KD5170, a novel mercaptoketone-based histone deacetylase inhibitor, exerts ant myeloma effects by DNA damage and mitochondrial signaling

Rentian Feng,1 Huihui Ma,1 Christian A. Hassig,2 Joseph E. Payne,2 Nicholas D. Smith,2 Markus Y. Mapara,1 Jeffrey H. Hager,2 and Suzanne Lentzsch1

1Division of Hematology-Oncology, Department of Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania and 2Kalypsys, Inc., San Diego, California

Abstract
Histone deacetylase inhibitors have emerged as promising anticancer drugs. Using an unbiased ultrahigh throughput screening system, a novel mercaptoketone-based histone deacetylase inhibitor series was identified that was optimized to the lead compound, KD5170. KD5170 inhibited the proliferation of myeloma cell lines and the viability of CD138+ primary myeloma cells by induction of apoptosis, accompanied by an increase of acetylation of histones and activation of caspase-3, caspase-8, and caspase-9. Treatment with KD5170 caused a loss of mitochondrial membrane potential resulting in release of apoptogenic factors such as cytochrome c, Smac, and apoptosis-inducing factor. Furthermore, KD5170 induced oxidative stress and oxidative DNA damage in myeloma cells as evidenced by the up-regulation of heme oxygenase-1 and H2A.X phosphorylation. Combination of KD5170 with proteasome inhibitor bortezomib or tumor necrosis factor–related apoptosis-inducing ligand synergistically enhanced the ant myeloma activity. We further found that resistance of myeloma cells to KD5170 was associated with activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway under treatment with KD5170. Pretreatment with the mitogen-activated protein kinase inhibitor U0126 restored sensitivity to KD5170, suggesting that the combination of KD5170 with U0126 could overcome drug resistance. Growth of myeloma tumor xenografts in KD5170-treated nude mice was significantly inhibited and survival was prolonged. Histone acetylation was increased in spleen and tumor tissues of animals treated with KD5170. Our data indicate that KD5170 has potent ant myeloma activity in vitro and in vivo, which is mediated by DNA damage and mitochondrial signaling and subsequent induction of apoptosis. [Mol Cancer Ther 2008;7(6):1494–505]

Introduction
Although many recent advances in the knowledge and treatment of multiple myeloma have been made, it currently remains an incurable plasma cell malignancy with a median survival of 3 to 5 years (1). It is characterized by aberrant proliferation of terminally differentiated plasma cells and impairment in apoptosis capacity. Over the last several years, a diverse spectrum of novel agents has shown therapeutic potential in myeloma, including thalidomide, lenalidomide, arsenic trioxide, and bortezomib, but high relapse rates and drug resistance continue to plague these therapies. Thus, there is an urgent need for new compounds that specifically target pathways critical for the survival of multiple myeloma cells and overcome resistance.

Histone deacetylase (HDAC) has been recognized as a key molecular target for transcription regulation (2). Aberrant altered activities of HDAC are associated with numerous malignancies (3, 4). In human hematologic malignancies, HDAC is involved in mediating the function of oncogenic translocation products. Some transcriptional repressors, such as BCL6 and AML1-ETO fusion protein, induce lymphoid oncogenic transformation through recruitment of HDAC activity (5–7). This transcriptional repression can pharmacologically be reversed by agents that inhibited HDAC activity.

HDAC inhibitors are a diverse group of agents that potentially activate differentiation-related or tumor suppressor genes by reversing transcriptional repression in various types of cancer cell lines. Several HDAC inhibitors have shown the anticancer activity and most of them are hydroxamic acid derivatives, including suberoylanilide hydroxamic acid, trichostatin A, and NVP-LAQ824 (8–11). However, concerns of poor pharmacokinetics and severe toxicity with hydroxamic acid agents have been proposed (12). In addition, these agents have failed to generate high objective response rates; hence, there is considerable interest in developing novel, non-hydroxamate-based HDAC inhibitors as cancer therapeutics (12).

Recently, using an unbiased ultrahigh throughput screening biochemical screen of 600,000 compounds, a structurally novel mercaptoketone series of HDAC inhibitors has been developed (13). To determine the
clinical potential of the lead compound KD5170, we focused on (a) investigating the inhibition of myeloma cell proliferation and induction of cell apoptosis using both multiple myeloma cell lines and primary patient material, (b) exploring the underlying mechanisms by which KD5170 induces myeloma cell death, and (c) evaluating the in vivo activity of KD5170 against multiple myeloma cells in a mouse xenograft myeloma model. These studies provide the preclinical rationale for clinical protocols using KD5170 to improve patient outcome in multiple myeloma and provide further insight on how HDAC inhibitors exert their anti–multiple myeloma effects.

