

**The tricyclic antidepressant amitriptyline inhibits D-cyclin transactivation and induces myeloma cell apoptosis by inhibiting histone deacetylases : *in vitro* and *in silico* evidence**

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**ABBREVIATIONS:**

5-HT, 5-hydroxytryptamine; Amit, Amitriptyline; DMSO, dimethylsulfoxide; GAFF, general AMBER force field; H3, Histone3; HAT, histone acetyltransferase; HDAC, histone deacetylase; MM, multiple myeloma; MOI, monoamine oxidase inhibitor; PBSC, peripheral blood stem cell; PI, propidium iodide; PME, particle mesh Ewald; SERT, serotonin transporter; SNRI, serotonin norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TSA, trichostatin A.

## Abstract

Amitriptyline is a classic tricyclic antidepressant (TCA) and has been used to treat depression and anxiety of cancer patients but its relevance to cancer cell apoptosis is not known. In the present study, we demonstrated that amitriptyline inhibited cyclin D2 transactivation and displayed potential anti-myeloma activity by inhibiting histone deacetylases (HDACs). Amitriptyline markedly decreased cyclin D2 promoter-driven luciferase activity, reduced cyclin D2 expression and arrested cells at the G0/G1 phase of the cell cycle. Amitriptyline-induced apoptosis was confirmed by Annexin v staining, cleavage of caspase-3 and PARP-1. D-cyclin expression is reported to be epigenetically regulated by histone acetylation. Thus, we examined the effects of amitriptyline on Histone (H3) acetylation and demonstrated that amitriptyline increased acetylation of H3 and expression of p27 and p21. Further studies indicated that amitriptyline interfered with HDAC function by downregulation of HDAC3, -6, -7 and -8, but not HDAC2 and by interacting with HDAC7. Molecular docking analysis and molecular dynamics simulations revealed that amitriptyline bound to HDAC7 and formed strong van der Waals interactions with five residues of HDAC7, including Phe162, His192, Phe221, Leu293 and His326, thus inhibiting HDAC activity. Therefore, we found that amitriptyline inhibited cyclin D2 transactivation and HDAC activity and could be a promising treatment for multiple myeloma.

## Introduction

Antidepressants are a large class of psychoactive drugs that include tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors (MAOI). These agents have been used to treat patients' psychiatric disorders, such as major depression and anxiety. These antidepressants are prescribed for cancer patients as a supportive treatment (Mishra et al., 2008). Recent studies found that antidepressants kill cancer cells (Argov et al., 2009; Stepulak et al., 2008). For example, antidepressants such as fluoxetine can induce cancer cell apoptosis, and displayed anti-cancer activity in various cancers such as glioma, bladder cancer and blood cancers (Argov et al., 2009; Stepulak et al., 2008). SSRIs and TCAs such as sertraline and paroxetine display potent anti-leukemia/anti-lymphoma activity in *in vitro* models and markedly enhanced the effects of both vincristine and doxorubicin (Amit et al., 2009). Sertraline was found to induce apoptosis and possess anti-colon cancer activity in both colon cancer cell lines and in colorectal cancer-xenografted mice by inducing the expression of tumor suppressor p53 and inhibiting anti-apoptotic BCL2 (Gil-Ad et al., 2008).

Amitriptyline is a representative of TCAs, and has been approved for the treatment of depression. Amitriptyline is also prescribed to modify pain and neuropathic symptoms. In addition, some biologic studies suggest that amitriptyline may have significant anti-cancer effects. For example, a recent study indicated that amitriptyline inhibits cellular respiration at concentrations of 0.14 mM-0.5 mM and

induces death in glioma cells (Higgins and Pilkington, 2010; Pilkington et al., 2008). Another study indicated that amitriptyline at 50  $\mu$ M decreased proliferation of human colon carcinoma cell HT29 (Arimochi and Morita, 2006). However, there are no reports evaluating amitriptyline in multiple myeloma (MM).

Multiple myeloma is a hematological malignancy derived from terminal plasma cells. Myeloma is currently incurable and is treated in a comprehensive regimen, including chemotherapy, immunotherapy, and stem cell transplantation. Chemotherapy is the mainstay of myeloma treatment but remission are limited even with newer agents such as lenolidomide and bortezomib (Richardson et al., 2010). Thus, it is necessary to develop novel drugs for this disease.

Molecularly, multiple myeloma is associated with elevated D-cyclins. At least one of the cyclin Ds is dysregulated in multiple myeloma (Bergsagel and Kuehl, 2003; Bergsagel et al., 2005). Of them, cyclin D2 is associated with a poor prognosis in myeloma (Bergsagel et al., 2005). Cyclin D inhibitors are potential therapeutics that induced cell death in both cell and animal models (Tiedemann et al., 2008).

To identify novel therapeutic agents for the treatment of myeloma, we employed our previous successful drug screening system established in NIH3T3 cells stably expressing cyclin D2-promoter driven luciferase as a reporter gene and used this system to screen an in-house library composed of on- and off-patent psychoactive drugs. Through this screen, we identified the tricyclic antidepressant amitriptyline that inhibited cyclin D2 expression and induced myeloma cell apoptosis. Further studies

indicated that amitriptyline-induced cell apoptosis was associated with decreased histone deacetylase activity.

## **Materials and Methods**

### ***Cell lines***

Human MM cell lines were maintained in Iscove Modified Dulbecco Medium (IMDM, Gibco, Invitrogen, CA). Leukemia cell lines were maintained in RPMI-1640 medium. All media were supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 100 µg/mL penicillin, and 100 units/mL streptomycin (Hyclone, Logan, UT).

### ***Primary multiple myeloma samples***

Bone marrow from MM patients was obtained from Princess Margaret Hospital, University Health Network (UHN), Toronto with UHN institutional review board approval and in accordance with the Declaration of Helsinki. Primary normal hematopoietic cells were obtained from healthy volunteers donating their peripheral blood stem cells (PBSCs) for allotransplantation at the First Affiliated Hospital of Soochow University, Suzhou, China. Mononuclear cells were isolated from the samples by Ficoll density centrifugation. Primary cells were cultured at 37°C in IMDM supplemented with 10% FCS, 1 mM of L-glutamine, and appropriate antibiotics.

### ***Chemicals and compiling of antidepressant library***

Psychoactive drugs were selected from LOPAC chemical collection (Sigma-Aldrich, St. Louis, MO) and arrayed them in a 96-well plate as a sublibrary.

### ***Identification of Amitriptyline***

To identify cyclin D2 inhibitors with psychoactive activity, we conducted a chemical screen for inhibitors of the D-cyclin promoter similar to previously described (Mao et al., 2007). Briefly, NIH3T3 cells stably expressing the cyclin D2 promoter driving firefly luciferase (10,000 cells per well) were plated in 96-well plates by a Biomek FX liquid handler (Beckman, Fullerton, CA). After the cells had adhered, they were treated with aliquots of the sublibrary at a final concentration of 5  $\mu$ M and 0.1% dimethyl sulfoxide (DMSO) at 37°C for 20 hours. After incubation, cyclin D2 transactivation was assessed by luciferase assay.

### ***Luciferase assay***

Luciferase activity was assessed using Bright-Glo Luciferase substrate according to the manufacturer's instructions (Promega, Madison, WI) and previously described (Mao et al., 2007).

