TP53 Genomic Status Regulates Sensitivity of Gastric Cancer Cells to the Histone Methylation Inhibitor 3-Deazaneplanocin A (DZNep)

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Abstract

Purpose: DZNep (3-deazaneplanocin A) depletes EZH2, a critical component of polycomb repressive complex 2 (PRC2), which is frequently deregulated in cancer. Despite exhibiting promising anticancer activity, the specific genetic determinants underlying DZNep responsiveness in cancer cells remain largely unknown. We sought to determine molecular factors influencing DZNep response in gastric cancer.

Experimental Design: Phenotypic effects of DZNep were evaluated in a panel of gastric cancer cell lines. Sensitive lines were molecularly interrogated to identify potential predictors of DZNep responsiveness. The functional importance of candidate predictors was evaluated using short hairpin RNA (shRNA) and siRNA technologies.

Results: DZNep depleted PRC2 pathway components in almost all gastric cancer lines, however, only a subset of lines exhibited growth inhibition upon treatment. TP53 genomic status was significantly associated with DZNep cellular responsiveness, with TP53 wild-type (WT) lines being more sensitive (P < 0.001). In TP53-WT lines, DZNep stabilized p53 by reducing ubiquitin conjugation through USP10 upregulation, resulting in activation of canonical p53 target genes. TP53 knockdown in TP53-WT lines attenuated DZNep sensitivity and p53 target activation, showing the functional importance of an intact p53 pathway in regulating DZNep cellular sensitivity. In primary human gastric cancers, EZH2 expression was negatively correlated with p53 pathway activation, suggesting that higher levels of EZH2 may repress p53 activity.

Conclusion: Our results highlight an important role for TP53 genomic status in influencing DZNep response in gastric cancer. Clinical trials evaluating EZH2-targeting agents such as DZNep should consider stratifying patients with gastric cancer by their TP53 genomic status. Clin Cancer Res; 18(15): 4201–12. ©2012 AACR.

Introduction

Gastric cancer remains the second leading cause of global cancer death accounting for 700,000 deaths annually (1).
transcriptional silencing of tumor suppressor genes (TSG), such as p57 in ovarian and breast cancer (13, 14) and RUNX3 and CDH1 in gastric cancer (15). For these reasons, EZH2 may be an appealing gastric cancer therapeutic.

Recently, the S-adenosylhomocysteine hydrolase inhibitor, 3-deazaneplanocin A (DZNep), was identified as a histone methylation inhibitor (16–18) and a promising example of histone methylation epigenetic therapy. Treatment of cancer cell lines with DZNep induced EZH2, SUZ12, and EED depletion, reduction of H3K27me3 repressive marks, growth inhibition, and apoptosis (16–18). However, despite exhibiting promising anticancer activity, little is currently known about specific genetic determinants regulating tumor responses to DZNep. For example, it has suggested that BRCA1 status may contribute to DZNep sensitivity in breast cancer cells (19). Identifying additional genetic determinants regulating DZNep response in different cancer types will likely contribute to our understanding of the mechanism of action of this promising drug candidate and may suggest strategies for patient stratification and selection in subsequent clinical trials.

In this study, we sought to determine genetic factors influencing DZNep response in gastric cancer cells. Analyzing a panel of gastric cancer cell lines (20), we discovered a subset of gastric cancer cell lines and that DZNep-sensitivetargets exhibited a significant tendency to carry wild-type TP53 genes (P < 0.001). Using functional assays, we show the existence of a novel mechanism of p53-dependent DZNep cellular sensitivity, involving p53 stabilization via USP10 upregulation. Finally, we show that in primary gastric cancers, EZH2 expression is negatively correlated with p53 pathway activity, highlighting a potentially important role for EZH2 in repressing p53 activity. We propose that future clinical trials evaluating EZH2-targeting agents such as DZNep in gastric cancer should preselect patients by their TP53 genomic status.

Translational Relevance

Expression of the polycomb-complex member EZH2 is increased in gastric cancer where it acts to suppress gastric cancer–related tumor suppressor genes. Here, we investigated the cellular and molecular effects of inhibiting EZH2 in gastric cancer cell lines, using a recently described small molecule (3-deazaneplanocin A, DZNep) that induces EZH2 depletion. We found that DZNep significantly suppressed the growth of only a subset of gastric cancer cell lines and that DZNep-sensitivetargets exhibited a significant tendency to carry wild-type TP53 genes (P < 0.001). Using functional assays, we show the existence of a novel mechanism of p53-dependent DZNep cellular sensitivity, involving p53 stabilization via USP10 upregulation. Finally, we show that in primary gastric cancers, EZH2 expression is negatively correlated with p53 pathway activity, highlighting a potentially important role for EZH2 in repressing p53 activity. We propose that future clinical trials evaluating EZH2-targeting agents such as DZNep in gastric cancer should preselect patients by their TP53 genomic status.

