Bis(4,7-dimethyl-1,10-phenanthroline) Sulfatooxovanadium(IV) as a Novel Antileukemic Agent with Matrix Metalloproteinase Inhibitory Activity

Rama Krishna Narla, Yanhong Dong, Dan Klis, and Fatih M. Uckun

Parker Hughes Cancer Center, Drug Discovery Program [R. K. N., Y. D., D. K., F. M. U.], and Departments of Experimental Oncology [R. K. N., D. K., F. M. U.] and Chemistry [Y. D.], Parker Hughes Institute, St. Paul, Minnesota 55113

ABSTRACT

We have examined the in vitro anticancer activity of METVAN (bis[4,7-dimethyl-1,10 phenan- throline] sulfatooxovanadium(IV); VO(SO₃)(Me₂-Phen)₂) against acute lymphoblastic leukemia (ALL; NALM-6 and MOLT-3), acute myeloid leukemia (AML; HL-60), Hodgkin’s disease (HS445), and multiple myeloma (ARH-77, U266BL, and HS-SULTAN) cell lines as well as primary leukemic cells from patients with ALL, AML, and chronic acute myeloid leukemia (CML). METVAN induced apoptosis in NALM-6, MOLT-3, and HL-60 cells in a concentration-dependent fashion with EC₅₀ values of 0.19 ± 0.03 μM, 0.19 ± 0.01 μM, and 1.1 ± 0.2 μM, respectively. METVAN induced apoptosis at low micromolar concentrations in primary leukemic cells from patients with ALL, AML, and CML. METVAN inhibited the constitutive expression of matrix metalloproteinase (MMP)-9 protein and its gelatinolytic activity in HL-60 cells and MMP-2 as well as MMP-9 gelatinolytic activities in leukemic cells from ALL, AML, and CML patients. Furthermore, METVAN inhibited the leukemic cell adhesion to the extracellular matrix proteins laminin, type IV collagen, vitronectin, and fibronectin and the invasion through Matrigel matrix. Further preclinical development of METVAN may provide the basis for the development of more effective chemotherapy programs.

INTRODUCTION

Vanadium can be found in both anionic and cationic forms with oxidation states ranging from −1 to +5 (1–4). Vanadium complexes with oxygen states +4 (IV) and +5 (V) have been shown to modulate cellular redox potential, regulate enzymatic phosphorylation, and exert various other effects in multiple biological systems (5–11). A number of vanadocene complexes were reported to possess promising antitumor activity (9, 12, 13). In addition to the ability of vanadium to assume various oxidation states, its coordination chemistry also plays a key role in its interactions with various biomolecules. As part of a systematic effort aimed at developing organometallic compounds with anticancer activity, we recently identified METVAN as an active apoptosis-inducing agent with potent in vitro antitumor activity (14, 15). At nanomolar concentrations, METVAN induced apoptosis in leukemic cell lines, MM cell lines, and solid tumor cell lines derived from breast cancer, glioblastoma, and testicular cancer patients (14, 15). Apoptosis induced by treatment with METVAN is mediated by the generation of reactive oxygen species, depletion of glutathione and depolarization of mitochondrial membranes (16). The purpose of the present study was to examine if METVAN affects matrix metalloproteinases and to evaluate the antileukemic activity of METVAN against primary leukemic cells from patients with ALL, AML, and CML. METVAN killed primary leukemic cells by inducing apoptotic cell death at nanomolar concentrations. METVAN also inhibited integrin-mediated leukemic cell adhesion to the ECM proteins laminin, type IV collagen, vitronectin, and fibronectin, as well as leukemic cell invasion through Matrigel matrix. The inhibition of invasion by METVAN was associated with the attenuation of the enzymatic activities of MMP-9 and MMP-2.