Materials and Methods

Cells and Reagents

Multiple myeloma cell lines U266, H929, and RPMI-8226 were purchased from the American Type Culture Collection. OPM2 was provided by Dr. Michael Kuehl (NIH) and dexamethasone-sensitive MM.1S human multiple myeloma cell line from Dr. Steven Rosen (Northwestern University). Cells were maintained in RPMI 1640 containing 10% fetal calf serum (Invitrogen).

Human bone marrow cells were obtained from either multiple myeloma patients or healthy donors. Mononuclear cells were isolated by separation on Hypaque-Ficoll gradients (Amersham) as described in the manufacturer’s instruction. CD138 + bone marrow cells from multiple myeloma patients were purified by CD138 (syndecan-1) microbeads using a magnetic cell sorting system (Miltenyi Biotec) as described by Lee et al. (14). Multiple myeloma patient sample cells were incubated with CD138 microbeads for 15 min and loaded onto a positive selection column for 1 mmol/L EDTA, 0.05% digitonin, and proteases inhibitors. Cell lysates containing 0.5% bovine serum albumin and 0.002 mol/L permeabilization with 0.05% digitonin as described previously (15, 16). Briefly, multiple myeloma cells (3 × 10^6) were washed with 1 mL PBS containing 0.5% bovine serum albumin and 0.002 mol/L EDTA, CD138 + cells were eluted from the column. The purity of the myeloma cells, as assessed by CD138/CD45 staining, was >95%. The University of Pittsburgh Institutional Review Board approved all studies.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was purchased from R&D Systems. KD7150 was dissolved in DMSO as stock solutions, provided by Kalypsys and the structure is shown in Potential

Assessment of Apoptotic Cell Death

Apoptosis was quantified morphologically by nuclear condensation and fragmentation using Hoechst 33342 staining as described previously (15, 16). The treated multiple myeloma cells were incubated with 2.5 μg/mL Hoechst 33342 (Molecular Probes) and/or 2.5 μg/mL propidium iodide for 30 min followed by examination under filter-combined fluorescence microscope (Olympus CKX41) equipped with a ×20/0.40 numeric aperture objective lens (Olympus) to distinguish apoptotic cells from necrotic cells. Cells (~200-300) per condition were randomly selected and assessed. Hoechst 33258–positive cells with apoptotic bodies and/or condensed and fragmented nuclei were considered as apoptotic cells regardless whether they were propidium iodide positive or not, whereas propidium iodide–stained cells without apoptotic nuclear changes were considered as necrotic cells.

Caspase activity was measured using 15 μg (caspase-3) or 20 μg (caspase-8 and caspase-9) proteins and 20 μmol/L fluorescent substrates (Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC for caspase-3, caspase-8, and caspase-9, respectively; Biomol). The fluorescence signals were detected by a fluorometer (Tecan GENios; Tecan US) at excitation and emission wavelengths of 400 and 510 nm, respectively (15). Caspase cleavage activation was analyzed by immunoblot assay using the indicated caspase antibodies.

Releases of apoptogenic factors from mitochondria and Bax translocation to the mitochondria were analyzed by immunoblot assay using the cytosolic and membrane fractions prepared from treated cells. Subcellular fractionation was conducted using limited plasma membrane permeabilization with 0.05% digitonin as described previously with minor modifications (15, 16). Briefly, multiple myeloma cells (3 × 10^6) were washed with PBS and gently lysed for 30 s in 50 μL ice-cold buffer [250 mmol/L sucrose, 1 mmol/L EDTA, 25 mmol/L Tris (pH 6.8), 1 mmol/L DTT, 0.05% digitonin, and proteases inhibitors]. Cell lysates were centrifuged for 2 min at 12,000 × g. Proteins from the supernatant (cytosolic fraction) and pellet (membrane fraction) were measured and resolved on SDS-15% PAGE mini-gels.

Analysis of Changes in Mitochondrial Membrane Potential

The treated cells (2 × 10^5) were stained with 2 μmol/L JC-1 from MitoProbe JC-1 assay kit (Molecular Probes) in PBS at 37°C for 20 min following the manufacturer’s procedures. Changes of cellular mitochondrial membrane potential were quantified by flow cytometric analysis of the decrease in the 590 nm (red)/527 nm (green) fluorescence intensity emission ratios using Beckman Coulter Epics XL-MCL and analyzed with the EXPO32 software. The well-known mitochondrial membrane potential disruptor, carbonyl cyanide 3-chlorophenylhydrazone, was used as a positive control and followed the same staining procedures.

