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Discovery of an Orally Active Subtype-Selective HDAC Inhibitor, Chidamide, as an Epigenetic Modulator for Cancer Treatment

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Abstract

Tumorigenesis is maintained through a complex interplay of multiple cellular biological processes and is regulated to some extent by epigenetic control of gene expression. Targeting one signaling pathway or biological function in cancer treatment often results in compensatory modulation of others, such as off-target drivers of cell survival. As a result, overall survival of cancer patients is still far from satisfactory.

Epigenetic-modulating agents can concurrently target multiple aberrant or compensatory signaling pathways found in cancer cells. However, existing epigenetic-modulating agents in cancer treatment have not yet fully translated into survival benefits beyond hematological tumors. In this article, we present a historical rationale for use of chidamide (CS055/Epidaza), an orally-active and subtype-selective histone deacetylase (HDAC) inhibitor of the benzamide chemical class. This compound was discovered and successfully developed as mono-therapy for relapsed and recurrent peripheral T cell lymphoma (PTCL) in China. We discuss the evidence supporting chidamide as a durable epigenetic modulator that allows cellular reprogramming with little cytotoxicity in cancer treatments.

Introduction

Components of epigenetic regulation, including those involved in regulation of DNA methylation, histone modification, and nucleosome remodeling, function cooperatively to determine chromatin configuration and unique transcriptional profiles in cells. Thus, they play a central role in normal development and pathological processes such as cancer.[1, 2]

DNA methylation of the 5-carbon on cytosine residues (5mC) in CpG dinucleotides was the first characterized epigenetic modification of chromatin. This DNA methylation occurs within CpG islands, which are present in about 70% of all mammalian promoters and plays an important role in transcriptional regulation. It is commonly altered during malignant transformation. Chromatin histone acetylation and deacetylation are also key steps in epigenetic regulation. These two reversible processes, jointly regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC), are in dynamic equilibrium under normal physiological conditions. The acetylation of chromatin histones neutralizes the positive charge on lysine and consequently may weaken the electrostatic interaction between histones and negatively charged DNA, thus forming a more "open" chromatin conformation. Histone lysine acetylation also serves as the nidus for the binding of various proteins with bromodomains and tandem plant homeodomain (PHD) fingers to determine local chromatin conformation and transcriptional activity. Additionally, it is well established that several nonhistone proteins, including many important oncogenes and tumor suppressors such as MYC, p53, and PTEN, are dynamically acetylated. [3]

Epigenetic drugs affect multiple cellular signaling pathways that are abnormally changed in cancer cells, including immune response and evasion, apoptosis, cell survival, and DNA-damage repair. [4-6] These inter- or intra-compensations occur among the abnormal signaling pathways in cancer and are the main challenges for existing treatments. Emerging epigenetic therapy provides novel arsenal for more comprehensive cancer treatment.[7]

There are four approved drugs considered to be epigenetic-modulating agents: 5-azacytidine and decitabine (inhibitors of DNA methytransferase for myelodysplastic syndrome), and vorinostat and romidepsin [HDAC inhibitors for refractory cutaneous T cell lymphoma (CTCL) or peripheral T cell lymphoma (PTCL)]. These epigenetic agents have shown success in hematological malignancies as single agent treatments. Among the targets, HDACs represent a family of enzymes with four major classes and up to 18 subtypes, essential for several biological pathways. However, the biological roles of individual HDAC subtypes are not fully understood; subtype-selective HDAC inhibitors may provide more specific targeting, allowing epigenetic-modulating programs to be used in combination treatment regimens. [8]

Peripheral T-cell lymphoma is an uncommon and aggressive form of non-Hodgkin's lymphoma that is associated with poor prognosis. PTCL comprises 23 pathological subtypes that differ in morphology, biology, and prognosis.[9] The distribution of pathological subtypes of PTCL in the Chinese population, especially NK/T lymphoma with the worst prognosis, is significantly higher than in North

America and Europe. There has been no approved treatment in China for PTCL patients except for conventional chemotherapy.[10] The CHIPEL (Phase II Study of Chidamide in Patients with Relapsed or Refractory Peripheral T-cell Lymphoma) trial was an open-label, single-arm study in the Chinese population to evaluate the efficacy and safety of orally administrated chidamide in relapsed or refractory peripheral T-cell lymphoma patients who had failed at least one prior systemic therapy.[11] The CHIPEL trial reached an objective response rate (ORR) of 28% as evaluated by an independent review committee [11] and gained approval from the Chinese Food and Drug Administration (CFDA).

