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Chemo-resistance to depsipeptide FK228 is mediated by reversible MDR1 induction in human cancer cell lines

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Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; MDR1, multidrug resistance protein or P-glycoprotein; MRP1, multidrug resistance-associated protein 1; ABC, ATP-binding cassette; ChIP, chromatin immunoprecipitation. FK228, depsipeptide or FR901228, (E)-(1S, 4S, 10S, 21R)-7-[(Z)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,22-pentanone; TSA, trichostatin A: SAHA, suberoylanilide hydroxamic acid.

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ABSTRACT

Histone acetylation status, an epigenetic determinant of gene transcription, is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The potent HDAC inhibitor FK228 is a substrate for MDR1 and MRP1, both of which mediate FK228 resistance. To determine the mechanisms underlying acquired FK228 resistance, we developed four FK228 resistant cell lines from HCT-15, IGROV1, MCF7 and K562 cells by stepwise increases in FK228 exposure. Parent and resistant cells were characterized using a 70-oligomer cDNA microarray, real time RT-PCR, Western blot, and cytotoxicity assays. At both mRNA and protein levels, MDR1, but not MRP1 or other potential resistance genes, was strongly upregulated in all resistant cell lines. HAT or HDAC activities were unaffected in resistant cells, consistent with a lack of cross-resistance to HDAC inhibitors that are not MDR1 substrates. FK228 was found to reversibly induce MDR1 expression by HDAC inhibition and subsequent histone hyperacetylation at the MDR1 promoter, as shown by real time RT-PCR, Western blot, and chromatin immunoprecipitation. This study reveals a significant role of histone acetylation in MDR1 transcription, which appears to mediate FK228 resistance.

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INTRODUCTION

The eukaryotic nucleosome consists of 146 base pairs of DNA wrapped around a histone octamer core, which is arranged as a $(H_3\text{-}H_4)_2$ tetramer and two $H_{2A}\text{-}H_{2B}$ dimers (Davie and Chadee, 1998). Transcriptional competence is governed by histone acetylation status, which is determined by two families of enzymes, histone acetyltransferases (HATs) (Grant and Berger, 1999) and histone deacetylases (HDACs) (Cress and Seto, 2000). HATs promote acetylation of lysines on histone proteins, and thereby destabilize the static bonds between DNA phosphates and histones, resulting in an open DNA conformation for gene transcription. In contrast, HDACs promote histone deacetylation, which results in gene silencing. Depending on cell type, the inhibition of HDACs in cancer cells can lead to transcriptional activation and silencing of ~2% of human genes (Weidle and Grossmann, 2000). HDACs work synergistically with promoter DNA methyltransferases, both of which result in gene silencing (Zhu et al., 2001). The HDAC-mediated silencing of specific tumor suppressor genes appears to play a role in cancer pathophysiology (Cress and Seto, 2000).

FK228 (FR901228, depsipeptide), a potent histone deacetylase (HDAC) inhibitor (Nakajima et al., 1998), is currently in Phase I/II clinical trials against various malignancies (Marshall et al., 2002; Sandor et al., 2002). Following intracellular bioactivation (Furumai et al., 2002; Xiao et al., 2003), FK228 specifically inhibits class I HDAC enzymes (Furumai et al., 2002), and results in the induction (e.g. p21) or suppression (e.g. c-Myc) of various target genes (Sandor et al., 2000b). An *in vitro* screening conducted at the National Cancer Institute (NCI) indicated that

FK228 is a MDR1 (P-glycoprotein, ABCB1) substrate (Scala et al., 1997). Recently, we have shown that FK228 is a substrate of multidrug resistance-associated protein 1 (MRP1 or ABCC1) (Xiao et al., 2005). Cancer cells with either MDR1 or MRP1 over-expression were significantly more resistant to FK228 (Xiao et al., 2005).

MDR1 and MRP1 are well-characterized ATP-binding cassette (ABC) transporters responsible for multi-drug resistance (Tan et al., 2000). While these proteins show an overlapping substrate specificity (Seelig et al., 2000), their different tissue distribution profiles and unsynchronized expressions in cancer cells (Huang et al., 2004) suggest that their expression is controlled by different mechanisms. Transcription of MDR1 gene has been reported to be controlled primarily by promoter DNA methylation. Hypermethylation at the MDR1 promoter CpG islands leads to MDR1 silencing. On the other hand, hypomethylation is associated with MDR1 transcription activation (Jin and Scotto, 1998; Nakayama et al., 1998; Baker and El-Osta, 2003). Histone acetylation appears to act merely as a secondary control mechanism (Jin and Scotto, 1998; Nakayama et al., 1998; Baker and El-Osta, 2003). It was reported that HDAC inhibition activates MDR1 transcription only when the MDR1 promoter is hypomethylated. However, the effect of DNA methylation and histone acetylation on MRP1 expression has yet to be studied.

The overall clinical response rate to FK228 has been low, although clinical resistance to FK228 has yet to be demonstrated (Marshall et al., 2002; Sandor et al., 2002). We found that HCT-15 colon carcinoma cells readily acquired FK228 resistance, which can be reversed by MDR1 inhibition (Xiao et al., 2005). This raises several questions: 1) whether FK228 can induce

resistance to itself in cancer through MDR1 and MRP1 upregulation; 2) whether the resistance is due to the sub-population selection of cancer cells with pre-existing high MDR1 and MRP1 expression, as reported for both paclitaxel (Schondorf et al., 2003) and doxorubicin (Abolhoda et al., 1999), or due to rapid MDR1 and MRP1 induction by FK228-mediated HDAC inhibition; 3) and in the case of induction, what is its molecular mechanism?

To address these questions, we developed four FK228-resistant cell lines from human colon carcinoma cell line HCT-15, human breast carcinoma cell line MCF7, human ovarian carcinoma cell line IGROV1 and human chronic myelogenous leukemia cell line K562. All of these cell lines are MDR1(-)/MRP1(-) except HCT-15, which is MDR1(+)/MRP1(-), based on our previous microarray analysis (Huang et al., 2004). The parental and resistant cells were subsequently characterized using a 70-oligomer cDNA microarray, real time RT-PCR, Western blot, cytotoxicity assays and HDAC and HAT activity assays. Additionally, the association between MDR1 promoter histone hyperacetylation and MDR1 induction was established by chromatin immunoprecipitation (ChIP).

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MATERIALS AND METHODS

Cell Culture. HCT-15, MCF7, IGROV1 and K562 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in RPMI 1640 medium containing 25 mM HEPES buffer and L-glutamine, supplemented with 10% fetal bovine serum, 100 U/mL sodium penicillin G and 100 μg/mL streptomycin at 37°C under 5% CO_2 atmosphere. HCT-15 is MDR1(+)/MRP1(-), while the others are MDR1(-)/MRP1(-). The cells were rendered resistance to FK228 by stepwise exposures with increasing FK228 concentrations. The treatment with FK228 started at 100 nM for HCT-15 cells and 1 nM for the other parental cells, and the concentrations were increased by 20 to 100% every time the medium was changed. Within 4 weeks, all cell lines developed FK228 resistance and were designated as HCT-15/FK228, MCF7/FK228, IGROV1/FK228 and K562/FK228 cells, respectively. IGROV1/FK228 cells were routinely maintained in medium containing 750 nM FK228, while the other FK228 resistant cell lines in a medium containing 1000 nM FK228. K562/Dox cell line (a gift from J.P. Marie, INSERM, E9912, University of Paris 6, France) was also cultured as the parental cell line and stimulated with 0.1 μM doxorubicin once a month.

Custom cDNA Microarray. We used a 70-oligomer custom microarray, previously developed in our laboratories (Huang et al., 2004), comprising of 1070 probes targeting 640 transporter genes (including 48 ABC transporters), ion channel genes, and 430 genes belonging to families of growth factors and receptors, cell adhesion molecules and signal transduction (the list of these genes is available upon request). For some genes of special interest (*e.g.* MDR1 and MRP1), two

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different probes were designed and printed onto the arrays. Total RNA was extracted from the parental and daughter cells with TRIzol (Invitrogen, Carlsbad, CA, USA), and further purified using the Rneasy Mini column (Qiagen, Carlsbad, CA, USA). Eighteen μg total RNA was used for cDNA synthesis and labeled with Cy5 (green fluorescence) or Cy3 (red fluorescence) dyes by amino-allyl coupling. The protocol is available at http://derisilab.ucsf.edu/pdfs/amino-allyl-protocol.pdf. A paired and dye-swap design was applied, in which cDNA samples of parental and resistant cell lines were first labeled with Cy3 dye and Cy5 dye, respectively, and then labeled in reversed order in the dye-swap experimental group. The Cy3 and Cy5-labeled samples were then mixed and hybridized to the array slides for 16 h at 65ºC. Slides were washed, dried and scanned in an Affymetrix 428 scanner to detect Cy3 and Cy5 fluorescence.

