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Letter

Nicotinamide Phosphoribosyltransferase (NAMPT) is a New Target of Antitumor Agent Chidamide

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Nicotinamide Phosphoribosyltransferase (NAMPT) is a New Target of Antitumor Agent Chidamide

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KEYWORDS: Chidamide, histone deacetylase, nicotinamide phosphoribosyltransferase, dual inhibitors

ABSTRACT: Chidamide is a histone deacetylase (HDAC) inhibitor, which is currently used to treat cutaneous T-cell lymphoma in clinic. Herein nicotinamide phosphoribosyltransferase (NAMPT) was identified to be a new target of chidamide on the basis of the pharmacophore analysis, molecular docking, biological assays, inhibitor design and structure-activity relationship study. The polypharmacology of chidamide will provide important information for better understanding its antitumor mechanism. Also, design of dual NAMPT/HDAC inhibitors may serve as an effective strategy to develop novel antitumor agents.

The balance between acetylation and deacetylation of histone plays an essential role in maintaining cell homeostasis.[1] These two processes are regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) which increases and decreases gene transcription, respectively.[2] It has been reported that HDAC is overexpressed in various tumor cells.[3, 4] HDAC inhibitors (**Figure 1**) can decrease the acetylation level to modify the gene expression and induce death of cancer cells.[5] HDAC inhibitors (*e.g.* vorinostat, romidepsin, belinostat, and panobinostat) have been widely used in clinic for the treatment of cutaneous T-cell lymphoma and multiple myeloma.[6-8] Chidamide (**1**) is a benzamide HDAC inhibitor, which was marketed in China for the treatment of cutaneous T-cell lymphoma in 2015.[9] Chidamide showed good inhibitory activity against class I (HDAC1-3) and class IIb HDACs (HDAC10), whereas it was poorly effective towards other class I, IIa, and IV HDAC isoforms.[10] Although its antitumor potency and antitumor mechanism has been widely investigated,[11-14] the research for target profiling of chidamide is still rare, which limits deeper understanding its antitumor mechanism.

Nicotinamide adenine dinucleotide (NAD+) plays an essential role in cellular physiological processes.[15] There are four synthetic routes of NAD+, including the *de novo* pathway synthesized from tryptophan (Trp), the alternative salvage pathway synthesized from nicotinic acid (NA) or nicotinamide ribose (NR) and the primary salvage pathway synthesized from nicotinamide (NAM). In mammalian cells, NAD+ relies on the primary salvage pathway using NAM as the precursor, in which nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme[16] (**Figure 1A**). Recently, NAMPT is recognized as a promising target for the development of novel antitumor agents. Although two NAMPT inhibitors (FK866 and CHS828) have been progressed into clinical trials for treatment of cutaneous T-cell lymphoma and metastatic melanoma.[17] However, further drug development was hampered due to significant side effects, which inspired the discovery of novel NAMPT inhibitors. Previously, we identified a series of new NAMPT inhibitors through high-throughput screening.[18-20] Moreover, novel NAMPT/HDAC dual inhibitors were rationally designed on the basis of the synergistic effects between NAMPT and HDAC, which showed excellent *in vitro* and *in vivo* antitumor efficacy toward human colon cancer cell HCT116.[21] Herein NAMPT was proven to be a new target of chidamide by pharmacophore analysis, molecular docking, inhibitor design and biological assays, which provided new insights for the antitumor mechanism of chidamide and important information for new antitumor drug development.

The pharmacophore of HDAC inhibitors consists of three parts (**Figure 1B**): cap, linker and zinc binding region (ZBG, hydroxamic acid or o-phenylenediamine)[22]. Similar to HDAC inhibitors, the pharmacophore of NAMPT inhibitors also includes cap, linker and hydrophobic tails (**Figure 1C**). For chidamide, its (*E*)-3-(pyridin-3-yl)acrylamide ZBG could be regarded as a bioisostere of the hydrophobic

tail in NAMPT inhibitors. Thus, we envisioned that chidamide might be a NAMPT inhibitor.

