Array-based analysis of the effects of trichostatin A and CG-1521 on cell cycle and cell death in LNCaP prostate cancer cells

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Abstract

Previous studies comparing the effects of two histone deacetylase (HDAC) inhibitors, trichostatin A (TSA) and CG-1521, have shown that these compounds selectively inhibit HDAC and induce differentially acetylated p53 isoforms and assembly of mutually exclusive transcriptional complexes on the *p21* promoter. To determine whether the differential transcriptional regulation seen in *p21* gene is unique or whether it is representative of the genome-wide effects of these two HDAC inhibitors, we have used microarray and Ingenuity pathway analysis to compare the effects of TSA and CG-1521 on gene expression on LNCaP cells. Gene array analysis confirmed by quantitative real-time PCR shows that CG-1521 modulates the expression of a highly circumscribed group of genes involved in cell cycle progression and cell death. In contrast, TSA appears to induce widespread trans-repression of many genes and does not modulate the expression of the same cohort as CG-1521. These data show that the selective effects of CG-1521 and TSA on the assembly of transcription complexes are not unique to the *p21* gene and suggest that selective inhibition of HDAC can lead to significant changes in gene expression through the acetylation of transcription factors including but not limited to p53. [Mol Cancer Ther 2008;7(7):1931–9]

Introduction

The importance of histone acetylation in the epigenetic regulation of gene expression has been recognized for many years (1), and the role of histone acetylases and histone deacetylases (HDAC) in regulating chromatin conformation has been studied extensively (2, 3). It has also become apparent over the last 5 years that many nuclear and cytoplasmic substrates are also acetylated. This includes transcription factors such as p53 (4, 5), the forkhead transcription factors (6), and nuclear steroid receptors (7, 8), structural proteins such as tubulin (9, 10), and signaling molecules such as PTEN (11), leading to the suggestion that acetylation is an important intracellular signaling pathway analogous to phosphorylation (4).

The potential to modulate gene expression through manipulation of the acetylation status of histones has spurred the development of small-molecule inhibitors of histone deacetylation. These molecules can be broadly classified as carboxylic acids, such as sodium butyrate, phenylbutyrate, and valproic acid, and hydroxamic acids, such as suberoylanilide hydroxamic acid, trichostatin A (TSA), and CG-1521, as well as several natural products including trapoxin B and depsipeptide (12). Although many of these molecules are effective inhibitors of histone deacetylation, it is not clear which of the HDAC enzyme activities are inhibited by each of these compounds because detailed information about the protein structure is not available for most of these enzymes.

LNCaP cells express several histone acetylases including P/CAF, p300, CBP, and Tip60 (13, 14) as well as the zinc-dependent class I and II HDAC 1, 2, 3, and 4 (5, 15) and the NAD^+-dependent class III enzymes such as Sirt1 (16). Thus, the biological effects of individual HDAC inhibitors represent the composite effects of the inhibitors on the activity of all the isoenzymes present in the cells. Comparison of the effects of CG-1521 and TSA, two hydroxamic acid–based inhibitors, has shown that, despite the similarity in structure, these compounds have very different effects on LNCaP cells (5). CG-1521 induces G_2-M arrest and apoptosis, whereas TSA induces G_1-S arrest but does not induce apoptosis. These inhibitors also induce different effects on the acetylation status of p53: CG-1521 stabilizes Ac-Lys^{373} p53, whereas TSA stabilizes Ac-Lys^{382} p53. This difference in acetylation status results in markedly different downstream consequences because Ac-Lys^{373} p53 recruits p300, forkhead transcription factors, the androgen receptor, and Brg-1. When assembled on the p53REs in the *p21* promoter, this complex induces the assembly of TBP, RNA polymerase II, and TFIIH on the basal promoter, initiating p21 transcription. In contrast, Ac-Lys^{382} p53 recruits CBP and Brm-1 to the same p53REs in the proximal p21 promoter, but the resulting complex does not recruit TBP, RNA polymerase II, or TFIIH to the basal promoter, suggesting that Ac-Lys^{382} p53 promotes the assembly of a futile, nonfunctional complex on the p21 promoter (8).

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To determine whether this remarkable difference in transcription complex assembly is unique to the *p21* gene or whether it is a more general genome-wide process, we have compared the effects of TSA and CG-1521 on the transcriptome of LNCaP cells. Confirmatory quantitative real-time PCR (qPCR) and pathway analysis shows that the many of the divergent effects of the two inhibitors are mediated through the differential stabilization of acetylated p53 isoforms.

Materials and Methods

Cell Culture

LNCaP human prostate cancer cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (Atlas). Cells were routinely passaged every 3 to 4 days.

Drug Treatments

LNCaP cells were treated with 7.5 μmol/L CG-1521 (Errant Gene Therapeutics) or 5 μmol/L TSA (Sigma) dissolved in DMSO as described previously (5).