Western Blot Analysis

Western blotting was done as previously described (17). The treated cells were harvested, washed in PBS, and lysed with radioimmunoprecipitation assay buffer (Pierce)
containing phosphatase and/or protease inhibitors (Halt Protease Inhibitor Cocktail Kit; Pierce). Lysates (30 μg) were subjected to 12% or 15% (for cytochrome c) SDS-PAGE and transferred to polyvinylidene fluoride membrane. Following probing with the specific primary antibodies against caspase-3, caspase-8, caspase-9, acetyl-H2A, acetyl-H3, acetyl-H4, phospho-H2A.X, phospho-c-Jun NH2-terminal kinase, MEK1/2, phospho-p38 mitogen-activated protein kinase, Bcl-2, Bax, Bir, Smac (Cell Signaling), or apoptosis-inducing factor (AIF; Santa Cruz Biotechnology) plus horseradish peroxidase–conjugated secondary antibody, the protein bands were detected using Amersham Enhanced Chemiluminescence-Western blotting detection reagents (GE Healthcare).

**Xenograft Murine Model**

Beige-nude-xid mice were purchased from Charles River Laboratories at age 5 to 6 weeks and cared for in accordance with the guidelines of University of Pittsburgh.

**Figure 1.** KD5170-induced acetylation of histones correlates with drug sensitivity in multiple myeloma cells. **A,** molecular structure of KD5170. **B,** multiple myeloma patient’s BMSC (3 × 10^3 per well) were plated in 96-well plates and cultured for 24 h. U266, H929, or MM.1S cell lines were seeded on BMSC layer and exposed to KD5170 at indicated concentrations (0-1 μmol/L). [3H]TdR uptake assays were carried out 24 h later as described in Materials and Methods. Mean [3H]TdR uptake with 95% confidence intervals from four experiments. **C,** multiple myeloma cell lines were treated with KD5170 at indicated doses for 16 h or indicated time at 1 μmol/L for U266 cells. Percentages of the apoptotic cell death with typical apoptotic nuclear morphology were determined by staining the cells with 10 μmol/L bis-benzamide Hoechst 33258 fluorochrome (Molecular Probes) for 20 min. Nuclear morphologic changes of U266 cells before and 16 h after treatment with KD5170. **D,** multiple myeloma cell lines were treated with KD5170 at indicated doses for 16 h or indicated time at 1 μmol/L for U266 cells. Acetylation of histones H2A, H3, and H4 was determined by Western blotting with proper anti-acetyl-histone H antibodies. β-Actin was used as a loading control. **E,** KD5170 potentiates the antimyeloma effect of bortezomib in primary multiple myeloma cells. Primary CD138+ and CD138−/C0 bone marrow cells were isolated by using CD138 (syndecan-1) microbeads and treated for 78 h with KD5170, bortezomib, or their combination. The viability of multiple myeloma cells (CD138+) or CD138− mononuclear marrow cells was determined using trypan blue staining assay. Representative results of two independently d experiments. *, P < 0.05, with respect to corresponding coculture (B) or vehicle control (C). Mean ± SD.
Cancer Institute. For the human tumor xenograft studies, 2 × 10^7 H929 myeloma cells in 75 μL RPMI 1640 together with Matrigel basement membrane matrix (Becton Dickinson) were injected s.c. as described previously (18–20). When tumors were measurable, mice were assigned randomly into the treatment group receiving 55 mg/kg KD5170 orally daily for the first 5 days and every other day thereafter or into the control group receiving vehicle alone (sterile water). Mice were weighed every other day and observed daily for any changes in behavior or condition. Tumor sizes were measured daily with a caliper and calculated by the formula: 0.5 × width^2 × length, representing the three-dimensional volume of an ellipse. Animals from both cohorts were euthanized when their tumors reached 2 cm in one diameter or were ulcerated. Survival was evaluated from the first day of treatment until animals were euthanized. This was defined as time to endpoint. Tumor growth was evaluated using caliper measurements from first day of treatment until day of death.

**Statistical Analysis**

For in vitro and in vivo studies, all data are presented as mean and SD. The significance of differences between experimental variables was determined using Student’s t test. The results were considered to be statistically significant at a value of P < 0.05. All statistical tests were two tailed.