### ***Cell Viability***

Cell viability was assessed with the CellTiter96® Aqueous Non-Radioactive Assay kit (MTS assay) according to manufactures' instructions (Promega). Apoptosis was measured by flow cytometry with Annexin-V-FITC and propidium iodide (PI) staining (Biovision, Mountain View, CA, USA) as previously described (Mao et al., 2007).



For primary myeloma cells, purified cells were labeled with both CD138-PE (eBioScience, San Diego, CA, USA) and Annexin V-FITC after amitriptyline treatment and analyzed on a flow cytometer as previously described (Tiedemann et al., 2008).

### ***Immunoblotting***

Whole cell lysates were prepared from myeloma and leukemia cells as described previously (Mao et al., 2007). Protein concentrations were determined by the BCA assay. Equal amount (25  $\mu$ g) of proteins was subjected to fractionation by SDS-PAGE electrophoresis followed by transfer to PVDF membranes. Membranes were probed with antibodies including monoclonal anti-human cyclin D1 (1:200, v/v), polyclonal anti-human caspase-3 (1:5,000, v/v), anti-human poly(ADP riboxyl)Polymerase (PARP-1) (1:1000, v/v), anti-human p21 and p27 (1:1000, v/v), polyclonal anti-human XIAP (1:1000, v/v) from Cell Signaling Technology, Inc; polyclonal anti-human cyclin D2 (1:400, v/v), polyclonal anti-human p53 (1:500, v/v), and polyclonal anti-Acetylated lysine (1:1000, v/v) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; polyclonal anti-human HDAC2, -3, -6, -7, -8 (1:600, v/v) from Biovision, Mountain View, CA), monoclonal anti-beta actin (1:10,000, v/v) (Sigma) followed by secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:10,000 v/v) or anti-rabbit IgG (1: 5,000, v/v) (Amersham Bioscience UK, Little Chalfont, England). Detection was performed by the Enhanced Chemical Luminescence (ECL) method (Pierce, Rockford, IL).

### ***Cell cycle Analysis***

Cells were treated with various concentrations of amitriptyline for 48 hr, harvested, washed with cold PBS, suspended in 70% cold ethanol and incubated overnight at -20°C. Cells were then treated with 100 ng/ml of DNase-free RNase (Invitrogen) at 37°C for 30 min, washed with cold PBS, and resuspended in PBS with 50 µg/ml of propidium iodine. DNA content was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Palo Alto, CA). The percentage of cells in each phase of the cell cycle was calculated with ModiFit software (Becton Dickinson).

### ***Molecular Docking***

Amitriptyline was docked into the binding site of HDAC7 using the *CDOCKER* module in Discovery Studio molecular simulation package (version 2.5). The crystal structure of HDAC7 complexed with Trichostatin A (TSA) (PDB entry: 3C10) was used as the template for molecular docking. All water molecules in the crystal structure were removed. The binding pocket occupied by TSA was defined as the binding site using the *Define and edit binding site* command in Discovery Studio for molecular docking (2009).

### ***Molecular Dynamics (MD) Simulations***

The HDAC7-amitriptyline complex predicted by molecular docking studies was used as the initial structure for the following MD simulations. The atomic partial

charges of amitriptyline were derived by semi-empirical AM1 geometry optimization and subsequent single-point Hartree-Fock (HF)/6-31G\* calculations of the electrostatic potential, to which the charges were fitted using the RESP technique (Bayly et al., 1993) applied in Gaussian03. Partial charges and force field parameters of amitriptyline were generated automatically using the *antechamber* program in AMBER9.0 (Gohlke et al., 2003). In molecular mechanics minimizations and MD simulations, the AMBER03 force field (Duan et al., 2003) was used for HDAC7 and the general AMBER force field (*gaff*) was used for amitriptyline. The whole system was immersed in a rectangular box of TIP3P water molecules. The water box was extended 12 Å from solute atoms in all six dimensions. In molecular mechanics minimization and MD simulations, particle mesh Ewald (PME) was employed to treat the long-range electrostatic interactions (Darden et al., 1993). Before MD simulations, the complexes were relaxed by 5,000 cycles of minimization procedure (500 cycles of steepest descent and 4500 cycles of conjugate gradient minimization). After minimization, the system was gradually heated in the NVT ensemble from 10 K to 300 K over 30 ps. Initial velocities were assigned from a Maxwellian distribution at the starting temperature. We then performed 2 ns NPT MD simulations with a target temperature of 300 K and a target pressure of 1 atm. The SHAKE procedure was employed to constrain all hydrogen atoms, and the time step was set to 2.0 fs. The coordinates of the complex were saved every 2 ps. The molecular mechanics optimization and MD simulations were accomplished by using the *sander* program in AMBER9.0 (Gohlke et al., 2003).

### **Free energy Decomposition Analysis**

The interaction between amitriptyline and HDAC7 was decomposed by using the MM/GBSA decomposition procedure implemented in AMBER10 (Chang et al., 2008; Gohlke et al., 2003). We calculated van der Waals ( $\Delta E_{vdw}$ ), electrostatic ( $\Delta E_{ele}$ ), and desolvation ( $\Delta G_{GB} + \Delta G_{SA}$ ) energies between ligand and each of the protein residues. The polar contribution ( $\Delta G_{GB}$ ) of desolvation was computed using the modified GB model developed by Onufriev and coworkers. (Onufriev et al., 2004). The non-polar contribution of desolvation ( $\Delta G_{SA}$ ) was computed using the surface area. The charges used in GB calculations were taken from the AMBER parameter set. All energy components were calculated using 150 snapshots from 500 ps to 2 ns.

### ***Statistical Analysis***

Results were expressed as mean  $\pm$  SD. Treatment effects were compared using Student's *t* test and differences were considered to be significant when  $P < 0.05$ . All *in vitro* experiments were repeated at least three times.

## Results

### *Amitriptyline was identified as an inhibitor of cyclin D2 transactivation.*

To find inhibitors of cyclin D2 transactivation from a sublibrary of psychoactive agents, 5  $\mu\text{M}$  of each drug from our in-house library of 29 drugs (Supplementary Figure 1a) was applied to NIH3T3 cells stably expressing cyclin D2 promoter-driven luciferase. Cell viability and activity of luciferase were then examined after 24 hr incubation with each drug. Among the on- and off-patent antidepressants, amitriptyline was found to be the most potent inhibitor of cyclin D2 transactivation (Supplementary Figures 1a and 1b). Secondary studies were conducted to validate this hit and demonstrated that amitriptyline inhibited cyclin D2 transactivation in a concentration-dependent manner (Supplementary Figure 1c).

### *Amitriptyline inhibited cyclin D2 expression and arrested cells at G0/G1 phase of cell cycle.*

To evaluate the ability of amitriptyline to inhibit cyclin D2 transactivation in myeloma cell lines, we examined the effects of amitriptyline on cyclin D2 expression in MM cell lines (OPM2, LP1, KMS11, OCI-My5, and U266) with a spectrum of genetic abnormalities and gene overexpression. MM cells were incubated with 25  $\mu\text{M}$  of amitriptyline for 40 hrs. After incubation, levels of cyclin D2 were analyzed by immunoblotting. Amitriptyline decreased cyclin D2 in all of the tested cell lines (Figure 1a). We also demonstrated reduction in a dose-dependent manner (Figure 1b). As D-type cyclins are critical factors regulating cell cycle progression from G1 to

S phase, we examined changes in cell cycle after amitriptyline treatment. Cell cycle was measured by propidium iodide staining and analyzed by flow cytometry. Consistent with its effect on cyclin D2 protein expression, amitriptyline arrested cells in the G0/G1 phase (Figure 1c) in a dose-dependent manner. The arrest at G0/G1 phase was accompanied by the decrease of cell fractions at the S phase (Table 1).

