HDAC4-Regulated STAT1 Activation Mediates Platinum Resistance in Ovarian Cancer


Abstract

Ovarian cancer frequently acquires resistance to platinum chemotherapy, representing a major challenge for improving patient survival. Recent work suggests that resistant clones exist within a larger drug-sensitive cell population prior to chemotherapy, implying that resistance is selected for rather than generated by treatment. We sought to compare clinically derived, intrapatient paired models of initial platinum response and subsequent resistant relapse to define molecular determinants of evolved resistance. Transcriptional analysis of a matched cell line series from three patients with high-grade serous ovarian cancer before and after development of clinical platinum resistance (PEO1/PEO4/PEO6, PEA1/PEA2, PEO14/PEO23) identified 91 up- and 126 downregulated genes common to acquired resistance. Significantly enhanced apoptotic response to platinum treatment in resistant cells was observed following knockdown of histone deacetylase (HDAC) 4, FOLR2, PIK3R1, or STAT1 (P < 0.05). Interestingly, HDAC4 and STAT1 were found to physically interact. Acetyl-STAT1 was detected in platinum-sensitive cells but not in HDAC4 overexpressing platinum-resistant cells from the same patient. In resistant cells, STAT1 phosphorylation/nuclear translocation was seen following platinum exposure, whereas silencing of HDAC4 increased acetyl-STAT1 levels, prevented platinum-induced STAT1 activation, and restored cisplatin sensitivity. Conversely, matched sensitive cells were refractory to STAT1 phosphorylation on platinum treatment. Analysis of 16 paired tumor biopsies taken before and after development of clinical platinum resistance showed significantly increased HDAC4 expression in resistant tumors [n = 7 of 16 (44%); P = 0.04]. Therefore, clinical selection of HDAC4-overexpressing tumor cells upon exposure to chemotherapy promotes STAT1 deacetylation and cancer cell survival. Together, our findings identify HDAC4 as a novel, therapeutically tractable target to counter platinum resistance in ovarian cancer. Cancer Res; 71(13): 4412–22. ©2011 AACR.

Introduction

One of the greatest areas of unmet need compromising the successful treatment of ovarian cancer is the acquisition of clinical resistance to platinum chemotherapy. Platinum-based compounds are standard first-line agents for ovarian cancer and initial response rates are high (1). However, subsequent relapse with acquired platinum resistance is frequent and closely linked to the poor survival associated with this cancer. Multiple mechanisms for platinum resistance have been described and are reviewed elsewhere (2–4).

A recent genomic analysis of a cell line series derived from 3 cases of serous ovarian cancer both before and after acquisition of clinical platinum resistance revealed that in addition to shared genomic features, sensitive and resistant tumor cells from the same patient also exhibit mutually exclusive genomic characteristics, indicating that rather than a direct linear evolution of resistance from sensitive disease in response to platinum challenge, platinum-resistant clones are present from the outset at low abundance within the sensitive presenting tumor (5). In this model, the minor resistant clone persists despite effective killing of the dominant sensitive population and subsequently expands causing relapse. This is in contrast to alternative hypotheses of acquired resistance whereby mutations are proposed to arise in sensitive cells in response to treatment with chemotherapy. In vitro derivation of acquired resistance by treatment of a sensitive cancer cell line with platinum agents is likely to mimic this alternative hypothesis producing adaptive linear responses, which may not accurately reflect clinical resistance. As such, we focused...
our analysis here on clinically derived models of resistance. Henceforth, for brevity, we refer to this selection hypothesis as acquired platinum resistance, as it describes the known clinical entity of relapse within 6 months of last platinum therapy after previous remission/response.

Here, we report the first linked gene expression profiling and functional analysis of intrapatient paired pre- and post-clinically acquired platinum resistance in ovarian cancer. Our analysis used ovarian cancer cell line series described previously (5, 6), identifying several novel modulators of platinum response and focuses on a previously unreported functional mechanism that behaves in a fundamentally different manner between clinically platinum-sensitive and -resistant cells from the same patients. In addition, we noted that this mechanism operates to produce resistance independently of pre-existing established changes in platinum response caused by functional reversion of a germ line BRCA2 truncating mutation (7). This work identifies therapeutic targets with implications for the management of ovarian cancer.