Chidamide is a benzamide-type selective HDAC inhibitor and exhibits unique biological responses against cancer cells *in vitro* and *in vivo*, though the structure does not suggest favorable medicinal chemistry properties. For example, chidamide is a far less potent inducer of cell cycle arrest and tumor cell apoptosis than the hydroxamates *in vitro*, but is as potent as other chemical classes for induction of histone acetylation and gene expression. The accumulation of acetylated histone by chidmide is very long lasting. The gene expression profile of cells is also distinct in the presence of chidamide and other similar chemical class inhibitors. In the clinic, the efficacious concentration of chidamide induces activation of anti-tumor immune responses and epigenetic reprogramming without triggering general cellular cytotoxicity. Preclinical evidence and current clinical efficacy data on chidamide in different cancer types supports chidamide as a promising anti-cancer agent. Further optimization of its clinical use against a variety of tumor types as an epigenetic

modulator, either in mono- or combinatory therapy, is warranted.

Chidamide is a benzamide class and subtype-selective HDAC inhibitor

We initiated a discovery program targeting HDAC inhibition as a potential differentiation-inducing agent for cancer therapy in 2002, with limited scientific information available at that time. Among various chemical structures synthesized and screened, hydroxamates and cyclic peptides were the most potent inhibitors in nuclear extract-based HDAC enzymatic activity measurements and cell proliferation assays *in vitro,* while the benzamide and aliphatic acid classes were less potent. We simulated a molecular docking study and demonstrated that the benzamide class of HDAC inhibitors (HDACi) such as MS-275 binds to HDLP, the bacterial prototype of class Ⅰ HDACs, differently than Trichostatin A (TSA), a hydroxamate HDACi.[12] MS-275 blocks the entrance to the HDLP active pocket rather than directly chelating with zinc as TSA does, suggesting that different interactions among different chemical structures might result in different biological responses. We observed different transcriptional regulation patterns among benzamide, hydroxamate, and other structure types of HDACi in chemical-genomic based drug screening. Interestingly, the benzamide class of HDACi exhibited preferential induction of epithelial differentiation- and death receptor 6 (DR6)-related apoptosis genes and repression of drug resistant and protein modification/degradation pathways (unpublished screening data). These findings led us to focus on determining the benzamide structure of HDAC inhibition as a potential drug lead.

We designed and screened a series of compounds with a benzamide scaffold structure and obtained their SAR data using *in vitro* enzymatic and cellular

screening.[12, 13] One of the benzamide compounds, CS055 (chidamide), was identified as having a clear subtype selectivity and distinct regulation of gene expression.[14]

Chidamide initially demonstrated less potency in inhibition of total HDAC enzymatic activity (IC_{50} as 7.16 μM from unpublished screening data) than hydroxamate compounds (e.g., 0.60 μM for SAHA from unpublished screening data) when evaluated *in vitro* using cell nuclear extract preparations (Figure S1 and ref.14). The concentration required for induction of acetylated histone H3, an effect of HDAC inhibition in cell lines, however, was surprisingly similar between chidamide, MS-275, and SAHA even at concentrations as low as 0.1 μM.[14] However, no increased α-tubulin acetylation mediated by the HDAC6 of class Ⅱb subtype was observed for chidamide even at concentrations as high as 10 μM (Figure S1). These results suggested that chidamide functions as a potent subtype selective HDACi. Using *in vitro* enzymatic assays against individual HDAC subtypes, we also then demonstrated that chidamide strongly inhibited class I HDACs 1, 2, and 3, as well as class IIb HDAC10, with the IC_{50} in the nanomolar range.[14]