Microarray data analysis. A background subtraction and calculation of median pixel measurements per spot was carried out using GenePix Software 3.0 (Foster City, California). Spots were filtered out if they had both red and green intensity <500 units after subtraction of the background, or if they were flagged for any visual reason (odd shapes, background noise, etc). Data normalization was carried out using the statistical software package R (www.r-project.org). The plot of M = log2 R/G vs. A = $\log_2\sqrt{R*G}$ shows dependence of the log ratio M on overall spot intensity A, where R is the red fluorescence intensity of Cy5 and G is the green fluorescence intensity of Cy3. To correct intensity- and dye-bias, we used location and scale normalization methods, which are based on robust, locally linear fits, implemented in the SMA R package (Huang et al., 2004). This method is based on the following transformation:

$$R/G \rightarrow \log_2 R/G - c_j(A) = \log_2 R/k_j(A)^* G \rightarrow (1/a_j)^* \log_2 R/k_j(A)^* G \qquad \text{Equation 1}$$

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where $c_j(A)$ is the Lowess fit of the *M vs. A* plot for spots on the j^{th} grid of each slide, and a_j is the scale factor for the j^{th} grid (to obtain equal variances along individual slides).

We calculated the differential MDR1 mRNA levels based on the *R/G* ratios for each primer (4 prints/primer) and for each dye-swap group (2 groups/primer). The values were averaged and a standard deviation was calculated. In the case when 2 primers were used for one gene, the MDR1 mRNA levels were individually calculated for each primer.

Real Time RT-PCR. Total RNA was extracted from sample cells with TRIsol as indicated in the microarray study. The RNA was precipitated by isopropyl alcohol and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified RNA using oligo-dT priming (Thermoscript RT Kit, invitrogen, Carlsbad, CA, USA), as described previously (Anderle et al., 2004), followed by CYBR-green real time PCR (ABI Prism 7700 Sequence Detection System, Foster City, CA). The primers for MDR1 were 5'-CAGCAAAGGAGGCCAACATAC-3', and 5'-TGAGGCTGTCTAACAAGGGCA-3'. The primers for β-actin were 5'-CCTGGCACCCAGCACAAT-3' and 5'-GCCGATCCACACGGAGTACT-3'. The threshold cycle for PCR products was defined as the cycle at which the SYBR-green fluorescent signal was 20 standard deviations above background. Relative quantification of gene expression was performed using the comparative CT method (or DDCT, method available in the user bulletin of ABI Prism 7700 Sequence Detection System) with beta-actin as the control. K562/Dox cell line was used as a MDR1(+) control. Melting dissociation was performed to evaluate the purity of the PCR product.

Western Blot. For Western blot analysis, cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 950 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.15% Igepal CA-630 and 1.5 mM PMSF). Equal amounts of proteins (100 μg) were size fractionated on 6% or 15% SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% nonfat milk, 200 mM NaCl, 50 mM Tris and 0.05% Tween-20) at room temperature for 2 h. The blocked membrane was then incubated with primary antibodies at 4°C overnight. The membrane was washed 3 times with TBS-T buffer (20 mM Tris, 500 mM NaCl and 0.05% Tween-20) for 15 min, and incubated with secondary antibody at room temperature for 1 h. The detection of specific protein binding was performed with the ECL chemiluminescent Western blotting detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were mouse monoclonal JSB-1 anti-human P-glycoprotein antibody (Research Diagnostics Inc.,1:50), mouse monoclonal AC-15 anti-β-actin antibody (Abcam Inc., 1:5000), and peroxidase-conjugated AffiniPure donkey anti-mouse IgG (Research Diagnostics Inc., 1:10,000), rabbit polyclonal anti acetylated histone H3 antibody (Upstate, 1:500), rabbit anti acetylated histone H4 antibody ChIP grade (Upstate, 1:500), and peroxidase-conjugated donkey anti-rabbit IgG (Upstate, 1:2000).

Cytotoxicity Assay. To determine the role of MDR1 in the acquired FK228 resistance, cytotoxicity assays were conducted. Adhesion cells (HCT-15, HCT-15/FK228, MCF7, MCF7/FK228, IGROV1, and IGORV1/FK228) were seeded in 96-well plates at 5000 cells/well

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in 100 μL medium, and allowed for adhesion at 37°C for 24 h. Fifty μL dosing solutions (4×) of either FK228, HDAC inhibitors TSA (trichostatin A) or SAHA (suberoylanilide hydroxamic acid) made by serial dilutions with blank medium were added to the wells. Also added was 50 μL 20 μM CsA solution (4×, containing 0.5% DMSO) or 50 μL blank medium, so that the total volume in each well was 200 μL. The plates were then incubated continuously for 72 h followed by SRB assay, which is used for adhesion cells (Skehan et al., 1990). K562, K562/FK228 and K562/Dox cells were evaluated under the similar procedure at a cell seeding density of 2000 cells/well by XTT method, which is commonly used for suspension cells (Goodwin et al., 1995). All resistant cells were cultured in FK228-free medium for 3 days before the cytotoxicity assays to avoid possible FK228 accumulation in cells. The 3-day culture in FK228-free medium did not lead to a significant decrease in MDR1 expression as will be discussed later.

HAT and HDAC activity measurement. Nuclear content was extracted from 8×10^6 cells selected from the cell lines of interest using a nuclear extract kit (Upstate Biotechnology, Inc., Waltham, MA) according to the manufacturer's protocol. The resistant cells were cultured in FK228-free medium for 3 days before nuclear extraction. The HAT and HDAC activities contained in the nuclear extracts were determined in triplicates using non-radioactive colorimetric and fluorescent kits, respectively (Upstate Biotechnology, Inc., Waltham, MA). The experiments were conducted according to the manufacturer's protocols.

MDR1 Induction Kinetics. MDR1(-)/MRP1(-) IGROV1, MCF7 and K562 cells were treated with 1, 10 and 100 nM FK228 for 4, 8, 24, and 48 h, followed by real time RT-PCR to determine

MDR1 induction as described above. K562 cells were treated with two other non-MDR1-substrate HDAC inhibitors (TSA and SAHA) at 100 and 1000 nM for 8, 24 and 48 h, followed by real time RT-PCR to determine MDR1 induction. Untreated cells were used as controls.

Chromatin immunoprecipitation (ChIP). K562/FK228 cells and K562 cells treated with 10 nM FK228 for 0, 2, 4, 8, and 24 h were collected. ChIP was performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Inc., Waltham, MA) according to the manufacturer's protocol with the antibodies to acetyl-histone H3 and acetyl-histone H4 (Upstate Biotechnology, Inc.). Immunoprecipitated chromatin was analyzed by PCR with primers specific for MDR1 promoter: 5'-ACAGCCGCTTCGCTCTCTTTG-3' and 5'-AGAAGCCCTTCTCCCGTGAAG-3'. The cycle number and the amount of templates were varied to ensure that results were within the linear range of the PCR.

RESULTS

Development of FK228 resistant cell lines. Exposure to stepwise increasing concentrations of FK228 resulted in rapid acquisition of FK228 resistance in all 4 cell lines (≤30 days). The FK228-resistant daughter cell lines, HCT-15/FK228, MCF7/FK228, IGROV1/FK228 and K562/FK228, were cultured in medium containing high concentrations of FK228 as specified previously in the method section. The resistant cells were growing, with no observable change in

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their proliferation rates as compared with their parental cells. No apparent morphological change in the resistant cell lines was observed under a microscope.

MDR1 is upregulated in drug resistant cells. To characterize the resistant cell lines, we used a custom 70-oligomer cDNA microarray to identify mRNAs that were differentially expressed between the parental and daughter cell lines. Figure 1 shows that MDR1 (ABCB1), but not other genes (e.g. MRP1), is primarily upregulated among all four resistant cell lines. This suggests that MDR1 upregulation may play a major role in the acquired FK228 resistance. Since no MRP1 upregulation was found, we focused only on MDR1 in the subsequent studies.

To confirm the microarray results, we performed real time RT-PCR in all resistant-parental cell line pairs. Table 1 summarizes the relative upregulation of MDR1 mRNA in all FK228 resistant cell lines as compared with their parental counterparts. The MDR1 mRNA level in MCF7 cells (the lowest among all cell lines) was arbitrarily defined as unity. As shown, the resistant cells showed a 4 to 18,728-fold increase in the MDR1 mRNA level as compared with their parental counterparts. A positive control using K562/Dox cells was also included, which showed a high MDR1 mRNA level.

