biotechnology (Aarhus, Denmark) for gene expression analysis using Illumina BeadChip expression arrays (HumanHT-12v4.0). Array data processing, background correction, normalization, and quality control checks were performed using the R package “Lumi” (bioconductor.org). Probe intensity values were converted to variance stabilized data. Robust spline normalization was used as an array normalization method. Poor-quality probes (detection threshold < 0.01), and probes that are not detected at all in the remaining arrays were removed prior to downstream analysis. The remaining probe normalized intensity values (18,634) were used in the differential expression analysis. The data discussed in this article have been deposited in NCBI’s Gene Expression Omnibus (GEO; ref. 32) and are accessible through GEO Series accession number GSE75197 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75197).

RNA extraction and qRT-PCR
Complementary DNA was generated using the Promega Reverse Transcription System (A3500, Promega) as described by the manufacturer. qRT-PCR was carried out using SYBR green RT-PCR master mix (Life Technologies) as per the manufacturer’s guidelines and the following primers (5′–3′): CDRNA1F (forward) (F-TCTGGTCAGAACACATTG, reverse) (R)-AAAGTGGAAATG-TCCATTGGCCT), TP53INP1 (F-TCTTCGATCGTCTGGTAGTACA, R-GTGCGGTGTCATACACAGGCTC), BTG2 (F-CCTGGTCCTGGT-GACCCCTAT, R-GGCGCTCTGGCTCAGGACAGG), MD2 (F-CAG-TACATCGATCCATGCAGAAG, R-CCGATCCATTGGATGAC), R-GATCC-AGATCCATGCAGAAG, R-GATCGGCTCCCTGGAGAT). A total of 50 ng/μL of the cDNA samples per 10 μl final reaction volume, with the standard cycling parameters (stage 1: 50°C for 2 minutes, stage 2: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute), were set and carried out on an ABI 7900HT sequence detection system. Data were presented as mean ± SEM relative quantities of four independent repeats where GAPDH was used as endogenous control and DMSO used as the calibrator for each independent repeat with the formula 2^-ΔΔCt. Analysis was carried out using SDS 2.2 software (Applied Biosystems).

Site-directed mutagenesis and p53 overexpression
The plasmid vector used in this study was pCDNA3.1 (+/− Life Technologies, Cat No. V790-20 and V795-20) and full-length human TP53 cDNA cloned into this backbone. The Gozani laboratory protocol for site-directed mutagenesis (33) was used to generate the p53^Ser15^ mutants. Primers used: p53^51S^S (F-CTGCG-AGCCCTCTGTGCTCAGGAAACATTTTCA, R- TGGAAATGTTT-TCTCTGAGCGCAGAGGGGTCGTGAC) and p53^51S^S (F-CTGAG-GCCCTCTGTGCTCAGGAAACATTTTCA, R-TGGAAATGTTT-CTCTGAGCGCAGAGGGGTCGTGAC). HCT116^-T^ cells were transfected with Lipofectamine 2000 and plasmid DNA 12 hours before lysates were collected at different time intervals.

Flow cytometry
After treatment, floating and adhered cells were pooled and incubated in propidium iodide solution [150 μmol/L propidium iodide (Calbiochem), 1.46 μmol/L DNease free-RNAse A (Sigma), 3.88 mmol/L sodium citrate (Sigma), and 0.3% Triton-X 100 (Sigma)] for 10 minutes at 25°C and then FACS was carried out using a FACScalibur (BD Biosciences). CellQuest software was used to establish cell-cycle distribution and gated histograms.

Statistical analysis
Statistical tests were carried out in GraphPad Prism 6 software and all P values represent two-tailed paired t tests of three or more independent repeats unless otherwise stated. For microarray differential expression analysis, R-statistical software was used. Microarray data was processed using R Bioconductor package limma (34). Probes intensity values were transformed using variance stabilizing transformation implemented in the limma package before data normalization. The robust spline normalization was used as a normalization method. Poor quality probes (detection threshold < 0.01), and probes that were not detected at all in the remaining arrays were removed. Differential expression analysis was performed using R Bioconductor package limma (35).