Chidamide displays distinct gene regulation pattern compared with hydroxamates

The main effect of HDAC inhibition is thought to be the regulation of gene expression through modification of both chromatin histone and transcription factors (e.g., p53, NHRs, STATs). HDACs do not directly bind DNA but act via a complex formed with transcription factors and co-factors (e.g., CoREST, NuRD, Sin3, NCoR).[15,16] These complexes define the cell-type specific effects of HDAC

inhibition, which explain why most HDACi affect only a small proportion of expressed genes in individual cell lines in our screening and reports from others. [17]

We performed gene expression profiling on *in vitro* cultured tumor cells treated with representative HDACi and chemotherapy agents. Two-dimension clustering analysis of gene expression changes was generated using data representing expression changes from at least one treatment (a total of 664 genes). The expression patterns induced by HDACi are clearly distinct from those observed from conventional chemotherapeutic drugs and EMS, a DNA damage agent. This suggests that HDACi have an anti-cancer mechanism distinct from conventional chemotherapeutic agents. Importantly, the two benzamide classes of HDACi, MS-275 and CS055, induced a very similar pattern of gene expression in cell lines tested; the hydroxamate HDACi TSA showed less similarity in gene expression profiles compared to those induced by the two benzamides in A549 cells (unpublished screening data and Figure S2). These results are similar to a previous report that identified the expression profile differences between MS-275 and two hydroxamates.[17]

In addition, genes in several functional clusters were differently regulated by the two benzamides than by TSA and fluorouracil (5-FU), e.g., genes involved in apoptosis, epithelia differentiation, drug resistance-related transporters, protein modification/degradation, and components of epigenetic machinery. These data were confirmed by RT-PCR (unpublished screening data, Table S1, and Figure S3). The distinct gene expression profiles observed in drug screening for chidamide appear to

be HDAC subtype-related because the same subtype selective inhibitor MS-275 shared very similar expression profiles, both of which are different from that of non-selective hydroxamate HDACi. It has recently been reported that benzamides preferentially inhibit the HDAC-NCoR complex over HDAC-Sin3,[15] which underlies the differential bioactivity among the compounds of different structural classes. Such a mechanism-directed regulation in gene expression profiles by chidamide may well bring a differential bioactivity towards future clinical applications.

Unique binding and induction of durable acetylation in vivo by chidamide

Conventionally, benzamide compounds are structurally less favorable for consideration in pharmaceutical development due to their shortfalls in PK/PD profile. Chidamide in solid dispersion was evaluated in preclinical studies to meet the oral availability, efficacy, and safety needs. Significant and durable induction of histone acetylation and subsequent gene regulation, two key steps in epigenetic modulation, were obtained in animal models with submicromolar concentrations of chidamide. These data indicate that binding of pharmacological concentrations of chidamide to HDAC subtypes is sufficient to generate HDAC inhibition *in vivo*.[14, 18]

We previously reported that MS-275 binds to bacterial HDLP with a mechanism different from that of TSA in a molecular docking simulation.[12] The crystal structure of human HDAC2 bound with SAHA and other benzamide compounds has revealed large differences in the mode of occupancy between the two chemical structures with

the exception of similar chelation with zinc.[19] Therefore, we performed a docking simulation with chidamide using this crystal model (Figure 1a). Chidamide binds to the catalytic active pocket of HDAC2 by a similar zinc-chelating mechanism as SAHA via known classical residues, including His183, Asp181, Asp269, and Tyr308, as well as forming a hydrogen bond with Asp104. However, there remain significant differences between the two interaction modes. Compared to SAHA, new π-π stacking interactions of chidamide were observed: a phenyl ring attached to a benzamide moiety with Phe155 and Phe210 (part 1 circled in Figure 1a). A halogen bonding interaction was also found between 4-fluorine in the terminal phenyl ring and Gly305 (part 2 circled in Figure 1a). These unique interactions may play an important role in stabilizing chidamide in the HDAC2 catalytic pocket, similar to what has been previously suggested for other benzamide class compounds.[19, 20]