The MDR1 upregulation was further confirmed by Western blot (Figure 2). Little MDR1 expression was detected in HCT-15, MCF7, IGROV1 and K562 parental cells, while thick bands were detected for their resistant daughter cell lines. A clear band was detected for the K562/Dox

cells, confirming the specificity of the Western assay. Contrary to the high MDR1 mRNA level in K562/Dox cells (Table 1), Western blot showed a distinct but relatively weak MDR1 band for K562/Dox cells (Figure 2). This discrepancy may be due to the use of periodical doxorubicin stimulation, which caused a fluctuation in MDR1 expression. Virtually no MDR1 was detectable for MDR1(+) HCT-15 cell line, probably due to the low sensitivity of Western blot, which prevents correlation of the FK228 cytotoxicity with MDR1 protein expression level.

Higher MDR1 levels decrease FK228 cytotoxicity. To investigate the effects of MDR1 expression on the cytotoxicity of FK228, we conducted a series of cytotoxicity assays (Table 2). All MDR1(-)/MRP1(-) parental cell lines (i.e., MCF7, IGROV1, and K562) showed low IC50 values of FK228 (range, 1.8–2.7 nM) and were insensitive to MDR1 inhibition by 5 μM CsA. Parental HCT-15 cells showed higher IC50 values of 378 nM, which decreased to 7.4 nM with MDR1 inhibition, consistent with its being a MDR1 (+) cell line. All resistant daughter cell lines showed much higher IC50 values of FK228 than their parental counterparts (range, 865–7139 nM). MDR1 inhibitor CsA at 5 μM reversed FK228 resistance in all resistant daughter cell lines, indicating that MDR1 upregulation is a major mechanism for the acquired FK228 resistance. This is further supported by the linear correlation between the IC50 values and the MDR1 transcription levels (Figure 3). The FK228 IC50 values are proportional to the MDR1 mRNA level on the logarithmic plot with $R^2 > 0.9$ for the eight cell lines under investigation. The logarithmic plot was chosen because of the wide range of both IC50 values and mRNA levels and the clustering of points on the lower end. Only a partial reversal resistance was achieved in K562/FK228 cells. This is probably due to the enormously high MDR1 expression in the resistant cells (Table 2, Figure 2B). Indeed, even a 99% inhibition of MDR1 in these resistant

cells would still leave significantly MDR1 function and higher IC50 values as compared with its parental MDR1(-) counterparts.

HAT and HDAC activities are not changed in the resistant cells. The lack of complete reversal in MCF7/FK228 and K562/FK228 cells may also suggest that the resistance is due to some defect in the transcription machinery in the resistant cells, so that the resistant cells were less responsive to FK228. For this reason, we determined the cytotoxicity of two non-MDR1-substrate HDAC inhibitors TSA and SAHA (Margueron et al., 2003) in these cell lines. No cross-resistance was found in the resistant daughter cell lines in comparison to their parental counterparts (Figure 4), suggesting that the target of HDAC inhibition and histone acetylation/deacetylation machinery remained intact. To further confirm this, we determined HAT and HDAC activities (Figure 5) in these paired cell lines, and the results showed that there was no significant change in HAT and HDAC activities.

Reversible MDR1 induction is associated with histone modification on its promoter. In order to probe into the MDR1 induction mechanism, we studied the reversibility of MDR1 induction by FK228. K562/FK228 cells were cultured either continuously in medium containing 1000 nM FK228 or in FK228-free medium for 1 to 6 weeks. Then the MDR1 expressions were determined by Western blot. Consistent with FK228 being a reversible HDAC inhibitor (Furumai et al., 2002), the MDR1 protein level decreased over time in K562/FK228 cells in the absence of FK228 treatment (Figure 6).

It has been reported that short-time treatment with MDR1-substrate drugs, such as paclitaxel (Schondorf et al., 2003) and doxorubicin (Abolhoda et al., 1999), may result in rapid MDR1 upregulation. FK228, both a MDR1 substrate and HDAC inhibitor, causes a rapid acquisition of FK228 resistance in several cell lines. This raises a question of whether the MDR1 upregulation is due to selection of sub-populations of cancer cells with pre-existing high MDR1 expressions, or due to rapid MDR1 induction by FK228-mediated HDAC inhibition. To answer this question, we monitored the progress of MDR1 induction using real time RT-PCR. FK228 readily increased MDR1 mRNA levels in a concentration and time dependent manner (Figure 7). All three MDR1(-) parental cell lines showed similar trends in MDR1 induction.

To investigate if FK228 treatment is associated with histone hyperacetylation, we first determined global histone acetylation profiles in the paired cell lines (Figure 8). All resistant cell lines showed significant histone H3 and H4 hyperacetylation as compared with the parental counterparts. In order to find out if the MDR1 induction is caused by histone hyperacetylation at the MDR1 promoter region, we conducted a ChIP study using K562 and K562/FK228 cell pair as a model. The K562 and K562/FK228 cell pair was chosen as we are more interested in leukemia as a Phase I clinical trial of FK228 in chronic lymphocytic leukemia and acute myeloid leukemia patients was recently completed at our institute (Byrd et al., 2005). Significantly higher levels of histone H3 and H4 acetylation at the MDR1 promoter region in K562/FK228 cells were found as compared with K562 parental cells (Figure 9A). Additionally, we found that a short exposure of K562 cells to 10 nM FK228 (up to 24 hr) caused increases in histone H3 and H4 acetylation at the MDR1 promoter (Figure 9B). This suggests that FK228 induces MDR1 via

HDAC inhibition, histone hyperacetylation at the MDR1 promoter region, and formation of MDR1 euchromatin ready for transcription.

The fact that FK228 induces MDR1 by its HDAC inhibitory activity was further confirmed by similar induction in K562 parental cells following treatments with HDAC inhibitors TSA and SAHA, which are not MDR1 substrates (Figure 10). Both compounds were able to induce MDR1 mRNA within 8 h at either 100 or 1000 nM.

DISCUSSION

Methylation of cytosine residue in DNA CpG islands at the promoter region (Baylin et al., 1998) and histone deacetylation (Marks et al., 2000) are two important epigenetic mechanisms in transcription control. It was reported the transcriptional regulation of the MDR1 gene by histone deacetylase inhibitor TSA in the human colon carcinoma cell line SW620 (Abolhoda et al., 1999). However, transfection with a single reporter gene (XmaI-NheI MDR1 promoter-luciferase) could not predict the significance of this mechanism in other types of cancers. Subsequently, several groups found that MDR1 expression is controlled by MDR1 promoter methylation (El-Osta and Wolffe, 2001). Recently, El-Osta et al. reported that promoter methylation is the predominant mechanism for MDR1 transcriptional control (El-Osta et al., 2002). Promoter hypermethylation inhibits MDR1 transcription by either directly interrupting the binding of transcription factors to the promoter, or by recruiting HDACs through methyl DNA binding proteins (MBPs) (Baker and El-Osta, 2003). Thus, HDAC inhibition was thought to cause MDR1 expression only when the MDR1 promoter is hypomethylated (El-Osta et al., 2002;

Baker and El-Osta, 2003). Unfortunately, this study used only MDR1(-) CEM-CCRF with hypermethylated MDR1 promoter and its derivative MDR1(+) CEM-A7R with hypomethylated MDR1 promoter. Therefore, it was not clear how important these two mechanisms were in suppressing MDR1 transcription in MDR1(-) cancer cell lines, since either promoter hypermethylation or histone hypoacetylation could suppress MDR1 expression. For these reasons, studies using multiple cancer cell lines may provide more insight into the role of HDACs and histone deacetylation on MDR1 transcription.

By developing several model cell lines resistant to FK228, we demonstrated that the rapid acquisition of resistance was due to induction of MDR1. It appears that, even though promoter demethylation is believed to be an upstream event to histone acetylation for MDR1 transcription, the MDR1 repression in all tested MDR1(-) cancer cells of various origins can be induced by HDAC inhibition. This suggests that the role of histone acetylation in MDR1 transcriptional control has been previously underestimated. Recent progress in MDR1 promoter methylation mapping (David et al., 2004) reveals that MCF7 cells have hypermethylated MDR1 promoter. This is interesting, since according to the literature, HDAC inhibition should not result in MDR1 induction in cell lines with hypermethylated MDR1 promoters. This suggests that histone (de)acetylation around the promoter is not always secondary to DNA methylation in controlling MDR1 transcription. To test this hypothesis, we evaluated whether IGROV1 and K562 cells also possess hypermethylated MDR1 promoters. We treated MCF7, IGROV1 and K562 cells with 2.5 μM of 5-azacytidine, a demethylating agent, for 24 h. The MDR1 mRNA level was determined by real time RT-PCR. Interestingly, MCF7, IGROV1 and K562 cells showed 4.0, 701, and 437-fold increases in MDR1 transcription after the treatment, respectively (data not shown). The

demethylating agent-inducible MDR1 transcription suggests that MDR1 transcription is suppressed by promoter DNA hypermethylation in all three MDR1(-) cell lines. The fact that MDR1 can be induced in MDR1(-) cell lines by either demethylating agents or HDAC inhibitors indicates a dynamic interaction between these two mechanisms. Therefore, the role of histone (de)acetylation in controlling MDR1 transcription may have been underestimated.

