Materials & Methods
CELL CULTURE

Human leukemic cell lines U927, HL-60, K562 and B16F10 were purchased from National Facility of Animal Tissue and Cell Culture, Pune, India. Leukemic cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS (GIBCO BRL, USA) and antibodies in appropriate conditions. B16F10 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37°C in a humidified atmosphere under 5% CO₂. In all the experiments untreated leukemic cells were used as control.

CHEMICALS

RPMI 1640 medium, fetal bovine serum (FBS), HEPES, streptomycin, penicillin, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DAPI (4′,6-diamidino-2-phenylindole), 7-AAD (7-Aminoactinomycin D), JC-1(5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide, Sigma), propidium iodide (PI), Annexin V-FITC (sigma) and general reagents were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bcl-2, Bax, Bcl-xL, PARP, Bid, pro-caspase 3, pro-caspase 8, caspase 9, pro-caspase 10, cytochrome c, TNF-R1, TNF-R2, TRADD, Beta-actin, PDK1, p-PDK1, AKT, p-AKT (ser-473 and Thr-450), MEK, p-MEK, ERK, p-ERK, p-38, pp-38, JNK1/2, and p-JNK1/2 were purchased from Cell Signaling (USA). Inhibitors for ERK (U0126) and for JNK1/2 (SP600125) and p38 (SB203580) were purchased from Sigma and Calbiochem respectively. All other chemicals and solvents were of high purity grade and purchased from local firms.
Methods

CELL VIABILITY ASSAY

**Principle:** Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology. The MTT assay is based on the cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan in presence of an electron-coupling reagent “Succinate reductase system”, carried out by active mitochondria. The water insoluble formazan salt produced has to be solubilized with DMSO. Cell viability was determined by the trypan blue exclusion assay (O’Brien and Gottlieb-Rosenkrantz, 1970), while MTT assay (Kawada et al., 1999) was performed to determine the cytotoxicity of COR-D induced leukemic (U937, HL-60, K562) and melanoma (B16F10) cells.

**Method:** Cells (1×10^4 per well) seeded in triplicate in 96-well plates were treated with varying concentrations of COR-D for determination of cell viability. For trypan blue exclusion assay at the indicated times, cells were collected by centrifugation (1000×g for 5 min), one-fourth volume of trypan blue dye solution (0.4% w/v) was added to cell suspension, and the mixture was allowed to sit briefly at room temperature. Cells were scored for dye uptake under light microscope. For MTT assay after at the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for another 4 h. The supernatant in each well was replaced with 100 μl of DMSO to solubilise the MTT formazan precipitate and optical density (OD) was measured immediately at 490 nm using microplate manager (Reader type: Model 680XR, Bio-Rad Laboratories Inc). The assay was performed in triplicate and in three independent experiments. Percentage inhibition of growth was calculated and expressed as mean ± SEM. For the confirmation of viability, cells (1×10^6) were cultured and treated with IC_{50} concentration of COR-D for 24 h, then processed and stained with 7-AAD. After incubation in dark for 15 min, these were subjected to flow-cytometry. For each sample 1×10^4 cells were acquired and the data were analyzed by Cell Quest Pro software of BD.
FACS Calibur (Martin et al., 1995). Control cells were then supplemented with complete media.

**CONFOCAL MICROSCOPY**

*Principle:* The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove (Kubista et al., 1987). Binding of DAPI to dsDNA produces ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove (Barcellona et al., 1990).

*Method:* After treatment with COR-D at the IC$_{50}$ concentration, the cells were harvested, washed with phosphate buffer saline (PBS), and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS, stained with 2.5 µg/ml of DAPI solution for 10 min at room temperature, washed twice with PBS, and analyzed using a confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany) with excitation/emission wavelengths of 358/461 nm.

**DETECTION OF EXPOSED SURFACE PHOSPHATIDYLSEERINE (PS) BY FLOW-CYTOMETRY**

*Principle:* Apoptotic cells were quantified by Annexin V-FITC and propidium iodide double staining using the Annexin V-FITC Apoptosis Detection Kit purchased from BD Biosciences and following the manufacturer’s instruction. In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer (Koopman et al., 1994). PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis (Martin et al., 1995). However, in necrosis, PS becomes accessible due to the disruption of membrane integrity. Apart from necrosis and apoptosis, PS also becomes accessible in activated platelets, in certain cell anomalies like sickle cell anemia, in
erythrocyte senescence, upon degranulation of mast cells, and in certain stages of B cell differentiation. PS exposure also serves as a trigger for the recognition and removal of apoptotic cells by macrophages. Annexin V is a 35 kDa phospholipid-binding protein as well as a major cell membrane component of macrophages and other phagocytic cell types. Annexin V has a high affinity to PS in the presence of physiological concentrations of calcium (Moss et al., 1990). Apoptotic cells are stained positively for Annexin V-FITC that binds to phosphotidylserine (Vermes et al., 1995), but are negative for staining with propidium iodide (PI). Dead cells are stained positive for Annexin V-FITC and PI, whereas viable cells are negative for both Annexin V-FITC and PI.