Results

Increased expression of EZH2, a PRC2 component, in gastric cancer cells

To confirm previous reports that EZH2 exhibits increased expression in gastric cancer cells (7, 15), we analyzed 100 primary normal and 192 cancerous gastric samples (Supplementary Table S1 provides clinical characteristics). EZH2 transcript levels were significantly elevated in tumors (P < 0.001, Supplementary Fig. S1A). Specifically, in 61% of gastric tumors, EZH2 was expressed greater than 1.5-fold above the mean level of normal gastric tissues. Similar results were observed for SUZ12 and EED (Supplementary Fig. S1B and S1C). In vitro, gastric cancer cell lines also exhibited higher EZH2 expression levels than nonmalignant gastric mucosa (Fig. 1A) and similarly for SUZ12 and EED (Supplementary Fig. S1D and S1E). The increased expression of EZH2 in gastric cancers suggests that EZH2-targeting drugs may prove useful in gastric cancer.

Gastric cancer cell lines exhibit common EZH2 depletion but differential growth inhibition after DZNep treatment

We treated 16 gastric cancer cell lines with 5 μmol/L DZNep for 72 hours, using previously reported dosages (16, 17). Across the lines, DZNep induced EZH2 protein depletion and both H3K27me3 and H4K20me3 histone marks were reduced (Fig. 1B and Supplementary Fig. S2). DZNep also caused SUZ12 and EED protein depletion in the majority of lines (Supplementary Fig. S3). However, despite reports describing inverse correlations of H3K27me3 and H3K27ac in certain genes (21), DZNep did not alter global H3K27 acetylation patterns (Supplementary Fig. S2). It is possible that the transient depletion of EZH2 by DZNep may not be
sufficient to increase global H3K27 acetylation. Taken together, DZNep is thus capable of depleting EZH2 in a wide variety of gastric cancer cells regardless of individual cell line differences.

To explore the in vitro growth response of gastric cancer cell lines to DZNep, we treated the lines with increasing concentrations of DZNep from 0.5 μmol/L to 100 μmol/L, and determined GI50s, referring to the drug concentration required for 50% growth inhibition after 72 hours treatment. MCF7 breast cancer cells, for which DZNep responses have been previously characterized (16, 22), were included as a positive control (Fig. 1C). In contrast to the seemingly common effects on EZH2 depletion (Fig. 1B), DZNep caused very distinct growth responses among the gastric cancer cells. Specifically, IM95, YCC6, AZ521, and AGS cells showed the highest sensitivity to DZNep (GI50 = 0.3–2.0 μmol/L) whereas other lines such as TMK1, YCC7, Ist1, and MKN7 did not exhibit cell growth alterations upon DZNep treatment even at the maximal dose (GI50 >100 μmol/L). There was more than 180-fold difference in sensitivity between the most DZNep-sensitive and -resistant lines, with a mean GI50 value of 15.8 μmol/L.

We selected 4 sensitive (IM95, YCC6, AZ521, and AGS) and 4 resistant lines (TMK1, YCC7, Ist1, and MKN7) for further characterization. Five micromolar was chosen as a working concentration based upon previous reports (16, 17). Similar to the GI50 analysis, sensitive lines exhibited significant growth inhibition at both 48 and 72 hours after

Figure 1. Molecular and cellular effects of DZNep on gastric cancer cells. A, EZH2 mRNA expression in gastric cancer (GC) cell lines. EZH2 expression is increased in gastric cancer cell lines (gray columns) relative to normal gastric mucosa (N. mucosa) sample (white column). B, DZNep treatment causes EZH2 protein depletion and trimethylated H3K27 reduction in gastric cancer cell lines. After 72 hours of DZNep treatment, cells were monitored for cell viability. Each point represents the mean ± SD from 3 independent experiments. C, differential growth responses are observed between sensitive and resistant cell lines. DZNep-sensitive and -resistant cell lines are highlighted for each cell line. The MCF7 GI50 value is very similar to concentrations obtained in C (5 μmol/L). D, proliferation effects of DZNep in a gastric cancer cell lines compared with the mean GI50 set as 0; blue and red color bars: DZNep-sensitive and -resistant cell lines. DZNep (5 μmol/L) and control- 

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DZNep treatment whereas resistant lines were unaffected ($p < 0.05$; Fig. 1D). These results suggest that other factors, either parallel or downstream to EZH2 depletion, may influence cellular responses to DZNep.