Our finding may have several clinical implications. 1) Frequent FK228 treatments could result in MDR1 upregulation and FK228 resistance; 2) FK228 refractory patients should not be treated with drugs being MDR1 substrates, such as paclitaxel or doxorubicin; 3) For future combination therapy, the use of FK228 prior to other anticancer drugs that are MDR1 substrates may not be suitable due to the possible rapid MDR1 induction exerted by FK228. Indeed, some recent combination studies showed sequence-dependent outcomes. The combination of paclitaxel and FK228 produced a synergistic effect against human prostate DU-145 carcinoma cells when used simultaneously or sequentially with paclitaxel dosing first. However, exposure to FK228 followed by paclitaxel showed an antagonistic effect (Naoe et al., 2004); 4) Since several HDAC inhibitors (e.g. TSA and SAHA) induce MDR1 transcription, MDR1 induction should be considered in the development of future HDAC inhibitors.

The decrease in P-glycoprotein expression in K562/FK228 cells in the absence of FK228 is consistent with FK228 being a reversible HDAC inhibitor (Marks et al., 2000). However, this process was unexpectedly slow and took more than 6 weeks to return to the baseline level (Figure 6). A similar result was found in HCT-15/FK228 cells (Xiao et al., 2005). It is well

known that histone acetylation and deacetylation are rapid procedures with half-lives in minutes for histone proteins that participate in epigenetic transcription modulation (Waterborg, 2002). Therefore, the slow decrease is unlikely due to a delay in histone deacetylation.

Stability studies of P-glycoprotein, the product of MDR1 gene, revealed that degradation half-life of P-glycoprotein is only about 14-17 h in cells (Muller et al., 1995). However, the degradation process can be disturbed significantly by multiple factors (Zhang et al., 2004). For example, N-glycosylation, a post-translational modification necessary for P-glycoprotein's function, was reported to contribute to P-glycoprotein stability (Schinkel et al., 1993). Additionally, since P-glycoprotein undergoes endocytosis, recycling and ubiquitination-mediated degradation (Zhang et al., 2004), any change in these processes might potentially alter the P-glycoprotein expression profile in K562/FK228 cells. Since genes that code the proteins involved in the above processes were not included in our microarray, the mechanism for the slow decrease of MDR1 in K562/FK228 cells in the absence of FK228 remains to be clarified.

Since the acquired FK228 resistance is mainly due to an elevated MDR1-mediated efflux, it would be expected that the intracellular levels of FK228 in the resistant cells would be minimal, even though the media contained prohibitively high FK228 concentrations. Paradoxically, all resistant cells showed global hyperacetylation on both histone H3 and H4 (Figure 8), suggesting effective intracellular exposures. This may be explained by that FK228 is a prodrug, which undergoes bioactivation intracellularly (Furumai et al., 2002; Xiao et al., 2005).The major active metabolites have open ring structures, very different from that of FK228, and are thus unlikely to

be MDR1 substrates. This is consistent with the fact that FK228 is a reversible HDAC inhibitor, and its sustained intracellular exposure is required for maintaining high expression of MDR1. Contradictory to the well-accepted association between HDAC inhibition-caused histone hyperacetylation and cytotoxicity (Vigushin and Coombes, 2002), the resistant cells were still living well with global histone hyperacetylation. There may be two possible explanations for this phenomenon. 1) Pathways downstream to histone hyperacetylation that lead to apoptosis may be blocked in the resistant cells; or 2) FK228 induced cancer cell apoptosis is not associated with histone hyperacetylation. The lack of cross-resistance to TSA and SAHA in the FK228-resistant cells (Figure 4) suggests that the downstream pathways were not blocked assuming that all these HDAC inhibitors share the same apoptosis pathway. Therefore, it seems that FK228-induced apoptosis is not triggered by global histone hyperacetylation. Recent studies in HDAC-inhibition-induced apoptosis suggested that other mechanisms, such as abnormal mitosis and G2/M arrest (Sandor et al., 2000a) and anti-angiogenesis (Kwon et al., 2002), may play important roles in FK228-caused cytotoxicity. Therefore, it seems necessary to re-examine the relationship between HDAC inhibition and cancer cell apoptosis.

It is interesting to note that MDR1 was the major gene upregulated from more than 1000 genes according to our microarray study. Assuming that expressions of 2% of genes are governed by histone acetylation/deacetylation status (Weidle and Grossmann, 2000), we would expect about 20 genes differentially expressed on each array, which was clearly not the case (Figure 1). One possible explanation would be that alteration of gene expressions other than MDR1 would not lead to survival advantage in FK228-containing medium. Therefore only cells with upregulation of favorable genes (e.g. MDR1) survive. We did not find any MRP1 upregulation in all four

resistant cell lines. This suggests that MRP1 is not suppressed by histone hypoacetylation in the selected cancer cell lines.

In conclusion, we developed and characterized four FK228-resistant cell lines. The acquired resistance is due to a reversible MDR1 induction caused by FK228-induced histone hyperacetylation at the MDR1 promoter. Since FK228 is a MDR1 substrate, the rate and extent of induction may be important for the clinical prognosis of patients receiving FK228 treatments. In addition, the role of HDACs in silencing MDR1 may have been previously underestimated. Additionally, the validity of the use of histone hyperacetylation as a surrogate marker for HDAC-inhibitor-induced cytotoxicity needs to be re-examined.

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Legends For Figures

Figure1: The mRNA expression levels in FK228 resistant and parental cell lines as determined by a custom 70-oligomer cDNA microarray. MDR1 (ABCB1) is the only gene that is consistently upregulated at the mRNA level in all four resistant cell lines.

Figure 2: MDR1 expression levels in the resistant and parental cell lines as determined by Western blot. All resistant cells have significantly higher MDR1 expressions than their parental counterparts. (A) HCT-15 and HCT-15/FK228. (B) MCF7, MCF7/FK228, IGROV1, IGROV1/FK228, K562, K562/FK228, and MDR1(+) K562/Dox. Beta actin was used as the loading control.

Figure 3: Correlation between the MDR1 mRNA level and the FK228 IC50 values. MDR1 mRNA levels of the 4 parental and 4 resistant cell lines showed linear correlation with their IC50 values of FK228 on the logarithmic plot. K562/Dox cell line was not included due to its time-dependent MDR1 expression.

Figure 4: Cytotoxicity of (A) TSA and (B) SAHA in the parental and the resistant cells ($n = 3$). No consistent cross-resistance to these two non-MDR1-substrate HDAC inhibitors was found in the resistant cell lines. IGROV1/FK228 cells showed cross-resistance to SAHA ($p < 0.005$), but not to TSA.

Figure 5: Determination of HAT and HDAC activities in the parental and resistant cell lines. Neither (A) HAT nor (B) HDAC activity is significantly changed in resistant cell lines as compared with their parental counterparts.

Figure 6: Reversible MDR1 induction by FK228 as determined by Western blot. K562/FK228 cells were either cultured in medium containing 1000 nM FK228 (lane 1) or in the absence of FK228 for 1 to 6 weeks (lane 2-7). The MDR1 expression decreased over time and was barely detectable at the end of 6 weeks. Beta actin was used as the loading control.

Figure 7: MDR1 induction in IGROV1, MCF7 and K562 parental cells as measured by real time RT-PCR after FK228 treatments. Cells were exposed to FK228 at 1, 10 or 100 nM for 4, 8, 24 or 48 h, followed by real time RT-PCR. FK228 readily induced MDR1 in a concentration and time dependent manner. The MDR1 mRNA levels in the parental cell lines were arbitrarily defined as unity.

Figure 8: Global histone acetylation status in the parental and resistant cells as determined by Western blot. Resistant cells showed significant global hyperacetylation of histone H3 and H4 as compared with their parental cell lines.

Figure 9: MDR1 promoter histone hyperacetylation in K562/FK228 cells and in K562 cells treated with 10 nM FK228 as determined by ChIP. (A) K562/FK228 (lane 1) showed significant hyperacetylation of both histone H3 and H4 on the MDR1 promoter as compared with untreated K562 (lane 2) cells. (B) FK228 induced histone H3 and H4 hyperacetylation in K562 cells at the MDR1 promoter in a time dependent manner. The inputs total DNA without immunoprecipitation were used as loading controls.