Results
GSK2830371 potentiates the response to MDM2 inhibitors Nutlin-3 and RG7388 in a p53-dependent manner
Growth inhibition assays were carried out on a panel of TP53 wild-type and mutant/null cell line pairs differing in their PPM1D genetic status, to assess their sensitivity to the selective allosteric WIP1 inhibitor GSK2830371 and its ability in turn to sensitize cells to MDM2 inhibitors (Fig. 1A). GSK2830371-sensitive MCF-7 cells were used as a positive control for the growth-inhibitory activity and biochemical effect of this compound. GSK2830371 had a 50% growth inhibitory concentration [GI50] of 2.65 μmol/L ± 0.54 (SEM) in MCF-7 cells. The growth inhibition curve for GSK2830371 plateaued in MCF-7 cells at doses 2.5 to 10 μmol/L, suggesting that a subpopulation of MCF-7 cells are resistant to growth inhibition and apoptosis in response to maximal WIP1 inhibition (Fig. 1A). All other cell line pairs were not sensitive to growth inhibition by GSK2830371 alone, with GI50 > 10 μmol/L irrespective of their PPM1D or TP53 genetic status (Fig. 1A). Basal expression of WIP1 across the panel of cell line pairs was...
Figure 1.
A, the effect on growth of a panel of p53 wild-type (green) and mutant/null (maroon) cell line pairs with different PPM1D genetic status to 0.08–10 μmol/L GSK2830371 exposure for 168 hours, using Sulforhodamine B growth inhibition assays. B, basal expression of WIP1 and p53 in cell lines (SE, short film exposure; LE, long exposure). C, the sensitivity of a panel of p53 Wt (green) and mutant/null (maroon) cell line pairs with different PPM1D genetic status to 0.08–10 μmol/L Nutlin-3 and 0.008–1 μmol/L RG7388 in 168 hours Sulforhodamine B growth inhibition assays in the presence and absence of the highest nongrowth inhibitory dose of GSK2830371 (2.5 μmol/L). The U2OS cell line pair was treated with MDM2 inhibitors/C6 1.25 μmol/L GSK2830371. FL-WIP1, full-length WIP1; S-WIP1, WIP1 shorter isoform; S/C3-WIP1, the shortest band detected by the F-10 antibody; T-WIP1, truncated WIP1 mutants; WIP1i, GSK2830371; WIP1 L450X in HCT116 cells or WIP1 R458X in U2OS cells.
assessed by immunoblotting with an antibody that detects WIP1 (FL-WIP1), its previously described shorter isoform (S-WIP1; ref. 36), and the two WIP1 gain-of-function mutant proteins in HCT116 and U2OS cell line pairs (Fig. 1B). Transient knockdown of WIP1 using four different anti-PPM1D siRNA constructs resulted in a reduction in the intensity of all the bands detected by the WIP1 antibody (F-10), which suggests that all of the bands detected in these conditions correspond to WIP1 (Supplementary Fig. S2A). This includes a further WIP1 isoform detected by F-10 (=55 kDa), which is referred to here as S’-WIP1. PPM1D-amplified MCF-7 cells (37) showed the highest basal expression of WIP1 and its isoforms, consistent with their sensitivity to GSK2830371 single treatment (Fig. 1B; see ref. 38). NGP cells, with PPM1D copy number gain (21), and its otherwise isogenic TP53-mutant daughter cell line, N20R1, were insensitive to <10 μmol/L GSK2830371 despite showing the second highest expression of full-length WIP1 after MCF-7 cells. The SISA-1 and SN40R2 TP53 wild-type and mutant pair, with wild-type PPM1D, showed the least WIP1 protein expression among all cell line pairs in the panel. Shorter bands corresponding to the previously reported truncated and activating WIP1-mutant proteins WIP1 L450X and WIP1 R458X were detected in lysates derived from HCT116 and U2OS cell line pairs, respectively (Fig. 1B; ref. 39).