**DZNep induces distinct cellular responses in sensitive gastric cancer cells**

Inhibition of cell proliferation can be due to various mechanisms, including cell cycle inhibition, apoptosis, and senescence. We sought to identify which of these mechanisms might be responsible for the growth inhibition effects of DZNep in gastric cancer cells.

We exposed 3 sensitive lines (IM95, AGS, and AZ521) and one resistant line (TMK1, as a negative control) to 5 μmol/L DZNep for 48 hours, and conducted apoptosis, DNA content, and senescence-associated β-galactosidase assays (Fig. 2A). In IM95 cells, we observed a drastic increase in the sub-G1, cellular population and proportion of Annexin V/7-aminoactinomycin D (7-AAD) double-positive apoptotic cells after DZNep treatment, indicating that IM95 cells undergo apoptosis upon DZNep exposure (Fig. 2A, top left). In contrast, DZNep-sensitive AGS and AZ521 cells displayed mild apoptosis and exhibited a G1 population increase after DZNep treatment. Morphologically, AGS and AZ521 became enlarged and flat and exhibited increased populations of senescence-associated β-galactosidase (SA-βGal) stained cells (20%–25%) after DZNep exposure (Fig. 2A, top right, bottom left and B), suggesting the induction of senescence. No significant changes in DNA content, apoptosis, and senescence characteristics were observed in resistant TMK1 cells after DZNep treatment (Fig. 2A, bottom right). These results suggest that DZNep is likely to elicit highly individualized cellular responses in sensitive cell lines, all culminating in growth inhibition.

**EZH2 gene silencing phenocopies DZNep treatment**

Two possible models could explain why DZNep can cause common EZH2 depletion but cellular responses in only a subset of gastric cancer cells. Firstly, the molecular determinants regulating DZNep cellular response could lie downstream of EZH2 but may only be selectively activated in sensitive cell lines. Alternatively, DZNep may act in an "off-target" fashion in an EZH2-independent manner. Indeed, besides EZH2 and H3K27me3, DZNep is also known to regulate other epigenetic marks (17). To distinguish between these 2 models, we genetically silenced EZH2 in the gastric cancer lines using a validated EZH2 siRNA both sensitive and specific to EZH2 depletion (23). Treatment of gastric cancer cells with EZH2 siRNA successfully caused EZH2 protein depletion (Fig. 2C). Similar to DZNep treatment, EZH2 gene silencing caused a significant increase of the sub-G1 population in IM95 cells, G1 population increases in AGS and AZ521 cells, but no significant alterations in TMK1 cells (Fig. 2A and D). These results suggest that the cellular effects induced by DZNep in gastric cancer cells are likely mediated through EZH2 and are not due to an off-target effect. Moreover, these findings implicate EZH2 downstream mechanisms in the selection of DZNep-induced cellular phenotypes.

**TP53 genomic status is a potential predictive marker for DZNep response in gastric cancer cells**

Initial gene expression analysis of DZNep-sensitive and -resistant cell lines yielded thousands of genes differentially regulated after DZNep treatment (Supplementary Table S2), rendering it difficult to pinpoint genes specifically responsible for DZNep cellular sensitivity. We thus adopted a pathway-specific approach, focusing on the TP53 tumor suppressor which is frequently mutated in several cancers including gastric cancer (24, 25). In normal cells, p53 protein is maintained at low levels by MDM2-mediated ubiquitination (26). During cellular stress (DNA damaging, oncogenic stress, hypoxia), p53 protein is stabilized, resulting in p53 binding to target promoters and induction of various cell context-specific genes related to apoptosis (target genes FAS, DR5), growth arrest (p21, GADD45α), and senescence (p21; ref. 27). The striking resemblance of the diverse cellular responses induced by DZNep to p53 biologic outcomes motivated us to explore relationships between TP53 genomic status and DZNep cellular responses.

Sequencing all TP53 exons in the gastric cancer lines (Supplementary Table S3), we found that 9 of 16 (56%) of the gastric cancer lines were TP53-mutated, a frequency comparable with the published literature (24). We observed a TP53 deletion in Ist1 and 2 hot spot mutations in the gastric lines (R175H and R282W). All TP53 mutations in the gastric cancer cells found were inactivating (28). Importantly, we found that 6 of 7 (86%) of the DZNep-sensitive gastric cancer cell lines harbored wild-type TP53 genes, whereas 8 of 9 (88%) of the DZNep-resistant gastric cancer cells were mutated in TP53 (Fig. 3A, P < 0.001 by Fisher exact test). This association between DZNep sensitivity and TP53 genomic status suggests that in gastric cancer cells, TP53 status could be a potential predictive factor for DZNep and EZH2 inhibitor responses.