**Results**

**Sensitivity of Multiple Myeloma Cells to KD5170 Correlated with the Acetylation Status of Histones**

Within the bone marrow microenvironment, interactions between myeloma cells and BMSC provide growth and survival signals to multiple myeloma cells and confer protection against drug-induced apoptosis (21, 22). Inhibition of multiple myeloma cell proliferation by KD5170 was determined by coculturing BMSC with multiple myeloma cells and subsequently measuring [3H]TdR uptake. The presence of stromal cells greatly enhanced multiple myeloma cell proliferation over multiple myeloma cells alone. [3H]TdR incorporation into DNA of two human multiple myeloma cell lines (H929 and U266) was strongly inhibited by exposure to KD5170. The median inhibitory concentration (IC50) was 0.1 to 0.2 μmol/L for H929 and U266 cells (Fig. 1B). We observed that MM.1S was resistant to KD5170 when cultured alone (data not shown) or in coculture with BMSC at doses up to 4 μmol/L (Fig. 1B). KD5170 modestly inhibited the growth of the multiple myeloma cell line OPM2 (Fig. 1B). The rank order of inhibitory activity of KD5170 on the multiple myeloma cells tested was H929 > U266 > OPM2 > MM.1S.

Next, we investigated whether the cell growth inhibition was due to induction of cell death. Exposure of multiple myeloma cells to KD5170 resulted in dose- and time-dependent apoptosis as evidenced by canonical nuclear morphology changes after staining with dye Hoechst 33342 (Fig. 1C). The spontaneous cell death of multiple myeloma cell lines was <5%. Median effective concentration (EC50) was 0.2 to 0.5 μmol/L for H929 and U266, suggesting that growth inhibition of the most sensitive cell lines is caused by apoptosis induction. Consistent with the lack of effect on [3H]TdR uptake, KD5170 only modestly induced apoptosis of the OPM2 cell line while MM.1S cells were resistant even at highest concentration tested (4 μmol/L; Fig. 1C). To determine if this differential effect on cell proliferation and apoptosis was likely a function of a target proximal event (histone acetylation) or a downstream phenomena, the acetylation status of histones H2A, H3, and H4 was monitored by immunoblot assay in KD5170-treated multiple myeloma cell lines (Fig. 1D). Interestingly, levels of induction of histone acetylation of these treated cells correlated with sensitivity to KD5170: H929 > U266 > OPM2 > MM.1S (Fig. 1B-D). Histone acetylation was induced in the time- and dose-dependent manners (Fig. 1D).

Next, we studied the effects of KD5170 on the viability of human primary multiple myeloma cells (CD138+1) and CD138− mononuclear marrow cells from multiple myeloma patients. Following 78-h exposure to KD5170, the viability of CD138− bone marrow cells dose-dependently decreased as monitored by trypan blue staining. Combination with proteasome inhibitor bortezomib increased antmyeloma effects observed with either agent alone (Fig. 1E). However, neither KD5170 (0-2 μmol/L) nor bortezomib alone or in combination affected the viability of CD138− cells derived from the same patients (Fig. 1E) or healthy donors (Supplementary Fig. S1), indicating the relative selectivity of KD5170 on the malignant plasma cells. This is in accordance with our previous findings showing that bortezomib alone up to a concentration of 10 nmol/L is not toxic to primary CD138− cells (23).

**KD5170 Induces Caspase Activation in Multiple Myeloma Cells**

To further understand the mechanism of action of KD5170-induced apoptosis, we examined caspase activation in both sensitive U266 and resistant MM.1S cells using biochemical and immunoblot assays. After treatment with 0 to 4 μmol/L KD5170 or vehicle for 16 h, U266 and MM.1S cells were harvested and analyzed for caspase-3, caspase-8, and caspase-9 activities. All three enzymes were markedly activated in U266 cells treated with KD5170. In contrast, enzyme activities stayed at the baseline in MM.1S cells even at the highest concentration tested (Fig. 2A). Notably, both caspase-8 and caspase-9 were induced by KD5170, suggesting that the extrinsic death receptor pathway and the intrinsic mitochondrial pathway are involved in mediating cell death.

To confirm the results obtained with the biochemical assay, we used an immunoblot assay to detect native and cleaved (activated) caspase enzymes. Following 16-h incubation of varying concentrations of KD5170, cleaved

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3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
fragments of caspase-3, caspase-8, and caspase-9 were detected in U266 cells (Fig. 2B). However, in MM.1S cells, no cleavage products were detectable for caspase-3 and caspase-9 and a very minor increase in activated caspase-8 over that observed in vehicle-treated cells.

To identify the dependence of KD5170-induced cell death on caspase activation, we pretreated the two sensitive cell lines, U266 and H929 cells, with the broad-spectrum caspase inhibitor z-VAD-fmk at the optimal concentrations from 0 to 40 μmol/L. Treatment with z-VAD-fmk inhibited KD5170-mediated apoptosis in both U266 and H929 cells (Fig. 2C), further supporting the critical role of caspase activation in KD5170-mediated apoptosis.