***Amitriptyline decreased proliferation in myeloma but not leukemia cell lines.***

Reductions in D-cyclins and cell cycle arrest in G0/G1 can lead to decreased cell proliferation (Tiedemann et al., 2008). Therefore, we examined the effects of amitriptyline on the proliferation of myeloma and leukemia cells. Myeloma and leukemia cell lines were treated with increasing concentrations of amitriptyline and cell growth and viability were measured by the MTS assay at 24, 48 and 72 hours after treatment. Amitriptyline decreased the proliferation and viability of myeloma but not leukemia cells in a time- (Supplementary Figure 2) and dose- dependent manner (Figure 2a). The IC<sub>50</sub>s were approximately 25  $\mu$ M after 72 hr treatment for all tested myeloma cell lines including H929, JLN3, KMS11, KMS12, KMS18, LP1, OPM2 and RPMI-8226 (Figure 2a). In contrast, the drug was less active in the leukemia cell lines. Leukemia cells such as OCI-AML2 and Jurkat remained viability more than 80% after 72 hr treatment with 30  $\mu$ M of amitriptyline (Figure 2a).

***Amitriptyline induced cell apoptosis in myeloma cell lines and primary samples.***

To examine whether amitriptyline induced apoptosis in the myeloma cells, we

treated myeloma cells with 20  $\mu$ M amitriptyline for 24, 48 and 72 hrs, respectively, and then measured cell death and apoptosis by Annexin v-FITC and PI staining. Cell death and apoptosis was detected within 24 hours of amitriptyline treatment and > 50% of cells were apoptotic/dead within 48 hr of treatment in 4/4 cell lines, and > 90% cells became apoptotic/dead within 72 hr of treatment in 3/4 cell lines (Figure 2b). We also analyzed the effects of amitriptyline on primary myeloma samples. Bone marrow cells from MM patients were treated with increasing concentrations of amitriptyline and cell death and apoptosis was measured by staining with anti-CD138-PE and Annexin V-FITC and flow cytometry. Amitriptyline preferentially induced death and apoptosis in primary CD138 positive myeloma cells versus CD138 negative normal hematopoietic cells within 72 hours of treatment (Figure 2c). Notably, amitriptyline was not cytotoxic to normal blood cells (Figure 2c).

To further determine whether cell death is occurring via the apoptotic pathway, we examined apoptotic executive enzyme caspase-3 and associated PARP-1. Immunoblotting assays indicated that both caspase-3 and PARP-1 were cleaved in a concentration-dependent manner (Figure 2d). We also evaluated the effects of amitriptyline on p53 and XIAP by immunoblotting assay. The tumor suppressor protein p53 was increased in LP1 (Supplementary Figure 3). Amitriptyline also induced apoptosis in KMS11 cells in which p53 was absent, thus amitriptyline-induced apoptosis was p53-independent. Compared with p53, the inhibitor of apoptosis XIAP was only weakly decreased by amitriptyline (Supplementary Figure 3).

***Amitriptyline-induced cell apoptosis was synergistic with dexamethasone but partially blocked by serotonin.***

Dexamethasone is a primary treatment for myeloma and is used alone or in combination with other agents. To examine the effects of the co-treatment of dexamethasone and amitriptyline for myeloma, we treated myeloma cells JJN3 and OPM2 with amitriptyline (10  $\mu$ M) and dexamethasone (10  $\mu$ M) alone or in combination for 48 hr. When amitriptyline was used in combination with dexamethasone, cell viability was significantly decreased ( $P < 0.05$ , Figure 3a) and the reductions in viability with the combination were greater than the cell death induced by each individual agent.

Amitriptyline acts as an inhibitor of serotonin transporter (SERT) and SERT are frequently expressed in B-cell clones of diverse malignant origin including multiple myeloma (Meredith et al., 2005). Moreover, serotonin (or 5-hydroxytryptamine, 5-HT) was markedly elevated in the plasma of myeloma patients (Kurup et al., 2003). Therefore, we questioned whether amitriptyline-induced cell apoptosis could be associated with serotonin. Both KMS11 and KMS12 cells were treated amitriptyline, serotonin alone or the combination for 72 hr. Amitriptyline alone induced cell death, and the addition of serotonin partially abrogated this effect of amitriptyline in MM cells (Figure 3b). Taken together this result suggests that amitriptyline induced myeloma cell apoptosis at least partly by interfering with the serotonin pathway.



***Amitriptyline increased acetylation of Histone 3 and expression of p21 and p27.***

D-type cyclins can be epigenetically regulated by histone acetylation and HDAC inhibition resulting in decrease of D-cyclin expression and cell cycle arrest at the G1-S-transition (Bhaskara et al., 2008; Hu and Colburn, 2005). When HDACs are inhibited, their target proteins such as Histone 3 (H3) are hyperacetylated. To examine the inhibition of HDACs by amitriptyline, we analyzed the acetylation status of H3. Myeloma cell lines RPMI-8226 and LP1 were treated with amitriptyline at increasing concentrations for 40 hr. Whole cell lysates of these cells were then subjected to acetylated-H3 analysis by immunoblotting. We found that acetylated H3 was accumulated in both RPMI-8226 and LP1 cells by amitriptyline (Figure 4a). HDAC inhibition increased the expression of tumor suppressor genes such as p21 and p27. Therefore, we evaluated the abundance of p21 and p27 after amitriptyline treatment and found both p21 and p27 were induced by amitriptyline (Figure 4b).

***Amitriptyline downregulated histone deacetylase HDACs in myeloma cells.***

Increase of H3 acetylation can result from the downregulation of HDACs and/or inhibition of HDAC activity. We tested whether amitriptyline down-regulates HDAC expression and analyzed the expression of HDACs after 40 hr treatment with amitriptyline. These enzymes included Class I (HDAC2, -3 and -8) and Class II (HDAC6, -7) members. Results indicated that amitriptyline had no effect on HDAC2, but decreased at least one of the other 4 HDACs in tested myeloma cells (Figure 5). HDAC3 was decreased in cell lines LP1, KMS11, and OPM2 at 15  $\mu$ M and became

almost undetectable in KMS11 and OPM2 at 30  $\mu$ M. In KMS12 cells, although there was no HDAC3 mRNA (data not shown) or protein detected, HDAC6, -7 and -8 were decreased by amitriptyline (Figure 5) at 30  $\mu$ M.

***Amitriptyline was an inhibitor of histone deacetylase.***

We next investigated the direct inhibition of amitriptyline on HDACs using HDAC7 as a model by a series of molecular simulation techniques, including molecular docking studies, MD simulations, and free energy decomposition analysis. The HDAC7-amitriptyline complex predicted by molecular docking was submitted to MD simulations. The stability of the MD trajectory was monitored and was confirmed by the analysis of atom root mean square deviation (RMSD) as a function of time. As shown in Supplementary Figure 4, RMSD values for the amitriptyline-HDAC7 complex showed a rise in the first 500 ps, and then became stable for the remaining simulations.