Materials and Methods

Cell lines and reagents

The paired high-grade serous ovarian carcinoma cell lines PE01 versus PE04/PE06, PEA1 versus PEA2, and PEO14 versus PEO23 were obtained from Dr. Simon Langdon (Edinburgh, UK) and have been described elsewhere (5–7). Cell line verification was by Identifiler kit (Applied Biosystems). In the matched pairs, the first set of cell lines (PE01, PEA1, PEO14) were derived prior to and the second set (PE04/PE06, PEA2, PEO23), following the onset of acquired clinical platinum resistance. SKOV3 cells were obtained from European Collection of Animal Cell Cultures (ECACC). Caspilatin response was measured by sulforhodamine B (SRB) assay as described (8). All cell lines have confirmed TP53 mutations (5). BRCA1/2 sequencing was done as described (ref. 9; see also Supplementary Methods). All lines were maintained in RPMI 1640 media with 10% fetal calf serum (FCS), penicillin, streptomycin at 37°C/5% CO₂. Antibodies used were FOLR2, HDAC4, HSXIAPAF1, ITGB2, LAMA4, MYC, PIK3R1, PRKCBP1, STAT1, TAPI, VEGFA and control siRNAs, LAMIN A/C, non-targeting and siCONTROL-TOX (100 nM/L final concentration; Dharmacon). Cells were retransfected after 48 hours. The siRNAs in 1× siRNA buffer were mixed with 2 μl transfection reagent #(1) (Dharmacon) per transfection in a total volume of 400 μL with OptiMEM media. Following 30 minutes incubation, siRNAs were added to 1,600 μL antibiotic-free RPMI 1640/10% FCS on cells. Twenty-four hours after second transfection, cells were reseeded. Cells reseeded in 6-well trays were incubated for 48 hours and protein and RNA samples prepared. RNA extraction was by RNeasy Mini Kit (Qiagen). Cells reseeded into clear and opaque 96-well trays were treated identically: for each transfection condition, 24 hours after seeding, 3 replicate wells were treated with 25 μmol/L cisplatin and 3 wells left untreated. After 24 hours, cells caspase activation was measured by caspaseGlo 3/7 assay (Promega) and viable cell numbers inferred by MTT assay as described elsewhere (11). Caspase activity was normalized to cell density for each treatment.

Immunofluorescent microscopy

Coverslips (VWR International) were treated with 1 mol/L HCl before cell seeding and incubation for 24 hours at 37°C/5% CO₂. After indicated treatment, cells were washed with PBS then fixed/permeabilized at 37°C for 30 minutes with 4% paraformaldehyde/1.8% TritonX-100/PBS. Coverslips were blocked in 10% goat serum/2% bovine serum albumin (BSA)/PBS for 30 minutes, then washed with PBS, and incubated with primary antibodies overnight at 4°C. Coverslips were washed in PBS and incubated with 1:500 dilutions of fluorescein isothiocyanate (FITC) anti-goat IgG, FITC anti-rabbit IgG, Alexa Fluor 555 anti-mouse IgG, and Alexa Fluor 488 goat anti-rabbit IgG; Invitrogen] and directly labeled actin stain (Alexa Fluor 633 phalloidin; Invitrogen) in blocking buffer for 1 hour. Cells were rinsed 3 times in PBS for 5 minutes and mounted onto slides using Vectashield media containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Slides were visualized on an inverted confocal microscopy system (Axiovert 200M, Zeiss and TCS SP5, Leica).

Pharmacologic inhibition of HDAC4

PE04 and SKOV3 cells were seeded at 25,000 cells per well in 96-well plates in a volume of 50 μL per well. After 24 hours, cells were either treated with 40 μL of fresh culture media (untreated control) or 40 μL fresh media containing HDAC inhibitor (HDACi) aroyl-pyrrolyl-hydroxy-amide 4a (APHA4a) at final concentration, calculated for 50 μL final volume, of 5 μmol/L and 10 μmol/L. After 1 hour, 10 μL of cisplatin was added to a final concentration of 25 μmol/L in 50 μL; 10 μL of fresh culture media was added to control cells. Caspase 3/7 and MTT assays were conducted at 24 hours post-cisplatin treatment as described above.