**Mitochondria Depolarization and KD5170 Treatment**

Mitochondrial membrane permeabilization (MMP) is an important marker of both intrinsic and extrinsic pathways of apoptosis induction (24). MMP induces the release of multiple apoptogenic proteins (e.g., cytochrome c, Smac/DIABLO, and AIF) into the cytoplasm, a crucial event for the onset of downstream caspase activation and subsequent apoptosis (25, 26). By using the mitochondria-sensitive dye, JC-1, which probes the changes of mitochondrial membrane potential, we showed that KD5170 treatment caused loss of mitochondrial membrane potential in sensitive U266 and H929 cells in a time-dependent manner (Fig. 3A; data not shown). The decline in membrane potential was detected as early as 6 h. Consistent with lack of effect on apoptosis and caspase activation, KD5170 failed to induce mitochondrial depolarization in the resistant MM.1S cells. This suggests KD5170-induced MMP may be an essential event for induction of cell death resulting in the release of the apoptogenic factors that are resident in the mitochondria.

**Bax Translocation and Apoptogenic Factors Release from Mitochondria**

Bax is a proapoptotic protein that has a key role in the intrinsic pathway of programmed cell death and is activated via conformational change and mitochondrial translocation. MMP can be mediated by the voltage-dependent anion channel in the mitochondrial outer membrane by insertion and oligomerization of activated Bax in response to apoptotic stimuli (27). We therefore examined the ability of KD5170 to induce Bax expression and translocation and apoptogenic factor release into the cytosol. First, Western blot analysis revealed that total levels of Bel-2 and Bax in the whole-cell lysates were not significantly altered by KD5170 treatment (data not shown). However, following KD5170 treatment of U266 cells, there was a marked increase in mitochondrial membrane bound Bax (Fig. 3B, top) that was accompanied by a decrease in the level of cytosolic Bax protein level (Fig. 3B, bottom). The constitutive mitochondrial binding of Bax in MM.1S was not affected by KD5170 treatment (Fig. 3B).

Mitochondrial translocation of Bax is associated with the liberation of apoptogenic proteins from the intermembrane space (27). After exposure to 0.75 μmol/L KD5170 for 6 to 16 h, there was a significant increase in cytosolic cytochrome c, Smac, and AIF (a mediator of the caspase-independent apoptotic pathway) in U266 cells (sensitive) but not in MM.1S cells (resistant; Fig. 3B, bottom). These results suggest that KD5170 leads to Bax activation and translocation into mitochondrial membrane, which causes MMP and subsequent apoptogenic factor release, confirming involvement of the mitochondrial pathway during KD5170-induced apoptosis. The fact that AIF is released into the cytosol of KD5170-treated U266 cells coupled with the data that the pan-caspase inhibitor z-VAD-fmk only partially inhibited KD5170-induced cell death (Fig. 2C) suggests that KD5170-induced apoptosis involves both caspase-dependent and caspase-independent pathways.

**KD5170-Induced Oxidative Stress and DNA Damage**

HDAC inhibition has been shown to increase oxidative stress via generation of reactive oxygen species, which plays a role in engaging cell death pathways (28). We investigated the role of oxidative stress in KD5170-induced apoptosis by examining oxidation-sensitive markers. First, heme oxygenase-1, which is sensitive to oxidative stress, was up-regulated by exposure to KD5170 in as little as 1 h and maintained thereafter (16 h) in U266 cells (Fig. 4A). Second, because oxidation was involved in oxidative DNA damage and previous studies indicated that suberoylanilide hydroxamic acid may enhance DNA damage (29), we monitored H2A.X phosphorylation at Ser\(^{139}\) as a marker of DNA double-strand breaks (30). After KD5170 treatment, phosphorylation of H2A.X was strongly induced in time- and dose-dependent manners in U266 cells (Fig. 4B) but not in MM.1S cells (Supplementary Fig. S2).\(^{3}\) Phosphorylation of H2A.X was detected as early as 30 min after KD5170 treatment (Fig. 4B) and increased and plateaued by 16 to 24 h. It should be noted that no apoptotic cell death was observed after exposure to KD5170 at up to 6 h, suggesting that phospho-H2A.X may serve as an early indicator of apoptosis-inducing events. Consistent with an increase in oxidative stress, the stress kinases c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase were also activated by KD5170 in U266 cells (Fig. 4A) but not in MM.1S cells (Supplementary Fig. S2).\(^{3}\) Moreover, there was a decrease in phosphorylation (activation) of the survival kinase MEK1/2 in treated U266 cells (Fig. 4A). In contrast, extracellular signal-regulated kinase (ERK) activation was markedly induced by KD5170 only in resistant multiple myeloma cells such as MM.1S (Fig. 4C), suggesting that ERK activation might, in part, confer resistance to KD5170 in MM.1S cells. We next investigated whether blocking ERK activation by treatment with a MEK-specific inhibitor (U0126) could restore sensitivity of MM.1S cells to KD5170 treatment. We pretreated the cells with U0126 for 1 h followed by exposure to KD5170 for another 16 h. Interestingly, U0126 significantly increased the percentage of apoptotic cells over that observed with KD5170 alone (Fig. 4D). The inhibitory effect of U0126 on ERK

