# Online supporting data

## *Materials and methods*

### *Compounds and Cell lines*

Chidamide and MS-275 were synthesized by Chipscreen biosciences. Other chemicals were purchased from Sigma.

All tumor cell lines were from American Type Culture Collection (ATCC, Rockville, MD, USA) and Human primary cells were obtained from the Cell Culture Center of Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured following the vendor's instructions.

### *In Vitro Cell Growth Inhibition Assay*

Cells were seeded into 96-well plates at 5-10×$10^3$ cells/well (according to the growth rate of individual cell lines used). 24 h later, chidamide was added at 7 serial dilutions and continuously incubated for 72 h. Cells viability were evaluation by MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) following the manufacturer's instruction. The concentration that inhibited cell growth by 50% over the control was determined as $GI_{50}$. All samples were evaluated in duplicate, and each experiment was repeated at least three times.

### *In vitro HDACs inhibition assay*

General HDACs inhibition was evaluated with HeLa nuclear extract by using HDAC Colorimetric Activity Assay/Drug Discovery Kit (BIOMOL, NY, USA), just following the provider's instruction. Briefly, test compounds in serial dilutions were respectively mixed with HeLa nuclear extracts, HDAC colorimetric substrate and

other components, the reactions were incubated 37℃ for 1 h. And then Lysine developer agent was added in and incubated at 37℃ for 30 min. The signal was read on an ELISA plate reader (BioTek, VT, USA) at 405 nm. Remaining HDAC activity can be expressed as the relative O.D. value, the 50% inhibition of HDACs activity ($IC_{50}$) for test compounds was calculated with O.D. values against their serial diluted concentrations.

***Histone and α-Tubulin Acetylation Assay***

HeLa cells were seeded into 10-cm culture dishes ($2x10^6$/dish). 24 h later, cells were treated with the tested compounds at different concentrations for different times. The nuclear histones or whole cell lysates were isolated following a previously described method. [1]

For western blot analysis of histone H3, nuclear histones samples were transferred onto Hi-Bond PVDF membranes (Thermo Scientific, Waltham, MA, USA), probed with anti-acetylated H3 antibody (Upstate Biotechnology, NY, USA) and re-probed with anti-total histone H3 antibody (Abcam, MA, USA).

For western blot analysis of α-tubulin, proteins from whole cell lysates were transferred onto Hi-Bond PVDF membranes (Thermo Scientific, Waltham, MA, USA), probed with anti-acetylated α-tubulin antibody (Santa Cruz, CA, USA) and re-probed with anti-α-tubulin antibody (Santa Cruz, CA, USA).

***Molecular docking simulation (MDS)***

The co-crystal structure of HDAC2 and hydroxamate-based SAHA (PDB entry 4LXZ, 1.85Å) was chose for docking template.[2] The ligand SAHA was extracted and

crystallographic water molecules in the structure were deleted. Charges and protonation states were assigned according to the standard SYBYL-X 1.1 (Tripos Inc.) procedure. Chidamide was sketched and added all hydrogen atoms based on the same procedure. By its default parameters, then the molecule was energy-minimized using Tripos force field with the Powell energy minimization algorithm. All those prepared mol2 and pdb format files would be used for the subsequent docking.

Molegro Virtual Docker (MVD 2010.4.0.0) program was employed for docking simulation of flexible Chidamide into the target protein HDAC2 model. The docking space was identified by the binding site for ligand SAHA in HDAC2 crystal structure, and it was defined as a sphere 10Å in radius with center X 25.97, Y 15.79, Z 1.09. Some residues close to the space were minimized by standard MVD procedure. The final docking simulations were performed by the following settings: MolDock SE algorithm, number of runs being 10, Max iterations being 1500 and Max poses being 5. The docking poses which were used for prediction of protein-ligand interactions were ranked by energy-based criteria using the embedded scoring function in MolDockScore. In our model, the highest MolDockScore value representing the lowest energy for simulation was -159.204 kJ $mol^{-1}$, which indicated a strong interaction between Chidamide and HDAC2.

***Microarray Analysis and RT-PCR validation***

Human cDNA library was purchase from the IMAGE consortium of Mammalian Gene Collection (MGC), and cDNA microarray chips were manufactured internally, which represents 8064 sequenced genes and ESTs on the chip.