Because of the role of WIP1 in the homeostatic feedback regulation of the p53 network, we aimed to assess whether GSK2830371 (WIP1i) can potentiate the response to MDM2 inhibitors. Treatment with a combination of the highest nongrowth-inhibitory dose of GSK2830371 (2.5 μmol/L) potentiated the response to MDM2 inhibitors Nutlin-3 and RG7388 in a p53-dependent manner in cell lines that were not sensitive to growth inhibition by GSK2830371 alone (Fig. 1C). TP53 wild-type parental cell lines HCT116+/−, NGP, and SISA-1 showed a 2.4-fold (P = 0.007), 2.1-fold (P = 0.039), and 1.3-fold (P = 0.017) decrease, respectively, in their Nutlin-3 GI50 values in the presence of 2.5 μmol/L GSK2830371. In contrast, Nutlin-3 GI50 did not change for their TP53 null/mutant matched pairs HCT116−/−, N20R1, and SN40R2. However, pertinent to the widening of RG7388 thera-
0.5 × Nutlin-3 GI₅₀ significantly decreased (P = 0.008) when GSK2830371 was present at 2.5 μmol/L (Fig. 3C).

In NGP cells, pp53Ser15 was not affected by the GI₅₀ dose of Nutlin-3 or 2.5 μmol/L GSK2830371 alone, whereas in combination treatment with 2.5 μmol/L GSK2830371 results in degradation of full-length and truncated WIP1 in HCT116+/− and U2OS cells. D, lysates obtained from HCT116+/− cells treated with 20 μmol/L proteasome inhibitor MG132 and 2.5 μmol/L GSK2830371 + 3.0 μmol/L Nutlin-3 overnight underwent immunoprecipitation with anti-ubiquitin antibody (Ub-Ab) and the precipitates probed for WIP1 and p53 by western blot analysis. Input samples (left panel) are western blots of total lysate before IP for comparison with the IP results on the right-hand panel. An aliquot of MG132-treated lysate was precipitated with rabbit IgG instead of Ub-Ab as a negative control. Ub-Ab, anti-ubiquitin antibody; WIP1i, GSK2830371; WIP1 (Wt), wild-type WIP1; WIP1 L450X, truncated WIP1.

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Figure 2.
A, GSK2830371 (2.5 μmol/L) treatment of MCF-7 cells over 8 hours shows WIP1 degradation over time, p53 stabilization, and Phospho-p53Ser15 (pp53Ser15) accumulation. B, pp53Ser15 phosphorylation in MCF-7 cells 30 minutes after 2 Gy ionizing radiation in the presence or absence of 2.5 μmol/L. GSK2831371 inhibits WIP1 catalytic activity independent of WIP1 protein levels. C, 4-hour treatment with 2.5 μmol/L GSK2830371 results in degradation of full-length and truncated WIP1 in HCT116+/− and U2OS cells. D, lysates obtained from HCT116+/− cells treated with 20 μmol/L proteasome inhibitor MG132 and 2.5 μmol/L GSK2830371 + 3.0 μmol/L Nutlin-3 overnight underwent immunoprecipitation with anti-ubiquitin antibody (Ub-Ab) and the precipitates probed for WIP1 and p53 by western blot analysis. Input samples (left panel) are western blots of total lysate before IP for comparison with the IP results on the right-hand panel. An aliquot of MG132-treated lysate was precipitated with rabbit IgG instead of Ub-Ab as a negative control. Ub-Ab, anti-ubiquitin antibody; WIP1i, GSK2830371; WIP1 (Wt), wild-type WIP1; WIP1 L450X, truncated WIP1.
Figure 3.
A, dose-dependent increase in caspase 3/7 activity of NGP cells after 24 hours treatment with Nutlin-3 (Nut-3 GI50 ≈ 3.0 μmol/L) alone or in combination with 2.5 μmol/L GSK2830371. B, increase in caspase-3/7 activity in NGP and SJSA-1 cells and their TP53-mutant daughter cell lines 48 hours after treatment with Nutlin-3 ≈ 2.5 μmol/L GSK2830371. C, reduction in clonogenic efficiency of HCT116þ/þ cells following exposure to 0.5 × GI50 concentration of Nutlin-3 in the presence of 2.5 μmol/L GSK2830371 compared with either inhibitor alone over 10 days. D, immunoblot of NGP cells showing Nutlin-3-dependent phosphorylation of p53 at Ser15 is markedly enhanced by GSK2830371 at 4- and 24-hour exposure and leads to increased caspase-3 cleavage at 48 hours. E, time-course of NGP response to 3.0 μmol/L Nutlin-3 (≈GI50) ≈ 2.5 μmol/L GSK2830371 over 24 hours. WIP1i, GSK2830371; *, P ≤ 0.05; **, P ≤ 0.005.
GSK2830371 increases RG7388-induced p53-dependent transcription of growth inhibitory and proapoptotic genes