**DZNep causes p53 protein accumulation and activation of cell context–specific p53 pathways in TP53 wild-type but not mutant cells**

To investigate whether the cellular responses induced by DZNep might be due to activation of wild-type p53, we analyzed p53 proteins in DZNep-sensitive and -resistant gastric cancer lines. We also included MCF7 cells, which are DZNep sensitive and TP53 wild-type (16). After 6 hours of DZNep treatment, we observed p53 protein accumulation in TP53 wild-type sensitive lines (including MCF7) but no changes in TP53-mutated TMK1 cells, which already express high levels of mutant p53 protein (Fig. 3B). Quantitative PCR analysis of TP53 mRNA transcripts confirmed that the p53 protein accumulation was posttranscriptional (Supplementary Fig. S4). To assess the functionality of this p53 accumulation, we investigated whether DZNep treatment also caused the transcriptional activation of p53 target genes specifically related to apoptosis, cell-cycle inhibition, and...
Figure 2. EZH2 depletion induces diverse cellular responses in sensitive cell lines. A, DZNep induces G1 population increases, apoptosis, and senescence in sensitive gastric cancer cells after 48 hours of treatment. Sensitive gastric cancer lines (IM95, AGS, and AZ521) were compared against a representative resistant line (TMK1, bottom right). DNA content analysis: DZNep and control-treated cells were analyzed by flow cytometry (representative red histograms under cell line names, all histograms are in same scale). Black arrows indicate changes in DNA content. Cell percentages representing sub-G1 (<G1) and G1 fractions are shown beside histograms. Apoptosis analysis (IM95, representative blots at top left): levels of apoptotic IM95 cells were determined using an Annexin V/7-AAD assay and flow cytometry (density plots). An increase in the apoptotic cell fraction (from 7%–46%) is observed after DZNep treatment (red arrow). Senescence analysis: morphology changes of DZNep and control-treated cells were monitored under white light microscopy. Levels of cell senescence were determined in DZNep and control-treated cells using senescence-associated SA-beta Gal (photomicrographs). DZNep-sensitive lines AGS and AZ521 exhibit enlarged and flattened cellular morphologies and an increase in senescent populations following treatment (black arrows in photomicrographs). No senescent phenotypes are seen in DZNep-resistant TMK1 cells (bottom right). Photos are shown at ×20 magnification. Data are based on 3 independent experiments. B, quantification of cell senescence. Bar charts show the average percentage of SA-beta Gal–stained cells of AGS and AZ521 cells in control (DMSO) and DZNep-treated cells. Significant increases in SA-beta Gal–positive cells are observed after 48 hours of DZNep treatment (P < 0.05, Student t test). C, EZH2 knockdown efficiency. Sensitive gastric cancer lines (IM95, AGS, and AZ521) and resistant TMK1 cells were treated with scrambled (Scr) or validated EZH2 siRNAs (siEZ) for 48 hours. EZH2 protein levels were examined by immunoblotting. β-Actin was used as a loading control. D, cell-cycle responses of scrambled and EZH2-silenced cells after transfection. DNA content of scrambled or EZH2 siRNA–treated cells were determined by propidium iodide (PI) staining and flow cytometry. All histograms are plotted according to the same scale. Black arrows indicate the DNA content changes induced by EZH2 silencing. Percentages of cells in sub-G1 (<G1) and G1 population are shown in the right corner of each histogram. EZH2 knockdown caused significant induction of sub-G1 population in IM95 cells and G1 induction in AGS and AZ521 cells. No similar changes were observed in resistant TMK1 cells.
Figure 3. DZNep sensitivity is correlated with wild-type TP53 genomic status and p53 protein accumulation after treatment. A, TP53 genomic status in DZNep-sensitive (blue columns) and resistant (red columns) lines. Sensitive lines are more likely to harbor a wild-type (P < 0.001, Fisher exact test). B, DZNep causes p53 protein accumulation in sensitive cell lines. Cells treated with DMSO control (−) or DZNep (+) after 6 hours were normalized against a TBP control. Cells were treated with DZNep for 6 hours and normalized to DMSO-treated controls. C, mRNA and protein analysis of p53 and p53 downstream targets in sensitive (IM95, AGS, and AZ521) and resistant (TMK1) cells. mRNA measurements (bar chart). qPCR mRNA measurements were normalized against a TBP control. Cells were treated with DZNep for 6 hours and normalized to DMSO-treated controls. *, P < 0.05 relative to DMSO control-treated cells. Three independent experiments were conducted for each line. Protein measurements (blots). Cells treated with DMSO (−) or DZNep (+) at 6 hours and probed by immunoblotting. DZNep activates distinct sets of p53 downstream targets in TP53 wild-type sensitive lines but no activation is observed in resistant TMK1 cells which are TP53-mutated. β-Actin was used as a loading control.

senescence. We targeted the following canonical p53 target genes for analysis by quantitative PCR and immunoblotting: FAS and DR5 are transcriptional targets of p53 encoding death receptors (27) whose upregulation can cause apoptosis (29, 30). p21 and GADD45a are p53 target genes involved in p53-mediated growth inhibition and senescence (31). We also included the MDM2 E3 ubiquitin ligase, as a general p53 activation marker, as MDM2 is upregulated in both growth inhibited and also in apoptotic cells upon p53 activation.