Human hepatocarcinoma cell HepG2 and human lung cancer cell A549 were treated with different compounds at the dose of corresponding $GI_{25}$ predetermined by MTS assay and incubated for 12 hours (for A549) or 16 hours (for HepG2), DMSO as mock control treatment. Total RNAs were isolated and purified by using RNeasy minikit (Qiagen). 20μg total RNA from control or compound treatments were transcribed and incorporated with Cy3 or Cy5 fluorescence dye in produced cDNA fragments as manufacture suggested (Amersham Pharmacia). Same amount Cy3 and Cy5 labeled probes were mixed for human cDNA microarray hybridization. Slides were scanned and transferred to digital data with ArrayVision 6.0. Signals from two fluorescence channels were firstly screened by detection criteria, which defined by serials of negative control spots on microarray, and then normalized by LOWESS regression. Gene expression changes were determined as signal ratio between samples from compounds treatments against corresponding controls. The expression data for each compound treatment were averaged from three independently repeated experiments.

Interested genes were picked up from microarray data and applied for validation by Semi-quantitative RT-PCR. Briefly, 5 μg total RNA from A549 and HepG2 with different drug treatments were used for reverse transcription and PCR amplification, expression changes were analyzed by agarose electrophoresis of PCR productions from different samples and compared to corresponding microarray data. GAPDH used as internal control to adjust experimental variation. Representative result pictures were showed from three independently repeated experiments.

### *Statistical Analysis*

The significance of differences was analyzed by using a Student's t-test. Results were considered statistically significant when $P < 0.01$ or $P < 0.05$.

a

| | $IC_{50}$ ( μ M) |
|---|---|
| SAHA | 0.60 |
| CHI | 7.16 |
| MS-275 | 8.44 |

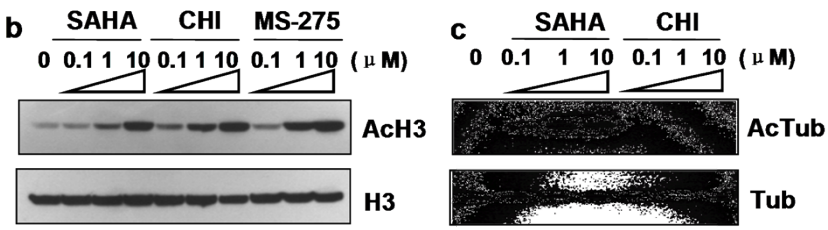


**Figure S1 *in vitro* inhibition of HDACs activity by SAHA, Chidamide (CHI) and MS-275.**

**a**. $IC_{50}$ of individual HDAC inhibitors on total HDAC activity was determined with nuclear extracts from human Hela cells by using the HDAC Colorimetric Activity Assay/Drug Discovery Kit (BIOMOL). **b&c**. Induction of acetylated histone (**b**) or tubulin (**c**) accumulation by different HDAC inhibitors. HeLa cells were incubated with SAHA, chidamide (CHI), or MS-275 at the concentrations indicated for 24 h. Nuclear histones or whole-cell lysates were extracted and applied for immunoblot against histone H3 and acetylated H3 (AcH3) (**b**) or α-tubulin and acetylated α-tubulin (AcTub) (**c**). Fig. 1b was extracted from original graphs that appeared in ref. 3.