MATERIALS AND METHODS

Leukemic Cells. Human B-lineage ALL cell line NALM-6 (17), T-lineage ALL cell line MOLT-3 (18), AML cell line HL-60 (19), Hodgkin’s disease cell line HS445, and MM cell lines ARH-77, U266BL, and HS-SULTAN were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. All of the tissue culture reagents were obtained from Life Technologies Inc, Gaithersburg, MD. Cell lines were cultivated for a minimum of two passages after thawing prior to experimentation. In addition, we used primary leukemic cells freshly isolated from four patients with ALL (Cases 1–4), two patients with AML (Cases 5 and 6), and one patient with CML in lymphoid blast crisis (Case 7). All of the patients’ bone marrow samples were used following the guidelines of the Parker Hughes Institute Committee on the Use of

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1 To whom requests for reprints should be addressed, at Parker Hughes Institute, 2665 Long Lake Road, Suite 330, St. Paul, MN 55113. Phone: (651) 697-9228; Fax: (651) 697-1042; E-mail: fatih_uckun@ih.org.

2 The abbreviations used are: METVAN, bis[4,7-dimethyl-1,10-phenanthroline] sulfatooxovanadium(IV); ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic acute myeloid leukemia; MMP, matrix metalloproteinase; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MM, multiple myeloma; TUNEL, terminal deoxynucleotidyl-mediated dUTP nick-end labeling; ECM, extracellular matrix.
Human Subjects in Research for secondary use of pathological or surgical tissue.

**Cytotoxicity Assays.** The cytotoxicity of METVAN against leukemia and Hodgkin’s disease and MM cell lines was tested using the MTT assay (Sigma, St. Louis, MO) as described previously (12, 18). Briefly, exponentially growing tumor cells were seeded into a 96-well plate at a density of $4 \times 10^4$ cells/well and incubated with medium containing METVAN at concentrations ranging from 0.1 to 100 $\mu$M for 48 h at 37°C in a humidified 5% CO$_2$ atmosphere. To each well, 10 $\mu$l of MTT (0.5 mg/ml final concentration) were added, and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well was measured in a microplate reader (Labsystems) at 540 nm and a reference wavelength of 690 nm. The IC$_{50}$ values were calculated by nonlinear regression analysis using Graphpad Prism v2.0 (Graphpad Software, Inc., San Diego, CA), and slides were viewed with a confocal laser scanning microscope. Nonapoptotic cells do not incorporate gated dUTP. Cells were washed with PBS to remove unbound reagents, and the coverslips were mounted onto slides with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA), and slides were viewed with a confocal laser scanning microscope. Nonapoptotic cells do not incorporate significant amounts of dUTP because of a lack of exposed 3-hydroxyl ends and, consequently, emit much less fluorescence than do apoptotic cells, which have an abundance of fragmented DNA with exposed 3’-hydroxyl ends. In control reactions, the terminal deoxynucleotidyl transferase enzyme was omitted from the reaction mixture.

**In Situ Detection of Apoptosis.** The demonstration of apoptosis was performed as described earlier (13, 20) by the in situ nick-end-labeling method using an in situ cell-death detection kit (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer’s recommendations. Cells were seeded in 6-well tissue culture plates and incubated with fresh medium containing METVAN, dexamethasone, or vincristine for 24 h. The cells were then fixed in 2% paraformaldehyde, washed with PBS, and transferred to Superfrost-plus slides. The cells were permeabilized with 0.1% Triton X-100 in 0.1% citrate buffer and incubated for 1 h at 37°C with the reaction mixture containing terminal deoxynucleotidyl transferase and FITC-conjugated dUTP. Cells were washed with PBS to remove unbound reagents, and the coverslips were mounted onto slides with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA), and slides were viewed with a confocal laser scanning microscope. Nonapoptotic cells do not incorporate significant amounts of dUTP because of a lack of exposed 3-hydroxyl ends and, consequently, emit much less fluorescence than do apoptotic cells, which have an abundance of fragmented DNA with exposed 3’-hydroxyl ends. In control reactions, the terminal deoxynucleotidyl transferase enzyme was omitted from the reaction mixture.