Additional methods

See Supplementary Data for additional methods.
Results

Coupled gene expression and functional analysis of upregulated transcripts in clinically acquired platinum resistance

To confirm that acquired clinical resistance to platinum-based chemotherapy was maintained in long-term platinum-free cell culture, we carried out in vitro cisplatin sensitivity assays (Fig. 1A). Average values from at least 3 replicate experiments revealed a between 4- and 9-fold increase in cisplatin IC_{50} values on acquisition of clinical resistance. Stable resistance in the absence of cisplatin suggests that clinical mechanisms of resistance are genetically or epigenetically determined: consistent with the description of distinct genomic differences between the sensitive and resistant paired lines studied here (5). Mutations in BRCA2 have been described previously in the cell lines PEO1 and PEO4 (7). We sequenced BRCA1 and BRCA2 in all paired cell lines and identified previously described alterations in our stocks of PEO1 and PEO4 that are associated with functional BRCA2 (discussed in detail in Supplementary Fig. S1). No further mutations were seen in BRCA1 or BRCA2 in other cell lines indicating that the clinical platinum resistance phenotype here is not accounted for by BRCA mutation/reversion. RNAs from cell line pairs were cohybridized to cDNA microarrays. Normalized data were filtered as described identifying 91 unique upregulated and 126 downregulated genes in association with clinically acquired platinum resistance (Supplementary Fig. S2 and Supplementary Table S1A and B). We hypothesized that silencing of genes overexpressed in resistant cells might lead to resensitization by re-engaging apoptotic response to cisplatin treatment and thus may directly reveal novel therapeutic targets for clinical reversal of resistance. Hence, the overexpressed genes formed the focus of our onward strategy. Thirteen upregulated candidate genes were selected on the basis of either magnitude of overexpression or cellular function and were taken forward for functional assessment (Supplementary Table S2). We combined an siRNA-based approach, using the platinum-resistant PEO4 cell line, to a caspase 3/7 activation-based apoptosis assay to evaluate the effect of each gene on the cellular response to platinum. To quantify the difference in platinum-induced apoptosis between control and test genes, the ratio of caspase3/7 induction in control transfected cells on cisplatin treatment was compared with that of target gene siRNA-transfected cells. Six of the 13 genes showed a 1.5-fold or greater increase in the ratio of caspase induction in the target gene siRNA compared with the control gene siRNA (platinum resensitization ratio; Supplementary Table S2) and these were taken forward for further analysis. Assays were repeated a further 3 times for these 6 genes and the average values plotted (Fig. 1B). The platinum resensitization ratios were calculated as averages subjected to Student’s t test for statistical significance (Table 1). Knockdown of control and test genes was confirmed by Western blotting (Fig. 1C). Four genes, FOLR2, PIK3R1, HDAC4, and STAT1, emerged as significantly resensitizing platinum-resistant ovarian cancer cells. We focused here on 2 of these genes, HDAC4 and STAT1, as recent
Table 1. Knockdown and resensitization assays were carried out a total of 4 times by using cisplatin-resistant PEO4 cells for genes selected following first round analysis (see Supplementary Table S2 for results of first round analysis)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Platinum resensitization ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAK</td>
<td>1.38</td>
<td>0.07</td>
</tr>
<tr>
<td>FOLR2</td>
<td>1.40</td>
<td>0.004</td>
</tr>
<tr>
<td>HDAC4</td>
<td>1.55</td>
<td>0.02</td>
</tr>
<tr>
<td>MYC</td>
<td>1.40</td>
<td>0.25</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>1.97</td>
<td>0.04</td>
</tr>
<tr>
<td>STAT1</td>
<td>1.73</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

NOTE: Platinum resensitization ratio is defined. Quadruplicate data were analyzed by Student’s t test for significant difference in cisplatin-induced caspase 3/7 induction between test gene siRNA-transfected cells and siRNA controls.

reports indicate that the transcription factor STAT1 can be regulated by acetyl modifications (12, 13) suggesting the possibility that STAT1 may transcriptionally control the switch from sensitivity to resistance in a manner that may be modulated clinically using HDACi, which are well developed and indeed approved for use in cutaneous T-cell lymphoma (14).