After 6 hours of DZNep treatment, MDM2, DR5, and FAS mRNA transcripts and proteins were consistently induced (up to 6-fold at the mRNA level) in IM95 cells which undergo apoptosis (Fig. 3C, top left). Conversely, AGS and AZ521, which exhibit growth inhibition after DZNep treatment, showed significant MDM2 upregulation (up to 3-fold) and upregulation of the other growth inhibition related markers p21 and/or GADD45a at both the mRNA and protein level (Fig. 3C, top right and bottom left). In contrast to TP53 wild-type cells, we neither observed MDM2 induction, nor activation of any of the other p53 target genes, in TP53-mutated TMK1 cells (Fig. 3C, bottom right). These targeted results were further confirmed at a more global level by using p53 pathway PCR gene expression arrays containing 84 p53-related genes. Similar to our original results, we confirmed upregulation of several p53 target genes after DZNep treatment in DZNep-sensitive (AGS, IM95) but not in resistant lines (TMK1) including MDM2, PPM1D, TNFRSF10B, BTG2, PIDD, SENS1, SENS2, and GADD45a (Supplementary Table S4). These results suggest that DZNep-stabilized p53 is functionally active in sensitive cells and
may operate to activate target genes related to the observed cellular phenotypes.

To confirm that DZNep-mediated p53 activation involved EZH2 and is not an off-target effect, we then used siRNA transfection technique to deplete EZH2. Similar to DZNep treatment, EZH2 knockdown caused p53 protein stabilization in a posttranscriptional manner and MD洮 transcript increases in TP洮 wild-type sensitive cells (Supplementary Fig. S5). In contrast, EZH2 depletion neither triggered p53 stabilization nor MDM洮 upregulation in TP洮-mutated TMK1 cells (Supplementary Fig. S5). These results provide evidence that DZNep treatment can cause p53 protein accumulation and activation in TP洮 wild-type cells, most likely by depleting EZH2.

DZNep reduces p53 ubiquitination levels possibly through USP10 upregulation

Ubiquitination is a critical process governing p53 stability, as ubiquitinated p53 is rapidly degraded by the 26S proteasome (26). We investigated whether DZNep might stabilize p53 by inhibiting p53 ubiquitination. To visualize ubiquitinated p53, we cotreated gastric cancer cells with MG132, a 26S proteasome inhibitor compound (32). DZNep reduced ubiquitin conjugates on p53 in TP洮 wild-type gastric cancer cells but not in mutant cells (Fig. 4A, Left). Similar results were obtained using EZH2 siRNAs instead of DZNep (Fig. 4A, right). These findings raise the possibility that DZNep most likely stabilizes p53 by inhibiting p53 ubiquitination.