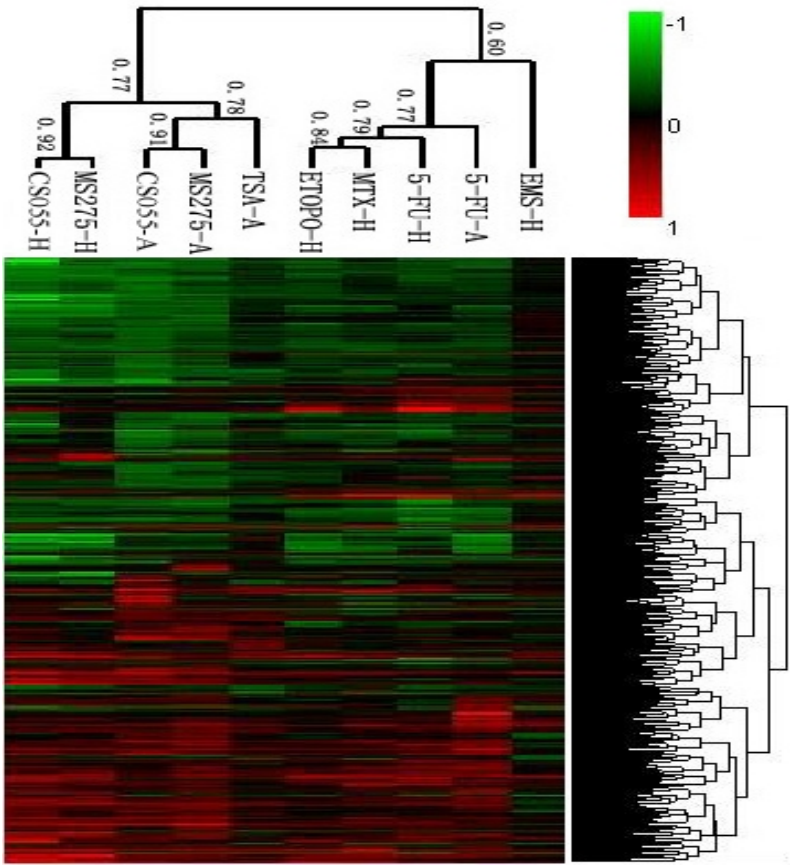


**Figure S2 Hierachical clustering of gene expression profile induced by selected compound treatments in HepG2 (H) and A549 (A) cells.**

Human hepatocarcinoma cell HepG2 (H) and human lung cancer cell A549 (A) were treated with Methotrexate (MTX, 50 μM), Etoposide (ETOPO, 20 μM), Ethyl mathanesulfonate (EMS, 5mM), Fluorouracil (5-FU, 30 μM on HepG2 and 60 μM on A549), chidamide (CS055, 2 μM on HepG2 and 4 μM on A549), MS-275 (2 μM on HepG2 and 5 μM on A549) and TSA (100 nM on A549) for 12 (A549) or 16 (HepG2) hours at the concentrations of their $IC_{25}$ predeterminded by MTS assay. Total RNA samples were isolated and applied for gene expression profiling with human cDNA microarray, gene expression changes were determined as normalized signal ratio between samples from compounds treatments against controls. The expression data for each compound treatment were averaged from three independently repeated experiments. A total of 664 genes with expression changes over 2-fold were selected for hierarchical clustering analysis between different compound treatments by using Ward's (minimum variance) method and presented result picture was made with DMVS 2.0 software (chipscreen

biosciences). The values besides each dendrite show the overall similarity (R value) between the columns of each other.

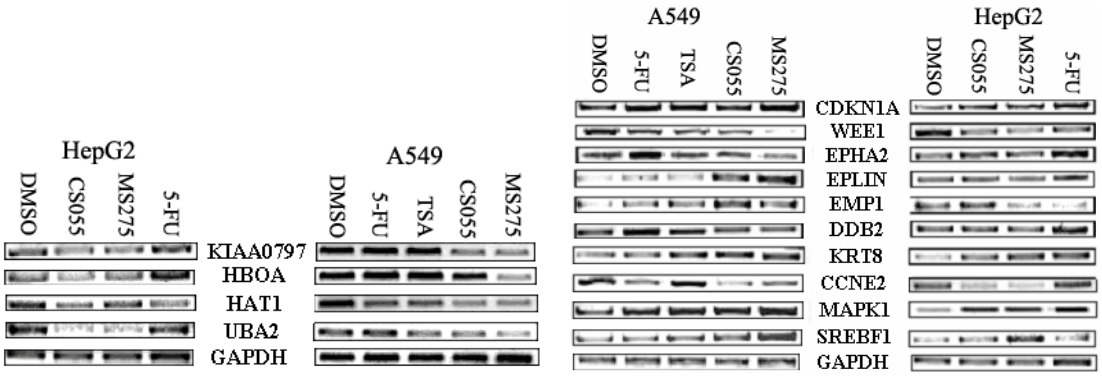


**Figure S3 Validation of expression changes of representative genes selected from gene profiling data.**

A549 and HepG2 cells were treated with Fluorouracil (5-FU, 30 μM on HepG2 and 60 μM on A549), chidamide (CS055, 2 μM on HepG2 and 4 μM on A549), MS-275 (2 μM on HepG2 and 5 μM on A549) and TSA (100 nM on A549) for 12 (A549) or 16 (HepG2) hours. Total RNA were isolated and applied for RT-PCR detection of expression levels of selected genes, which were significantly regulated by chidamide in microarray study (listed in supplementary Table 1), following methods described in supplementary online materials. The agarose electrophoresis pictures were taken as representative results from three independent experiments.

