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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ABSTRACT

Amitriptyline is a classic tricyclic antidepressant (TCA) and has been used to treat the depression and anxiety of patients with cancer, 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 antmyeloma 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, and cleavage of caspase-3 and poly(ADP-ribose) polymerase-1. D-Cyclin expression is reported to be epigenetically regulated by histone acetylation. Thus, we examined the effects of amitriptyline on histone 3 (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 down-regulation 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, serotonin norepinephrine reuptake inhibitors, and monoamine oxidase inhibitors. These agents have been used to treat patients' psychiatric disorders, such as major depression and anxiety. These antidepressants are prescribed for patients with cancer as a supportive treatment (Mishra et al., 2008). Recent studies found that antidepressants kill cancer cells (Stepulak et al., 2008; Argov et al., 2009). For example, antidepressants such as fluoxetine can induce cancer cell apoptosis and displayed anticancer activity in various cancers such as glioma, bladder cancer, and blood cancers (Stepulak et al., 2008; Argov et al., 2009). Selective serotonin reuptake inhibitors and TCAs such as sertraline and paroxetine display potent antileukemia/antilymphoma activity 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 anticancer activity in both colon cancer cell lines and in colorectal cancer-xenografted mice by inducing

ABREVIATIONS: TCA, tricyclic antidepressant; 5-HT, 5-hydroxytryptamine; H3, Histone3; HDAC, histone deacetylase; MM, multiple myeloma; PBSC, peripheral blood stem cell; PI, propidium iodide; TSA, trichostatin A; PBS, phosphate-buffered saline; PARP-1, poly(ADP-ribose) polymerase-1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; FITC, fluorescein isothiocyanate; MD, molecular dynamics; XIAP, X-linked inhibitor of apoptosis protein; NF-κB, nuclear factor κB.
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 biological studies suggest that amitriptyline may have significant anticancer effects. For example, a recent study indicated that amitriptyline inhibits cellular respiration at concentrations of 0.14 to 0.5 mM and induces death in glioma cells (Pilkington et al., 2008; Higginson and Pilkington, 2010). Another study indicated that amitriptyline at 50 μM 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 remissions are limited even with newer agents such as lenalidomide and bortezomib (Richardson et al., 2010). Thus, it is necessary to develop novel drugs for this disease.

Multiple myeloma is molecularly 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 used 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’s modified Dulbecco’s medium (Invitrogen, Carlsbad, CA). Leukemia cell lines were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 100 μg/ml penicillin, and 100 U/ml streptomycin (HyClone Laboratories).

Primary Multiple Myeloma Samples. Bone marrow from patients with MM was obtained from Princess Margaret Hospital, University Health Network, Toronto, with University Health Network, Toronto’s 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 Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, and appropriate antibiotics.

Chemicals and Compiling of Antidepressant Library. Psychoactive drugs were selected from Library of Pharmacologically Active Compounds 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 the method described previously (Mao et al., 2007). In brief, NIH3T3 cells stably expressing the cyclin D2 promoter driving firefly luciferase (10,000 cells/well) were plated in 96-well plates by a Biomek FX liquid handler (Beckman Coulter, Fullerton, CA). After the cells had adhered, they were treated with aliquots of the sublibrary at a final concentration of 5 μM and 0.1% dimethyl sulfoxide at 37°C for 20 h. 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 as described previously (Mao et al., 2007).

Cell Viability. Cell viability was assessed with the CellTiter 96 Aqueous Non-Radioactive Assay kit (MTS assay) according to the manufacturer’s instructions (Promega). Apoptosis was measured by flow cytometry with Annexin-V-FITC and propidium iodide (PI) staining (BioVision, Mountain View, CA) as described previously (Mao et al., 2007). For primary myeloma cells, purified cells were labeled with both CD138-PE (eBioScience, San Diego, CA) and Annexin-V-FITC after amitriptyline treatment and analyzed on a flow cytometer as described previously (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 μg) of proteins was subjected to fractionation by SDS-polyacrylamide gel electrophoresis followed by transfer to polyvinyldene difluoride membranes. Membranes were probed with antibodies including monoclonal anti-human cyclin D1 [1:200 (v/v)], polyclonal anti-human caspase-3 [1:5000 (v/v)], anti-human poly-ADP-ribose) polymerase 1 (PARP-1) [1:1000 (v/v)], anti-human p21 and p27 [1:1000 (v/v)], and polyclonal anti-human XIAP [1:1000 (v/v)] from Cell Signaling Technology (Danvers, MA); 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 (Santa Cruz, CA); polyclonal anti-human HDAC2, -3, -6, -7, and -8 [1:600 (v/v)] from BioVision; and monoclonal anti-β-actin [1:10,000 (v/v)] (Sigma-Aldrich) followed by secondary horseradish peroxidase-conjugated goat anti-mouse [1:10,000 (v/v)] or anti-rabbit IgG [1:5000 (v/v)] (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Detection was performed by the enhanced chemical luminescence method (Thermo Fisher Scientific, Waltham, MA).