HDAC4 knockdown resensitizes a panel of clinically derived platinum-resistant ovarian cancer cell lines

We used quantitative real time PCR (qRT-PCR) and Western blotting to validate the upregulation of HDAC4 seen in the gene expression analysis (Fig. 2A). This indicated an increase in expression between sensitive and resistant cells in each pair tested. We showed that the effect seen in the 24-hour caspase3/7 activation assay is maintained at 72 hours by SRB assay following HDAC4 siRNA knockdown (Fig. 2B). We then considered the effect of siRNA knockdown of HDAC4 in the clinically derived platinum-resistant cell lines PEA2, PEO23, and SKOV3. Figure 2C and Supplementary Figure S4 show significantly increased apoptotic response to cisplatin following siRNA knockdown of HDAC4, compared with control siRNAs. Conversely, silencing of HDAC4 expression in sensitive cells (PEO1, PEA1, PEO14) did not significantly enhance platinum sensitivity, in keeping with their already relatively low HDAC4 expression levels (Supplementary Fig. S3). However, overexpression of HDAC4 in platinum-sensitive PEO1 cells resulted in decreased apoptotic response to cisplatin ($P = 0.032$) indicating that the platinum-resistant phenotype can be, at least partially, recapitulated by overexpression of HDAC4 alone (Fig. 2D). Deconvolution of the pool of 4 HDAC4 siRNAs confirmed target specific knockdown (Supplementary Fig. S4B).

Demonstration of a novel role for HDAC4 in acquired platinum resistance led us to consider possible targets for HDAC4-mediated deacetylation. Posttranslational modification of the transcription factor STAT1 by acetylation at lysine residues 410 and 413 has been reported previously in melanoma cells (12) and shown to promote dephosphorylation at tyrosine residue 701 (13) thereby acting as a control mechanism for this protein in the IFN response. Because STAT1 was identified as upregulated in resistant cells in our analysis and knockdown significantly resensitized cells to platinum (see above), we considered that STAT1 might be subject to acetyl regulation in acquired resistance and hence explored whether a functional interaction occurs between HDAC4 and STAT1.

STAT1 is activated and translocated to the nucleus in response to cisplatin treatment in acquired platinum-resistant but not -sensitive ovarian cancer cells

We examined the broader role of STAT1 in platinum resistance prior to more detailed mechanistic studies. STAT1 exists as 2 distinct isoforms, full-length STAT1α and STAT1β, a truncated form considered to act as a dominant-negative inhibitor of STAT1α. qRT-PCR in platinum-sensitive and -resistant lines showed that both STAT1α and STAT1β were upregulated to the same extent in the resistant lines (Fig. 3A), suggesting that differences between isoforms did not explain the alteration in platinum sensitivity.

Next, we carried out RNA interference (RNAi) knockdown of STAT1 in additional platinum-resistant ovarian cancer cell lines: PEA2, PEO23, and SKOV3. Figure 3B and Supplementary Figure S5A depict the enhanced apoptotic response following STAT1 siRNA knockdown. Deconvolution of the pool of 4 siRNAs to STAT1 validated target-specific phenotype (Supplementary data Fig. S5B).