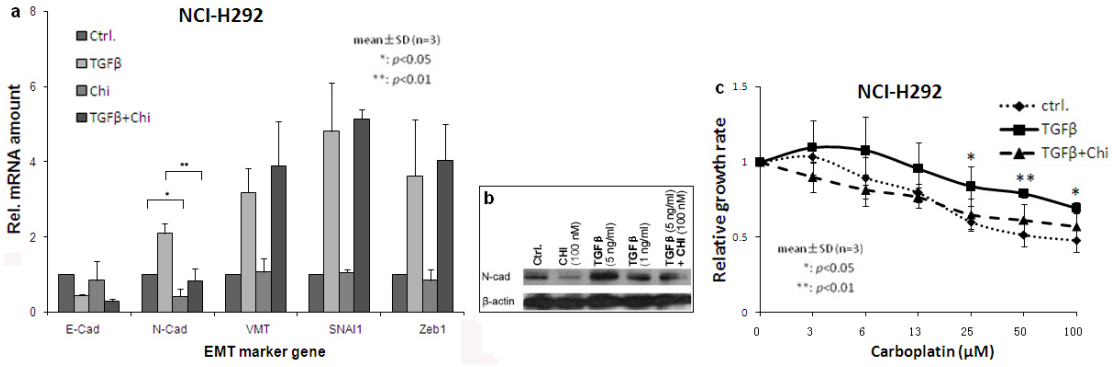


**Figure S4 Chidamide represses TGFβ induced Epithelial-Mesenchymal Transition and carboplatin resistance in non-small cell lung cancer cell line NCI-H292.**

**a & b.** chidamide represses TGFβ induced N-cadherin (N-cad) expression at both mRNA (a) and protein levels (b). NCI-H292 cells were incubated in serum-starving medium (0.1% FBS) for 3 days, and then treated with 0.1 μM chidamide with TGF-β (5 ng/ml) in presence or absence for another 48 hours. The relative expression change of target genes (E-cadherin, E-cad; N-cadherin, N-cad; Vimentin, VMT; SNAI1, Snail1; Zeb1) were evaluated by qRT-PCR and compared with vehicle control. Western immunoblot (Anti-N-Cadherin antibody (ab12221) was purchased from Abcam) was applied for detection of protein levels of N-cad in different treatments as indicated in insert. The gene expression changes were averaged from three independent experiments, Statistical significance was tested using Student's t-test with $P < 0.05$ (*) or 0.01 (**). **c.** chidamide represses TGFβ induced carboplatin resistance. NCI-H292 cells were incubated in serum-starving medium (0.1% FBS) for 3 days, and then treated with TGF-β (5 ng/ml) with 0.1 μM chidamide in presence or absence. Carboplatin was added in at serial dilutions. Cells were incubated for 72 hours and applied for viability evaluation by MTS assay. Concentration dependent growth curves upon carboplatin treatments were draw and comparedin presence of TGFβ alone, TGFβ togetherwith chidamide, or neither of them as the control. The represented data were averaged from three independent experiments, Statistical significance was tested using Student's t-test with $P < 0.05$ (*) or 0.01 (**).

**Supplementary table 1 list of representative genes in several functional clusters with significantly expression change by chidamide on two cell models in microarray study**