**Method:** Briefly, cells were seeded at a density of $1 \times 10^5$/ml, then treated with COR-D at different concentrations. After COR-D exposure, cells were washed twice with cold PBS, resuspended in 100 µl of 1X annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4) and then incubated with 5 µl of annexin V-FITC and 50 µg/ml propidium iodide (PI). After 15 min of incubation at room temperature in the dark, 400 µl of 1X annexin V binding buffer was again added and cells were analyzed within 1 h by flow cytometry. Data acquisition and analysis were performed using a BD Biosciences BDTM LSR flow cytometer equipped with Cell Quest software. Cells that appeared in the lower right quadrant were positively stained by annexin-V-FITC only (early apoptosis) and cells in the upper right quadrant were stained by both annexin-V-FITC and propidium iodide (late apoptosis); these were quantitated and both subpopulations were considered as overall apoptotic cells. At least $1 \times 10^4$ cells were examined and the data were analyzed by CellQuest Pro software (BD FACS Calibur) (Bandyopadhyay et al., 2004).

**CELL CYCLE PHASE DISTRIBUTION ANALYSIS BY FLOW-CYTOMETRY**

**Principle:** Propidium iodide, which can bind to DNA, provides a rapid and accurate means for quantitating both total nuclear DNA content and the fraction of cells in each phase of the cell cycle. The profiles of cellular DNA contents indicate the distribution of the cells in different phases of the cell cycle and reveal any possible DNA loss due to
DNA fragmentation during apoptosis. The fluorescence intensity of PI is directly proportional to the amount of DNA in each cell. So, the fluorescence intensity shown by PI stained cells in G2 and M phases should be double of that shown by similarly treated cells in G1 phase, while the S phase-fluorescence intensity will be in between. The cells with a DNA content less than that of a G1 phase cellular DNA were classified as sub-G1 population and considered as apoptotic cells. PI is unable to penetrate an intact membrane, so cells must first be fixed to permeabilize. PI intercalates into the major groove of double-stranded DNA. It can be excited at 488 nm with a broad emission centered around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for optimal DNA resolution (Nicoletti et al., 1991). Cell cycle analysis needs to be performed by flow cytometry to examine the occurrence of apoptotic sub-G0/G1 peak after compound treatment.

Method: Briefly, cells (1 \times 10^6 cells/ml) were pre-incubated in the presence of different concentrations of COR-D for 24 h, washed twice with cold PBS, and fixed overnight with 70% ice-cold ethanol (E-Merck India, Mumbai, India) in PBS at -20°C. Fixed cells were treated with 500 µg/ml RNase A for 1 h to destroy the cellular RNA-pool, incubated with 69 µM PI dissolved in 38 mM sodium citrate for 30 min at room temperature in dark, and analyzed immediately by flow cytometry using Cell Quest TM software (Becton Dickinson). A total of 10,000 events were acquired, and analysis of flow-cytometric data was performed using ModFit software. A histogram of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) was displayed (Surh et al., 1999).

DNA FRAGMENTATION ASSAY

Principle: Agarose gel electrophoresis is one of the most common and widely used methods. It is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes, and is a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs. Drug-induced apoptosis was further confirmed by a DNA fragmentation assay.
**Method**: cells (4 \times 10^6) were preincubated in the presence or absence of COR-D or with medium alone for 24 h. After treatment, cells were pelleted, washed twice with PBS, and incubated for 1 h at 50 °C in 400 μl of lysis buffer [10 mM EDTA, 50 mM Tris, pH 8, 0.5% (w/v) sodium dodecyl sulphate, 0.5 mg/ml proteinase K]. DNase-free RNase A was added (200 μl, 0.5 mg/ml) and the incubation was continued for an additional hour at 50 °C. DNA was extracted with 600 μl of phenol/chloroform/isoamyl alcohol (25:24:1,v/v/v). Then 0.1 volume of 3 mM sodium acetate and 2 volumes of ice-cold 70% ethanol were added, and incubation done overnight at -20 °C. Samples were pelleted by centrifugation at 11,000 g for 20 min and resuspended in Tris/EDTA (10 mM, pH 8.0) buffer. The pattern of DNA fragmentation was visualized by electrophoresis in 1.5 % (w/v) agarose gel containing ethidium bromide for 2 h at 75 V and photographed under trans-UV illumination (Eldadah et al., 1996).