In order to make a quantitative estimation of the amitriptyline-HDAC7 interactions, free energy decomposition analysis was conducted to decompose the total binding free energy into residue-ligand pairs, and the interaction spectrum of amitriptyline was shown in Figure 6. As shown in Figure 6a eleven residues gave favorable contribution to amitriptyline binding including Phe162, His192, Phe221, Leu293 and His326 (Figures 6a and 6b) and Phe221 was the most important one for amitriptyline binding ( $\Delta G_{\text{bind}} = -6.62$  kcal/mol) to HDACs. Further analysis of the complex structure indicated that the  $\pi$  system in amitriptyline was almost parallel to

that in Phe221 and formed a strong  $\pi$ - $\pi$  interactions that stabilized the amitriptyline-HDAC7 recognition. In addition to Phe221, another hydrophobic residue, Leu293, also formed effective van der Waals contacts with amitriptyline ( $\Delta G_{\text{bind}} = -5.56$  kcal/mol) and played an important role in the interaction.

Because zinc is the essential element in the active site of HDACs, we questioned whether amitriptyline interacts with zinc. The Zn ion probably formed ion-ion interactions with amitriptyline and three polar residues in HDAC7 including His192, Asp190 and Asp284 (Figure 6c). The fluctuation of the distance between Zn and the nitrogen atom in amitriptyline was shown in Figure 6d. This distance was 3.8 Å initially, and was decreased to ~2.5 Å after ~700 ps MD simulations and then remained relatively stable. Although the distance between Zn and the nitrogen atom in amitriptyline was stable, it should be noted that the coordinate bond between Zn and amitriptyline was weak. The analysis of the individual energy terms showed that the electrostatic interaction between Zn and amitriptyline was favorable (-6.82 kcal/mol). However, the unfavorable desolvation contribution could compensate the favorable electrostatic term (22.22 kcal/mol).

## Discussion

Amitriptyline is used for the treatment of psychiatric disturbances and pain in cancer patients (Fann et al., 2009). Although some studies have reported an association between antidepressants and the risk of breast cancer (Lash et al., 2010; Lawlor et al., 2003), other preclinical studies indicate that antidepressants display anti-cancer activities (Cloonan and Williams, 2010; Coogan et al., 2009; Xia et al., 1999). These discordant observations may relate to the activity of the individual antidepressants. Two classes of TCAs can be proposed based on their genotoxicity, and amitriptyline belongs to Class II, which are not genotoxic (Sharpe et al., 2002). In the present study, we demonstrated that amitriptyline is pro-apoptotic and displays anti-myeloma activity, but is not cytotoxic to normal blood cells.

Multiple signal pathways are possibly involved in amitriptyline-induced myeloma cell death, such as serotonin pathway and cell cycle progression, especially HDAC transcriptional regulation. Serotonin or 5-HT was found significantly elevated in the plasma of MM patients (Kurup et al., 2003), and its receptors such as 5-HT<sub>1A</sub> is expressed in T and B-lymphocytes. Addition of serotonin has also been shown increase the proliferation of B-cells (Abdoun et al., 2004), the origin of myeloma cells. We found that serotonin does not increase MM cell proliferation, but it partially reversed decreased cell proliferation of MM cells induced by amitriptyline, suggesting that serotonin is associated with amitriptyline-induced myeloma cell apoptosis.

Histone acetylation is an important modification for chromatin modeling by loosening chromatin and exposing DNA for transcription where acetylation is made

by histone acetyltransferases (HATs) and is reversed by HDACs. HDACs are a large family containing 11 members. Both Class I and Class II HDACs were downregulated by amitriptyline. Amitriptyline did not decrease HDAC2 expression, but decreased at least one of the other four HDACs. For example, HDAC3 was decreased in LP1, OPM2 and KMS11 but was undetectable in KMS12. HDAC3 is required for cell growth and apoptotic process via the regulation of pro-apoptotic genes (Karagianni and Wong, 2007) and responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4) that correlates with epigenetic repression (Karagianni and Wong, 2007). This deacetylation is involved in transcriptional regulation of genes important for cell cycle progression and development such as p21(WAF1/cip1)(Huang et al., 2006) which was induced by amitriptyline. HDAC6 is involved in  $\alpha$ -tubulin acetylation, cell motility and proteasomal function regulation (Wickstrom et al., 2010). HDAC6 knockdown causes a decrease in the steady-state level of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor alpha in A549 lung cancer cells (Kamemura et al., 2008). But its relevance to amitriptyline-induced myeloma cell apoptosis is yet to study.

In addition to decreasing the protein levels of HDACs, molecular docking simulations showed that amitriptyline was capable of binding to HDAC and interfering with their enzymatic activity. Amitriptyline interacts with HDAC7 and forms effective van der Waals contacts with HDAC7 by *in silico* analysis. Eleven residues of HDAC7 can interact with amitriptyline, and 5 of them, including Phe162,

His192, Phe221, Leu293 and His326, are the most important contributors in this interaction. Furthermore, the  $\pi$  system in amitriptyline forms strong  $\pi$ - $\pi$  interactions with HDAC and stabilizes the amitriptyline-HDAC7 recognition. Thus, our study indicates that amitriptyline interferes with HDAC function by two mechanisms, downregulation of HDAC expression direct inhibition on HDAC activity.

D-cyclins are functionally important for the pathogenesis and progression of myeloma and also predict patient outcome (Bergsagel and Kuehl, 2003; Bergsagel et al., 2005). Thus, downregulating D-cyclins could be important therapeutically for the treatment of myeloma. Amitriptyline downregulated cyclin D expression, especially cyclin D2, which is dysregulated in more than 50% of MM cell lines and primary patients. Amitriptyline decreased D-cyclin expression, thus arresting MM cells at G1 phase and decreasing fraction at S phase. Mechanistically, amitriptyline-mediated decreases in cyclin D expression were due to its inhibition of CCND2 promoter transactivation, or inhibition on cyclin D transcription via the HDAC pathway. Previous studies indicated that inhibition of HDACs leads to hyperacetylation of transcription factor NF $\kappa$ B (Hu and Colburn, 2005), a key regulator of cyclin D expression. Hyperacetylation of NF $\kappa$ B/p52 prevents NF $\kappa$ B/p65 binding to cyclin D promoter (Hu and Colburn, 2005). Another possible mechanism is NF $\kappa$ B/p65 acetylation, which is deacetylated by HDAC3 (Kiernan et al., 2003). NF $\kappa$ B/p65 acetylation will facilitate its removal from DNA and consequently its I $\kappa$ B $\alpha$ -mediated export from nucleus (Kiernan et al., 2003). Thus, amitriptyline downregulated HDACs and interfered with HDAC activity, which led to NF $\kappa$ B hyperacetylation and

decreased cyclin D transcription. The inhibition of HDACs also led to the induction of other key cell cycle regulators such as p21 and p27 in various cell lines, consistent with previous report (Hu and Colburn, 2005; Huang et al., 2006).

Thus, by *in vitro* and *in silico* investigations, we demonstrate that amitriptyline induces MM cell apoptosis by decreasing HDAC expression and inhibiting HDAC activity, and it might favor the therapy for MM patients as part of a supportive or chemotherapeutic regimen but further evaluation *in vivo* and in a clinical setting is necessary.

#### **Authorship Contributions**

Participated in research design: Mao, Schimmer, Hou.