We explored whether STAT1 is activated in response to cisplatin and thus examined the subcellular localization of STAT1. Immunofluorescence microscopy of platinum-sensitive and matched, resistant cells showed that in the absence of platinum, STAT1 is predominantly unphosphorylated and cytoplasmic. However, nuclear STAT1, phosphorylated at Y701, is observed following cisplatin treatment in resistant cells (PEO4/PEA2) but not matched, sensitive cells (PEO1/PEA1) where STAT1 remains largely unphosphorylated and cytoplasmic (Fig. 3C and Supplementary Fig. S6A). This is in keeping with the canonical pathway of STAT1 activation by phosphorylation, dimerization and nuclear translocation and suggests a platinum-induced, STAT1-mediated transcriptional response occurring specifically in acquired platinum-resistant cells. To corroborate these findings, we also looked at pSTAT1 Y701 levels in the sensitive and resistant cells by Western blotting. Figure 3D shows striking discordance between the phospho-STAT Y701 levels in the paired sensitive/resistant cell lines PEO1 and PEO4, with no detectable Y701 phosphorylation in sensitive cells. In contrast, phospho-STAT1 Y701 is detectable at baseline and is induced following platinum treatment in PEO4 cells. Expression of the STAT1 regulated gene IRF1 was measured in platinum-resistant PEO4 cells and showed cisplatin-mediated induction of IRF1 transcription which was abolished by STAT1 knockdown.
confirming functional activity of STAT1 in response to cisplatin exposure in resistant cells (Supplementary Fig. S6B).

We hypothesized that if HDAC4 and STAT1 had independent roles in the platinum-resistant phenotype, we would expect additivity or synergy in platinum resensitization if both were inhibited simultaneously. Therefore, we carried out dual knockdown of HDAC4 and STAT1 in resistant PEO4 cells. Data indicated no additional effect in apoptotic response to cisplatin over that seen following knockdown of either gene alone (Fig. 4A), implying redundancy between these 2 genes and supporting the notion that they may act in the same pathway in this phenotype.

Having shown a clear role for STAT1 in platinum resistance, we considered the hypothesis outlined above, that HDAC4 may be acting as a STAT1 deacetylase, altering the impact of cisplatin on STAT1 signaling.

**HDAC4 interacts with STAT1, modulating its acetylation, phosphorylation, and nuclear translocation, thereby abrogating sensitivity to cisplatin**

To assess STAT1 acetylation and the potential role of HDAC4 in STAT1 deacetylation, we carried out coimmunoprecipitation and showed an interaction between HDAC4 and STAT1 in
resistant cells (PEO4, PEA2, and SKOV3: Fig. 4B). We assayed for the presence of acetyl-STAT1 (ac-STAT1) by immunoprecipitation with anti-acetyl lysine antibody and Western blotting for STAT1. Ac-STAT1 was present in the platinum-sensitive cell lines PEO1 and PEA1 (expressing low levels of HDAC4) but was undetectable in their HDAC4 overexpressing matched platinum-resistant counterparts PEO4 and PEA2 and was similarly undetectable in platinum-resistant SKOV-3 cells. However, following HDAC4 knockdown, ac-STAT1 was detectable in all resistant lines (Fig. 4C) indicating that HDAC4 is required for the deacetylation of STAT1 and providing a novel mechanistic link between these 2 modulators of platinum resistance. Next, we considered the consequence of HDAC4-mediated deacetylation of STAT1 on its protein function and on the phenotype of clinically acquired platinum resistance.

We hypothesized that because acetylation of STAT1 has been shown to abrogate IFN-induced phosphorylation at Y701 (13), and that because the knockdown of HDCA4 has been shown here to increase acetylation of STAT1, that HDAC4 may influence the phosphorylation, and hence nuclear accumulation, of STAT1. Figure 4D shows that the cisplatin-induced accumulation of phospho-STAT1 observed in the presence of HDAC4 is abrogated following HDAC4 knockdown in platinum-resistant PEO4, PEA2, and SKOV3 cells.
Immunofluorescence staining of PEO4 cells showed that knockdown of HDAC4 is linked to loss of cisplatin-induced nuclear phospho-STAT1 accumulation (Fig. 5A).

Acquired platinum resistance can be reversed by treatment with pharmacologic inhibitors of HDACs

To consider the potential therapeutic utility of inhibiting HDAC4 or STAT1, we treated platinum-resistant cells with the hydroxamic acid–based HDACi APHA4a (15). This treatment elicited a restoration of platinum sensitivity, as determined by induction of caspase 3/7 activity, in the resistant cell lines PEO4 and SKOV3 when combined with platinum at concentrations that had little or no single agent effect in the same assay (Fig. 5B). Treatment with HDACi resulted in a similar blockade on the phosphorylation of STAT1 shown at the morphologic level by immunofluorescence microscopy. We also show loss of cisplatin-induced STAT1 Tyr701 phosphorylation following cotreatment with cisplatin/HDACi or HDAC4 siRNA and a loss of nuclear phospho-STAT1 localization indicating functional loss of transcription factor activity (Fig. 5A).