Previous reports have shown that the benzamide class of compounds have different *in vitro* enzymatic kinetics and pharmacodynamic characteristics compared with the hydroxamate class of compounds (e.g., SAHA), the 'tight fit' kinetics of benzamide versus the fast-on/fast-off kinetics of hydroxamate.[19,21] Consistently, chidamide demonstrated similar long-lasting pharmacodynamic characteristic as measured by acetylated histone H3 accumulation in peripheral blood cells from patients in the phase I clinical trial before and after chidamide administration.[18] A single dose of chidamide (25, 32.5, or 50 mg) increased acetylation of histone H3 in peripheral blood cells isolated from patients even at the first time point of 6 hours, Acetylation of histone H3 reached a peak between 24 and 48 hours, and was

maintained at high levels up to 72 hours post-chidamide administration; the half-life of chidamide in patients was about 24 hours. In contrast, the induction of histone acetylation by SAHA in patients was maintained for only a few hours.[22] This durable pharmacodynamic characteristic of chidamide supports the current therapeutic dosing schedule as twice weekly.[11]

Chidamide exerts clinical efficacy at concentrations far lower than its in vitro cytotoxic doses

Chidamide has been shown to have no detectable effect on the growth of normal human primary cells, yet inhibits the growth of a broad spectrum of tumor cells *in vitro* with 50% inhibition of cell growth (GI_{50}) values ranging from submicromolar to micromolar.[14] However, chidamide exhibits clinical efficacy in T cell lymphoma patients at a dose much lower than the concentrations needed to inhibit or arrest cell growth *in vitro*.[11,18] For example, chidamide given to patients at a single dose from 5 to 50 mg resulted in a maximum plasma concentration (C_{max}) of no more than 500 nM; no significant accumulation was observed with a twice weekly schedule.[18] This concentration is much lower than its GI_{50} against *in vitro* tumor cell lines; the medium value of GI_{50} ranged from 1.5 μM for a panel of the hematopoietic tumor cell lines, including several T lymphoma cell types, to over 6 μM for solid tumor cell lines (Table 1).[14] We have noticed a difference between chidamide and other marketed epigenetic drugs such as decitabine, vorinostat, and romidepsin. These marketed drugs had a pharmacologically relevant C_{max} in patients close to, or higher,

than their respective GI_{50} against a large panel of cell types tested *in vitro* (freely accessible GI_{50} Database at: http://dtp.nci.nih.gov/index.html.).[23-25] This may partly explain the adverse effects seen with these marketed drugs that are similar to typical chemotherapeutic agents, i.e., gastrointestinal side effects, which are not significant in patients treated with chidamide.[11,18] It has previously been shown that chidamide causes cell cycle arrest (at G0/G1 phase) and induces apoptosis only in leukemia cell lines at a concentration of 100 nM *in vitro*,[26] which may explain the decrease in white blood cells and granulocytes seen in chidamide-treated patients.[11, 18]

To date, there is limited clinical evidence that HDACi provide clear anti-tumor activity against hematopoietic malignancies other than T cell lymphomas by single agent administration. One plausible explanation could be that the growth inhibition or cell cycle arrest observed with HDACi is not relevant to their anti-T-lymphoma activities in patients.

There are published clinical trial results showing that HDACi as single agents may be beneficial for certain type of solid tumors. For example, one partial response (PR) and four stable disease (SD) responses were observed among five cases of adenoid cystic carcinoma (ACC) treated in a phase Ⅰ trial with SAHA.[27] Similarly, one PR and one SD were observed in two ACC cases treated in a phase Ⅰ trial with chidamide,[18] indicating that a specific mechanism might be responsible for the clinical benefits in this particular cancer type.[28,29] Although the mechanism behind improved outcomes with HDACi is unknown, understanding the therapeutic pathway would greatly accelerate treatment using epigenetic modulators. For

example, it has been shown that alterations in the components of HDAC complexes such as PML-RAR or AML-ETO fusion proteins have been found to be the drivers of certain subtypes of leukemia cells, and addition of HDACi to this type of tumor *in vitro* can promote degradation of the fusion proteins.[30-32]