Conducted experiments: Mao, Hou, Cao, Wang, Li, Hurren, Gronda.

Contributed new reagents or analytic tools: Hou, Chen, Fei, Wu, Trudel

Performed data analysis: Mao, Hou, Schimmer

Wrote or contributed to the writing of the manuscript: Mao, Schimmer, Hou.

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## Footnotes

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## Legends for the figures

Fig.1 Amitriptyline decreases cyclin D2 expression and arrested cell cycle at G0/G1 phase. **a**, amitriptyline (Amit, 25 $\mu$ M) was added to various MM cell lines and incubated for 40 hrs before cell lysates were prepared in RIPA buffer for immunoblotting against cyclin D2 specific antibody. **b**, amitriptyline decreased cyclin D2 protein in a concentration-dependent manner after 40 hr treatment. **c**, KMS11 and LP1 cells were treated with amitriptyline at indicated concentration for 40 hr, followed by PI staining for cell cycle analysis on a flow cytometer. G0/G1 phase cells were plotted against amitriptyline concentration.

Fig. 2 Amitriptyline decreases proliferation and induces apoptosis in MM cells. **a**, MM cell lines (H929, JJN3, KMS11, KMS12, KMS18, LP1, OPM2 and RPMI-8226) and leukemia cell lines (AML2, Jurkat) were incubated with increased amitriptyline for 72hrs. Cell viability was measured by MTS assay. **b**, MM cell lines were incubated with 20  $\mu$ M of Amit for 24, 48 or 72 hrs. Cells were then collected for Annexin V-FITC and PI staining before subjected to cytometric assay. **c**, Amitriptyline preferentially induced cell apoptosis in primary myeloma cells. Primary bone marrow species from MM patients and peripheral blood stem cells (PBSC) from healthy donors were purified as described in *Material and Methods* and then incubated with amitriptyline at indicated concentration for 72 hr. Cells were collected and

stained with anti-CD138-PE and annexin v-FITC. CD138+/Annexin V- myeloma cells underwent apoptosis and became CD138-/Annexin V+ after amitriptyline treatment. **d**, MM cell lines KMS11 and KMS12 were treated with Amit for 48 hrs. Cell lysates were prepared in RIPA buffer followed by SDS-PAGE and immunoblotting assay against human polyclonal antibody caspase-3 and PARP-1.

Fig. 3 Effects of Dexamethasone and 5-HT on amitriptyline-decreased MM viability.

**a**, Five MM cell lines were treated with Amit (10 $\mu$ M) and/or Dexamethasone (Dex, 10 $\mu$ M) for 48 hr, here JJN3 and OPM2 cell viabilities were reported as a representative. Viable cells with combined treatment of Dex and Amit were significantly decreased compared with those treated by Dex or Amit alone. **b**, KMS11 and KMS12 cells were treated with 20 $\mu$ M of Amit in the presence or absence of 20  $\mu$ M of 5-HT for 72 hr. Cells viability was measured by MTS assay.

Fig. 4 Amitriptyline increases acetylation of H3 and expression of p21 and p27. **a**,

Amitriptyline increased H3 acetylation. MM cells RPMI-8226 and LP1 were treated with amitriptyline at indicated concentration for 40 hrs, nuclear fragments were evaluated by immunoblotting using anti-acetylated H3 (Ac-H3). Tricostatin A (TSA) was used as a positive control and  $\beta$ -actin was used as a loading control. **b**, Amitriptyline induced expression of p21 and p27.

Myeloma cells were treated with Amit for 40 hrs at indicated concentrations followed by immunoblotting assay against specific human p21 and 27 antibodies.

Fig. 5 Amitriptyline decreases HDAC3, -6 and -7 but not HDAC2 or -8 in MM cells.

MM cell lines LP1, KMS11, OPM2 and KMS12 were incubated with amitriptyline for 40 hrs. Cells were then lysed and the nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) followed by immunoblotting assay using HDAC specific antibodies HDAC2, -3, -6, -7, -8 or tubulin. Relative signals were expressed as (density of the protein)/(density of tubulin) and normalized to the vehicle-treated controls.

Fig.6 Interactions between amitriptyline and HDAC7 predicted by molecular simulation techniques. **a**, amitriptyline-HDAC7 interaction spectrum given by the MM/GBSA free energy decomposition analysis. The x-axis denotes the residue number of HDAC7 and the y-axis denotes the interaction energy between the inhibitor and specific residues. **b**, Geometries of the residues within 5 Å of amitriptyline. Amitriptyline is colored in yellow and the five important residues which can form strong interactions with HDAC7 are labeled and colored in blue. **c**, The 2-D representation of the amitriptyline-HDAC7 interactions. **d**, The distance fluctuation between the Zn ion and the nitrogen atom in amitriptyline.

Table 1 Amitriptyline arrested myeloma cells at G0/G1 phase.

| KMS11<br>Amit ( $\mu$ M) | G0/G1             | S                 | G2/M             |
|--------------------------|-------------------|-------------------|------------------|
| 0                        | 52.66 $\pm$ 0.56  | 35.89 $\pm$ 0.52  | 11.45 $\pm$ 0.5  |
| 5                        | 56.5 $\pm$ 1.23   | 32.8 $\pm$ 0.68   | 10.7 $\pm$ 0.43  |
| 20                       | 64.21 $\pm$ 1.96* | 26.26 $\pm$ 1.2*  | 9.53 $\pm$ 0.98  |
|                          |                   |                   |                  |
| LP1<br>Amit ( $\mu$ M)   | G0/G1             | S                 | G2/M             |
| 0                        | 43.46 $\pm$ 2.31  | 43.42 $\pm$ 1.57  | 13.12 $\pm$ 1.1  |
| 5                        | 48.44 $\pm$ 1.9   | 39.26 $\pm$ 0.59  | 12.3 $\pm$ 0.89  |
| 20                       | 58.47 $\pm$ 2.45* | 28.94 $\pm$ 1.32* | 12.59 $\pm$ 1.12 |

Note: Cells were treated with Amitriptyline at indicated concentrations for 24 hr followed by ethanol fixation at 4°C. Cells were then stained with Propidium iodide and analyzed on a cytometer. \*  $p < 0.01$ , compared with vehicle-treated control cells.