Figure 10: MDR1 induction in K562 cells as measured at the mRNA level by real time RT-PCR after TSA and SAHA treatments. Both TSA and SAHA induced MDR1 in a concentration and time dependent manner. The MDR1 mRNA level in the parental K562 cells was arbitrarily defined as unity.

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Table 1: MDR1 mRNA levels in the parental and the resistant cell lines as determined by real time RT-PCR. Double delta cycle of threshold (ΔΔCT) method was used to calculate the MDR1 mRNA level relative to the control beta actin. $\Delta\Delta CT=(CT_{MDR1} - CT_{beta\ actin})^{resistant\ cell\ line}$ - $(CT_{MDR1} - CT_{beta\ actin})^{parental\ cell\ line.}$ Folds of MDR1 mRNA upregulation in resistant cells = $2^{-\Delta\Delta CT}$

		Relative MDR1 mRNA levels (n = 3)	Folds of upregulation (resistant/parental)
HCT-15	Parental	2894	–
	FK228/FK228	12292	4
MCF7	Parental	1	–
	FK228/FK228	18728	18728
IGROV1	Parental	11	–
	FK228/FK228	8991	789
K562	Parental	38	–
	FK228/FK228	19982	525
	FK228/Dox	19161	503

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Table 2: Summary of IC50 values of FK228 in the parental and the resistant cell lines in the absence and the presence of 5 μM CsA (n = 3).

Cell lines	Mean IC50 ± SD (nM) Without CsA	Mean IC50 ± SD (nM) With CsA	IC50 decrease%
HCT-15	378 ± 49	7.4 ± 1.4	98.0
HCT-15/FK228	7139 ± 813	11.4 ± 0.4	99.8
MCF7	1.8 ± 0.1	1.6 ± 0.1	11.1
MCF7/FK228	1363 ± 203	50.3 ± 10.6	96.3
IGROV1	2.5 ± 0.1	1.3 ± 0.0	48.0
IGROV1/FK228	865 ± 89	7.5 ± 0.3	99.1
K562	2.7 ± 0.19	1.9 ± 0.1	29.6
K562/FK228	4624 ± 360	1123 ± 79	75.7
K562/Dox	2274 ± 164	117 ± 9.5	94.9

Figure 1

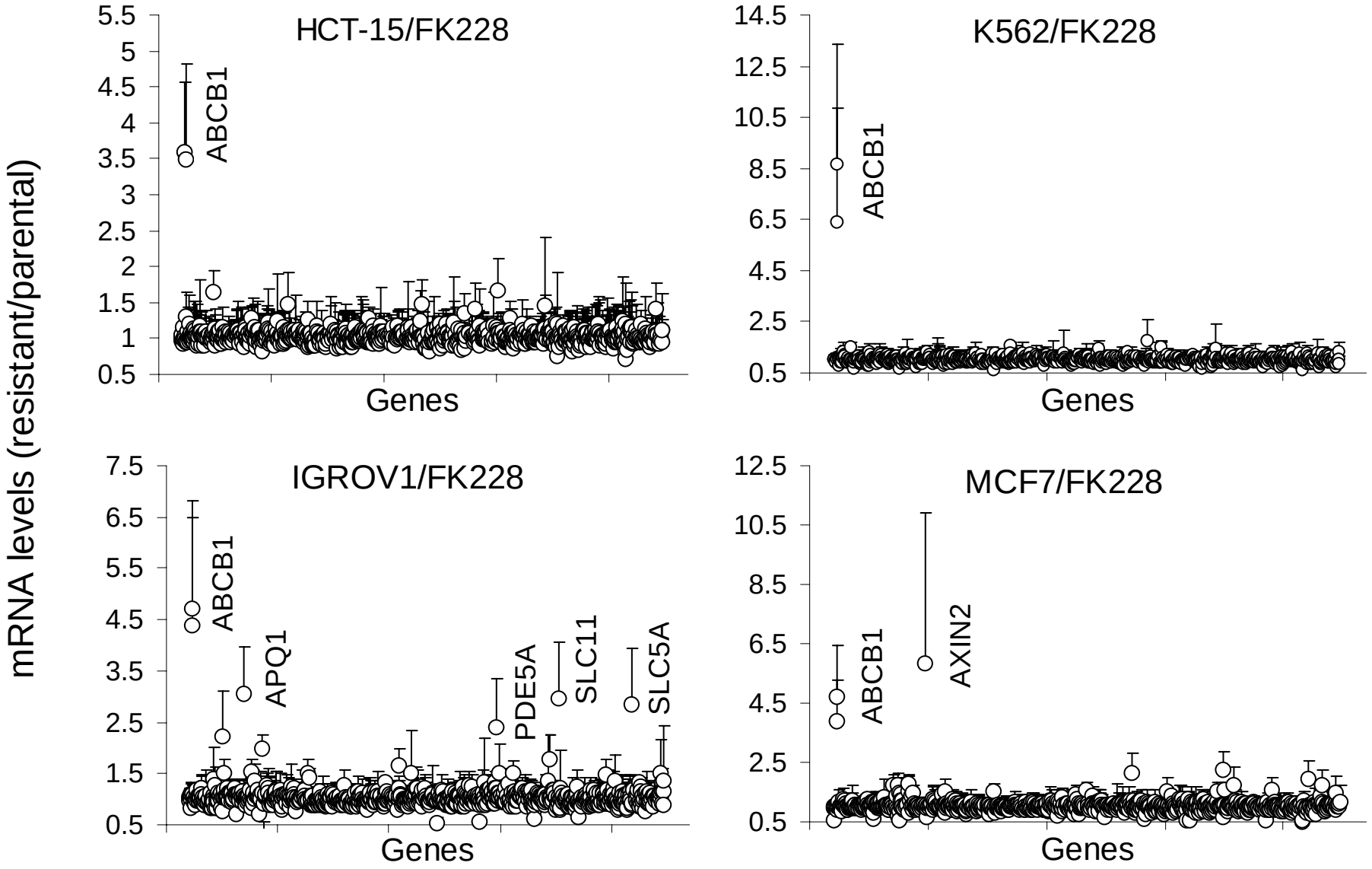
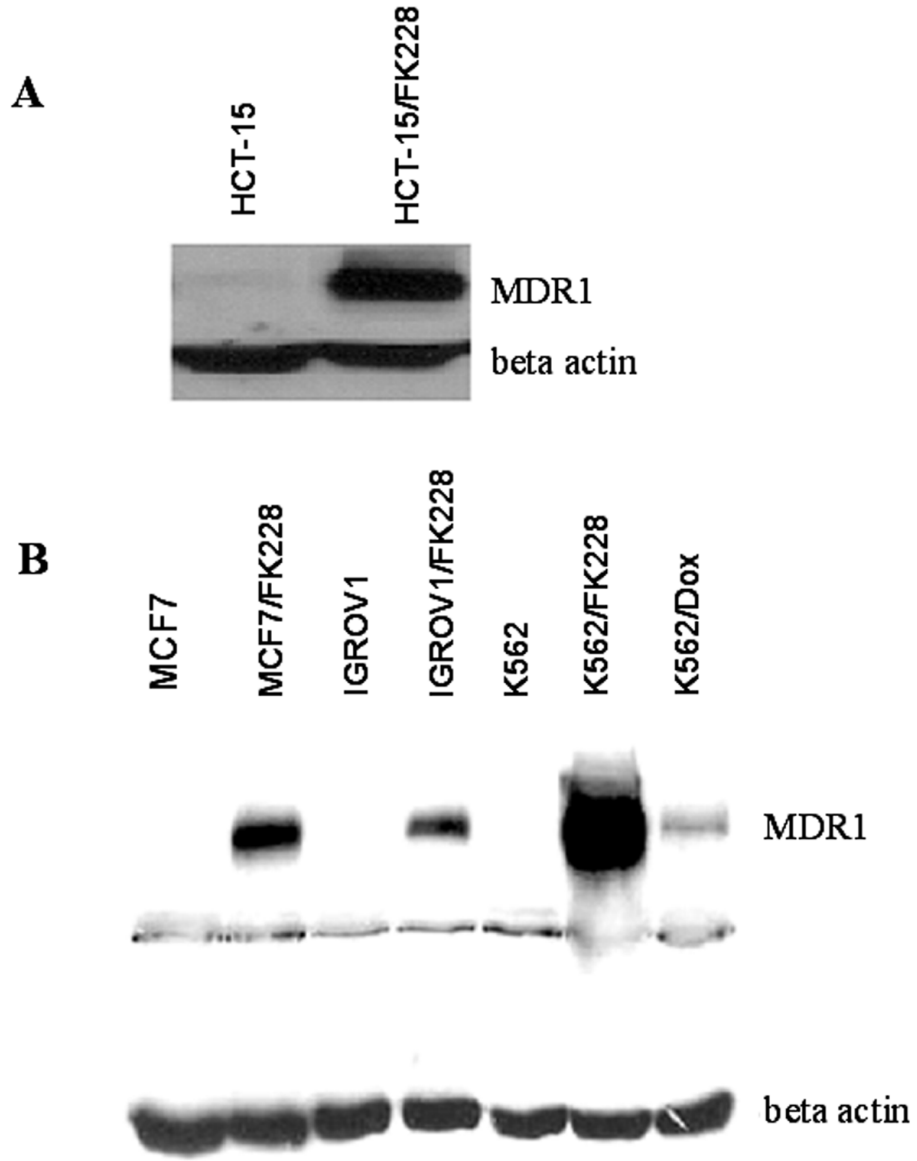