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

Molecular Docking. Amitriptyline was docked into the binding site of HDAC7 using the CDOCKER module in Discovery Studio molecular simulation package (version 2.5; Accelrys, San Diego, CA). The crystal structure of HDAC7 complexed with trichostatin A (TSA) (Protein Data Bank 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.

Molecular Dynamics Simulations. The HDAC7-amitriptyline complex predicted by molecular docking studies was used as the initial structure for the following molecular dynamics (MD) simulations. The atomic partial charges of amitriptyline were derived by
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 µM amitriptyline for 40 h. After incubation, levels of cyclin D2 were analyzed by immunoblotting. Amitriptyline decreased cyclin D2 in all of the tested cell lines (Fig. 1a). We also demonstrated a reduction in a dose-dependent manner (Fig. 1b). Because G0-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 (Fig. 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).

**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 µM concentration of each drug from our in-house library of 29 drugs (Supplementary Fig. 1a) was applied to NIH3T3 cells stably expressing cyclin D2 promoter-driven luciferase. Cell viability and activity of luciferase were then examined after 24 h of 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 Fig. 1a and b). Secondary studies were conducted to validate this hit and demonstrated that amitriptyline inhibited cyclin D2 transactivation in a concentration-dependent manner (Supplementary Fig. 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 µM amitriptyline for 40 h. After incubation, levels of cyclin D2 were analyzed by immunoblotting. Amitriptyline decreased cyclin D2 in all of the tested cell lines (Fig. 1a). We also demonstrated a reduction in a dose-dependent manner (Fig. 1b). Because G0-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 (Fig. 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).

**Fig. 1.** Amitriptyline decreases cyclin D2 expression and arrested cell cycle at G0/G1 phase. a, amitriptyline (Amit, 25 µM) was added to various MM cell lines and incubated for 40 h before cell lysates were prepared in radioimmunoprecipitation assay buffer for immunoblotting against cyclin D2-specific antibody. b, amitriptyline decreased cyclin D2 protein in a concentration-dependent manner after 40 h treatment. c, KMS11 and LP1 cells were treated with amitriptyline at indicated concentration for 40 h, followed by PI staining for cell cycle analysis on a flow cytometer. G0/G1 phase cells were plotted against amitriptyline concentration. *P < 0.05; **P < 0.01 compared with the vehicle controls.

**TABLE 1**

<table>
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<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<tr>
<td>KMS11</td>
<td></td>
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<tr>
<td>0 µM Amitriptyline</td>
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<td>35.89 ± 0.52</td>
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<td>32.8 ± 0.68</td>
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<td>20 µM Amitriptyline</td>
<td>64.21 ± 1.96*</td>
<td>26.26 ± 1.2*</td>
<td>9.53 ± 0.98</td>
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<tr>
<td>LP1</td>
<td></td>
<td></td>
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<tr>
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<td>43.42 ± 1.57</td>
<td>13.12 ± 1.1</td>
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<tr>
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<td>12.3 ± 0.89</td>
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<tr>
<td>20 µM Amitriptyline</td>
<td>58.47 ± 2.45*</td>
<td>28.94 ± 1.32*</td>
<td>12.59 ± 1.12</td>
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*P < 0.01 compared with vehicle-treated control cells.
Amitriptyline Decreased Proliferation in Myeloma but Not Leukemia Cell Lines. Reductions in D-cyclins and cell cycle arrest in G1/S 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 h after treatment. Amitriptyline decreased the proliferation and viability of myeloma but not leukemia cells in a time- (Supplementary Fig. 2) and dose-dependent manner (Fig. 2). The IC_{50} values were approximately 25 μM after 72 h treatment for all tested myeloma cell lines including H929, JJN3, KMS11, KMS12, KMS18, LP1, OPM2, and RPMI-8226 (Fig. 2a). In contrast, the drug was less active in the leukemia cell lines. Leukemia cells such as OCI-AML2 and Jurkat remained more than 80% viable after 72 h treatment with 30 μM amitriptyline (Fig. 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 μM amitriptyline for 24, 48, and 72 h, respectively, and then measured cell death and apoptosis by Annexin V-FITC and PI staining. Cell death and apoptosis was detected within 24 h of amitriptyline treatment and >50% of cells were apoptotic/dead within 48 h of treatment in four of four cell lines, and >90% cells became apoptotic/dead within 72 h of treatment in three of four cell lines (Fig. 2b). We also analyzed the effects of amitriptyline on primary myeloma samples. Bone marrow cells from patients with MM were treated with increasing concentrations of amitriptyline, and cell death and apoptosis were examined 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 h of treatment (Fig. 2c). It is noteworthy that amitriptyline was not cytotoxic to normal blood cells (Fig. 2c).