**DETERMINATION OF INTRACELLULAR ROS PRODUCTION**

**Principle**: 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to monitor intracellular hydrogen peroxide levels. H_2DCFDA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non fluorescent DCFH, which is then rapidly oxidized to the intensely fluorescent compound DCF in the presence of ROS. Thus, the DCF fluorescence intensity is proportional to the amount of peroxide formed intracellularly (Cathcart et al., 1983).

**Method**: Briefly, after treatment with varying concentrations of COR-D for the indicated lengths of time, cells (1 \times 10^6/ml) were sedimented at 1000 g, resuspended in phosphate-buffered saline (PBS) containing 20 μM H_2DCFDA, and incubated at 37°C for 15 min in the dark DCF fluorescence (excitation wavelength set at 488 nm and the emission wavelength at 530 nm) was measured by flow cytometry (240) and analyzed with Cell Quest software (Becton Dickinson, San Jose, CA) (Kuo et al., 2007).
MITOCHONDRIAL MEMBRANE POTENTIAL (MMP) ASSAY

**Principle:** The cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide), is useful for the detection of mitochondrial membrane depolarization occurring at an early stage of apoptosis (Salvioli et al., 1997). The JC-1 dye bearing a delocalized positive charge enters the mitochondrial matrix due to the negative charge established by the intact mitochondrial membrane potential (MMP). In living cells, JC-1 exhibits potential dependent accumulation in mitochondria leading to the concentration-dependent formation of red fluorescent J-aggregates. On depolarization, there is usually a reduction in orange-red staining. The red fluorescence is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape and density. Binding JC-1 monomer with mitochondrial membrane results in green fluorescence and such interaction is independent of the membrane potential. In healthy cells which have a normal membrane potential, JC-1 forms J-aggregates in the mitochondria and emits an orange-red fluorescence, but a collapse of the MMP in apoptotic cells result in cytoplasmic accumulation of the JC-1 monomer, which then emits a green fluorescence. Therefore, the red and green fluorescence of JC-1 reflect changes in the MMP of the mitochondrial membrane (Salvioli et al., 1997). Stock solution of JC-1 (1 mg/ml) was prepared in DMSO and freshly diluted with assay buffer before use.

**Method:** Cells (1 × 10^6) were seeded in 6-well plates and exposed to the desired concentrations of COR-D for specified time periods. After the treatment, cells were collected by centrifugation, washed in ice-cold PBS, and then incubated with medium containing JC-1 (25 μM) for 15 min at 37°C in the dark. These were then washed and re-suspended in 0.4 ml assay buffer and the percentage of red and green fluorescence was estimated by flow cytometry. Approximately 1×10^4 cells were acquired per sample for analysis by BD Cell Quest Pro software (Kuo et al., 2007).
DETERMINATION OF CASPASE ACTIVITY

**Principle:** Caspase activation occurs due to withdrawal of growth factor, exposure to radiation or chemotherapeutic agents, or initiation of the Fas/Apo-1 receptor mediated cell death process. Active caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic disassembly of dying cells. The biological substrates of caspases include poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), lamins, topoisomerases, Gas2, protein kinase C δ (PKC δ), sterol regulatory element binding proteins (SREBP), U1-70 kDa protein and Huntingtin protein (Kumar and Lavin, 1996; Vaux and Strasser, 1996). The colorimetric substrate (Ac-DEVD-pNA) provided in the CaspACE™ Assay System Colorimetric is labeled with the chromophore p-nitroaniline (pNA), which is released from the substrate upon cleavage by DEVDase. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. The amount of yellow color produced upon cleavage is proportional to the amount of DEVDase activity present in the sample (Alnemri et al., 1996).

**Method:** Colorimetric assays were performed using caspase 3, caspase 8 and caspase 9 activation kits according to the manufacturer's protocol (Bio Vision, USA). The kit utilizes synthetic tetrapeptides labeled with p-nitroanilide. Cells (1×10^4) were treated with COR-D at different concentrations for the indicated time, and then lysed in the supplied lysis buffer. The supernatants were collected and protein concentration was determined using a protein assay kit (Bangalore Genei). Two hundred microgram of protein from each sample was incubated with the supplied reaction buffer containing dithiothreitol (DTT) and substrates at 37°C. Caspase activity was determined by measuring changes in absorbance at 405 nm using the microplate reader. Fold increase in activity was determined by comparing the results of the treated samples with the level of uninduced control.
IMMUNOBLOTTING AND IMMUNOPRECIPITATION

**Principle:** Western blotting, also known as immunoblotting or protein blotting, is a core technique in cell and molecular biology. In most basic terms, it is used to detect the presence of a specific protein in a complex mixture extracted from cells. The procedure relies upon three key elements to accomplish this task: the separation of protein mixtures by size using gel electrophoresis, efficient transfer of separated proteins to a solid support, and the specific detection of a target protein by appropriately matched antibodies. Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film, or an imaging system (Towbin et al., 1979).