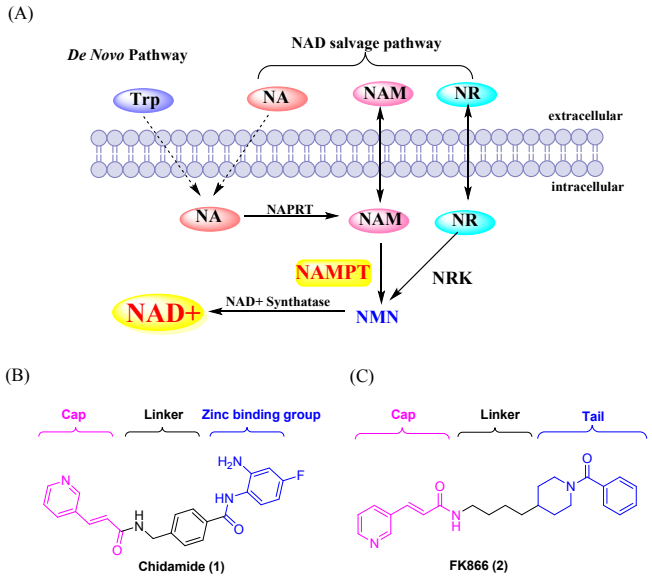


Figure 1. (**A**) Pathway of NAD+ biosynthesis; (**B**) Pharmacophore of HDAC inhibitors; (**C**) Pharmacophore of NAMPT inhibitors.

To validate the hypothesis, molecular docking was initially carried out to investigate whether chidamide shares a similar binding mode to NAMPT inhibitors. Chidamide was docked into the active site of NAMPT (PDB code: 2GVJ)[23] using docking software Gold[24]. The results showed that chidamide bound to the same pocket of FK866 in the active site of NAMPT (**Figure 2**). As shown in Figure **2A**, the pyridyl group of chidamide formed face to face π-π interactions with TYR18, PHE193 and ARG311, respectively, which were similar to that of FK866. The carbonyl oxygen and nitrogen atom of the pyridyl amide group formed two hydrogen bonds with SER275 and ASP219, respectively, while FK866 only formed a hydrogen bond with SER275. The results suggested that chidamide could bind to the active site of NAMPT. Thus, the inhibitory activity of chidamide against human recombinant NAMPT was tested using the fluorometric assay described in our previous studies.[19] As shown in **Table 1**, chidamide was proven to be a NAMPT inhibitor with an IC_{50} value of 2.1 μM.

Cellular thermal shift assay (CETSA)[21] was further performed to investigate whether NAMPT is the direct binding target of chidamide in HCT116 cells using FK866 as the positive control. The results indicated that the NAMPT expression level of cells treated with chidamide was more stable compared with the control, indicating a good binding affinity between the chidamide and NAMPT protein (**Figure 3**).

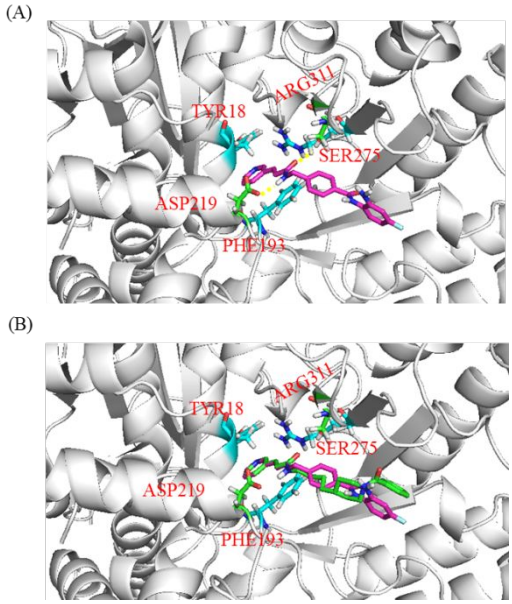


Figure 2. Predicted binding mode of chidamide in the active site of NAMPT (PDB: 2GVJ). (**A**) Predicted binding pose of chidamide in the active region of NAMPT. Hydrogen bonds (yellow) are represented with dash lines. The figure was generated using PyMol (http://www. Pymol.org/). (**B**) Superimposition of FK866 (green) and chidamide (purple) in the active region of NAMPT. The figure was generated using PyMol (http://www. Pymol.org/).