Figure 2



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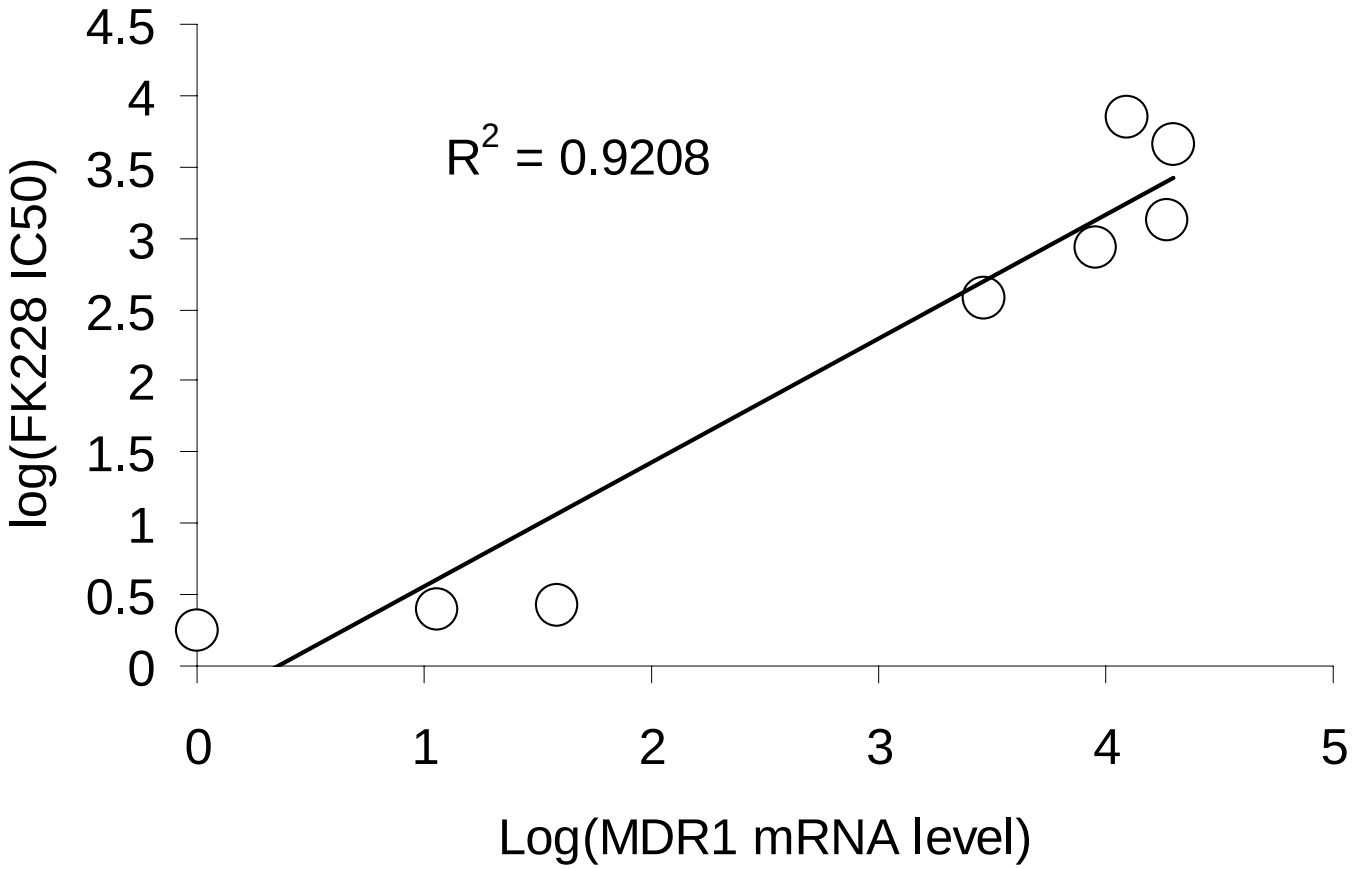

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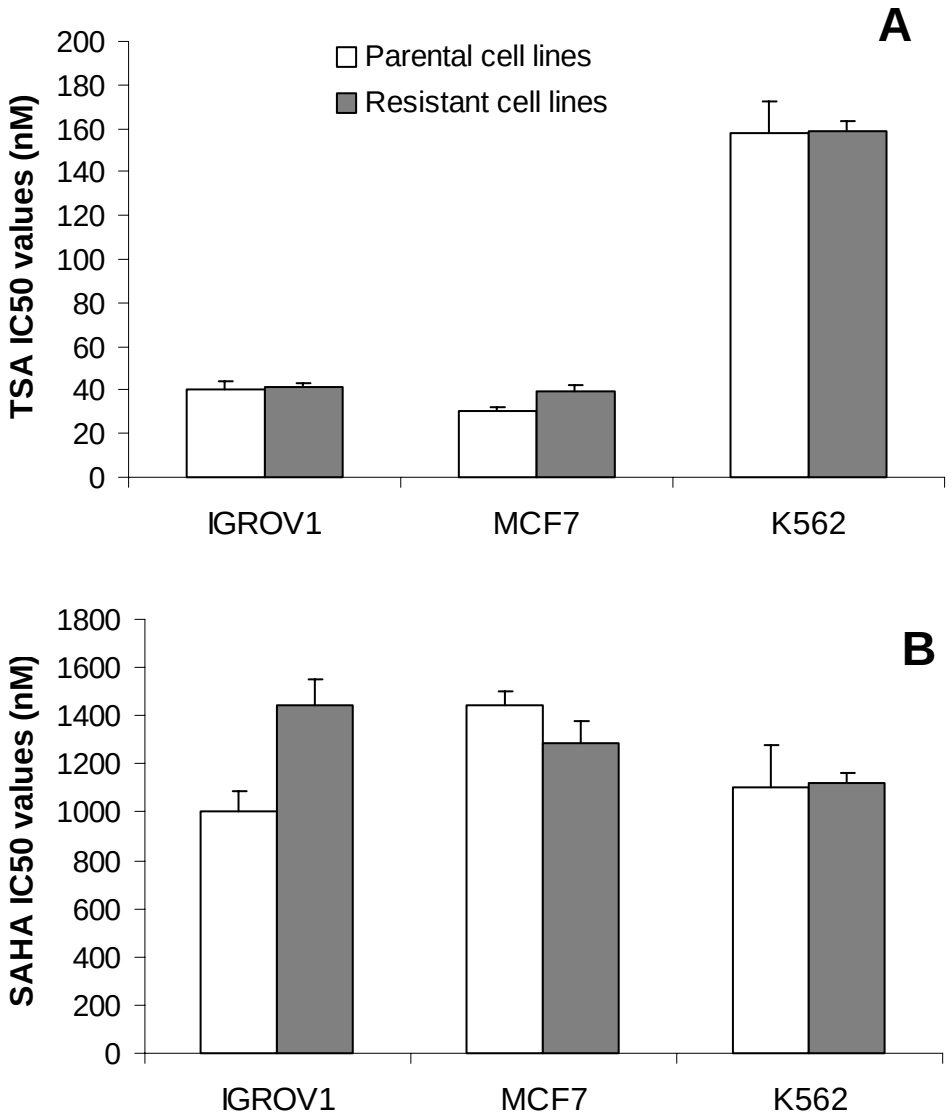