Figure 4. DZNep treatment causes p53 ubiquitination reduction, likely associated with USP10 upregulation by reducing EZH2 binding. A, effects of DZNep or EZH2 targeting siRNA on p53 ubiquitin conjugation. Cells were treated with (left) DMSO (-) or DZNep (+) for 6 hours or (right) scrambled siRNAs (Scr) or a validated EZH2 siRNA (++) for 48 hours and analyzed for levels of ubiquinated p53. EZH2 immunoblots confirm successful silencing (bottom). Cells were cotreated with the proteasomal inhibitor MG132 for 3 hours to visualize ubiquitinated bands. β-Actin was used as a loading control. Reduced levels of p53 ubiquitination are observed following 6 hours of DZNep or 48 hours of EZH2 siRNA exposure. This ubiquitination reduction is seen in DZNep-sensitive cells (IM95, AGS, and AZ521, all TP洮 wild-type) but not in resistant cells (TMK1, TP洮-mutated). B, DZNep causes USP10 protein upregulation in sensitive cells. Cells treated with DZNep for 6 hours were analyzed for USP10 protein expression. β-Actin was used as a loading control. Elevated levels of USP10 are observed in sensitive cells but not resistant TMK1 cells. C, DZNep reduces EZH2 binding to the USP10 promoter. CHIP assays were conducted on sensitive IM95 cells using ChIP-validated EZH2 antibodies and analyzed by qPCR amplifying EZH2 region of USP10 promoter. EZH2-free region was shown as a negative control. Relative enrichments of EZH2 binding were normalized to input controls. D, USP10 inhibition attenuates DZNep-induced p53 accumulation in sensitive cells. IM95 and TMK1 were treated with USP10 siRNA-1 for 72 hours and cotreated with DMSO (-) or DZNep (+) for 6 hours. Protein expression was analyzed by indicated antibodies. Numbers below each blot indicate specific band intensities relative to β-actin expression. USP10 knockdown using siRNA-1 sequence reduces p53 expression and attenuates DZNep-induced p53 accumulation in sensitive IM95 cells but not resistant TMK1 cells.
MDM2 is the major E3 ubiquitin ligase for p53 and the ability of MDM2 to ubiquitinate p53 requires direct interactions between MDM2 and p53 (26). Upon cellular stress, activated ATM kinase can phosphorylate p53 at Ser15, inhibiting MDM2 binding to p53, resulting in p53 stabilization (33). Surprisingly, our analysis suggests that DZNep-associated p53 ubiquitination reduction is unlikely to involve ATM and MDM2/p53 interactions. DZNep treatment neither induced γH2AX, a biomarker for DNA damage and ATM activation (ref. 34; Supplementary Fig. S6) nor p53 Ser15 phosphorylation beyond basal levels (Supplementary Fig. S6). Coimmunoprecipitation assays revealed that DZNep did not significantly hinder the binding of MDM2 to p53 in sensitive IM95 and AGS cells (Supplementary Fig. S7). Thus, DZNep treatment most likely inhibits p53 ubiquitination by an alternative pathway.

Recently, a nonclassical p53 modulator, USP10, was shown to counteract MDM2 ubiquitinase activity, resulting in p53 accumulation (35). We found that DZNep upregulated both USP10 mRNA and protein (at least around 2-fold increase) in sensitive cells but not resistant TMK1 cells (Fig. 4B and Supplementary Fig. S8A). Similarly, EZH2 silencing in IM95, AGS, and AZ521 cells but not TMK1 cells also caused higher USP10 mRNA expression, increasing approximately 2-fold compared with control cells (Supplementary Fig. S8B). Consistent with USP10 being a direct transcriptional target of EZH2, chromatin immunoprecipitation (ChIP) assays using EZH2 antibodies confirmed EZH2 binding on the USP10 promoter, which was significantly reduced after DZNep exposure (Fig. 4C).

To determine whether USP10 induction is functionally required for DZNep-induced p53 stability, we used siRNAs to deplete USP10 in sensitive IM95 and resistant TMK1 cells (Fig. 4D). USP10 silencing reduced p53 protein levels in IM95 cells consistent with previous findings (35). Importantly, DZNep induced significantly less p53 accumulation in USP10 siRNA–treated sensitive cells than in scrambled siRNA–treated cells. Similar results were observed when the USP10 silencing was repeated using a different nonoverlapping USP10 targeting sequence, indicating this is unlikely due to an off-target siRNA effect (Fig. 4D and Supplementary Fig. S9). USP10 silencing did not alter p53 stability in resistant TMK1 cells, suggesting that USP10 may only regulate wild-type p53 proteins. Collectively, these results provide evidence that DZNep-induced p53 stabilization may involve an alternative pathway associated with USP10 upregulation rather than the classical ATM/p53ser15/MDM2 pathway.

In a separate series of experiments, we also investigated whether DZNep might directly regulate p53 methylation levels which can also influence p53 transcriptional activity (36). Using an antibody specific to p53 monomethylated at residue K372, we immunoprecipitated methylated p53 before and after DZNep treatment. However, we did not observe any significant alterations in methylated p53 K372 levels after DZNep treatment, above and beyond general p53 stabilization (Supplementary Fig. S10). Levels of p53 methylation, at least at residue K372, thus remain unaltered upon DZNep treatment.