Amitriptyline acts as an inhibitor of serotonin transporter, and serotonin transporters 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, or the combination for 72 h. Amitriptyline alone induced cell death, and the addition of serotonin partially abrogated this effect of amitriptyline in MM cells (Fig. 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 (Hu and Colburn, 2005; Bhaskara et al., 2008). 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 h. 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 (Fig. 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 that both p21 and p27 were induced by amitriptyline (Fig. 4b).

Amitriptyline Down-Regulated Histone Deacetylase HDACs in Myeloma Cells. Increase of H3 acetylation can result from the down-regulation of HDACs and/or inhibition of HDAC activity. We tested whether amitriptyline down-regulates HDAC expression and analyzed the expression of HDACs after 40-h treatment with amitriptyline. These enzymes included class I (HDAC2, -3, and -8) and class II (HDAC6 and -7) members. Results indicated that amitriptyline had no effect on HDAC2 but decreased at least one of the other four HDACs in tested myeloma cells (Fig. 5). HDAC3 was decreased in cell lines LP1, KMS11, and OPM2 at 15 μM and became almost undetectable in KMS11 and OPM2 at 30 μM. In KMS12 cells, although there was no HDAC3 mRNA (data not shown) or protein detected, HDAC6, -7, and -8 were decreased by amitriptyline (Fig. 5) at 30 μM.

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 confirmed by the analysis of atom root mean square deviation as a function of time. As shown in Supplemental Fig. 4, root mean square deviation values for the amitriptyline-HDAC7 complex showed an increase in the first 500 ps and then became stable for the remaining simulations.
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 72 h. Cell viability was measured by MTS assay. b, MM cell lines were incubated with 20 μM amitriptyline (Amit) for 24, 48, or 72 h. 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 patients with MM and PBSCs from healthy donors were purified as described under Materials and Methods and then incubated with amitriptyline at indicated concentration for 72 h. 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 h. Cell lysates were prepared in radioimmunoprecipitation assay buffer followed by SDS-polyacrylamide gel electrophoresis and immunoblotting assay against human polyclonal antibody caspase-3 and PARP-1.
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 is shown in Fig. 6. As shown in Fig. 6a, 11 residues gave favorable contribution to amitriptyline binding including Phe162, His192, Phe221, Leu293, and His326 (Fig. 6a and b) and Phe221 was the most important one for amitriptyline binding ($\Delta G_{\text{bind}} = -6.62 \text{ kcal/mol}$) 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 ($\Delta G_{\text{bind}} = -5.56 \text{ kcal/mol}$) 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 zinc ion probably formed ion-ion interactions with amitriptyline and three polar residues in HDAC7, including His192, Asp190, and Asp284 (Fig. 6c). The fluctuation of the distance between zinc and the nitrogen atom in amitriptyline is shown in Fig. 6d. This distance was 3.8 Å initially and was deceased to $\sim 2.5 \text{ Å}$ after $\sim 700$-ps MD simulations and then remained relatively stable. Although the distance between zinc and the nitrogen atom in amitriptyline was stable, it should be noted that the coordinate bond between

![Fig. 3. Effects of dexamethasone and 5-HT on amitriptyline decreased MM viability.](image-url)

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![Fig. 4. Amitriptyline increases acetylation of H3 and expression of p21 and p27.](image-url)
zinc and amitriptyline was weak. The analysis of the individual energy terms showed that the electrostatic interaction between zinc 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 patients with cancer (Fann et al., 2009). Although some studies have reported an association between antidepressants and the risk of breast cancer (Lawlor et al., 2003; Lash et al., 2010), other preclinical studies indicate that antidepressants display anticancer activities (Xia et al., 1999; Coogan et al., 2009; Cloonan and Williams, 2011). 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 proapoptotic and displays antymyeloma 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 (5-HT) was found significantly elevated in the plasma of patients with MM (Kurup et al., 2002). In the present study, we demonstrated that serotonin is involved in the transcriptional regulation of genes important for cell cycle progression and development, such as HDACs. Amitriptyline down-regulated HDACs by amitriptyline. HDAC6 is involved in α-tubulin acetylation, cell motility, and proteasomal function regulation (Wickström et al., 2010). HDAC6 knockdown causes a decrease in the steady-state level of receptor tyrosine kinases, such as epidermal growth factor receptor and platelet-derived growth factor receptor-α in A549 lung cancer cells (Kamemura et al., 2008), but its relevance to amitriptyline-induced myeloma cell apoptosis has yet to be studied.