Gene Array

Cells were seeded in T-150 cell culture dishes at a density of 5×10^6 per dish and 24 h after plating were treated with 7.5 μmol/L CG-1521, 5 μmol/L TSA, or DMSO (as a time matched negative control) for 24 h. Total RNA was extracted from the treated or untreated cells after 24 h using Qiagen RNeasy Midi Kit (Qiagen). A total of eight independent pairs of treatment and control experiments for CG-1521 or TSA were done. Gene array from the total RNA was processed at Nimblegen Systems. Gene array data were analyzed using the GeneSpring GX 7.2 software (Agilent Technologies). The data sets from these experiments have been deposited in the National Center for Biotechnology Information curated Gene Expression Omnibus accession no. GSE8645.

Standardizing Gene Lists from Statistical Analysis of Gene Array Data after Treatment with CG-1521 or TSA

Only standardized gene expression fold changes of ≥1.8-fold, determined using GeneSpring GX 7.2 software (Agilent Technologies), were considered significant. *Post hoc* one-way ANOVA ($P < 0.05$) and Benjamini and Hochberg False Discovery Rate Multi-Testing Correction were used to eliminate genes showing large variations in response and false positives.

Reverse Transcription-PCR

Total RNA was extracted from LNCaP cells untreated and treated with 7.5 μmol/L CG-1521 or 5 μmol/L TSA for 0 to 72 h using the Qiagen RNeasy Midi Kit (Qiagen). Reverse transcription PCR were done with 1.5 μg RNA samples using Taqman Reverse Transcription Reagents (Applied Biosystems) and a DNA Engine Peltier Thermal Cycler (PTC-200; MJ Research). For each 100 μL reaction, a final concentration of 1× RT buffer, 5.5 mmol/L $MgCl_2$, 500 μmol/L per deoxynucleotide triphosphate, 2.5 μmol/L random hexamers, 0.4 units/μL RNase inhibitor, and 3.125 units/μL MultiScribe Reverse Transcriptase (500 units/μL) were used and final reaction volume was made up to 61.5 μL by addition of 38.5 μL RNase-free water and the appropriate volume of RNA sample. The reaction mixture was incubated for 10 min at 25°C, 1 h at 37°C, and 5 min at 95°C for 1 cycle and kept at 4°C until further analysis. Each sample was replicated three times from three independent sets of experiments.

Quantitative Real-time PCR

Real-time SYBR Green probes for target genes were designed using Primer Express 1.5 (Applied Biosystems) and synthesized by Integrated DNA Technologies. SYBR Green reactions were done using the SYBR Green PCR Master Mix (Applied Biosystems). For each 25 μL reaction, 7.5 μL DNase-free water, 12.5 μL SYBR Green PCR master mix, and 0.5 μL each of forward and reverse primers for each probe and 4 μL cDNA sample prepared as above were used. SYBR Green reactions were done using an ABI Prism 7700 Sequence Detector (Applied Biosystems) and incubated for 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 50 cycles. Relative expression levels of genes in real-time were analyzed using the 2^{-CT} method (17) and presented as ratio to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Each sample was replicated twice from three independent sets of experiments. Results are tabulated as mean ± SE of three independent experiments.

Pathway Analysis

p53 target genes were analyzed using Ingenuity pathway analysis software (Ingenuity). To infer association of p53 target genes after treatment with CG-1521 or TSA, this method uses the gene identities in conjunction with controlled, vocabulary-based data mining of literature associations, protein-protein interaction databases, and a metabolism pathway database. Separate cell cycle and cell death and/or apoptosis pathways were assembled by connecting genes regulated by either CG-1521 or TSA.

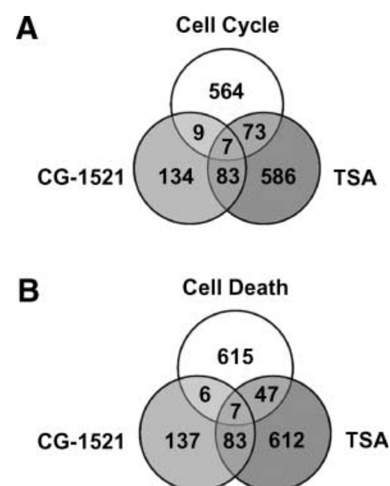

Figure 1. Global changes in gene expression induced in LNCaP cells after CG-1521 or TSA. Venn diagram representation of genes involved in cell cycle regulation (**A**) or cell death and/or apoptosis (**B**). The genes were assigned their functions by the Gene Ontology program in the Genespring 7.2 software from Agilent Technologies.