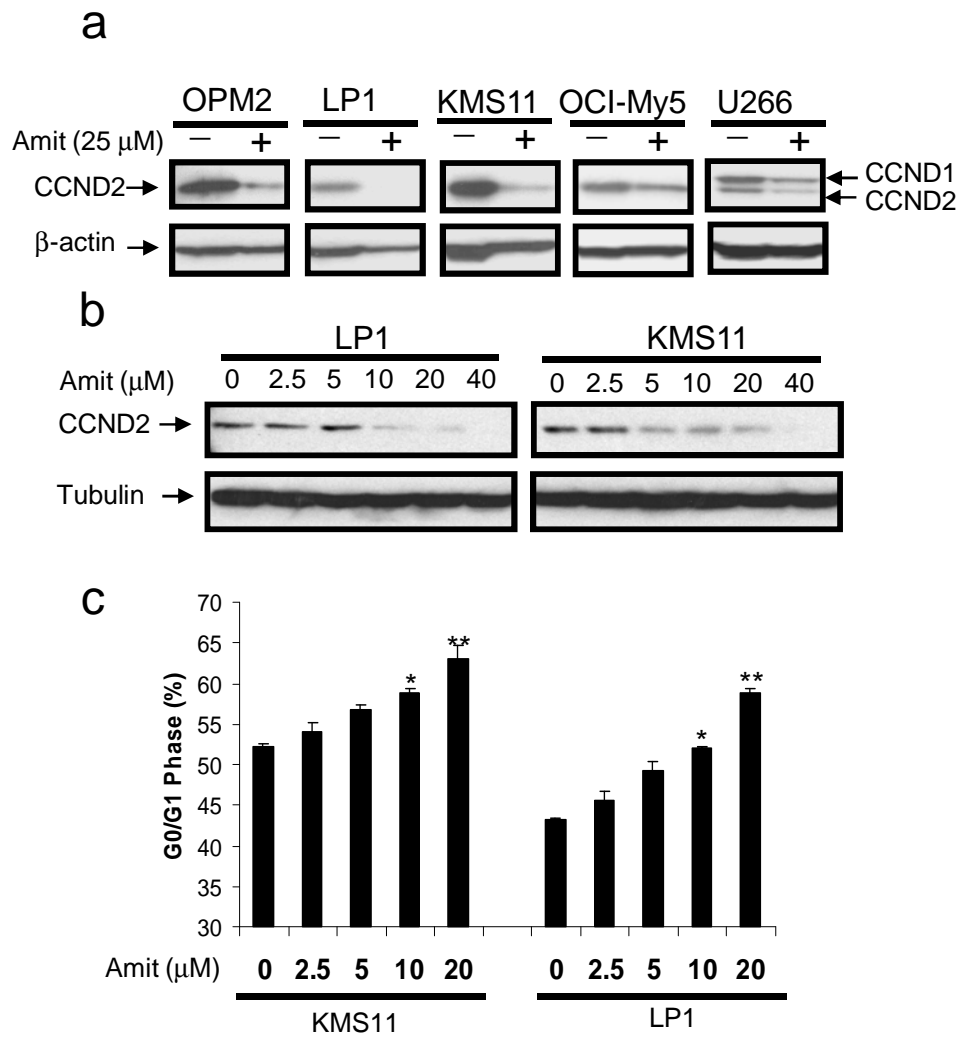


Figure 1



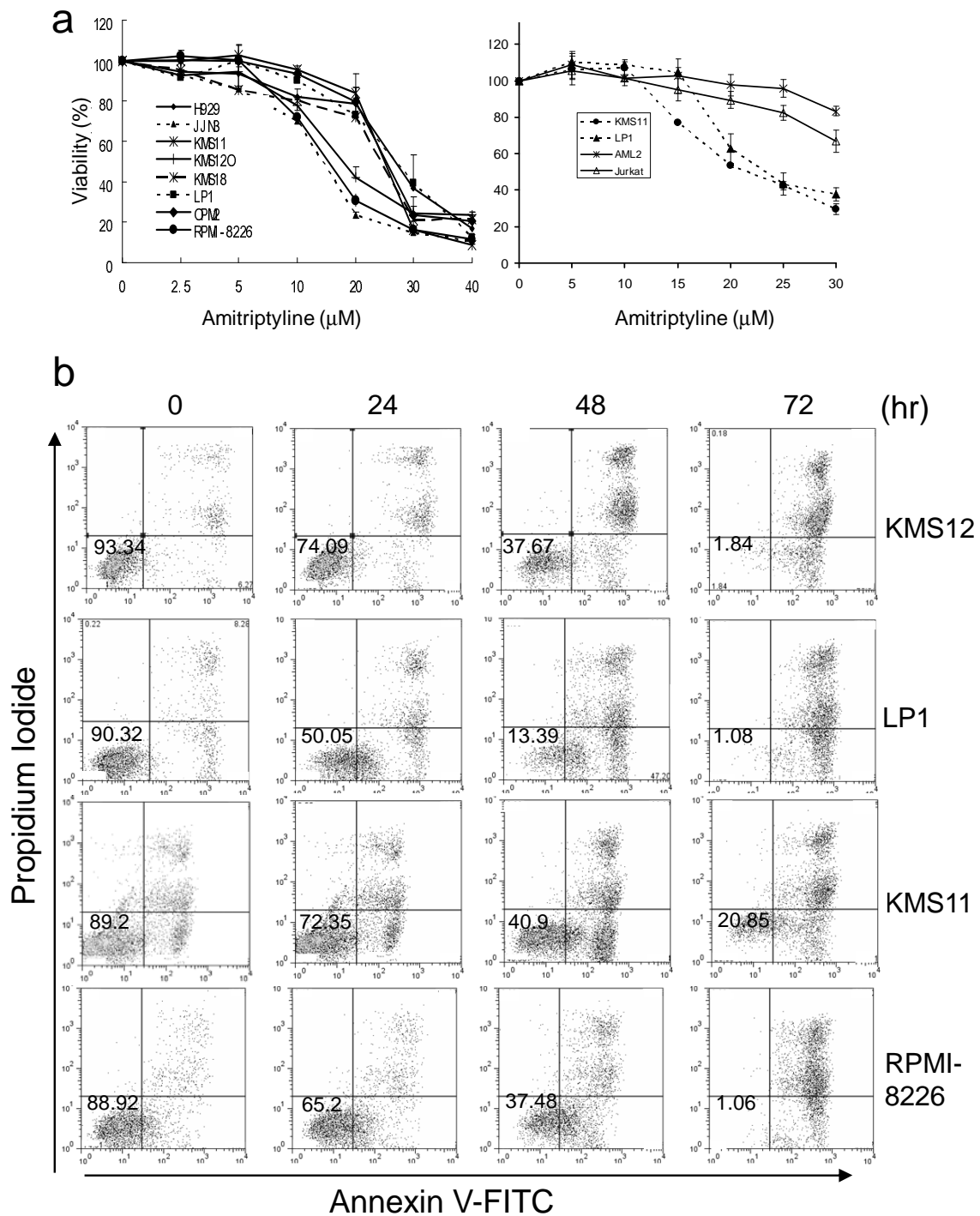


Figure 2

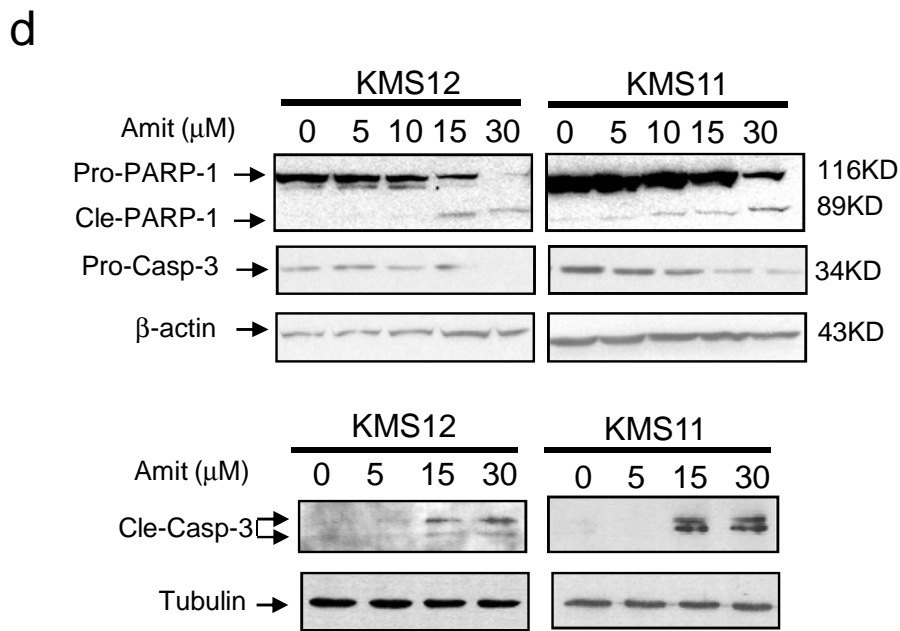
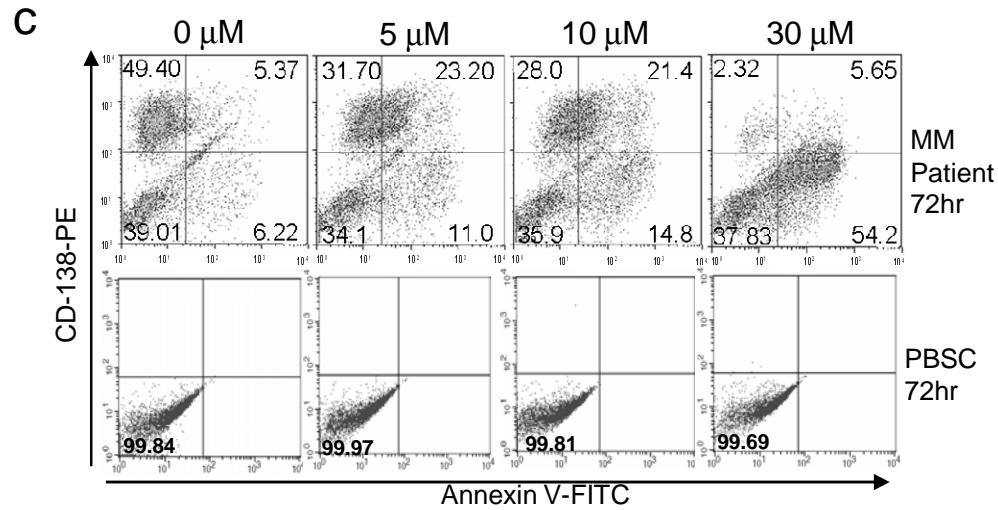