The unique aspects of HDAC inhibition that contribute to anti-cancer activity have been described, e.g., reprogramming distorted transcription patterns and signal pathways in tumor cells, and modulation of the tumor microenvironment and immune system.[4,5] For instance, benzamides induce MHC class II-like antigens (e.g. MICA, MICB, etc.) and the ligand of the natural killer receptor (NKG2D), which can stimulate innate anti-tumor immunity.[33] We have observed similar effects with chidamide on *in vitro* cell lines and in clinical samples.[14,18] Chidamide enhances the cytotoxic effects of human peripheral mononuclear cells *ex vivo* on K562 target cells via upregulation of proteins involved in NK cell functions (e.g., NKG2D and granzymes) that lead to the activation of NK cells. This NK activity was also observed in peripheral white blood cells derived from two lymphoma patients who responded to chidamide administration.[14, 18] It has been reported that chidamide induces expression of the leukemia-specific antigen PRAME (preferentially expressed antigen in melanoma) in both cell lines and leukemia blasts in bone marrow samples from patients, resulting in increased PRAME specific- and cytotoxic T lymphocyte (CTL)- mediated *in vitro* cytotoxicity against leukemia.[34] Most importantly, the pharmacological concentrations required to activate either NK-mediated or antigen-specific CTL activities by chidamide *in vitro*, *ex vivo*, and

in patients were between 100 and 500 nM, a concentration where cytotoxicity against normal cells was not evident. Inhibition of different subtypes of HDAC could have distinct effects on regulatory T cell (Treg) activity. For example, inhibition of class □ or Ⅳ subtypes 6, 9, and 11 may exaggerate Treg expansion while inhibition of class□subtypes 1, 2, and 3 may repress the Treg expansion, which in general favors the anti-tumor immune response by class □ selective inhibitors.[35-37]

Our *in vitro* studies also show that chidamide could sensitize non-small cell lung cancer (NSCLC) cells to DNA damage induction by carboplatin *in vitro* using a clinically relevant concentration, consistent with other reports that suggest the pivotal roles of HDACs in DNA damage and autophagy.[38, 39] Chidamide in combination with carboplatin strongly induces the phosphorylation of histone H2A.X (γH2A.X), a hallmark of the DNA damage response, increases cell cycle arrest at G2/M, and increases apoptotic cell death in NSCLC cell lines A549 and NCI-H157 at a non-cytotoxic concentration of 0.3 μM.[40]

TGFβ induces a coordinated program change in gene expression and subsequent growth of tumor cells related to epithelial-mesenchymal transitions (EMT), essential to epithelial tumor metastasis. Recent reports revealed an epigenetic regulatory component in the reversible process of EMT.[41-43] We have shown that chidamide can repress EMT and consequent drug resistance induced by TGFβ on the NSCLC cell line NCI-H292 (unpublished result). *In-vitro*, TGFβ downregulated the expression of the epithelial marker E-cadherin and upregulated the expression of the mesenchymal markers N-cadherin and vimentin, resulting in

less growth inhibition by carboplatin treatment in the NSCLC cell line NCI-H292, a cellular model for the EMT process.[44] In this experimental model, adding a non-cytotoxic concentration of chidamide to the cultured H292 cells antagonized the above-mentioned effect by repressing the induction of N-cadherin and restoring the carboplatin-induced growth inhibition in this cell line (Figure S4).

In summary, emerging data suggests there are three major mechanisms underlying the anti-cancer activities of HDACi: preferential induction of growth arrest and apoptosis in blood and lymphoid-derived tumor cells, activation of NK-mediated and CD8+ CTL-mediated antigen-specific cellular anti-tumor immunity, and partial reversal of EMT and drug-resistance of tumor cells as described in Fig. 2. However, the activation of NK- and antigen-specific CTL-mediated cellular anti-tumor immunity appears only to be associated with the benzamide type of selective HDACi such as chidamide (Figure 2).

Of note, the most common adverse effects of chidamide in clinical trials were reported as hematopoietic toxicities, e.g. thrombocytopenia and neutropenia.[11, 18] Knowing that HDAC 1 and 2 are essential for hematopoietic stem cell homeostasis [45, 46] and that thrombocytopenia and neutropenia are common adverse effects reported for all existing HDACi, these adverse effects are most likely to be the on-target effects of such agents.