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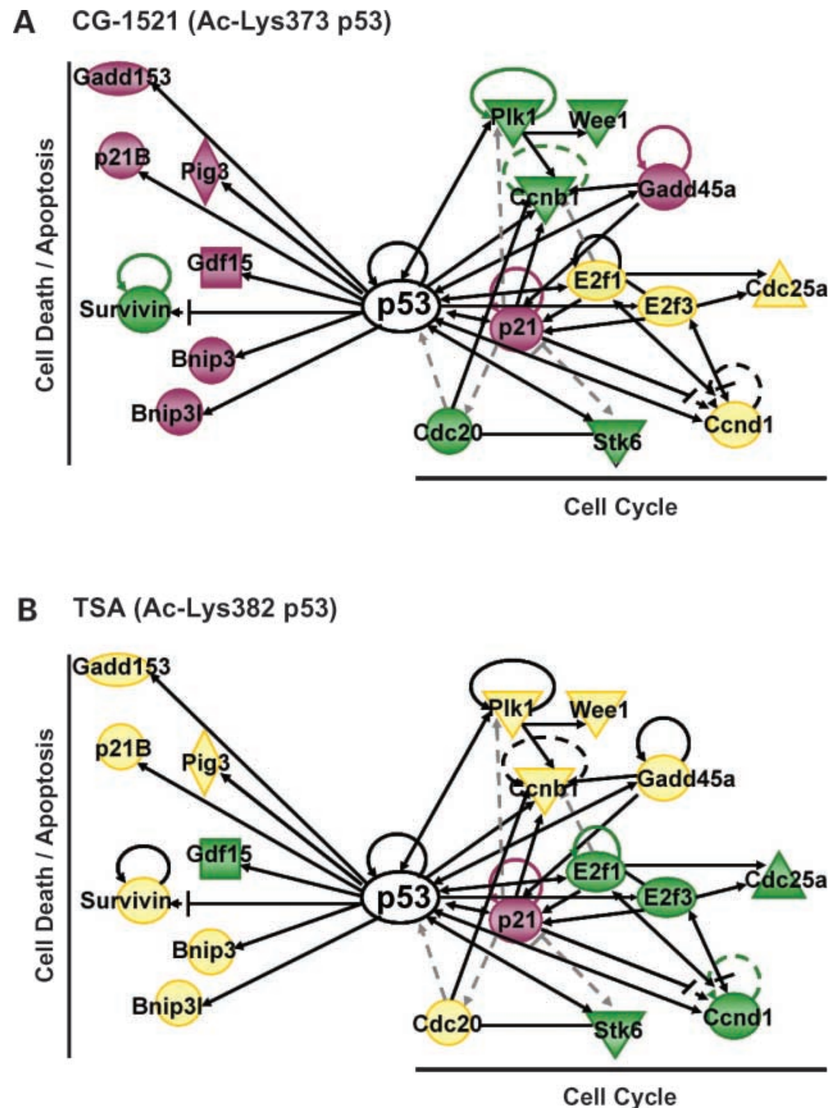

Figure 2. Pathway analysis of p53-regulated cell cycle and cell death/apoptosis genes. Ingenuity pathway analysis predicts that 14 of the genes associated with cell cycle or cell death are regulated by CG-1521 in a p53-dependent manner (**A**). In contrast, 7 genes associated with cell cycle or cell death are modulated by TSA (**B**) compared with cell cycle and cell death pathways. *Yellow fill,* unregulated; *magenta fill,* up-regulated genes; *green fill,* down-regulated genes. The Ingenuity network designates the following node shapes: ▭, cytokine; ◇, enzyme; △, phosphatase; ▽, kinase; ⬭, transcription regulator; ◯, transmembrane receptor; ⬭, other.

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Statistical Analysis

Data are expressed as mean ± SE. One-way ANOVA was used to assess statistical significance between means. Differences between means were considered significant when $P < 0.05$ using the Bonferroni post-test. All statistical analyses were done with the GraphPad Instat software (Intuitive Software for Science).

Results

After treatment with CG-1521, 729 genes pass the initial 1.8-fold change filter, whereas TSA modulates the expression of 7,007 genes by >1.8-fold. Following ANOVA and Benjamini and Hochberg multiple test correction, these numbers are reduced to 233 and 1,749 genes, respectively. Of the 653 genes associated with the regulation of cell cycle (GO:0051726) or its children in *Homo sapiens* by the Gene Ontology Consortium, CG-1521 regulates 16 genes and TSA regulates 80 genes (Fig. 1A). Seven genes are modulated by both treatments; however, their expression is antithetically regulated by CG-1521 and TSA.

CG-1521 regulates 13, whereas TSA regulates 54 of the 675 genes associated with cell death (GO:0008219) or apoptosis (GO:0006915) by the Gene Ontology consortium (Fig. 1B). The 7 genes that are affected by both drugs are also regulated antithetically.

Biological network analysis using the proprietary Ingenuity pathway analysis software shows that several of the genes modulated by CG-1521 and TSA are regulated through p53-dependent pathways. Filtering the gene array data to focus on the genes associated with cell cycle and cell death reveals a group of 18 genes whose transcription appears to be mediated by different acetylated isoforms of p53 (Fig. 2A and B).

To validate the gene array and pathway analysis, which was done at a single time point (24 h), we have further characterized the changes in expression of these genes associated with the regulation of the cell cycle and cell death over 96 h in LNCaP cells using qPCR.