HDAC4 is significantly overexpressed following acquired platinum-resistant relapse in clinical samples

To validate the observed increase in HDAC4 expression in platinum-resistant cells, we measured HDAC4 expression by immunohistochemistry in tumor biopsies from 16 clinically
platinum-sensitive ovarian cancer patients matched to second biopsies from the same patients subsequent to development of acquired platinum resistance. In addition, we utilized paired biopsies from 14 patients with platinum refractory disease; those biopsies obtained before and after platinum-based chemotherapy. Sections were stained and scored, blinded by 2 pathologists. Intensity of staining was scored on a 0 to 3 scale and percentage cells stained scored on a 0 to 4 scale. The product score was derived and used for further statistical analysis. This revealed an increase in HDAC4 protein expression in 7 of 16 (44%) acquired platinum-resistant biopsies compared with their matched platinum-sensitive biopsies (Fig. 6A and B; \( P = 0.0413 \); Wilcoxon rank-sum test).

Interestingly, the analysis of 14 paired sections from patients with platinum refractory disease revealed no increase in HDAC4 expression between the paired biopsies taken before and after ineffective first-line platinum treatment indicating that upregulation of HDAC4 is specifically an acquired resistance mechanism and is not involved in refractory disease (Supplementary Fig. S7). Clinical parameters are shown in Supplementary Table S4. This analysis suggests that up to 44% of patients with acquired platinum resistance may have an HDAC4-mediated resistance pathway.

Discussion

Resistance to platinum chemotherapy continues to be a major obstacle in the treatment of ovarian and other cancers. A recent genomic analysis of the cell lines used in this study has shown that resistance to platinum seems not to arise by mutational adaptation of the sensitive tumor cells in the presence of chemotherapy; rather the resistant cells are present in the initial prechemotherapy tumor as a minor population (5). The implication is therefore, that clinical mechanisms of acquired resistance may not be recapitulated in vitro by adaptive responses following exposure of homogeneous sensitive populations to chemotherapy. Mechanisms produced...
in vitro may reflect the artificial environment of the monolayer culture: a suggestion underscored by the lack of correlation between gene expression changes detected in a cisplatin-resistant cell line, PEO1cddp (16), produced in vitro from sensitive PEO1 cells and those seen in the clinically derived resistant PEO4 and PEO6 lines (r² values: PEO1cddp vs. PEO4 = 0.25; PEO1cddp vs. PEO6 = 0.23; PEO4 vs. PEO6 = 0.81; Supplementary Fig. S8). Interestingly, most in vitro derived platinum-resistant cell lines require periodic retreatment with platinum to maintain resistance, indicating that the mechanisms affecting drug response are transient and adaptive rather than being genomically established. Conversely, the clinically derived resistant cell lines used here exhibit stable platinum resistance in vitro, implying that the genomic differences reported between sensitive and resistant cells from the same patient (5) are likely to underpin the resistant phenotype. Such alterations between these cell line pairs are extensive, however, and preclude efficient identification of resistance mechanisms by DNA-level candidate selection. We therefore used transcriptional profiling to indicate changes in gene activity relating to acquired resistance.

Gene expression profiling has been used previously to attempt identification of better predictive biomarkers for clinically acquired platinum resistance and novel targets for therapy and has been applied to clinical material (17–19) and cell lines (20, 21). Jazaeri and colleagues identified expression changes related to chemoresistance and, although underpowered for statistical significance, suggested that distinct mechanisms may be responsible for intrinsic and acquired chemoresistance, as we have also indicated here (17). Spentzos and colleagues developed a 93-gene signature predictive of response to platinum-based chemotherapy (18). Hellerman and colleagues identified a set of 9 genes, predictive of intrinsic platinum resistance. These studies were focused on frozen clinical samples and as such were coherent rather than functional; however, they suggest that gene expression measurements are informative in this context (19). Cheng and colleagues compared global gene expression levels between isogenically matched, platinum-sensitive and -resistant ovarian cancer cell line pairs (21). The analysis identified a number of dysregulated genes; however, no functional validation was reported. Of note, the platinum-resistant cell lines used were not clinically derived but were created by in vitro exposure of sensitive cells to cisplatin (21).