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Figure 2. KD5170 induces caspase activation in drug-sensitive multiple myeloma cells. **A,** human multiple myeloma cell lines U266 (gray column) or MM.1S (black column) were exposed to KD5170 for 16 h at 0 to 4 μmol/L dose range. Cells were harvested and lysed with radioimmunoprecipitation assay buffer containing protease cocktail inhibitors. Caspase-3, -8, and -9 activities were measured using 15 μg (caspase-3) or 20 μg (caspase-8 and caspase-9) proteins and 20 μmol/L of the fluorogenic caspase substrates Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC, respectively. Incubation time was 2 h (for caspase-3) and 16 h (for caspase-8 and caspase-9), respectively. **B,** human multiple myeloma cell lines U266 and MM.1S were exposed to KD5170 for 16 h, harvested, and lysed by radioimmunoprecipitation assay buffer containing protease cocktail inhibitors. Whole-cell lysates were subjected to immunoblot assay and detected with indicated antibodies. Arrows, full-length and cleavage of caspase-3, caspase-8, or caspase-9. β-Actin served as an equal loading control. **C,** KD5170-sensitive multiple myeloma cells U266 and H929 were pretreated with caspase pan-inhibitor z-VAD-fmk for 30 min and then exposed to KD5170 at the indicated concentrations for 16 h. Apoptosis was determined with Hoechst 33342 as shown in Fig. 1B. Three experiments were done. *, P < 0.05, with respect to corresponding vehicle control (A) or KD5170 treatment only (C).
phosphorylation in MM.1S cells was confirmed by immunoblot assay (data not shown). In summary, KD5170 leads to oxidative stress and oxidative DNA damage in the sensitive U266 cells, which may have a role in inducing oxidation-associated kinase activation and mitochondrial injury resulting in the engagement of the apoptotic machinery.

Combination Interactions of KD5170 and TRAIL or Bortezomib on Sensitive and Resistant Multiple Myeloma Cells

Previously, it has been shown that up-regulation of death receptor induced by chemotherapeutic agents may cause synergistic cytotoxicity with TRAIL in vivo and in vitro (31, 32). To determine whether KD5170 can mediate similar interactions with TRAIL or bortezomib, we examined the effects of these agents in combination with KD5170 in sensitive and resistant myeloma cells.

Figure 3. KD5170 induces loss of mitochondrial membrane potential, mitochondrial release of apoptosis-inducing proteins, and Bax translocation. A, after exposure to KD5170 (U266: 0.75 μmol/L; MM.1S: 1 μmol/L) for either 6 or 16 h, multiple myeloma cells were stained with JC-1 (Molecular Probes) before flow cytometry analysis (FACSCalibur; Becton Dickinson). Data analyses were done with CellQuest software by measuring both green (530 ± 15 nm; FL-1) and red (585 ± 21 nm; FL-2) JC-1 fluorescence. Mitochondrial membrane potential loss was observed as the shift to lower JC-1 red fluorescence and/or an increase in JC-1 green fluorescence. At least 10,000 events were collected and counted per sample. Representative histograms. B, human multiple myeloma cell lines U266 and MM.1S were treated with KD5170 (0.75 μmol/L) for the indicated hours. Both cytosolic and mitochondrial-rich fractions were prepared and subjected to immunoblot analysis in 15% SDS-PAGE using the indicated antibodies. β-Actin (for cytosolic fraction) and COX-IV antibodies (for mitochondrial fraction) were used as loading controls.
effects, we carried out combination studies with recombinant human TRAIL. MM.1S, U266, and H929 cell lines were treated with TRAIL and KD5170 at concentrations that would have little effect alone. Hoechst 33342 nuclear staining revealed that KD5170 significantly enhanced the proapoptotic effect of TRAIL in U266 cells (additive interaction) and H929 cells (synergistic interaction; Fig. 5A). Interestingly, in the resistant MM.1S cells, apoptosis was greatly enhanced in the combination treatment over that observed with either agent alone. In addition, combination of KD5170 with the well-known proteasome inhibitor bortezomib synergistically inhibited the proliferation of U266 and another multiple myeloma cell line RPMI-8226 (Fig. 5B). Based on this work, KD5170 in combination with TRAIL or bortezomib should be further explored in the treatment of multiple myeloma.