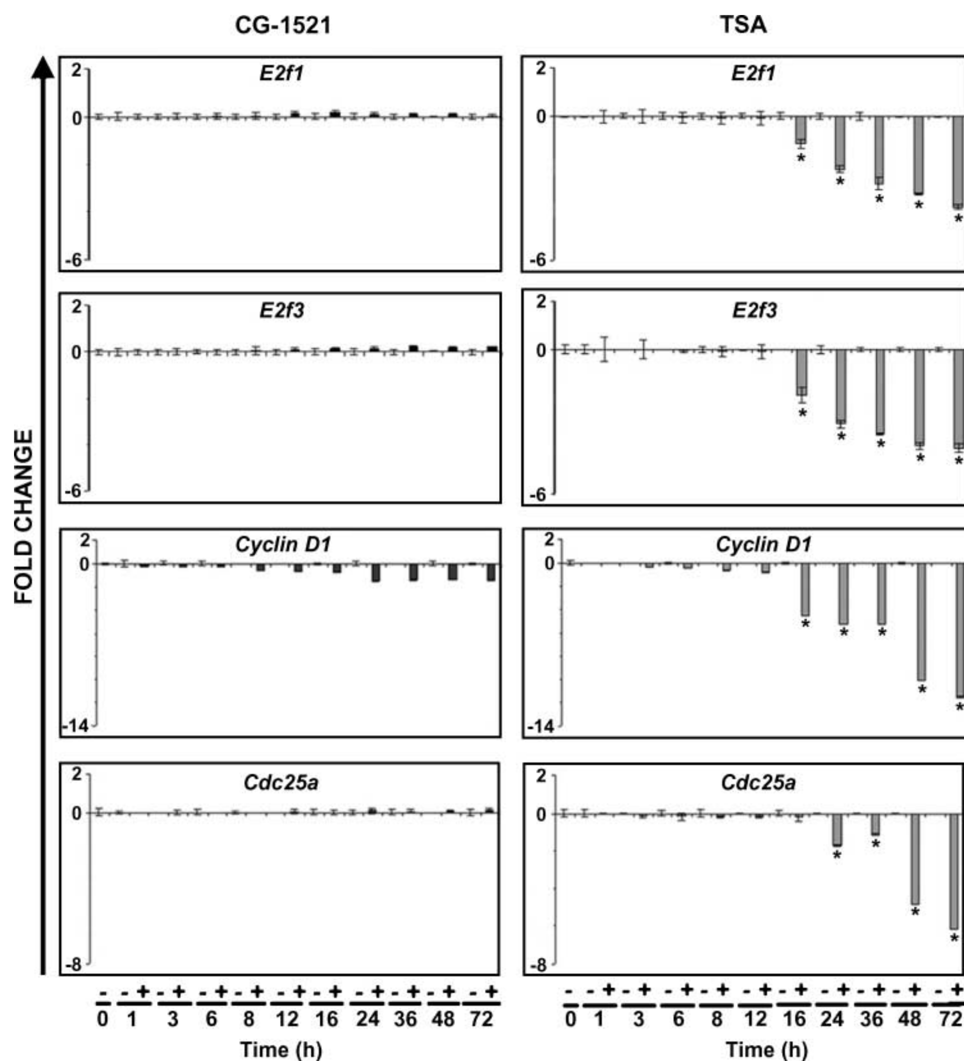

Figure 3. Changes in steady-state mRNA level of p53 target genes associated in G_1-S transition after treatment with CG-1521 or TSA. qPCR analysis of expression of *E2f1, E2f3, cyclin D1*, and *Cdc25a* in LNCaP cells treated with CG-1521 and TSA from 0 to 72 h of treatment. Mean ± SE of three independent experiments. Means were considered statistically significant at $P < 0.05$.

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The effects of CG-1521 and TSA on the steady-state levels of four mRNAs that have been implicated in the G_1-S transition in the cell cycle (GO:0000082) were characterized by qPCR (Fig. 3). TSA induces a significant decrease in the steady-state levels of *E2f1, E2f3*, and *cyclin D1*, starting at 16 h of treatment and reaching a nadir after 72 h of treatment. Cdc25a mRNA levels are also down-regulated, starting 24 h after treatment with TSA. In contrast, CG-1521 has no effect on the expression of the steady-state levels of these mRNAs.

Nine genes were chosen for further analysis based on their known association with G_2-M transition (GO:0000086; *p21, Gadd45a, cyclin B1*, and *Wee1*) or the association of their gene products with spindle organization and biogenesis (GO:0007051; *Stk6, Cdc20, Cks2*, and *Kntc2*) or the centrosome (GO:0005813; *Plk1*). CG-1521 significantly up-regulates the expression of *p21* as early as 6 h after treatment, gradually reaching its maximum expression after 72 h (20-fold higher than untreated cells; Fig. 4). The steady-state levels of *Gadd45a* and *Wee1* are also significantly increased after treatment with CG-1521 for 16 h. The steady-state levels of all three genes remain significantly elevated up to 72 h. In contrast, CG-1521 significantly down-regulates the steady-state levels of *cyclin B1, Cks2*, and *Cdc20* between 6 and 12 h after treatment. TSA has no effect on the steady-state mRNA levels of any of these genes.