Phosphorylation of p53Ser15 has been reported to increase p53-mediated transcriptional transactivation but not to be necessary for dissociation of p53 from MDM2 in response to DNA damage (24, 26). Also, reports in the literature have suggested that p53 posttranslational modifications can behave as variable barcodes and induce transcription of alternate sets of p53 target genes that could lead to different cell fates after p53 activation (42). As the greatest potentiation of MDM2 inhibitors by GSK2830371 was observed in NGS cells (5.8-fold), we assessed whether the subset of early genes activated in response to RG7388 in this cell line differed in the presence of 2.5 μmol/L GSK2830371, which produced a marked increase in pp53Ser15. Peak p53 transcriptional target expression (e.g., p21WAF1 and MDM2) as detected by western blotting is reached by 6 to 8 hours after treatment (Fig. 3E). Because later changes in transcription may be secondary effects and not directly p53-dependent, we assessed changes in global gene expression 4 hours following RG7388 (GI50 ≈ 75 nmol/L) ± 2.5 μmol/L GSK2830371 using the Illumina BeadChip expression array platform. A testament to the specificity of RG7388 in exclusively activating p53, 4 hours of exposure to a GI50 dose of RG7388 led to significantly increased mRNA expression of only 9 genes, all of which were known p53 transcriptional targets (Fig. 4A). The top 41 genes just below the statistical significance cut-off point (P > 0.05 after correction for multiple testing) were also mostly genes that are well established to be direct p53 transcriptional targets. Interestingly, in the presence of 2.5 μmol/L GSK2830371, the subset of statistically significant RG7388-mediated transcriptional changes increased from 9 to 24 genes, indicating that inhibition of WIP1 results in a significant increase of additional p53-mediated transcriptional activity at this early time point (Fig. 4B, for a list of genes refer to Supplementary Tables S1A and S1B).

To validate the results of the array, we assessed the expression of the top three genes with the highest odds ratio difference between the three gene sets of single and combination treatments [CDKN1A (p21WAF1), TP53INP1, and BTG2] and one of the genes that was exclusively induced in the combination treatment (MDM2) by qRT-PCR using the same mRNA samples used in the expression array experiment (Fig. 4C). All the genes tested showed significant increase in their mRNA expression in combination treatment compared with the RG7388 alone (Fig. 4C). TP53INP1/PS3DINP1 is a known proapoptotic p53 transcriptional target gene, the overexpression or induction of which following cellular stress has been associated with increased p53-mediated apoptosis (43). Among the 16 additional p53-regulated target genes induced exclusively in response to the combination treatment were TNF super family member 10B (TNFRSF10B) and p53-induced death domain protein 1 (PIDD1), two genes critical for extrinsic and intrinsic proapoptotic pathways, respectively (44–47). Interestingly, despite neither of the agents being genotoxic, one of the other genes that showed differential expression in response to the combination treatment was the DNA base excision repair gene xeroderma pigmentosum complementation group C (XPC), the increased basal expression of which has been reported to correlate with increased sensitivity to MDM2 inhibitors in a large panel of TP53 wild-type cell lines and predict a better clinical response to RG7112 and RG7388 in acute myeloid leukaemia patients (13).

The increase in the subset of genes expressed correlated with a marked increase in the proportion of pp53Ser15 to total p53 in immunoblots of lysates prepared in parallel to the mRNA samples used in the expression array experiment (Fig. 4D and see Supplementary Fig. S3A). These data suggest that the underlying mechanism for the observed potentiation of MDM2 inhibitors in combination with WIP1 phosphatase inhibition may be contributed to by the increased p53Ser15 phosphorylation that enhances p53-dependent proapoptotic gene transcription.