Further clinical development of chidamide as a durable epigenetic modulator

The important findings in recent years about HDAC inhibition includes an

understanding of its role in reversing irresponsiveness of tumor cells to previous drug treatments via epigenetic reprogramming,[47, 48] There is increasing evidence demonstrating a pivotal role of EMT in cancer heterogeneity and drug resistance.[43] Clinical trials have demonstrated the potential for overcoming drug resistance in patients with hematopoietic or solid tumors by combining conventional regimens with epigenetic modulators.[49-51] The ongoing clinical trials in advanced NSCLC patients with wild type EGFR using chidamide in combination with carboplatin and paclitaxel, as well as trials in metastasized breast cancer, may provide additional data.

The successful development of benzamide-type selective HDACi, such as chidamide, opens a new treatment avenue to activate anti-tumor immune responses and to separate the epigenetic-reprogramming effect from cytotoxicity in patients.[7] A combination of new-generation epigenetic modulators such as chidamide with existing treatment regimens may hold great promise for improving overall survival for cancer patients.

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Tables and Figures

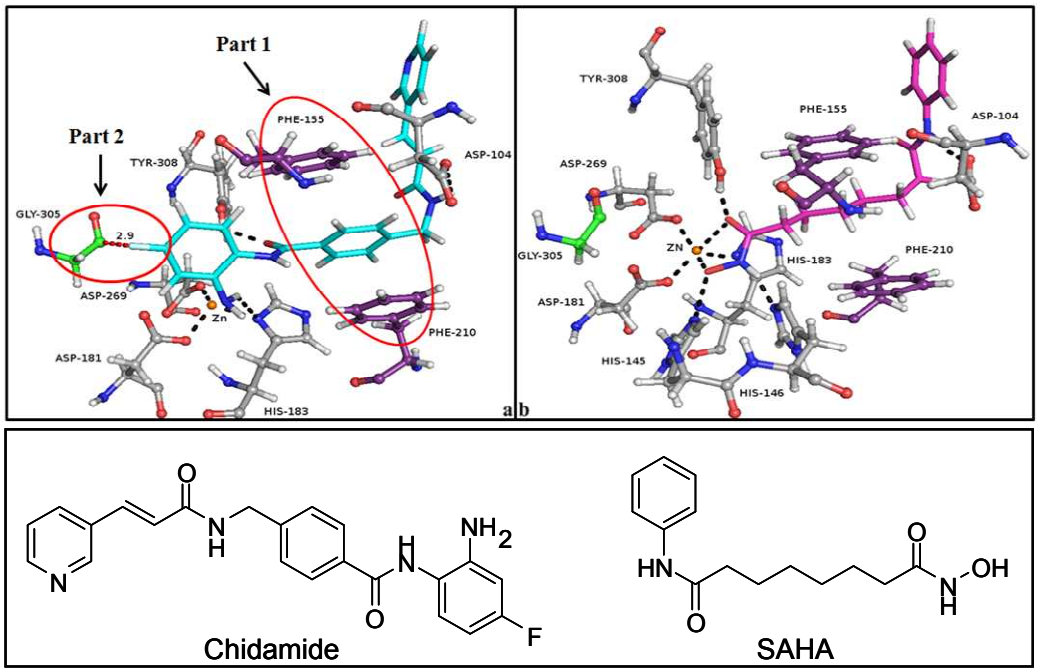

Fig. 1 Comparison of binding modes of chidamide versus SAHA binding to human HDAC2.