Plk1, Stk6, and *Kntc2*, the genes associated with spindle organization and biogenesis, are down-regulated by CG-1521 as early as 3 h after treatment and are continually repressed throughout the 72-h time course (Fig. 5). With the exception of *Stk6* mRNA, which is down-regulated after 16 h of treatment, TSA has no effect on the steady-state mRNA levels of any of the genes that are modulated by CG-1521.

Time-course changes in the steady-state mRNA level of seven selected genes associated with cell death (GO:0008219) or apoptosis (GO:0006915) are shown in Fig. 6. CG-1521 up-regulates the expression of several proapoptotic genes including *Gadd153, Bnip3, Bnip3L, Pig3, Gdf15*, and *p21B*, starting as early as 3 h, and sharply down-regulates the expression of the antiapoptotic gene, *survivin*,

starting at 8 to 12 h after treatment. TSA has no significant effect on the expression changes of *Gadd153, Bnip3, Pig3, Gdf15*, or *survivin* but, in marked contrast to CG-1521, down-regulates the expression of *Bnip3L* after 16 h of treatment.

Discussion

Previous studies have shown that the selective stabilization of acetylated p53 at Lys^{373} or Lys^{382} induces different cellular responses. In LNCaP cells, CG-1521 stabilizes $Ac\text{-}Lys^{373}$ p53 and induces both G_2-M cell cycle arrest

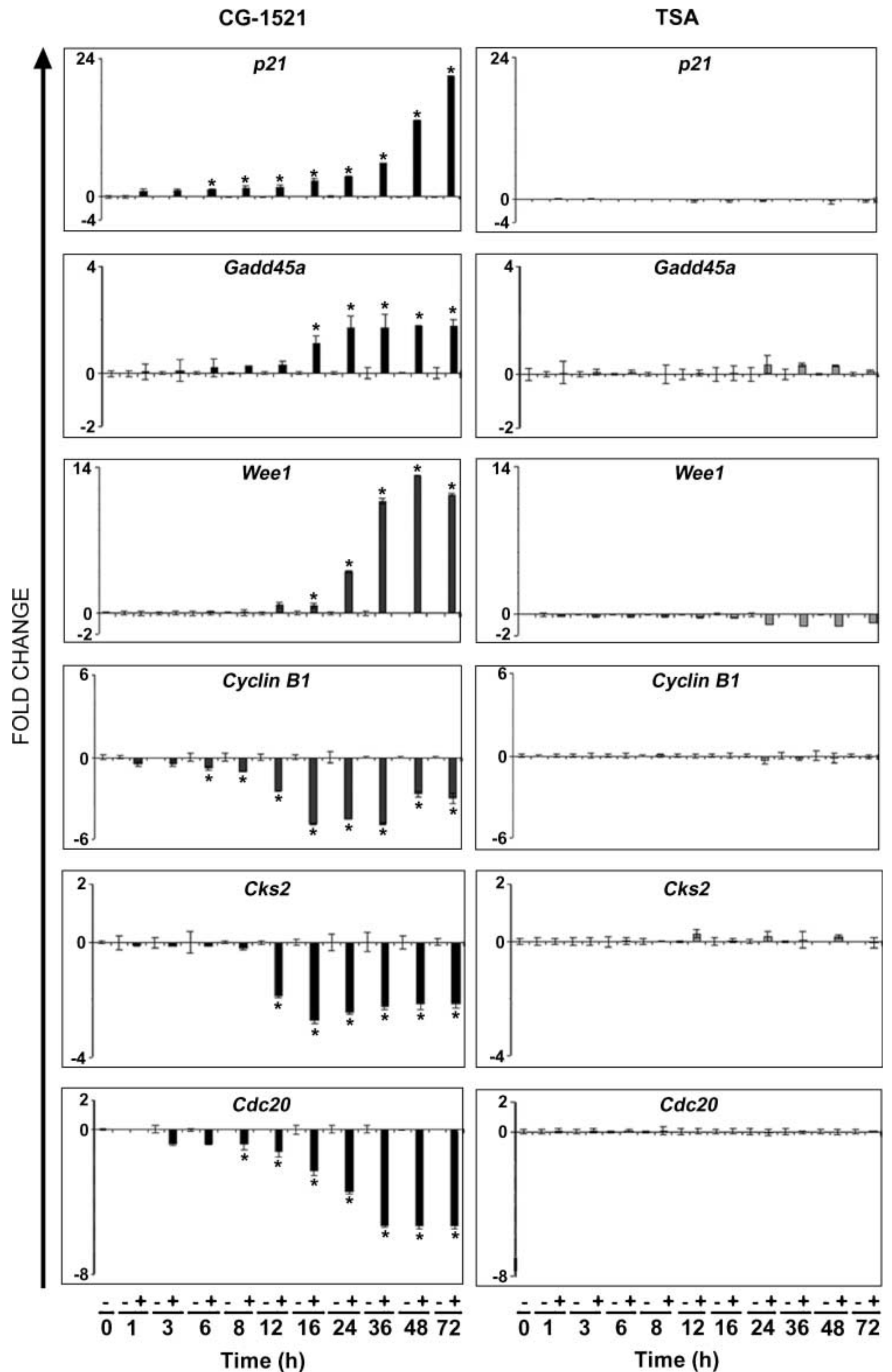

Figure 4. Changes in steady-state mRNA level of p53 target genes associated with G_2-M transition after treatment with CG-1521 or TSA. qPCR analysis of expression of *p21, Gadd45a, Wee1, cyclin B1, Cks2*, and *Cdc20* in LNCaP cells treated with CG-1521 and TSA from 0 to 72 h of treatment. Mean ± SE of three independent experiments. Means were considered statistically significant at $P < 0.05$.