Our analysis identified 4 genes, HDAC4, STAT1, FOLR2, and PIK3RI as overexpressed in clinically resistant cells, each of which also significantly potentiated cisplatin response when knocked down by siRNA. We were prospectively interested in whether our approach would identify unrelated mechanisms or common pathways of acquired resistance. It is of note that by using this discovery-based approach, we identified a novel relationship between HDAC4 and STAT1 whereby they physically interact to create conditions under which STAT1 can be activated following cisplatin treatment in cells with acquired clinical platinum resistance but not in matched sensitive cells from the same patient. As a surrogate of this resistance-specific activation, we observed phosphorylation and nuclear translocation of STAT1 upon platinum treatment in resistant cells only. We showed that overexpression of HDAC4 promotes deacetylation of STAT1 which facilitates STAT1 phosphorylation at Y701 and its subsequent nuclear translocation. In contrast, matched cisplatin-sensitive cells have lower endogenous HDAC4 levels and readily detectable ac-STAT1: consequently, cisplatin treatment is unable to induce phosphorylation of STAT1 (Y701) or STAT1 nuclear relocalization (model summarized in Supplementary Fig. S9).

The role of HDACs in deacetylation of nonhistone proteins is not a new concept. HDAC6, a class IIb HDAC found exclusively in the cytoplasm, deacetylates tubulin, contractin, and Hsp90 affecting cell morphology, adhesion, and migration (22). A sumoylation/acetylation cross-talk has been described for p53 that affects the transcriptional activity of this tumor suppressor (23), whereas activity of the FOXO proteins, downstream targets of AKT, can be modulated by p300/CBP-mediated acetylation and deacetylation by the class II HDAC, SIRT1 (24). Kramer and colleagues reported acetylation of STAT1 at K410 and K413 in melanoma cells and showed increased acetylation following HDAC inhibition or IFN-α treatment (12) although, HDAC4 was not analyzed in their study. Following activation at the cell surface, nuclear translocation of STAT1 occurs which has been reported to follow binding of STAT1 to importin-α5 via critical K410 and K413 (25, 26); the same residues as identified by Kramer and colleagues as the acetyl sites on STAT1 (12). This suggests a potential interference with the nuclear import machinery in platinum-sensitive cell lines shown here to have constitutively acetylated STAT1 and to remain in the cytoplasm following platinum treatment (Figs. 3C and 4C). Subsequent to their report of acetyl control of STAT1 (12), Kramer and colleagues described a cyclical control system whereby phosphorylated, nuclear STAT1 is acetylated by the histone acetyl transferase CBP marking it for subsequent dephosphorylation by the phosphatase TCP45 resulting in decreased DNA binding and target gene transcription (13). Deacetylation by HDAC3 and nuclear–cytoplasmic translocation returns STAT1 to a latent cytoplasmic state in readiness for reactivation by subsequent IFN stimulation (13, 27). Our data suggest a similar but distinct set of effects. In contrast to previous studies, which identified the involvement of the class I HDACs, HDAC1 and HDAC3 in STAT1 deacetylation, we identify the class II deacetylase, HDAC4, as having a key role in the cisplatin-mediated activation of STAT1, specifically in platinum-resistant cells that overexpress this HDAC. Importantly, we see that in both platinum-sensitive and -resistant lines, IFN treatment induces strong phosphorylation of STAT1 Y701 despite the observed differences in basal STAT1 acetylation levels between those platinum-sensitive and -resistant cells (Supplementary Fig. S10). In contrast, we only see STAT1 Y701 phosphorylation in response to cisplatin treatment in resistant lines. Our data indicate that both sensitive and resistant cells activate STAT1 in response to IFN-γ (Supplementary Fig. S10); however, only resistant cells activate STAT1 in response to cisplatin-induced DNA damage (Fig. 3D), suggesting that this effect is not mediated via IFN-γ.