**Adhesion Assays.** In vitro adhesion assays were performed to evaluate the effects of METVAN on the adhesive properties of leukemic cells as described previously (21). The plates for the adhesion assays were precoated with the ECM proteins laminin, fibronectin, vitronectin, or type IV collagen (each at a final concentration of 1 $\mu$g/ml in PBS) overnight at 4°C and dried. To study the effects of METVAN on NALM-6 cell adhesion, exponentially growing cells were incubated with the compound at concentrations ranging from 0.1 $\mu$M to 5 $\mu$M in 0.1% DMSO for 16 h in a humidified 5% CO$_2$ atmosphere. The cells were centrifuged, washed twice with serum-free medium, counted, and resuspended in serum-free medium to a final concentration of $5 \times 10^3$ cells/ml. One $\times 10^5$ cells were added to each well, and cells were allowed to adhere for 1 h at 37°C in a humidified 5% CO$_2$ atmosphere. The nonadherent cells were removed by gently washing the cells with PBS, and then the adherent fraction was quantitated using MTT assays as described above.

**In Vitro Invasion Assays.** The in vitro invasiveness of leukemic cells was assayed using a previously published method that uses Matrigel-coated Costar 24-well transwell cell culture chambers (“Boyden chambers”) with 8.0-$\mu$m-pore polycarbonate filter inserts (21). The chamber filters were coated with 50 $\mu$g/ml of Matrigel matrix, incubated overnight at room temperature under a laminar flow hood, and stored at 4°C. To study the effects of METVAN on the invasiveness of HL-60 cells, exponentially growing cells were incubated with the compound at various concentrations ranging from 0.1 $\mu$M to 5 $\mu$M in 0.1% DMSO overnight. The cells were washed twice with serum-free RPMI 1640 containing 0.1% BSA, counted, and resuspended at $1 \times 10^5$ cells/ml. An 0.5-mL cell suspension containing $5 \times 10^3$ cells in a serum-free RPMI 1640 containing METVAN or vehicle was added to the Matrigel-coated and rehydrated filter inserts. Next, 750 $\mu$L of NIH fibroblast-conditioned medium was placed as a chemoattractant in 24-well plates, and the inserts were placed in wells and incubated at 37°C for 48 h. After the incubation period, the invasive cells that migrated into the lower chamber were counted under a light microscope. The invasive fractions of cells treated with METVAN were compared with those of DMSO (0.1%)-treated control cells, and the percentage inhibition of invasiveness was determined.

**Gelatin Substrate Gel Zymography.** Gelatinolytic activities in conditioned media of HL-60 cells or of primary leukemic cells were determined according to Heussen and Dovvle (22). Briefly, $1 \times 10^6$ cells/well were plated and treated with various concentrations of METVAN in 24-well plates and incubated for 24 h at 37°C. After incubation, the cells were washed with serum-free medium, replated in 0.5 ml of serum-free medium, and incubated for 24 h at 37°C. The conditioned media were collected and centrifuged at 1500 rpm for 10 min to

**Fig. 1** Cytotoxic activity of METVAN against human ALL (MOLT-3), AML (HL-60), MM cells (ARH-77), and Hodgkin’s lymphoma (HS445) cells. Cells were incubated with increasing concentrations (0.1–100 $\mu$M) of METVAN for 48 h in 96-well plates, and the cell survival was determined by MTT assays. Data points, the mean values from three independent experiments; bars, SE.
Fig. 2. METVAN induces apoptosis in an ALL cell line MOLT-3 (A, A') and primary leukemic cells from ALL (B, B'; Case 1) and AML (C, C'; Case 5) patients. Cells were incubated with vehicle (A, B, C) or 1 μM (A') or 10 μM (B', C') METVAN for 24 h, fixed, permeabilized, and visualized for DNA degradation in a TUNEL assay using FITC-labeled UTP labeling. Red fluorescence, nuclei stained with propidium iodide. Green or yellow (i.e., superimposed red plus green), apoptotic nuclei containing fragmented DNA.
remove cell debris. Twenty μl of conditioned medium was applied to 10% SDS-polyacrylamide gels containing 0.1% gelatin (Novex, San Diego, CA). After electrophoresis, SDS was removed from gels by incubation in renaturing buffer containing 2% Triton X-100 for 30 min. The gels were incubated overnight at 37°C in developing buffer containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, and 5 mM CaCl₂. The gels were stained for 30 min with Coomassie Blue and destained for another 30 min. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin. The gelatin zymograms were subjected to densitometric scanning using the automated Eagle Eye system (Stratagene, La Jolla, CA), and for each concentration, the percentage of inhibition was determined by comparing the density of the gelatinolytic bands in baseline and treated samples using the formula: % inhibition = (Density of gelatinolytic band of baseline control sample - Density of gelatinolytic band of test sample) / Density of gelatinolytic band of baseline control sample × 100. The IC₅₀ values were determined using a Graphpad Prism v2.0.