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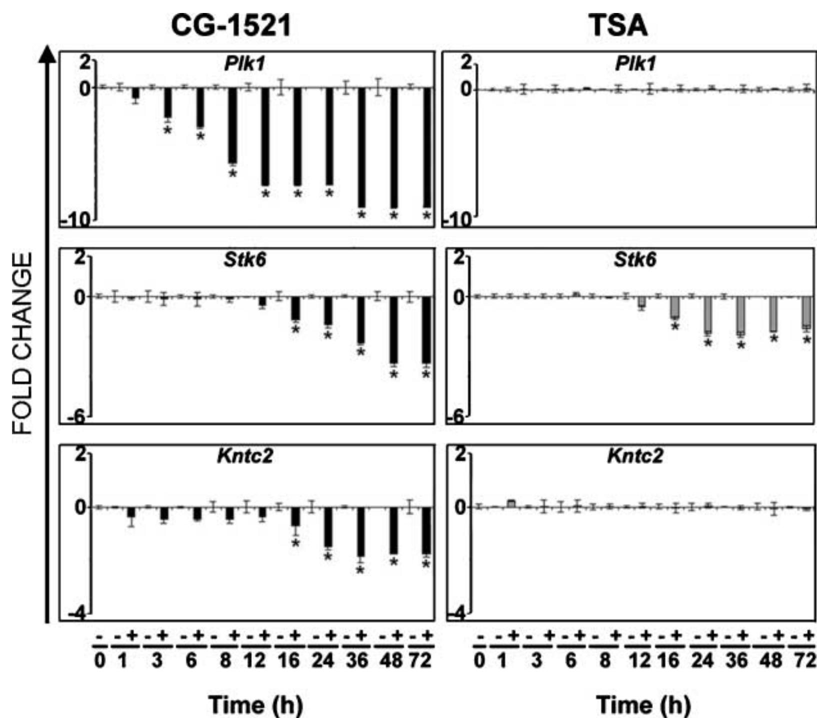

Figure 5. Changes in steady-state mRNA levels of p53 target genes associated with spindle or kinetochore formation. qPCR analysis of the expression of *Plk1, Stk6*, and *Kntc2* from LNCaP cells treated with CG-1521 and TSA from 0 to 72 h of treatment. Mean ± SE of three independent experiments. Means were considered statistically significant at $P < 0.05$.

and apoptosis, whereas TSA stabilizes Ac-Lys^{382} p53 and induces G_1-S cell cycle arrest (5). The different cellular effects seen with Ac-Lys^{373} p53 and Ac-Lys^{382} p53 are reflected in the differential recruitment and assembly of transcription complexes on the *p21* promoter (8). We have used gene array to determine whether the selective modulation of transcription by CG-1521 and TSA is restricted to the p21 gene or is more global in nature. The data presented here show that the expression of genes is differentially regulated by CG-1521 and TSA. CG-1521 modulates the expression of a highly circumscribed group of 233 genes, 29 of which are associated with the regulation of cell cycle and cell death by the Gene Ontology consortium. In contrast, TSA modulates the expression of >1,700 genes, 1,400 of which are down-regulated by >1.8-fold. Of these, 134 genes are associated with the processes of cell cycle and cell death. Ingenuity pathway analysis of the genes associated with cell cycle (GO:0051726) or cell death (GO:0008219) shows that the modulation of at least 18 genes is through the differential acetylation of p53, suggesting that many of the effects of the two HDAC inhibitors are independent of p53. However, many of the most important events associated with cell cycle and cell death appear to be mediated through the differential acetylation of the transcription factor. These include the p21 gene, which we have shown previously is up-regulated by CG-1521 that stabilizes Ac-Lys^{373} p53, whereas TSA (and Ac-Lys^{382} p53) has no effect on the transcription of the p21 gene. The role of p21 in inducing G_1-S-phase arrest through its interactions with cyclin E/Cdk2 is well characterized (18) and p21 induction has also been shown to induce G_2-M arrest (19), suggesting that CG-1521 induces cell cycle arrest in either G_1-S or G_2-M; however, the data presented here show that CG-1521 predominantly targets the expression of genes that have been implicated in G_2-M transition (GO:0000086). In particular, the steady-state level of cyclin B1, a well-characterized p53 target gene (20), is rapidly down-regulated by CG-1521, as is the steady-state level of *Cks2* kinase, which is responsible for the tyrosine phosphorylation of the *cyclin B-cdk1* complex (21, 22). CG-1521 simultaneously up-regulates the steady-state levels of the mRNA encoding the inhibitory *Wee1* kinase (23) and Gadd45a (DDIT-1), which acts as an inhibitor of cdk1 (24). In addition, CG-1521 significantly down-regulates *Cdc20* mRNA, which regulates the activity of the anaphase-promoting complex, and is itself regulated by cyclinB1/cdk1 complex (25). The combined effect of these changes would be predicted to block G_2-M transition (26).