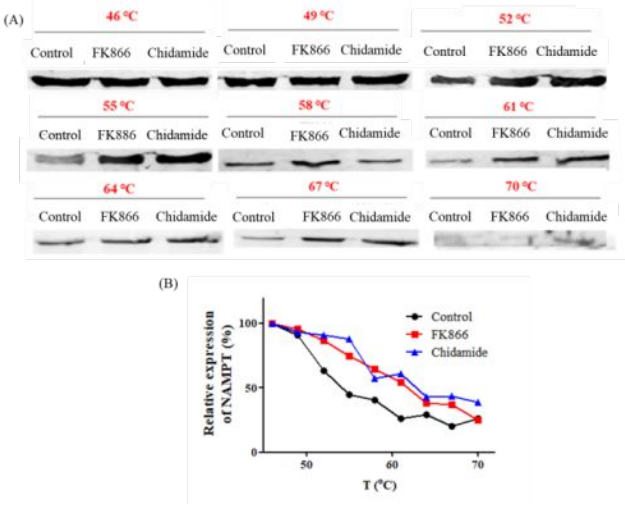


Figure 3. Binding of chidamide with NAMPT using CETSA. (**A**) Western blot of CETSA for NAMPT with FK866 (10 μM) and chidamide (10 μM) in HCT116 cells after the treatment for 2 h. (**B**) CESTA melt curves in HCT116 cells for NAMPT with FK866 and chidamide (at 10 μM).

The decrease in NAD+ level is a classic feature after inhibition of NAMPT activity.[21] Therefore, we measured the NAD+ variation qualitatively compared with the control group. As shown in **Figure 4A**, chidamide effectively decreased the cellular NAD+ level after incubation with human HCT116 cells for 24 h.

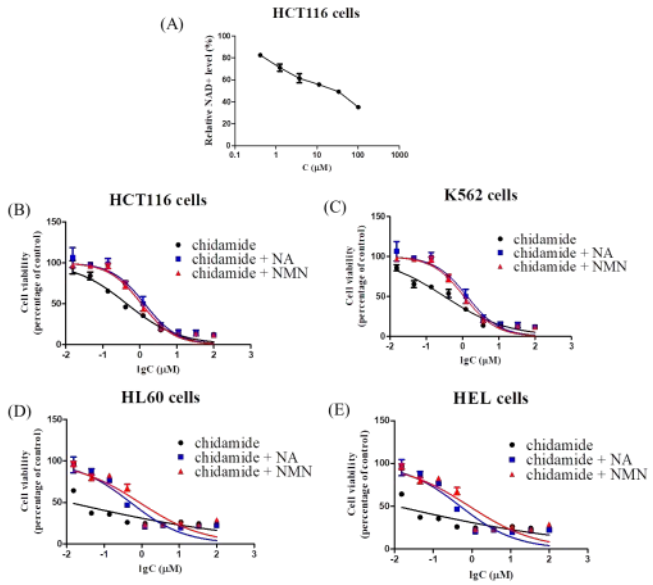
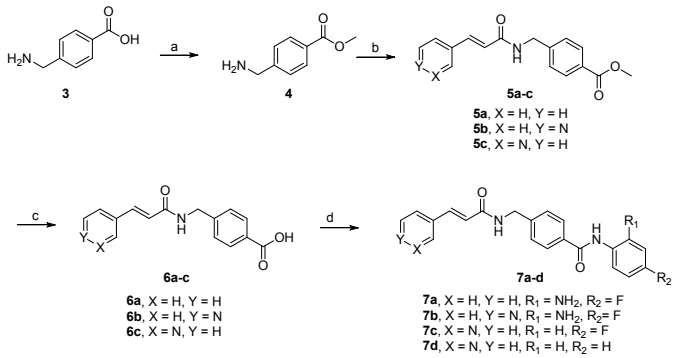