The work presented here shows a novel mechanism involving HDAC4 and places this interaction at the centre of the
response to platinum chemotherapy revealing clinically relevant phenotypic differences resulting from within-patient changes in HDAC4 gene expression. Our data indicate that an HDAC4 threshold level exists that creates a dichotomous STAT1 response that differentiates clinical platinum sensitivity from acquired clinical platinum resistance. Pharmacologic and clinical data (Figs. 5 and 6) suggest that this mechanism may be a frequent event and can be targeted to reverse resistance using HDACi, many of which are either in or near the clinic. It further suggests that HDAC4/class II HDACi may be more appropriate than pan-HDACi in this indication, with potentially lower toxicity/nonspecificity. This has implications for management of platinum-resistant disease, as a relatively modest reduction in HDAC4 can cause resistant cells to behave like their sensitive parental cells with respect to both the control of STAT1 and the cytotoxic effects of cisplatin. HDAC4 has not previously been shown to functionally modulate clinically acquired resistance to platinum-based treatment.

Although STAT1 is most commonly associated with proapoptotic signaling, it is also associated with cell survival in certain contexts. STAT1 is relocalized to the nucleus of breast cancer cells following doxorubicin treatment and is associated with increased apoptotic response (28) and enhances apoptotic response to DNA damage in mouse fibroblasts via down-regulation of mdm2, a negative regulator of p53 (29). Conversely, STAT1 can induce doctaxel resistance in prostate cancer cells (30) and cisplatin resistance in A2780 ovarian cancer cells (20). Roberts and colleagues carried out expression profiling of ovarian cancer cell lines with predefined response to a number of chemotherapeutics (20). Gene expression was correlated with drug IC50 values across the cell lines used and this identified an association between STAT1 and response to cisplatin and AMD473. Overexpression of STAT1 by transfection was shown to increase resistance to both agents; however, inhibition of upstream Janus kinase by treatment with AG490 (31) increased sensitivity to AMD473 but not cisplatin, perhaps suggesting that cisplatin-mediated activation of STAT1 may be independent of Janus-activated kinase (JAK) 2/3. The study included the cell line pair PEO1 and PEO4, however, interestingly the analysis did not take advantage of the matched nature of these lines. Our study shows that the behavior of STAT1 can differ fundamentally, even within a single patient’s tumor before and after the onset of clinical resistance to platinum, thus highlighting the importance of the cellular context in understanding STAT1 responses following stimulus.

In summary, we have shown that molecular profiling of appropriate, clinically derived model systems coupled to functional assays can reveal novel biological mechanisms underlying acquired platinum resistance. The identification of an HDAC4-mediated STAT1 response "switch" represents a novel mechanism of in vivo acquired platinum resistance that is demonstrably amenable to therapeutic modulation and can result in resensitization to platinum-based chemotherapy. The recent report that platinum-resistant clones exist within the platinum-sensitive presenting tumor also raises the possibility of therapeutically targeting these cells in front-line therapy to increase survival and/or delay the onset of resistance. Further work will determine the most appropriate predictive biomarker(s) for identifying patients who might benefit from this approach. Drug development and clinical trials around HDAC4 or STAT1 inhibition will address the therapeutic potential of re-engaging response to platinum-based treatment in resistant cells by disrupting this specific mechanism in ovarian and potentially other platinum-treated cancers. Further work is required to identify the downstream mechanistic functions of STAT1 that confer this resistant phenotype and to further integrate this novel mechanism in the context of other parallel signaling changes in acquired resistance identified here and elsewhere. In addition, it remains unclear how platinum-induced DNA damage leads to STAT1 activation. A comprehensive screen of ligands, receptors, and associated kinases (JAKs and others) in the context of platinum treatment would help to address this question. Establishment of a system-level understanding of acquired platinum resistance will allow us to better predict the behavior of each tumor and identify rational means of targeting it for re-engagement of apoptotic response to platinum chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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