In addition to the negative effects on the cyclin-mediated regulation of the G_2-M cell cycle transition, CG-1521 also down-regulates the expression of several genes, including *Plk1* (Polo like-kinase), *Kntc2*, and *Stk6* (Aurora kinase A) that together are responsible for kinetochore assembly and spindle checkpoint control. *Plk1*, which is down-regulated in response to CG-1521, is associated with the centrosome (27). It is also down-regulated by DNA damage and is critical for proper checkpoint response (28). In addition to its role in the regulation of M phase of cell cycle, *Plk1* inhibits p53 function through a physical interaction (29), providing a feedback mechanism for p53 activation. The *Kntc2* (or *Hec1*) gene, which is also down-regulated by CG-1521, is important for chromosomal segregation (30). Expression of *Stk6* (Aurora A kinase), which is

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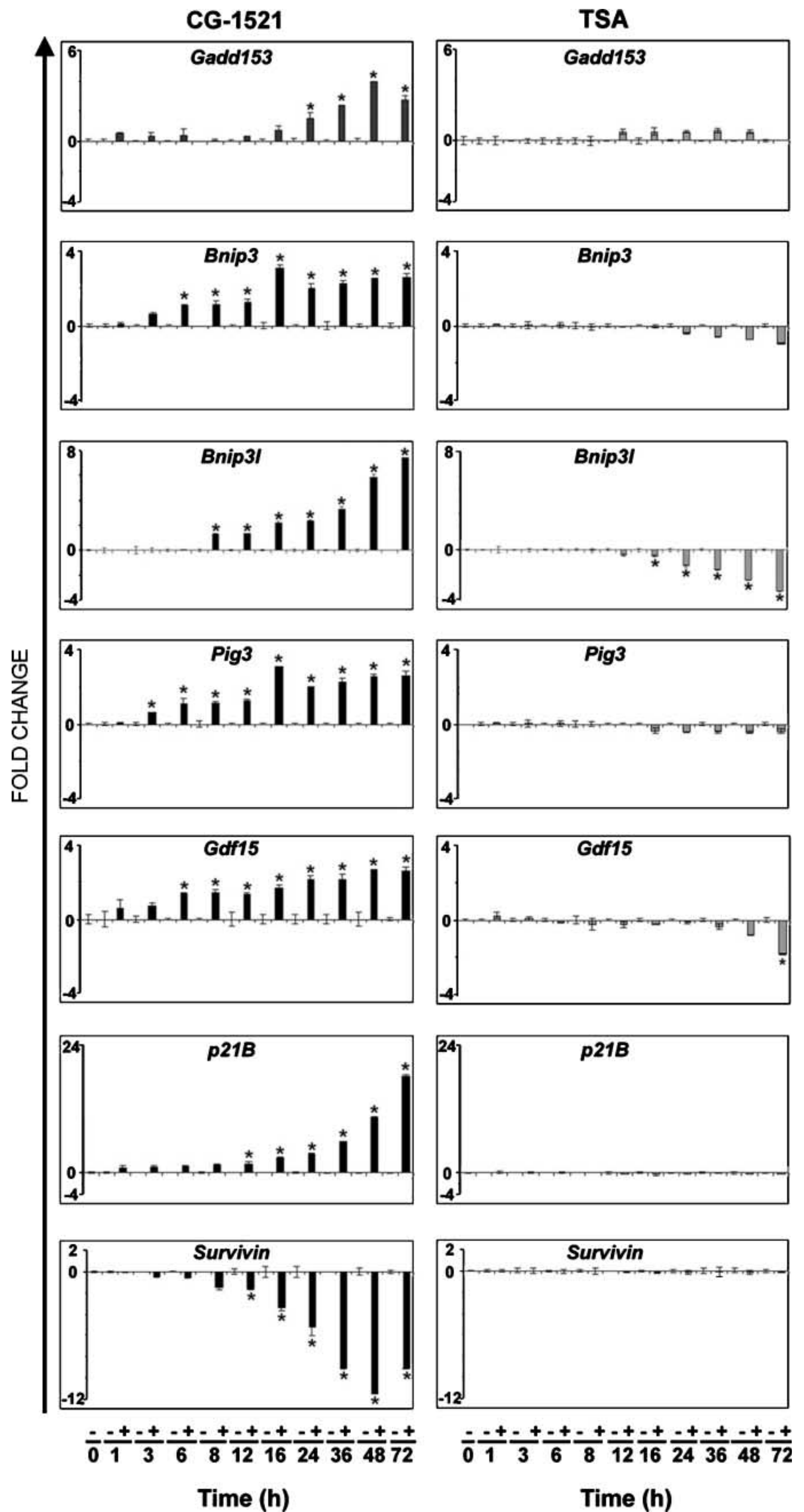