Figure 4. (**A**) Relative NAD+ level in HCT116 cells treated with chidamide at different concentrations for 24 h. Rescue studies with the addition of NA (4 μM) or NMN (10 μM) in HCT116 cells (**B**), K562 cells (**C**), HL60 cells (**D**) and HEL cells (**E**).

The addition of NA could activate the alternative rescue pathway to synthesize NAD+. Furthermore, the addition of the downstream product NMN could skip the exertion of activity of NAMPT to obtain NAD+. Herein, rescue studies[25] showed that addition of NA (4 μM) or NMN (10 μM) could significantly rescue cells from treatment with chidamide in HCT116 cells and human leukemia cells including K562, HL60 and HEL (Figure **4B-4E**), further confirming that NAMPT is a target of chidamide.

To investigate the role of NAMPT in exerting the antitumor activity of chidamide, four chidamide analogs **7a-d** (Scheme 1) were designed as the control molecules by removing the pharmacophores of HDAC (o-phenylenediamine) or NAMPT ((*E*)-3-(pyridin-3-yl)acrylamide) inhibitors. Enzyme inhibition and in vitro antitumor activity assay (**Table 1**) showed that compounds **7a** and **7b** without the pharmacophore of NAMPT inhibitors lost NAMPT inhibitory activity. Interestingly, their HDAC1 inhibitory activities were improved, while HDAC2 and HDAC3 inhibitory activities were comparable to chidamide (**Table 1**). For the antitumor activity, they retained good potency against K562 cell line, whereas the growth inhibitory activity against HCT116, HL60 and HEL cell lines were decreased. Similarly, after the removal of pharmacophore of HDAC inhibitors, compounds **7c** and **7d** lost HDAC inhibitory activity but retained the NAMPT inhibitory activity. However, their antitumor activities were significantly decreased. Compounds **7a-7d** and chidamide were also assayed cytotoxicity against cancer cells deficient in NAMPT using siRNA[26]. The results indicated that NAMPT inhibitors chidamide, **7a** and **7b** showed decreased inhibitory activity in NAMPT-deficient cells, further confirming that NAMPT is a target of chidamide. The detailed contribution of HDAC1-3 and NAMPT to the antitumor activity of chidamide still remains to be further explored.

Scheme1 Chemical synthesis of target compounds[a]



[a] **Reagents and conditions:** (a) CH_3OH, H_2SO_4, reflux, 6 h, yield 92%; (b) (*E*)-3-(pyridin-4-yl)acrylic acid or (*E*)-3-(pyridin-3-yl)acrylic acid or cinnamic acid, HATU, DIPEA, DMF, rt, 2 h, yield 85%-93%; (c) LiOH, THF/MeOH/H_2O, rt, 4 h, yield 80%-92%; (d) different substituted anilines, HATU, DIPEA, DMF, rt, 2 h, yield 76%-88%.