**TP53 knockdown rescues the growth inhibitory effects of DZNep**

To explore the role of the p53 pathway in DZNep-induced cellular responses, we then generated IM95, AGS, and AZ521 cells where p53 levels were significantly silenced using validated p53 shRNAs (short hairpin RNA; ref. 37, Supplementary Fig. S11A and S11B). In both the parental and TP53-knockdown lines, DZNep treatment induced similar EZH2 depletion (Supplementary Fig. S11C). However, in TP53-knockdown lines, DZNep stabilized the remaining p53 proteins to a significantly lesser extent (Supplementary Fig. S11C). Importantly, we found that TP53-knockdown cells were also now significantly more resistant to DZNep in terms of their cellular responses than parental cells. In TP53-knockdown IM95 cells, 80% of cells remained alive after DZNep treatment compared with 45% of parental IM95 cells (P < 0.05), and those DZNep-induced p53 downstream targets (MDM2, DR5, and FAS) were also no longer significantly upregulated (P < 0.05; Fig. 5A). Similarly, TP53-knockdown AGS and AZ521 cells displayed significantly reduced levels of G1 population induction (Fig. 5B and C) and attenuated transcriptional induction of p53 downstream targets (P < 0.05; Fig. 5A, bar charts on right). However, TP53 knockdown did not reduce USP10 mRNA induction by DZNep (Supplementary Fig. S11D). This further supports the notion that USP10 is upstream of p53 and that its upregulation is not a consequence of p53 stabilization following DZNep treatment. These results functionally confirm that the cellular phenotypes of DZNep are dependent, at least in part, on the presence of a wild-type p53 regulatory machinery.

**EZH2 may suppress p53 activity in gastric cancer**

Finally, the finding that pharmacologic EZH2 depletion by DZNep results in p53 activation led us to hypothesize that EZH2 might function to inactivate p53 activity, particularly in those gastric cancers with wild-type TP53 genes. We thus explored the relationship between EZH2 expression and p53 activity in primary gastric cancers.

We applied Bayesian factor regression modeling (BFRM; ref. 38) to predict p53 pathway activity in individual tumor samples using a TP53 gene expression signature (39). The reliability of BFRM to predict p53 activity was confirmed by comparing p53 activity levels across a cohort of 132 primary gastric cancers of known TP53 mutational status (exons 4–9)—TP53 wild-type gastric cancers showed significantly higher BFRM-p53 activation levels than TP53-mutated samples (P = 0.001; Fig. 6A). Additional confirmation was obtained when the BFRM-p53 analysis was applied to an independent set of 251 primary breast samples for which TP53 genomic status is known (P < 0.001; Supplementary Fig. S12A). We then correlated patterns of predicted p53 activity to levels of EZH2 expression in the same gastric...
tumor set, this time specifically focusing on the 105 tumors carrying wild-type TP53 genes. We observed a significant negative correlation between p53 activity and EZH2 expression ($R < -0.56$, $P < 0.001$; Fig. 6B), indicating that gastric tumors with increased EZH2 expression have significantly lower p53 activity. EZH2, in addition to its accepted role in transcriptionally silencing TSGs, may thus also contribute to tumorigenesis and cancer progression through repressing p53 activity (see Discussion).

**Discussion**

The EZH2 histone methyltransferase is highly expressed in many cancers, where it functionally acts to regulate several pro-oncogenic traits (4). EZH2 has been considered a promising potential target in cancer therapy, and DZNep, a recently identified histone methylation inhibitor that can deplete EZH2, has been tested for anticancer activity in many cancers, where it functionally acts to regulate several pro-oncogenic traits (4). EZH2 has been considered a promising potential target in cancer therapy, and DZNep, a recently identified histone methylation inhibitor that can deplete EZH2, has been tested for anticancer activity in various cancer types including breast (16), prostate (40), lung cancers (41) and leukemia (6). Currently, little is known about specific molecular factors determining DZNep response or resistance. However, emerging data suggest that different tumors are likely to respond to DZNep in a heterogeneous fashion—for example, estrogen receptor (ER)-negative breast cancers with low BRCA1 levels may be particularly DZNep-sensitive (19). These observations raise the need to identify specific biomarkers that can predict which subsets of patients with cancer might benefit most from EZH2-targeted therapies.

This is the first study investigating the effects of DZNep in gastric cancer. Confirming previous studies, we observed increased expression of EZH2 in primary gastric tumors and gastric cancer cell lines (7, 15). In an independent study, EZH2 expression levels have also been shown to be progressively increased during multistep gastric carcinogenesis (42), supporting EZH2 as a potential therapeutic target in gastric cancer. To investigate effects of DZNep on gastric cancer, we treated a panel of gastric cancer cell lines with a wide range of DZNep concentrations. Two notable findings emerged from this analysis. First, DZNep treatment caused a
near-uniform depletion of EZH2 protein and a reduction in PRC2-associated H3K27me3 and H4K20me3 histone marks in all or close to all of the cell lines tested, suggesting that histone alterations are likely to represent a general and stereotyped response to DZNep. Second, in contrast to these stereotyped effects, DZNep induced a diversity of responses in the gastric cells at the cellular level—for sensitive lines, we observed DZNep-induced apoptosis, G1 population induction, and senescence, whereas resistant lines exhibited no obvious cellular phenotypes while still displaying comparable EZH2 depletion and H3K27me3 reduction. Importantly, similar findings were observed when we conducted EZH2 silencing on the lines, showing that these effects are due to EZH2 depletion and are not an ‘off-target’ effect. Supporting our findings, others have reported distinct phenotypes in different cellular systems after DZNep treatment (12, 16, 18, 40)—for example Wu and colleagues have recently shown early changes in DNA content and sub-G1 increases upon EZH2 silencing in osteosarcoma cell lines (43).