The co-crystal structure of HDAC2 and hydroxamate-based SAHA (PDB entry 4LXZ, 1.85Å) was chosen for the docking template. The Molegro Virtual Docker (MVD 2010.4.0.0) program was used for docking simulation of flexible chidamide into the target protein HDAC2 model. The docking space was identified by the binding site for the ligand SAHA in the HDAC2 crystal structure; it was defined as a sphere 10Å in radius with center X 25.97, Y 15.79, and Z 1.09. The highest MolDockScore value representing the lowest energy for simulation was -159.204kJ/mol, which indicated a strong interaction between chidamide and HDAC2. **a.** The docking view of interactions between chidamide (cyan stick) and HDAC2 is represented. **b.** The co-crystal structure of SAHA (pink stick) in complex with HDAC2 (4LXZ) is shown. Key HDAC2 pocket residues are displayed as grey, purple, and green ball-sticks. Zinc is shown as an orange sphere. Protein-ligand interactions contain hydrogen bonding (dashed black lines), halogen bonding (dashed red line), and π-π stacking. All pictures were prepared with PyMol (DeLano Scientific LLC).

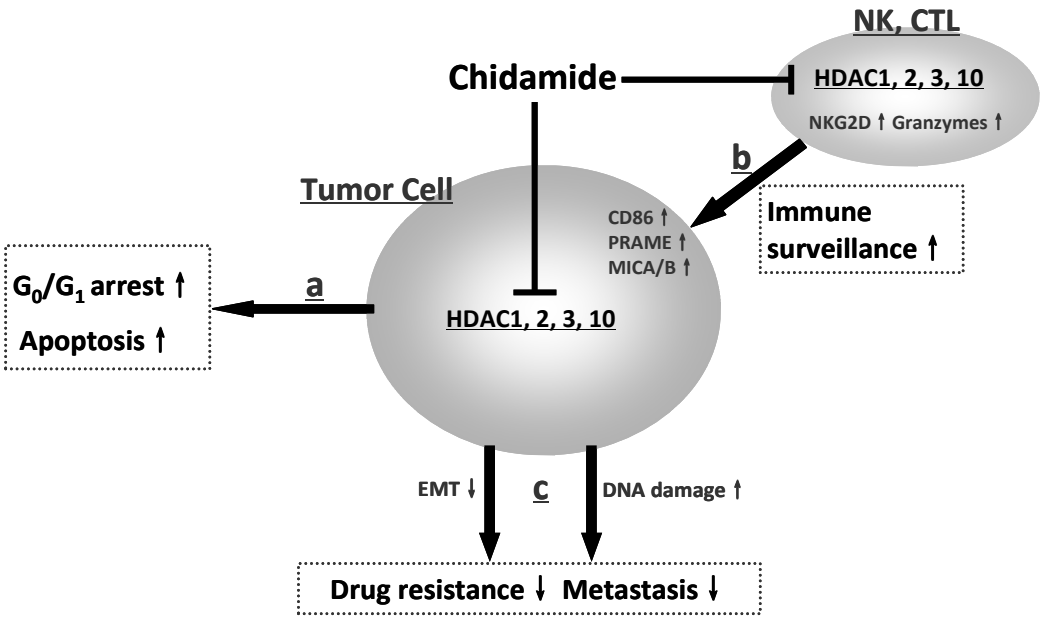

Fig. 2 Proposed manifold anti-tumor mechanisms of chidamide.

Chidamide exerts its anti-tumor activity via at least three mechanisms. a. Preferential induction of growth arrest and apoptosis in blood and lymphoid-derived tumor cells. b. Stimulation of innate and adaptive immune surveillance via induction of antigen representation of tumor cells and activation of cytotoxic activity of natural killer (NK) and cytotoxic T lymphocytes (CTL). c. Exhibiting a synergistic effect with chemotherapeutic agents or other target therapeutic agents via enhancing drug sensitivity and repressing EMT of tumor cells.,

Table 1 *in vitro* growth inhibition potency and clinical exposure of chidamide

		*Chidamide * (32.5 mg, PO)*
Clinical pharmacokinetic parameters (single dose drug administration)	Cmax (nM)	312±322
	AUC (nM·h)	2238±1309
In vitro growth inhibition on cell lines panel (medium GI_{50}, nM) [#]	Hematopoietic malignant cell lines (n=9)	1860
	Solid tumor cell lines (n=24)	6650

PO, oral administration. *Pharmacokinetic parameters of chidamide were cited from ref. 18. GI_{50} data were cited from ref. 14 and unpublished screening data.