Figure 2 continued

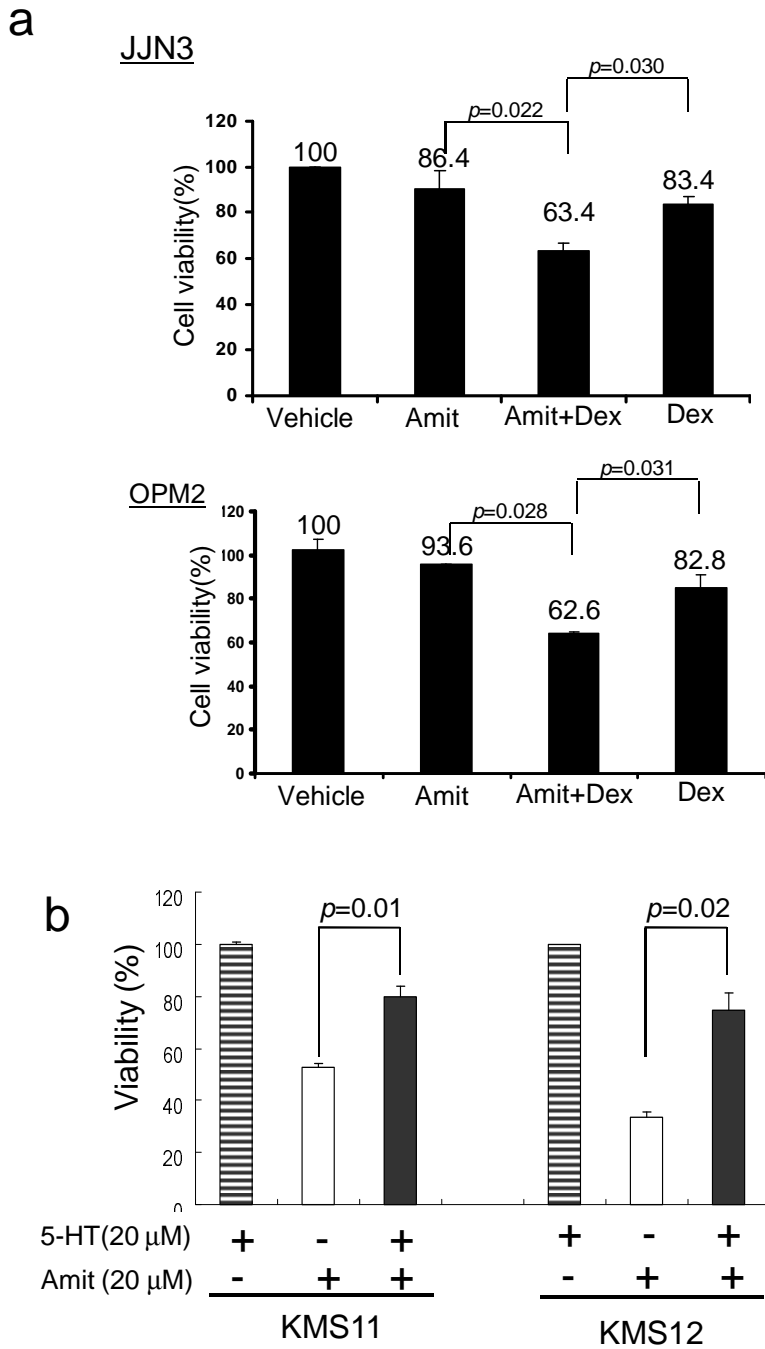


Figure 3

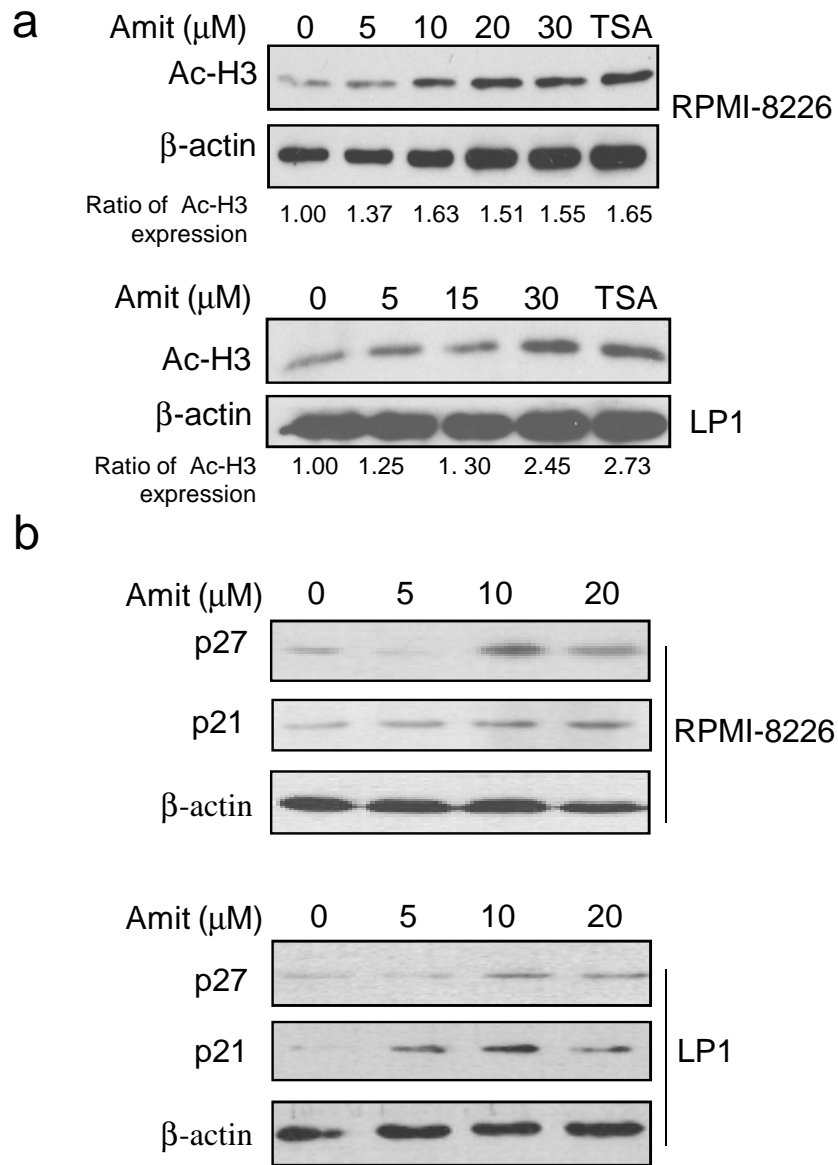