**Effect of KD5170 on Human Multiple Myeloma Xenograft Murine Model**

To evaluate the potential of KD5170 to inhibit multiple myeloma tumor growth in vivo, we tested KD5170 on H929 cells growing as a s.c. xenograft in immune compromised mice. We treated mice daily with 55 mg/kg for the first 5 days and then every other day for the duration of the study. Following 4 days of KD5170 administration, the mean tumor volume of the treatment group was significantly lower than that of the mice treated with vehicle only (P = 0.038). Significantly lower mean tumor volume for the KD5170-treated group in comparison with the control group continued to be observed on day 5 (P = 0.037), day 6 (P = 0.016), day 7 (P = 0.007), and day 8 (P = 0.009; Fig. 6A). Using Kaplan-Meier curves and log-rank analysis, the mean overall time to endpoint was 9 days (95% confidence interval) in the control cohort versus 17.5 days (95% confidence interval) in the treatment group. Prolongation of mean time to endpoint of treated animals compared with control mice was statistically significant (P = 0.009; Fig. 6B). The dose regimen was well tolerated with a modest body weight loss (≤10%) observed after 5 days of continuous treatment that was recovered when treatment was given every fourth day (data not shown). Importantly, our in vivo data also showed that histone acetylation markedly increased in tela in spleen and tumor tissues of the animals treated with KD5170 (Fig. 6C). These data are consistent with our cell-based data and further confirm that HDAC inhibition plays a primary role in KD5170-induced tumor cell death.

**Discussion**

Multiple myeloma is an incurable hematologic malignancy, with ~60,424 people in the United States living with myeloma. An estimated 19,900 new cases of myeloma will...
be diagnosed in the United States in 2007 (Facts 2007-2008, The Leukemia & Lymphoma Society). Therefore, novel therapeutics that effectively inhibit tumor growth and overcome conventional drug resistance are urgently needed. Chromatin remodeling agents, such as HDAC inhibitors, are novel drugs that have been shown to modulate gene expression in tumor cells and inhibit tumor growth and angiogenesis (3). In the present study, we show that KD5170, a novel mercaptoketone-based HDAC inhibitor, induces significant cytotoxicity in both multiple myeloma cell lines and patient multiple myeloma cells at submicromolar levels. We did not observe cytotoxicity of KD5170 on CD138+/CD0 mononuclear bone marrow cells from multiple myeloma patients or healthy donors at the same concentrations, suggesting selective cytotoxicity of KD5170 on multiple myeloma cells. Our data show that KD5170 inhibits HDAC activity resulting in increased acetylation of histones H2A, H3, and H4 in multiple myeloma cells. The relative sensitivity of multiple myeloma cells to KD5170 correlated with degree of histone acetylation: KD5170-resistant MM.1S cells showed no increase in acetylation of histones, whereas KD5170-sensitive cells H929 and U266 exhibited highly acetylated histones after KD5170 treatment. Intermediate response was evident in the OPM2 cell line, which was less sensitive to the antiproliferative effects of KD5170. These findings indicate that the primary molecular target of KD5170 is indeed HDAC and inhibition of this class of enzymes is necessary for antiproliferative effects. It follows that histone acetylation status may serve as a reliable predictor of KD5170

Figure 5. Combination of TRAIL or bortezomib augments KD5170-induced cytotoxic effects on human multiple myeloma cells. A, KD5170-sensitive multiple myeloma cell lines U266 and H929 and resistant MM.1S were treated with indicated doses of KD5170 and TRAIL alone or combination for 16 h. Cell death, shown as apoptotic morphology changes, was examined by Hoechst 33342 staining. B, coculture of multiple myeloma patient's BMSC with multiple myeloma cells (U266 or RPMI-8226) was done as described in Fig. 1. After treatment with indicated doses of KD5170 and bortezomib alone or combination for 24 h, [3H]Tdr uptake assays were carried out as described in Materials and Methods. Mean counts per minute (cpm) with 95% confidence intervals.

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sensitivity in multiple myeloma cells (3). The intimate interaction between malignant plasma cells and the bone marrow microenvironment is critical for tumor growth and protection from therapy-induced cell death (20). Our coculture data show that coculture of multiple myeloma cells with BMSC did not rescue multiple myeloma cells from KD5170-induced cell death.