**Western Blot Analysis.** The conditioned medium was concentrated with centricon chambers to 4X and suspended in Lamellie sample buffer, electrophoresed on 7.5% SDS-PAGE minigels, transferred to nylon membranes using a trans-blot cell (Bio-Rad). MMP-9 protein was detected by using a monoclonal antibody against MMP-9 (Chemicon, Tamecular, CA) at a dilution of 1:5000 and by using chemiluminescence reagents from Amersham Pharmacia Biotech.

**RESULTS AND DISCUSSION**

**Cytotoxicity of METVAN against Human Leukemia, Hodgkin’s Disease, and MM Cell Lines.** We have used MTT assays and in situ apoptosis assays to evaluate the cytotoxic activity of METVAN against human leukemia, Hodgkin’s disease, and MM cell lines. Fig. 1 shows the concentration-response curves for the cytotoxic activity of METVAN against four representative cell lines. METVAN killed NALM-6 (IC₅₀: 198 ± 36 nm), MOLT-3 (IC₅₀: 191 ± 16 nm), HL-60 (IC₅₀: 1.12 ± 0.19 μM), HS445 (IC₅₀: 521 ± 88 nm), ARH77 (IC₅₀: 810 ± 95 nm), U266BL (IC₅₀: 501 ± 24 nm), and HS-SULTAN cells (IC₅₀: 519 ± 53 nm) in a concentration-dependent fashion. TUNEL assay of exposed 3'-OH termini of DNA with FITC-conjugated dUTP was used to determine whether METVAN induces apoptosis in leukemic cells. As evidenced by the confocal laser scanning microscopy images depicted in Fig. 2, METVAN-treated leukemic MOLT-3 cells and primary leukemic cells from two representative patients with ALL and AML, examined for FITC-conjugated dUTP incorporation (green fluorescence) and propidium iodide counterstaining (red fluorescence), showed many apoptotic yellow nuclei with su-
perimposed green and red fluorescence at 24 h after treatment. To further quantitate the apoptotic nuclei of cancer cells treated with METVAN, the FITC-dUTP positive apoptotic nuclei were counted. As compared with DMSO-treated controls, the nuclei of all of the METVAN-treated cells showed marked increase in TUNEL-positivity in a concentration-dependent fashion. As shown in Fig. 3, METVAN was much more effective than the standard chemotherapeutic agents dexamethasone and vincristine in inducing apoptosis of primary leukemic cells from patients with ALL, AML, or CML.

**METVAN Inhibits Leukemic Cell Adhesion to and Invasion through ECM.** Expansion of leukemic cell populations residing within the bone marrow microenvironment involves adhesion of leukemic cells to bone marrow ECM proteins via cell surface integrin receptors and migration into the surrounding tissues (23–26). These ECM proteins to which tumor cells initially attach include laminin, fibronectin, type IV collagen, and vitronectin. Laminin, fibronectin, vitronectin, and collagen have been found in the basal lamina that promote the adhesion and invasion of tumor cells in situ (27–30). To determine whether METVAN affects the integrin-mediated leukemic cell adhesion to ECM, NALM-6 cells were incubated with various concentrations, and then the integrin-mediated cancer cell adhesion was examined. As shown in Fig. 4A, pretreatment of NALM-6 cells with METVAN inhibited their adhesion to laminin-, fibronectin- collagen-, and vitronectin-coated plates. The inhibition of cell adhesion by METVAN was concentration dependent. The IC50 values for adhesion to laminin, fibronectin, collagen, and vitronectin were 0.28 ± 0.01 μM, 0.15 ± 0.009 μM, 0.97 ± 0.05 μM, and 0.615 ± 0.01 μM, respectively. Matrigel matrix-coated Boyden chambers were used to examine the ability of METVAN to inhibit the invasiveness of HL-60 AML cells (31). The cells were treated with METVAN for 24 h and then placed in Matrigel matrix-coated Boyden chambers and allowed to invade for 48 h. We observed that 68.3 ± 4.5% of control HL-60 cells invaded through the Matrigel matrix, whereas pretreatment of cells with METVAN inhibited the invasiveness of HL-60 cells in a concentration-dependent manner with an average IC50 value of 0.61 ± 0.05 μM (Fig. 4B). HL-60 cells produce a novel ECM-degrading proteinase that is not inhibited by tissue inhibitors of MMPs (31). This may potentially explain why higher concentrations of METVAN are required to inhibit invasion versus induce apoptosis.