In line with this hypothesis, increased pp53Ser15 phosphorylation in HCT116/−/+ following combination treatment resulted in an increase in the p21WAF1 product of the CDKN1A gene in comparison with monotherapy with either drug (Fig. 4E). To confirm earlier findings showing that phosphorylation of p53Ser15 increases p53-mediated transcription in HCT116 cells, we overexpressed wild-type (Wt) p53, mutant p53S15A or p53S15D in HCT116/−/+ cells and assessed p53-mediated expression of p21WAF1 protein encoded by the CDKN1A gene, which had showed the biggest fold change in expression on the array following the combination treatment in NGS cells. Consistent with previous findings (24, 26), Wt p53 and phospho-mimetic p53S15D mutant constructs increased p53-mediated expression of p21WAF1 and MDM2 following transfection compared with the p53S15A mutant, which could not be phosphorylated on that residue (Fig. 4F). See Supplementary Fig. S3B and S3C for repeat and densitometry data.

The effect of combined MDM2 and WIP1 inhibition on cell-cycle distribution

Given that WIP1 inhibition potentiated the growth inhibitory and apoptotic response of TP53 wild-type cell lines to MDM2 inhibitors, and the highest fold increase in transcription was of the CDKN1A (p21WAF1) cyclin-dependent kinase inhibitor gene, we investigated changes in cell-cycle distribution following this combination treatment. In all cell lines, 2.5 μmol/L GSK2830371 alone did not significantly affect cell-cycle distribution throughout 72 hours of treatment (Fig. 5A and Supplementary Fig. S4). Changes in cell-cycle distribution after exposure to Nutlin-3 ± 2.5 μmol/L GSK2830371 were cell line-dependent. In SiSA-1 and NGS cell lines, 24 hours exposure to Nutlin-3 resulted in an increase in the proportion of cells in G1/G0 phases of the cell cycle. In SiSA-1 cells, this effect of Nutlin-3 remained unchanged in the following 48 hours treatment with Nutlin-3 ± GSK2830371. However, in NGS cells, the relative proportion of cells in G2/M and S-phase increased over the following 48 hours when Nutlin-3 and the WIP1 inhibitor were combined compared with Nutlin-3 alone. In HCT116/−/+ cells, Nutlin-3 resulted in an increase in the proportion of cells in G0/G1 and G2/M phases at 24 hours, which persisted to the 72 hours treatment time point (Fig. 5A), consistent with the increase in CDKN1A (p21WAF1) expression in response to the combination treatment (Fig. 4E). Cell-cycle distribution was not affected in HCT116/−/+ cells regardless of the treatment condition, suggesting that the changes in cell-cycle distribution observed in HCT116/−/+ cells are p53-dependent (Fig. 5A).

Sub-G1 changes

In response to the combination treatment compared with Nutlin-3 alone, the increase in sub-G1, FACS signal after exposure to Nutlin-3 was significantly augmented in the presence of
2.5 μmol/L GSK2830371 (WIP1i) in both SJSA-1 and NGP cell lines (Fig. 5B and Supplementary Fig. S4B). This is in keeping with the increased cleaved caspase-3/7 activity in NGP and SJSA-1 cells (Fig. 3A and B). Sub-G₁ signals were not significantly changed in HCT116−/− cells throughout the 72 hours of Nutlin-3 ± GSK2830371 treatment (Fig. 5B).

Figure 4.

A and B, volcano plots showing the significantly induced genes by RG7388 treatment of NGP cells for 4 hours in the presence or absence of 2.5 μmol/L GSK2830371 (RG7388 vs. DMSO and RG7388 + WIP1 (2.5 μmol/L) vs. DMSO). The results represent the mean of four independent biologic repeats. y-axis: −log₁₀ of the P value adjusted for false discovery rate using the Benjamini-Hochberg procedure; x-axis Log₂ FC: log₂ fold change in normalized mRNA expression. For a list of genes induced, see Supplementary Table S1A and S1B. Microarray data have been deposited in the NCBI GEO databank (accession number: GSE75197).