| Function cluster | Gene | A549 | | | | HepG2 | | |
|---|---|---|---|---|---|---|---|---|
| | | Chidamide (2 μM) | MS-275 (2 μM) | TSA (100 nM) | 5-FU (60 μM) | Chidamide (4 μM) | MS-275 (5 μM) | 5-FU (30 μM) |
| Transporters | ABCB10 | 0.35 | 0.36 | 0.49 | 0.52 | 0.30 | 0.18 | 0.30 |
| | ABCC2 | 0.53 | 0.38 | 0.94 | 1.01 | 0.75 | 0.79 | 1.28 |
| Cell differentiation | CDH1 | 2.17 | 1.68 | 0.93 | 1.15 | 5.89 | 4.62 | 0.90 |
| | EMP1 | 2.08 | 3.08 | 1.02 | 0.90 | NA | NA | NA |
| | EPHA2 | 1.29 | 1.46 | 1.13 | 2.30 | 2.00 | 1.49 | 4.18 |
| | EPLIN | 6.58 | 8.42 | 2.46 | 1.50 | 1.09 | 2.03 | 1.62 |
| | KRT8 | 3.31 | 4.21 | 3.20 | 3.57 | 2.46 | 3.63 | 2.21 |
| Histone modification | HAT1 | 0.46 | 0.37 | 0.64 | 0.42 | 0.42 | 0.29 | 0.57 |
| | HBOA | 0.52 | 0.45 | 0.97 | 0.78 | 0.49 | 0.41 | 0.82 |
| | MORF | 0.39 | 0.48 | 0.74 | 0.70 | 0.36 | 0.40 | 0.72 |
| Cell cycle and apoptosis | BUB1 | 0.57 | 0.64 | 0.94 | 0.28 | 0.38 | 0.34 | 0.56 |
| | BUB1B | 0.53 | 0.54 | 0.90 | 0.22 | 0.21 | 0.27 | 0.50 |
| | CCNA2 | 0.40 | 0.29 | 0.86 | 0.29 | 0.16 | 0.14 | 0.50 |
| | CCNB2 | 0.62 | 0.61 | 1.23 | 0.36 | 0.31 | 0.24 | 0.91 |
| | CCNE2 | 0.32 | 0.21 | 0.03 | 0.30 | NA | NA | NA |
| | CDKN1A | 2.00 | 4.90 | 1.51 | 9.96 | 3.62 | 6.94 | 39.93 |
| | CHEK1 | 0.53 | 0.43 | 0.77 | 0.57 | 0.20 | 0.18 | 0.39 |
| | DDB2 | 0.72 | 1.41 | 1.61 | 2.72 | NA | 1.64 | NA |
| | DR6 | 4.07 | 3.77 | 1.88 | 1.22 | 2.67 | 4.26 | 1.28 |
| | PCNA | 0.47 | 0.44 | 0.64 | 0.91 | 0.28 | 0.31 | 1.59 |
| | PMSCL1 | 0.34 | 0.28 | 0.77 | 0.32 | 0.19 | 0.20 | 0.43 |
| | PMSCL2 | 0.65 | 0.36 | 0.88 | 0.73 | 0.42 | 0.31 | 0.66 |
| | RAD23B | 0.54 | 0.34 | 0.64 | 0.60 | 0.49 | 0.41 | 0.50 |
| | WEE1 | 0.49 | 0.34 | 0.69 | 0.33 | 0.41 | 0.27 | 0.52 |
| Protein turnover | KIAA0797 | 0.38 | 0.23 | 0.61 | 0.86 | 0.47 | 0.35 | 0.80 |
| | UBA2 | 0.52 | 0.46 | 0.76 | 0.64 | 0.47 | 0.36 | 0.62 |
| | UBCH10 | 0.38 | 0.38 | 0.78 | 0.34 | 0.32 | 0.20 | 1.00 |
| | USP21 | 0.44 | 0.43 | 0.87 | 0.74 | 0.56 | 0.38 | 0.97 |
| miscellous | HDGF | 0.36 | 0.32 | 0.55 | 0.77 | 0.43 | 0.61 | 0.80 |
| | MAPK1 | 1.97 | 3.57 | 3.35 | 1.89 | 2.29 | 6.87 | 2.02 |
| | SREBF1 | 3.08 | 4.45 | 2.07 | 1.30 | NA | 3.06 | 1.04 |

Gene expression data were expressed as the fold change over the mock control in microarray study. Data were averaged from three independent microarray experiments. NA: no applicable data due to low invalid signal on microarray.

## References


1. Ning ZQ, Li ZB, Newman MJ, Shan S, Wang XH, Pan DS, Zhang J, Dong M, Du X, Lu XP. Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity. Cancer Chemother Pharmacol. 2012; 69(4):901-909.
2. Lauffer BE, Mintzer R, Fong R, Mukund S, Tam C, Zilberleyb I, Flicke B, Ritscher A, Fedorowicz G, Vallero R, Ortwine DF, Gunzner J, Modrusan Z, Neumann L, Koth CM, Lupardus PJ, Kaminker JS, Heise CE, Steiner P.. Histone deacetylase (HDAC) inhibitor kinetic rate constants correlate with cellular histone acetylation but not transcription and cell viability. J Biol Chem. 2013; 288 (37): 26926-43.
3. Dong M, Ning ZQ, Xing PY, Xu JL, Cao HX, Dou GF, Meng ZY, Shi YK, Lu XP, Feng FY. Phase I study of chidamide (CS055/HBI-8000), a new histone deacetylase inhibitor, in patients with advanced solid tumors and lymphomas. Cancer Chemother Pharmacol. 2012; 69(6):1413-22.