Mitochondria play a central role in the apoptosis process (33). The genomic responses in intracellular organelles after DNA damage are controlled and amplified in a complex signaling cascade via mitochondria resulting in programmed cell death in the form of both apoptosis and autophagy. Our data indicate that KD5170 mediates cell death through mitochondrial perturbation. It is known that proteins of the Bcl-2 family share one or several Bcl-2 homology regions and behave as proapoptotic or antiapoptotic proteins at the mitochondrial level. Insertion of activated Bax/Bak into the outer membrane in response to DNA damage and/or oxidative stress induces MMP via a voltage-dependent anion channel. This plays a crucial role in releasing small molecules such as cytochrome c, Smac, or AIF (20). Smac binds the caspase inhibitors (inhibitors of apoptosis protein) to inactivate caspases and AIF induces caspase-independent apoptosis. Cytochrome c together with caspase-9 and Apaf-1 interact to create the apoptosome for caspase-9 and caspase-3 activation. As expected from its action on the mitochondrial pathway, KD5170 provoked caspase-9 activation/cleavage and downstream caspase-3 activation, which in turn resulted in nuclear fragmentation. This was further supported by the blockade of nuclear condensation and/or fragmentation in cells pretreated with pan-caspase inhibitor z-VAD-fmk before exposure to KD5170. In addition, KD5170-induced caspase-8 activation (Fig. 2A and B) suggests the possible involvement of the death receptor pathways in KD5170-induced apoptosis. Combination of KD5170 with TRAIL, an apoptosis inducer through death receptor pathway, showed additive or synergistic induction of cell death in different multiple myeloma cell lines (Fig. 5A). This supports our hypothesis that KD5170 causes caspase-8 activation and DNA damage/mitochondrial depolarization, leading to caspase-dependent and caspase-independent cell death.

Nearly all patients with multiple myeloma relapse become refractory to treatment during the course of the disease. Several salvage therapies have been explored to overcome drug resistance, but the optimal combination regimen has not been defined (35). Our results show that multiple myeloma cells resistant to KD5170 exhibit high-level ERK phosphorylation under KD5170 treatment relative to the sensitive lines, suggesting that up-regulation of survival pathways such as MEK/ERK mediates resistance to HDAC inhibitors. We showed that pretreatment with MEK1/2 inhibitor U0126 partially restores the sensitivity of MM.1S to KD5170, providing evidence that KD5170-induced ERK activation is one of the factors conferring drug resistance in MM.1S cells. Thus, combining HDAC inhibitors with agents that modulate the MEK/ERK pathway may have clinical utility in treating multiple myeloma.
Previously, we and others have shown that the combination of HDAC inhibitors with bortezomib induces a synergistic anti–multiple myeloma effect (23, 35–37). This is in accordance with the findings of this study that combination of bortezomib with KD5170 synergistically inhibits multiple myeloma cell proliferation. Importantly, KD5170 strongly enhances bortezomib-induced cell death in primary multiple myeloma cells and apoptotic effects of TRAIL in all multiple myeloma cell lines tested. The synergy of KD5170 with TRAIL was also documented against multiple myeloma cells resistant to KD5170, suggesting that the molecular pathways underlying this synergy are independent of classic mechanisms of drug resistance in multiple myeloma cells. The mechanism by which KD5170 synergizes with TRAIL in multiple myeloma cell lines is not clear but may involve transcriptional up-regulation of the death receptor, DR5 (38). In addition, recent studies have implicated the role of Bax, which abrogates Bcl-2 and Bcl-xL activity, in TRAIL-induced apoptosis (39, 40). KD5170-induced Bax activation and translocation could be an important factor for their synergy. In addition, caspase-8 activation by both agents could result in enhanced caspase-8 cleavage and thus contribute to the synergistic effects.

The potent and selective proapoptotic effect of KD5170 on primary multiple myeloma cells and most multiple myeloma cell lines in vitro was confirmed by testing the in vivo anti–multiple myeloma activity of KD5170 in beige-nude-xid mice bearing human multiple myeloma xenografts. KD5170 significantly inhibited myeloma growth and prolonged survival without significant toxicity. We also observed an increased histone acetylation in the tumors of treated animals, supporting our in vitro findings that acetylation of histones mediates cell toxicity. Together, these data show that KD5170 is a potent, orally bioavailable HDAC inhibitor with demonstrable antimyeloma activity, in vitro and in vivo, and thus supports further preclinical and clinical investigations in this indication.

Disclosure of Potential Conflicts of Interest

C.A. Hassig, J.E. Payne, N.D. Smith, and J.H. Hager: Kalypsys employees. S. Lentzsch: Kalypsys grant. The other authors disclosed no potential conflicts of interest.

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Rentian Feng, Huihui Ma, Christian A. Hassig, et al.


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