Table 1. Enzyme inhibition and in vitro antitumor activity of target compounds (IC_{50}, μM)

Compounds	chidamide	**7a**	**7b**	**7c**	**7d**
NAMPT	2.1 ± 0.10	>100	>100	2.5 ± 0.20	3.9 ± 1.1
HDAC1	0.13 ± 0.0020	0.026 ± 0.004	0.033 ± 0.0070	>100	>100
HDAC2	0.11 ± 0.0004	0.14± 0.0016	0.15 ± 0.0015	>100	>100
HDAC3	0.33 ± 0.028	0.36± 0.018	0.32 ± 0.0064	>100	>100
HCT116	0.34 ± 0.064	2.6 ± 0.45	2.4 ± 0.87	>20	>20
K562	0.32 ± 0.063	0.38 ± 0.17	0.14 ± 0.08	0.70 ± 0.16	4.1 ± 1.2
HL60	0.0022 ± 0.0010	1.5 ± 0.56	0.37 ± 0.042	11 ± 1.2	8.4 ± 2.0
HEL	0.013 ± 0.0071	1.5 ± 0.55	1.2 ± 0.52	5.0 ± 0.67	4.7 ± 0.65
HCT116-siRNA	> 20	9.7 ± 1.9	6.1 ± 0.31	> 20	> 20
K562-siRNA	2.4 ± 0.26	0.40 ± 0.12	0.27 ± 0.06	> 20	> 20
HL60-siRNA	3.2 ± 0.40	0.89 ± 0.080	0.48 ± 0.03	> 20	> 20
HEL-siRNA	1.8 ± 0.23	2.3 ± 0.090	1.4 ± 0.21	> 20	> 20

In summary, NAMPT was identified to be a new target of chidamide on the basis of the similarity of pharmacophore between HDAC and NAMPT inhibitors. Chidamide had low micromolar inhibitory activity towards NAMPT and significantly decreased cellular NAD+ level, which shares a similar binding mode to NAMPT inhibitor FK866. The results are helpful for better understanding of antitumor mechanism of chidamide. The modification of chidamide by removal of HDAC pharmacophore and NAMPT pharmacophore significantly decreased its antitumor activity, indicating that dual inhibition of HDAC and NAMPT might lead to improved antitumor activity. In our previous studies, the balanced inhibitory activity against both targets was found to be important for the anti-tumor activity.[21] Considering the synergistic effects of HDAC and NAMPT, HDAC/NAMPT dual inhibitors might be a promising strategy for the development of novel antitumor agents.

ASSOCIATED CONTENT

Supporting Information

Chemical synthesis and structural characterization of the target compounds; protocols of biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

D.G. and S.C designed the experiments and revised the manuscript. W.Y. synthesized chidamide analogs and carried out the biological experiments. W.L., H.Y., C.S, and W.S. assisted in the biological experiments and preparing the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

NAMPT, nicotinamide phosphoribosyltransferase; HDAC, histone deacetylase; NAD+, nicotinamide adenine dinucleotide; NRK: nicotinamide ribose kinase; NAPRT, nicotinic acid phosphoribosyltransferase; Trp: tryptophan; NAM, nicotinamide; NMN, nicotinamide mononucleotide; ZBG, zinc-binding group; CETSA, cellular thermal shift assay; NA, nicotinic acid.

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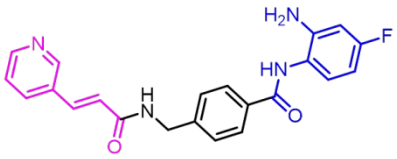
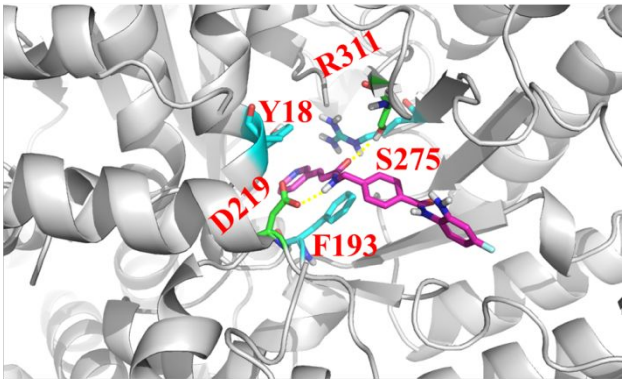
Table of Contents Graphic



chidamide



NAMPT