C, qRT-PCR validation of p53 target genes implicated in increased sensitivity to MDM2 inhibitors: TNFRSF10B, AEN, XPC, and CDKN1A using the same RNA samples as used for the microarray analysis. D, Western blot analysis for NGP cells showing changes in p53, pp53Ser15, MDM2, WIP1, and p21WAF1 protein expression in response to 75 nmol/L RG7388 (G₁,0) ± 2.5 μmol/L GSK2830371. Lysates were obtained from samples treated in parallel to the microarray experiment and represent the first three independent repeats. E, Western blot analysis of HCT116−/− cells 4 hours following treatment with the stated doses of Nutlin-3, GSK2830371 or their combination. F, Western blot analysis showing changes in pp53Ser15, MDM2, and p21WAF1 detection 12 hours after ectopic expression of wild-type p53 (Wt), p53 mutants Ser15Ala (S15A), and Ser15Asp (S15D) in HCT116−/− cells. WIP1i, GSK2830371; *, P ≤ 0.05; **, P ≤ 0.005; ***, P ≤ 0.0005.
Figure 5.
A, time-course of cell-cycle distribution changes over 72 hours of treatment using FACS analysis. B, percent of sub-G1 signals for NGP, SJSA-1, HCT116+/+, and HCT116−/− cells in response to the stated treatments at 72 hours. WIP1i, GSK2830371.
Discussion

Although mutant TP53 status is a dominant mechanism of resistance to MDM2-p53 binding antagonists, there is nevertheless a clinically relevant wide range of sensitivity to MDM2 inhibitors among TP53 wild-type cancer cell lines. Importantly, this variation of response is not exclusive to one class of MDM2 inhibitors and is clearly seen in panels of cell lines with validated wild-type TP53 status. Amgen has recently reported a wide range of sensitivity to their MDM2 inhibitor AMGMD53 among their carefully curated panel of TP53 wild-type and functional cell lines showing a 500-fold G_{100} difference between the least to most sensitive (8). These observations suggest that there are a diverse set of underlying genetic variables that determine cell fate following a dose of activated/stabilized p53. Here we have shown a non-growth-inhibitory dose of the selective orally bioavailable allosteric WIP1 phosphatase inhibitor, GSK2830371, can modulate the phosphorylation state of p53 and potentiate both the growth inhibitory and apoptotic response to MDM2 inhibitors in TP53 wild-type cell lines, especially those with increased WIP1 expression or activity. MDM2 inhibitor potentiation was at its greatest when the cell line harbored either PPM1D copy number gain/ elevated expression or gain-of-function truncating mutations, thus providing a rationale for specific combination treatment targeting of tumors with this genotype.

The argument in support of p53^{Ser15} phosphorylation increasing p53-mediated transcription (24, 26, 27) is compelling and consistent with our findings. In contrast to what was originally reported by Vassilev and colleagues, in 2004, phosphorylation of p53 following treatment with MDM2 inhibitors is observed; however, it is not as intense and immediate compared with p53^{Ser15} phosphorylation following DNA damaging agents of equivalent growth-inhibitory dose (7, 26). As shown by Loughery and colleagues, the basal activity of kinases involved in phosphorylation of p53^{Ser15} (e.g., ATM and ATR) in response to DNA damage are also likely responsible for, or contribute to, this posttranslational modification in response to MDM2 inhibitors (26). Our findings have shown that, in the presence of a selective WIP1 inhibitor, the minimal phosphorylation of p53^{Ser15} in response to MDM2 inhibitors is markedly accentuated, which correlates with potentiation of apoptotic and growth-inhibitory response to MDM2 inhibitors in TP53 wild-type cells, particularly in those with high WIP1 expression or activity. This was also associated with significantly increased transcript levels from an increased number of immediate p53 transcriptional target genes as compared with those induced by single-agent RG7388. Increased p53^{Ser15} phosphorylation in response to the combination treatment also resulted in increased p21^{WAF1} and MDM2 protein expression. Consistent with the reported role of p53^{Ser15} phosphorylation in increasing transcriptional activity of p53, we also confirmed that mutation of this residue influenced expression of p21^{WAF1} and MDM2 proteins. Thus, our current working model includes evidence for the role of enhanced p53 transcriptional activity in response to the combination of MDM2 inhibitors and GSK2830371 (Fig. 6). It is likely, however, that direct and/or indirect WIP1-mediated posttranslational modifications that effect the stability and function of stress response proteins and their cross-talk with the p53 network (as reviewed in ref. 15) may also contribute to MDM2 inhibitor potentiation in the presence of GSK2830371. Regardless of this, our data strongly suggest that increased pp53^{Ser15} can be considered a surrogate marker of p53 dissociation from MDM2 in response to single-agent GSK2830371 treatment or its combination with MDM2 inhibitors, as this modification coincides with p53 transcriptional activation that precedes the subsequent enhanced p53-mediated growth inhibitory or apoptotic response to each of these treatments.