Figure 6. Changes in steady-state mRNA levels of p53 target genes associated with cell death/apoptosis. qPCR analysis of the expression of *Gadd153, Bnip3, Bnip3L, Pig3, Gdf15, p21B*, and *survivin* from LNCaP cells treated with CG-1521 and TSA from 0 to 72 h of treatment. Mean ± SE of three independent experiments. Means were considered statistically significant at $P < 0.05$.

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down-regulated by CG-1521, has been implicated in cell cycle arrest through its effects on Brca-1 (31) and through phosphorylation of p53 at Ser[215], which may provide a feedback mechanism to regulate the transactivating function of p53 (32). The Stk6 gene product localizes to the spindle pole during mitosis and is particularly important for progression from prophase to anaphase. Thus, CG-1521 appears to effectively arrest cells in anaphase through at least two interrelated but complementary mechanisms: G_2-M arrest mediated by modulation of cyclin B–mediated cell cycle progression and inhibition of kinetochore and spindle assembly, blocking the transition of the cell through the spindle checkpoint.

The differential effects of CG-1521 and TSA on the expression of genes associated with cell death also mirrors the biological response of the cells to the two HDAC inhibitors and reflects the difference in the acetylation sites of p53.

It is particularly interesting that CG-1521, which stabilizes Ac-Lys[373] p53 isoform, up-regulates several p53 target genes that are well characterized proapoptotic genes. In particular, CG-1521 up-regulates the transcription of *p21B*, an alternate transcript from the *p21* locus that has been strongly implicated as a direct inducer of apoptosis (33) as well as *Bnip3* and *Bnip3L*, two proapoptotic mitochondrial members of the Bcl2 family that are central to the p53-dependent response to genotoxic stress (34, 35), hypoxia (36), and anthrax (37). In addition, the p53 target gene *Gdf15* (MIC-1 and prostate derived factor), which encodes a member of the transforming growth factor ligand superfamily (38), has been shown to have antiproliferative and proapoptotic effects in LNCaP cells (39). Although the expression of *Pig3*, a putative oxidoreductase (40), has not been documented previously in prostate cancer, it has been shown to be induced in KATO III human gastric cancer cells in response to another HDAC inhibitor, sodium butyrate, which stabilizes the Ac-Lys[373] p53 and/or Ac-Lys[320] p53 isoforms (41). CG-1521 also markedly down-regulates the expression of *survivin*, a p53 target gene that has been implicated in cell survival in the prostate (42) and many other tumor types (43). In contrast, TSA does not modulate the levels of *Bnip3, Pig3*, or *survivin* but down-regulates the expression of *Bnip3L* and *Gdf15*, which would be predicted to reduce the susceptibility of the treated cells to apoptosis.

These data show that the two HDAC inhibitors target different HDACs. Other than our previous observation that CG-1521 has a significantly marked effect on the stability of HDAC2 (8), the selectivity of the HDAC inhibitors for individual HDAC has not been rigorously characterized nor have the substrates for the individual HDAC been identified and catalogued. In the current context, it is likely that TSA induces cell cycle arrest at G_1-S through Ac-Lys[382] p53-mediated down-regulation of *E2f1, E2f3, cyclin D1*, and *Cdc25a*, whereas CG-1521 induces G_2-M arrest through an alternative subset of p53 target genes that respond to Ac-Lys[373] CG-1521. This isoform also appears to be responsible for the up-regulation of proapoptotic gene expression and down-regulation of antiapoptotic gene expression that leads to the initiation of cell death. Because both HDAC inhibitors alter the expression of many more genes than those identified as p53 target genes, it is probable that some of the biological effects of these inhibitors are mediated by p53-independent mechanisms. In fact, the observation that TSA down-regulates the expression of >1,400 genes in LNCaP cells suggests that the effects of this HDAC inhibitor are pleiotropic and represent extensive trans-repression similar to that noted in the p53-mediated response to hypoxia (44). Whether this is due to enhanced acetylation of a small subset of transcription factors, acetylation of cytoplasmic and nuclear structural proteins such as tubulin, or the alteration in global chromatin structure, remains to be determined. Regardless of which mechanism or combination of mechanisms is responsible for the non-p53-mediated effects of the HDAC inhibitors, there are at least nine different HDAC enzymes with varying cofactor requirements (and presumably selective substrates), which can serve as targets for the HDAC inhibitors. This further suggests that the identification of small-molecule inhibitors for specific HDAC holds out the promise of customized therapies based on the complement of HDAC enzymes present in the target cell and the desired outcome (cell cycle arrest and differentiation or cell death). Refinement of HDAC inhibitor-based therapeutics should add a powerful new arm to adjuvant therapies for prostate and other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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