**METVAN Inhibits MMPs in Leukemia Cells.** MMPs are a family of zinc-dependent endopeptidases with proteolytic activity for a large range of components of the ECM. These enzymes have been implicated in the physiological turnover of ECM as well as several pathological processes including tumor cell invasion (32–37). MMPs are synthesized as inactive proenzymes (zymogens) and, to be activated, require the removal of an 80-amino acid NH2-terminal domain. MMPs are expressed in leukemia cells (31, 38–41). The HL-60, AML cell line was shown to constitutively produce significant amounts of the 96-kDa MMP-9 or gelatinase B (31). As shown in Fig. 5A, a gelatinolytic activity at 96 kDa corresponding to the molecular mass of MMP-9 was detected by gelatin zymography in conditioned media from untreated HL-60 cells. Twenty-four h treatment of HL-60 cells with various concentrations of METVAN significantly reduced the extracellular MMP-9 activity with an average IC50 value of 531 ± 49 nm. To investigate whether the METVAN-mediated inhibition of MMP-9 gelatinolytic activity could be attributable to the inhibition of the release of MMP-9 protein, HL-60 cells were incubated with various concentrations of METVAN for 24 h, and the amount of MMP-9 protein released by HL-60 cells was assessed by immunoblotting. As shown in Fig. 5B, METVAN treatment markedly reduced the amount of MMP-9 protein in the conditioned medium.

Expression of MMPs and their response to METVAN were also examined by gelatin zymography using primary leukemic cells from two AML patients and one CML patient. As shown in Fig. 6, leukemic cells from both of the patients with AML constitutively released MMP-9 and MMP-2 and with gelatinolytic activities at 96 kDa and 72 kDa, respectively. The levels of MMP-2 activity from AML cells were much higher than those of MMP-9. In addition, leukemic cells from CML also constitutively expressed MMP-9 and MMP-2. In CML cells, the 96-kDa gelatinase seem to be more predominant than MMP-2. We further evaluated the effect of METVAN on the expression of MMPs in these AML and CML cells. A 24-h treatment with METVAN inhibited the gelatinolytic activity of MMP-9 as well as of MMP-2 in all of the leukemic cells examined. The effect of METVAN on MMP-9 appeared more dramatic than its effect on MMP-2.

Primary leukemic cells from two B-lineage ALL and two T-lineage ALL patients were also evaluated for the constitutive release of MMPs. As shown in Fig. 7, leukemic cells from both bone marrow and peripheral blood of patients with B-lineage...
ALL as well as T-lineage ALL constitutively released MMP-9. A weak MMP-2 band was detected in supernatants of both B-lineage ALL and T-lineage ALL cells. The MMP-9 activity was significantly diminished after treatment of the cells with METVAN for 24 h (Fig. 7).

Our results provide evidence that leukemic cells of AML, CML, B-lineage ALL, and T-lineage ALL origin produce gelatinases of molecular masses of 72 kDa and 96 kDa corresponding to MMP-2 and MMP-9, respectively. Although expression and dysregulated production of MMPs in lymphoid malignancies has been investigated (42–45), studies of MMP expression in hematological disorders have been scarce (44, 47, 48). In contrast to AML cells, which secreted predominantly MMP-2, ALL cells produced primarily MMP-9. The cultured promyelocytic HL-60 cells however, produced predominantly MMP-9.

In conclusion, our results indicate that the oxovanadium(IV) complex METVAN has potent activity against human cancer cells. METVAN may have clinical potential if it possesses pharmacokinetic, metabolic, and safety attributes that would yield a promising therapeutic index in vivo. Future preclinical development of METVAN will focus on the evaluation of its in vivo pharmacodynamic features.
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