Figure 5

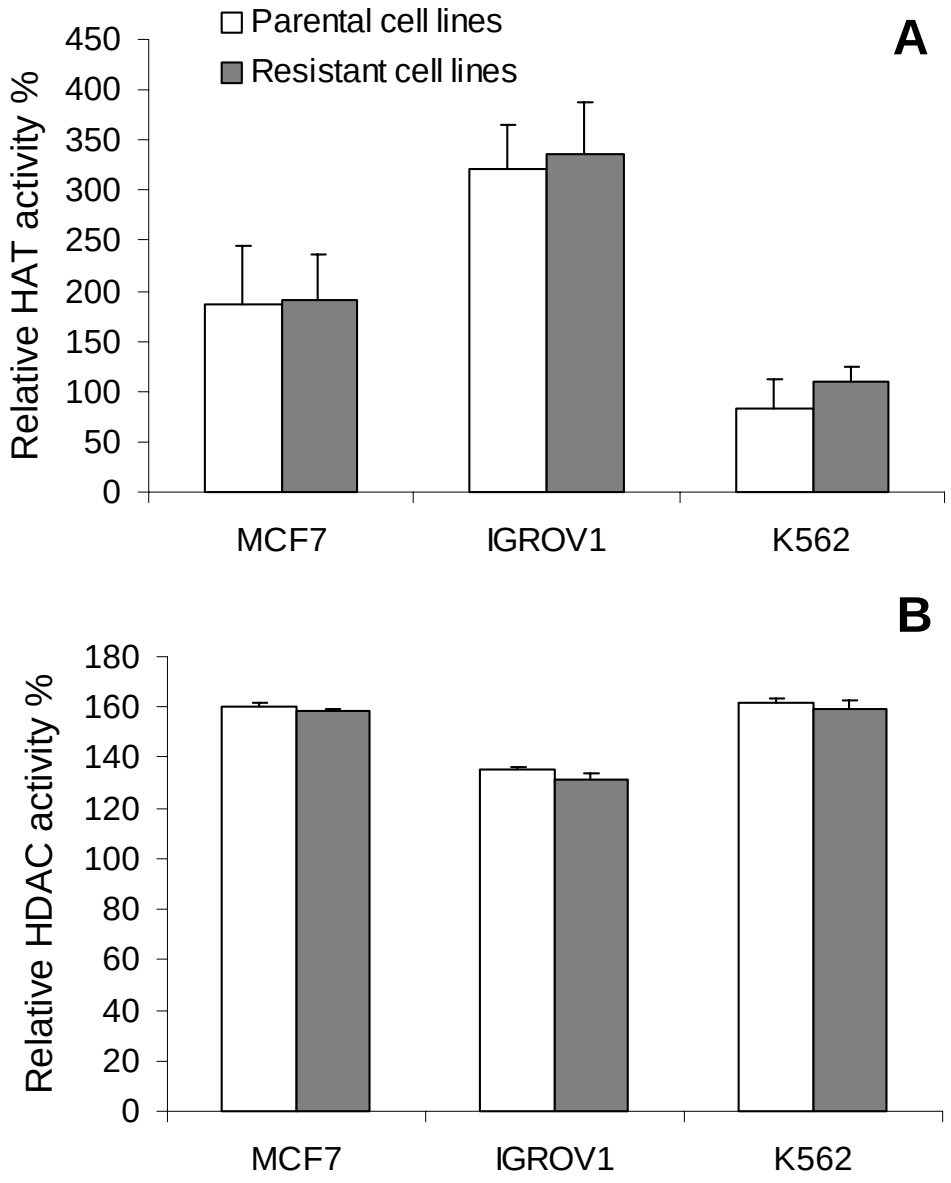

Figure 6

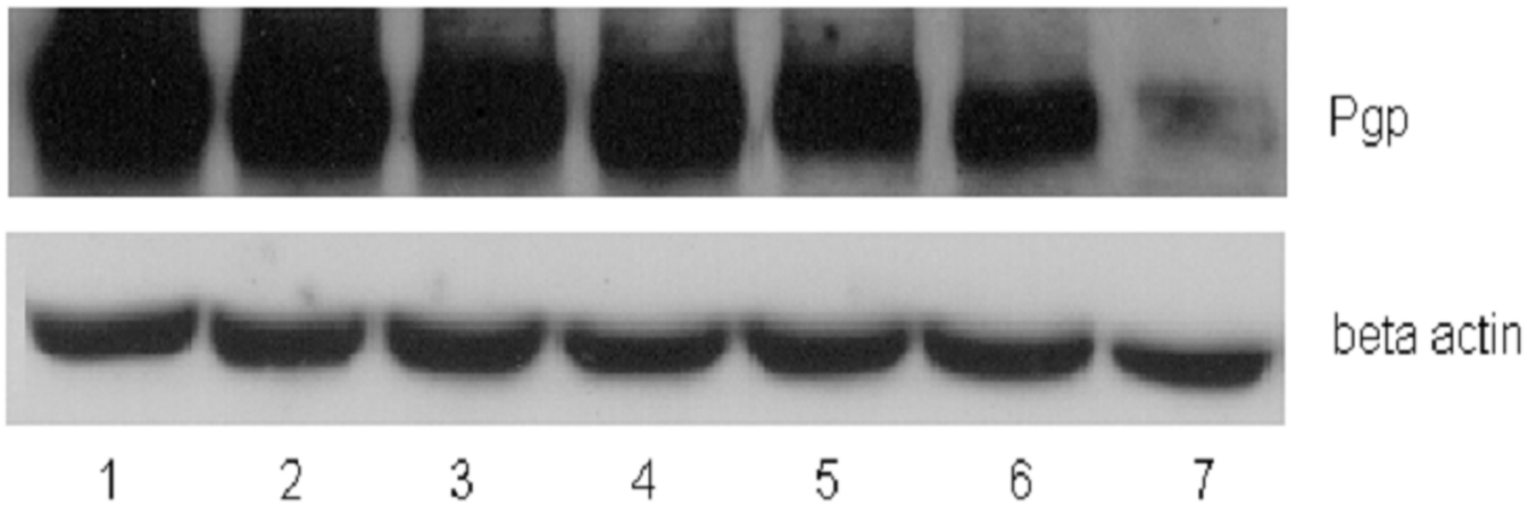

Figure 7