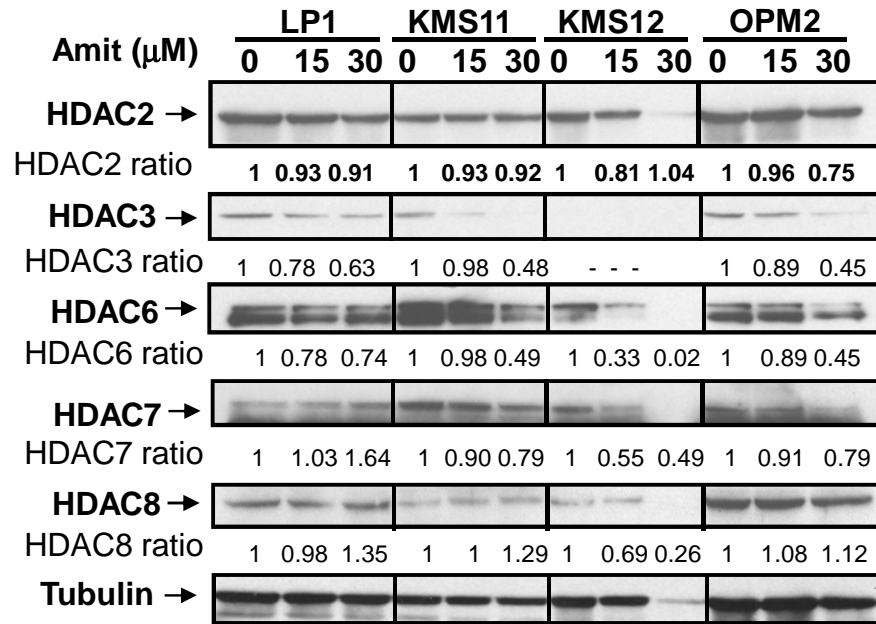


Figure 4



**Figure 5**

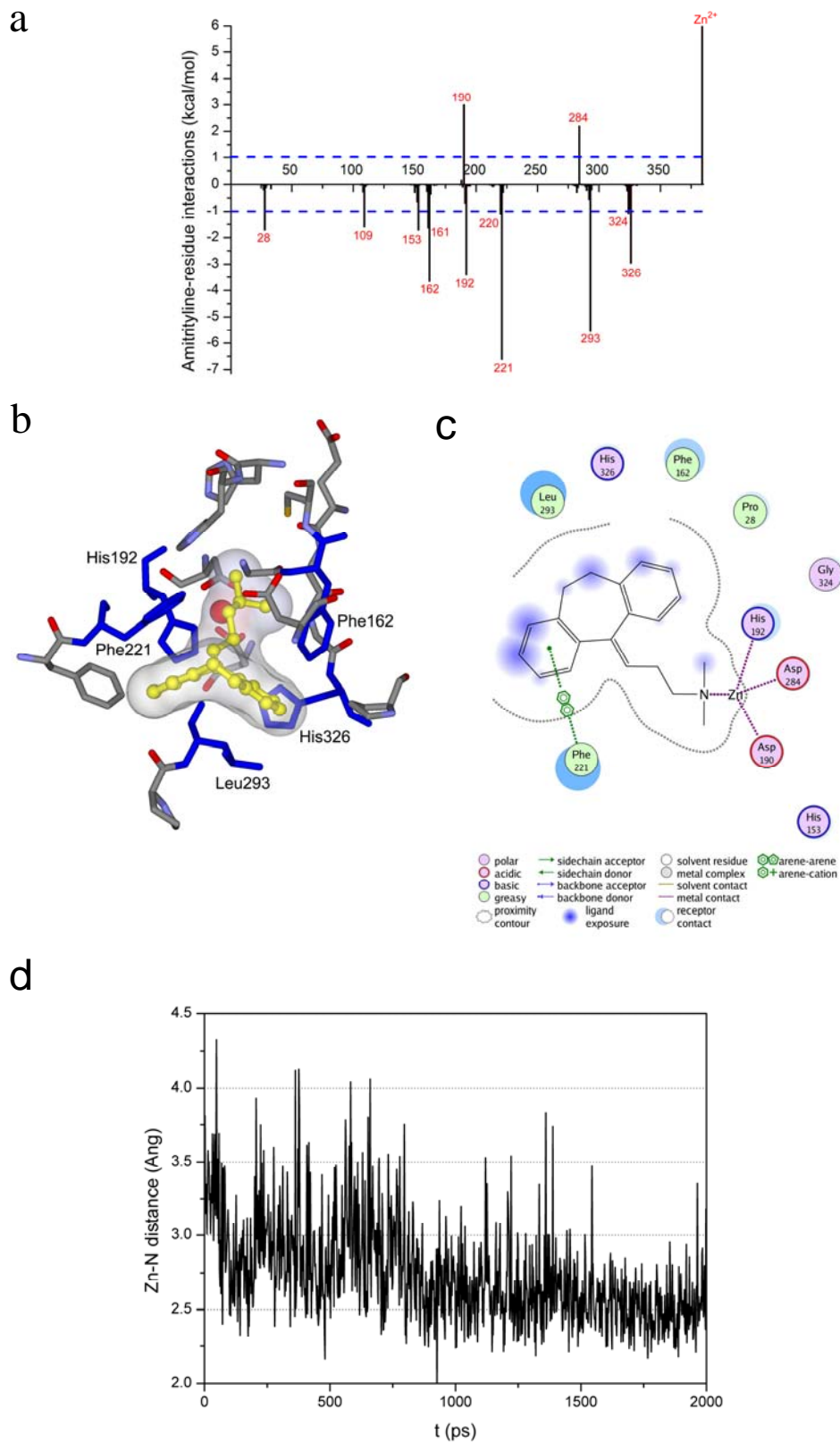


Figure 6