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 five of them, including Phe162, His192, Phe221, Leu293, and His326, are the most important contributors in this interaction. Furthermore, the π system in amitriptyline forms strong π–π interactions with HDAC and stabilizes the amitriptyline-HDAC7 recognition. Thus, our study indicates that amitriptyline interferes with HDAC function by two mechanisms: down-regulation of HDAC expression, and direct inhibition of 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, down-regulating d-cyclins could be important therapeutically for the treatment of myeloma. Amitriptyline down-regulated cyclin D expression, especially cyclin D2, which is dysregulated in more than 50% of MM cell lines and in primary patients. Amitriptyline deceased β-cyclin expression, thus arresting MM cells at G1 phase and decreasing fraction at S phase. Amitriptyline-mediated decreases in cyclin D expression were due to its inhibition of CCND2 promoter transactivation or inhibition of cyclin D transcription via the HDAC pathway. Previous studies indicated that inhibition of HDACs leads to hyperacetylation of transcription factor NF-κB (Hu and Colburn, 2005), a key regulator of cyclin D expression. Hyperacetylation of NF-κB/p52 prevents NF-κB/p65 binding to cyclin D promoter (Hu and Colburn, 2005). Another possible mechanism is NF-κB/p65 acetylation, which is deacetylated by HDAC3 (Kiernan et al., 2003). NF-κB/p65 acetylation will facilitate its removal from DNA and consequently its IκBα-mediated export from nucleus (Kiernan et al., 2003). Thus, amitriptyline down-regulated HDACs and interfered with HDAC activity, which led to NF-κB hyperacetylation and decreased cyclin D transcrip-

![Fig. 5. Amitriptyline decreases HDAC3, -6, -7, and -8, but not HDAC2 in MM cells. MM cell lines LP1, KMS11, OPM2, and KMS12 were incubated with amitriptyline for 40 h. Cells were then lysed, and the nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA) followed by immuno-blotting 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.](atp008703.jpg)
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 that can form strong interactions with HDAC7 are labeled and colored in blue. 

c. the two-dimensional representation of the amitriptyline-HDAC7 interactions. 

d. the distance fluctuation between the zinc ion and the nitrogen atom in amitriptyline.
tion. 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 reports (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 patients with MM as part of a supportive or chemotherapeutic regimen. Further evaluation in vivo and in a clinical setting is necessary.

Authorship Contributions

Participated in research design: Mao, Hou, and Schimmer.

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

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

Performed data analysis: Mao, Hou, and Schimmer.

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

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Gronda.


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

References


Suppl. Fig. 1 Identification of amitriptyline as a inhibitor of cyclin D2 transactivation. Amtriptyline was found to decrease cyclin D2-driving luciferase expression among an array of antidepressants. These compounds were purchased from Sigma-Aldrich Co. Ltd., St. Louis, MO. 1, Pirenzepine; 2, Amoxapine; 3, Bupropion; 4, Indatraline; 5, Alaproclate; 6, Trimipramine; 7, Citalopram; 8, Fluvoxamine; 9, Maprotiline; 10, Methiothepin; 11, Zipindine; 12, Protriptyline; 13, Mianserine; 14, Imipramine; 15, Fluoxetine; 16, Guipazine; 17, P-MPPF; 18, Ketanserin; 19, Ritanserin; 20, Desipramine; 21, Methysergide; 22, Metyergoline; 23, Reserpine; 24, trazodone; 25, Cyclobenzapine; 26, Piperezine; 27, Nortriptyline; 28, Clomipramine; 29, Amitriptyline. b, Amitriptyline chemical structure. c, Amitriptyline inhibited cyclin D2 promoter transactivation in a concentration-dependent manner. Amitriptyline at indicated concentration was added to NIH3T3 cells expressing pCCND2.Luci for 24 hrs, followed by luciferase activity assay using Bright-Glo agent as described in the Materials and Methods.

Suppl. Fig. 2. M cells (KMS11, OPM2, KMS12, LP1, My5 and RPMI-8226) were treated with 25 μM of amitriptyline for 24, 48 or 72 hr, followed by cell viability determination by MTS assays.

Suppl. Fig. 3. MM cell lines LP1 and KMS11 were treated with Amitriptyline (Amit) for 48 hrs followed by immunoblotting assay against human specific antibody for p53 and XIAP. The ratios of the protein levels after treatment against control were calculated.

Suppl. Fig. 4. Root-mean-square displacement (RMSD) of the heavy atoms of the HDAC-7-amitriptyline complex with respect to the first snapshot as a function of time.