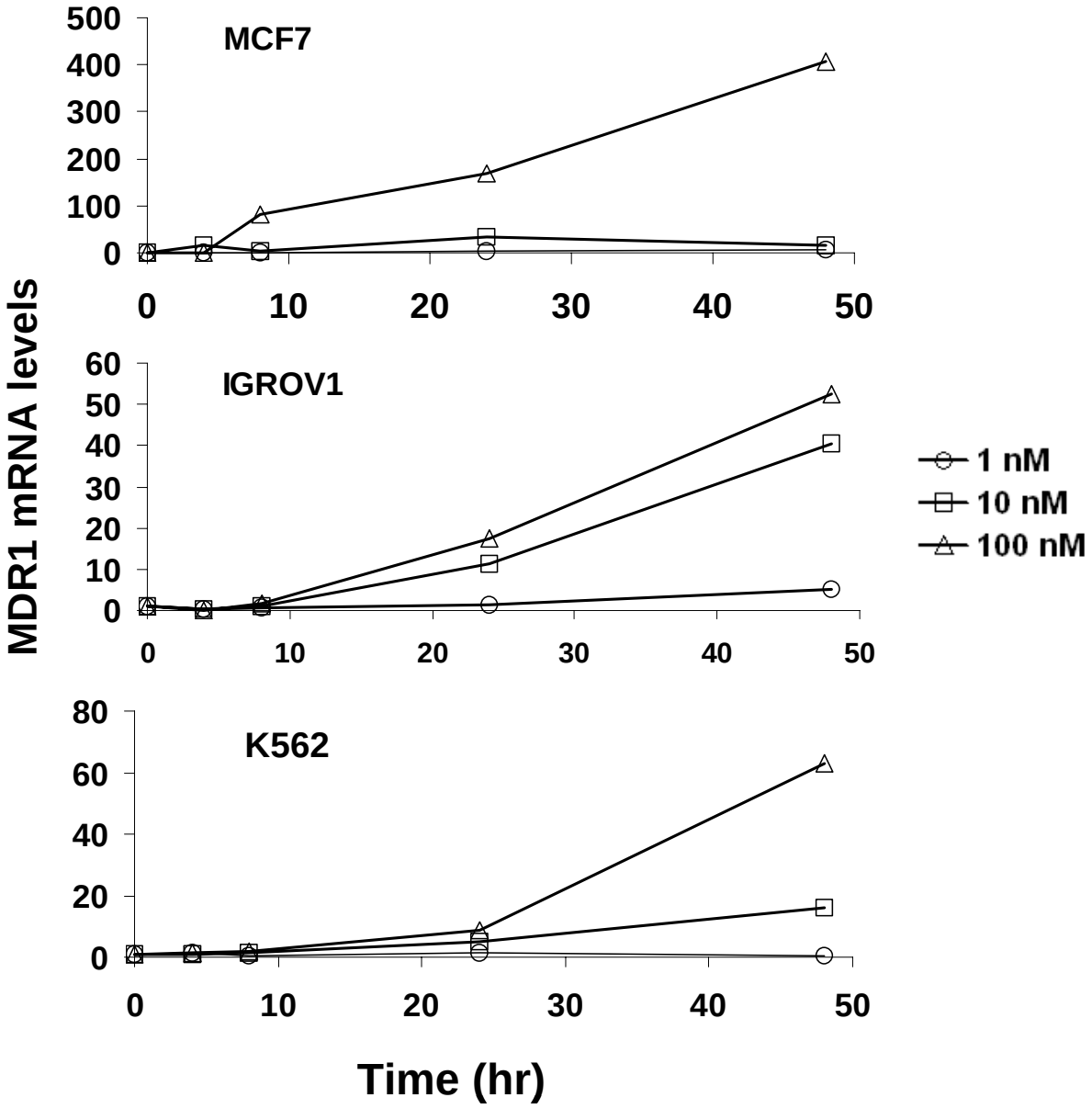

Figure 8

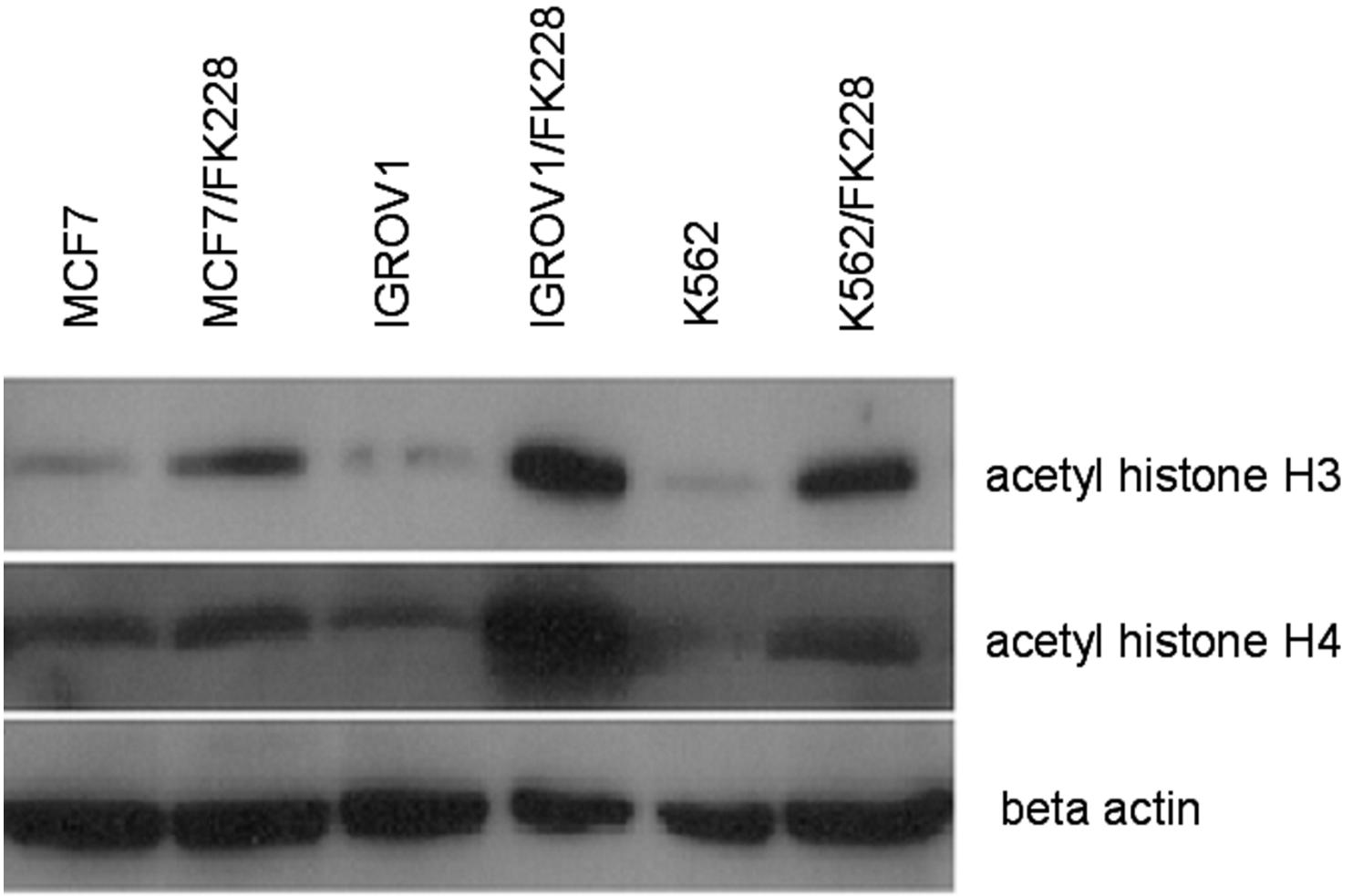

Figure 9

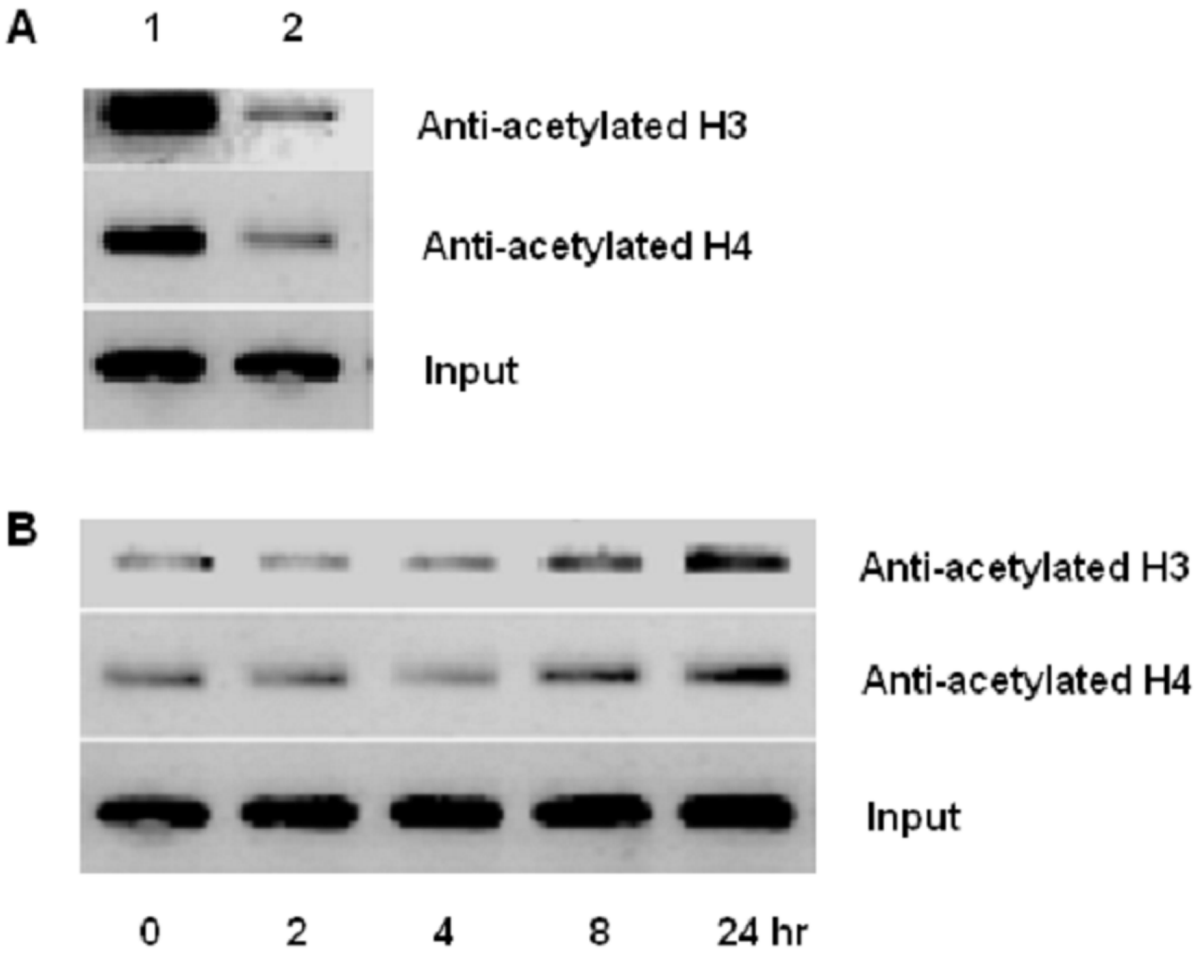

Figure 10