Nutlin-3–mediated changes in cell-cycle distribution were all enhanced in the presence of a dose of GSK2830371 that on its own did not affect cell-cycle distribution. The observed increase in sub-G_{1} FACS analysis signals with combination treatment of NGP cells is consistent with potentiation of apoptosis and growth inhibition in this cell line. Enhancement of Nutlin-3–mediated...
G1:S and G2:S ratios in the presence of GSK2830371 are also consistent with the increased CDKN1A (p21WAF1) expression observed at both transcript and protein levels and its importance in negative regulation of cell-cycle progression (48, 49). Kleiblova and colleagues, (39) had previously shown that transient knockdown of truncated PPM1D increases G1 checkpoint in response to ionizing radiation. Interestingly, in our current study, WIP1 inhibition and depletion by GSK2830371 in HCT116\(^{+/+}\) cells harboring a PPM1D L450X truncation mutation also resulted in an increase in p53-dependent G1 arrest following p53 activation by Nutlin-3, whereas this did not occur in NGP and SISA-1 cell lines that do not have PPM1D gain-of-function mutations. Lindqvist and colleagues (50) also reported that WIP1 knockdown ablates the competence of cellular p53-dependent G2 checkpoint recovery following cellular stress, although the authors were not aware of the gain-of-function WIP1 R458X mutation in U2OS cells used in their study, as it had not yet been reported. These findings suggest that the increase in G1:S ratio observed in HCT116\(^{+/+}\) cells treated with the combination of MDM2 inhibitors and GSK2830371 is likely due to inhibition of WIP1 L450X that would otherwise be negatively regulating p53 transcriptional activity in these cell lines. Of note, we have also shown that GSK2830371 increases the ubiquitin-mediated degradation of truncated WIP1 as postulated by Gilmartin and colleagues, (16). Interestingly, this increase in the ubiquitination of truncated WIP1 was reversed by inhibition of MDM2, which suggests that MDM2 is directly or indirectly involved in WIP1 ubiquitination following GSK2830371.

Wild-type TP53 genetic status is the most important determinant of response to MDM2 inhibitors, while being necessary but not sufficient for growth inhibitory response to WIP1 inhibition by GSK2830371. Following their promising clinical outcomes so far, MDM2 inhibitors will be explored in combination with other anticancer agents to optimize their therapeutic potential. Combination regimens of these nongenotoxic agents could minimize DNA damage to healthy tissue that does not express altered forms of PPM1D. Here we have shown that specific pharmacologic inhibition of WIP1 combined with MDM2 inhibitors is a promising therapeutic strategy in TP53 wild-type tumors that show increased WIP1 function, and that p53\(^{307/315}\) and PPM1D genotype are important both mechanistically and as predictive biomarkers for response to this combination treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Esfandiari, T.A. Hawthorne, S. Nakjang, J. Lunec
Writing, review, and/or revision of the manuscript: A. Esfandiari, J. Lunec
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Esfandiari
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Acknowledgments

The authors thank Prof. Nicola J. Curtin for providing the U2OS cell line pair and also acknowledge Mrs. Mahsa Azizyan and Drs. Olivier Binda and Richard Heath for their provision of reagents and advice on experimental procedures. The encouragement and provision of RG7388 by Prof. Newell and the NICR Drug Discovery team are gratefully acknowledged.

Grant Support

This work was supported by the grants from Cancer Research UK (C240/ A15751; to J. Lunec), Newcastle University and Northern Institute for Cancer Research (C0190R4011; to A. Esfandiari), and Newcastle University (C0190R4011; to S. Nakjang and T. Hawthorne).

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Received August 6, 2015; revised December 8, 2015; accepted December 23, 2015; published OnlineFirst February 1, 2016.

References

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