One model explaining why DZNep can uniformly induce EZH2 and H3K27me3 depletion in all lines but cellular responses in only a subset of lines is that the molecular determinants regulating the latter may lie downstream of EZH2. In this regard, our most significant finding was the discovery of a highly significant relationship ($P < 0.001$) between TP53 genomic status and DZNep responsiveness, where TP53 wild-type cell lines were more DZNep-sensitive. p53 is a well-known tumor suppressor, one of the most frequently mutated proteins in cancer (24), and p53 activation can result in the activation of downstream target genes in a cell-type specific manner leading to cell-cycle arrest, senescence, and apoptosis. These phenotypes mirrored our DZNep treatment results, and in our study we confirmed that DZNep treatment and EZH2 silencing can both stabilize p53 and activate distinct p53 pathways by activating distinct sets of canonical p53 downstream genes in p53 wild-type gastric cancer cells. Although a relationship between EZH2 and p53 has been previously proposed in other cancers (44), to our knowledge this is the first study to reveal functional associations between DZNep sensitivity and TP53 genomic status in gastric cancer. For example, shRNA-mediated knockdown of wild-type TP53 in significantly reduced the apoptotic and cell-cycle effects caused by DZNep in sensitive lines. These results suggest that future clinical trials evaluating EZH2 inhibitors in gastric cancer should strongly consider stratifying patients by their TP53 genomic status.

In this study, we found that DZNep treatment stabilizes p53 in gastric cancer by inhibiting p53 ubiquitination. Interestingly however, this ubiquitination inhibition appears to occur independently of p53 phosphorylation and primary p53-MDM2 binding, the most commonly known pathways of regulating p53 ubiquitination. Rather, we found that DZNep-induced p53 stabilization likely involved the upregulation of USP10, an ubiquitin specific peptidase known to inhibit p53 ubiquitination without interfering with p53-MDM2 binding. Knockdown experiments confirmed that USP10 is functionally required in DZNep-mediated p53 stabilization. However, our data does not rule out the possibility that EZH2 may also act to repress other p53-modulating genes similar to USP10 to regulate p53 activity.

At present, it is unclear to what extent the EZH2-p53 relationship discovered in gastric cancer can also be extended to other cancer types. At the protein level, positive correlations between EZH2 expression and high p53 expression, a hallmark of inactive or mutated TP53 (45), has been reported in breast cancer (44), and we also observed a similar correlation in gastric cancer (Supplementary Fig. S13). However, at the gene expression level in both breast and gastric tumors with wild-type TP53 genes, we detected a weak negative correlation between p53 pathway activation scores and EZH2 expression levels in breast tumors
(Supplementary Fig. S12B) and a strong negative correlation in gastric cancer (Fig. 6B). A study in leukemia reported p21 activation upon EZH2 knockdown (6), and recently, Fan and colleagues reported that EZH2 knockdown can activate p21, albeit in a p53-independent manner, in melanoma (46). In osteosarcoma, EZH2 knockdown does not appear to stabilize p53 nor activate p21 and may instead transcriptionally reactivated a p21 regulator which directs p21 degradation (43). These findings suggest that the effects of DZNep treatment and EZH2 silencing on p53 stabilization and expression of target genes such as p21 may have significant tissue specificity.

In conclusion, our results suggest that in gastric cancers where expression of EZH2 is increased, wild-type p53 is potentially functionally inactivated by EZH2. Upon exposure to DZNep, EZH2 is depleted and ubiquitination of these wild-type p53 proteins is inhibited, resulting in p53 stabilization and activation of downstream p53 pathways involved in apoptosis, cell-cycle arrest, and senescence (Supplementary Fig. S14). Given that TP53 is mutated in only approximately 50% of gastric cancers (24)—a substantial fraction of patients with gastric cancer are thus TP53 wild-type and may thus be potentially targetable by EZH2-targeting epigenetic agents. Analogous to RITA and the nutlins that can reactivate p53 by modulating MDM2/p53 binding (47), DZNep may thus represent a novel nongenotoxic pharmacologic strategy to reactivate p53 activity in gastric cancer cells.

References

Disclosure of Potential Conflicts of Interest
S. Rozen is an employee for Duke University. No potential conflicts of interest were disclosed by other authors.

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TP53 Genomic Status Regulates Sensitivity of Gastric Cancer Cells to the Histone Methylation Inhibitor 3-Deazaneplanocin A (DZNep)

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