**Immunoprecipitation** is one of the most widely used methods for antigen detection and purification. The principle of an IP is very straightforward: an antibody (monoclonal or polyclonal) against a specific target protein forms an immune complex with that target in a sample, such as a cell lysate. The immune complex is then captured or precipitated on a beaded support to which an antibody-binding protein is immobilized (such as Protein A or G), and any protein not precipitated on the beads is washed away. Finally, the antigen (and antibody, if it is not covalently attached to the beads and/or when using denaturing buffers) is eluted from the support and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often followed by Western blot detection to verify the identity of the antigen (Bonifacino et al., 2001).

**Method:** Cells (1×10⁶) were treated with different concentrations of COR-D for 24 h. After cell lysate preparation, an equivalent amount of protein (40 µg) from each sample was resolved by SDS-PAGE (10%) and electro-transferred into PVDF membrane. The membrane was blocked by dipping in 3% TBST-BSA (TBS containing 0.05% Tween 20), incubated with appropriate primary antibody overnight at 4°C, washed with PBS containing 0.1% Tween 20, and incubated with the appropriate HRP-conjugated secondary antibody. The immune complexes were detected by Super Signal West Pico Chemiluminescent substrate kit (Pierce). To determine the approximate molecular weight of desired protein bands, 5 µl of Bio-Rad protein molecular weight marker (High range,
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Low range and Broad range) was run in parallel with protein samples in each gel. In the immunoprecipitation assay, whole cell lysate (300 μg) was incubated with specific antibody (1:100 dilution) and incubated overnight at 4°C. The immunocomplex was precipitated by protein A sepharose 4B, the resultant complex was washed and resolved in non-reducing SDS PAGE, and western blotting was carried out.

SUB CELLULAR FRACTIONATION

Following specific treatment, cytosolic and pellet (mitochondrial) fractions were generated using a reagent-based sub-cellular fractionation technique according to the manufacturer’s protocol (Pierce). Briefly, 1×10^7 cells were harvested by centrifugation at 800 g, washed in PBS, and re-pelleted. Reagent A (400 μl) was added to the cells, vortexed at medium speed, and incubated on ice for 2 min. Then 5 μl of reagent B was added and incubated on ice for 5 min. This was followed by the addition of 400 μl of reagent C and proper mixing. Following centrifugation at 800 g and 4°C for 10 min, the supernatant containing cytoplasmic protein was separated from the pellet comprising mitochondria and cellular debris, and further purified by centrifugation at 13,000 g and 4°C for 10 min. Then 400 μl of mitochondrial isolation reagent was added to the pellet and centrifuged at 12,000 g for 5 min. The supernatant was discarded and the mitochondrial pellet was maintained on ice before downstream processing. Cytochrome c and proteins of Bcl-2 family were detected by Western blot analysis.

IN VIVO ANTITUMOR ACTIVITY OF COR-D

B16F10 cells were injected subcutaneously (s.c.) on the right flank of BALB/c mice for tumor induction. Care and maintenance of animals were done in adherence to the guidelines of the Institutional Animal Care and Use Committee. In toxicity study, intraperitoneal (i.p.) injection of 25, 50, 100 and 200 mg/kg/dose of COR-D did not show any abnormalities. The animal were divided into three groups (n = 6). One group of tumor bearing mice was treated as control and another two groups were given i.p.
injections (25 and 50 mg/kg/day) of COR-D as per Wilcoxon method (Litfield and Wilcoxon, 1949). Tumor weight was measured with weighing balance.

**HAEMATOXYLIN EOSIN (H&E) STAINING OF THE HISTOLOGICAL SECTIONS**

*Principle:* Haematoxylin stains, commonly employed for histological studies, are employed to color the nuclei of cells (and a few other objects, such as keratohyalin granules) blue. The mordants used to demonstrate nuclear and cytoplasmic structures are alum and iron, forming lakes or colored complexes (dye-mordant-tissue complexes) the color of which will depend on the salt used. Aluminium salt lakes are usually colored blue-white while ferric salt lakes are colored blue-black. Eosin is a fluorescent red dye resulting from the action of bromine on fluorescein. It can be used to stain cytoplasm, collagen and muscle fibers for examination under the microscope. Structures that stain readily with eosin are termed eosinophilic. Eosin is most often used as a counterstain to haematoxylin in H&E (haematoxylin and eosin) staining. It stains red blood cells intensely red. Eosin is an acidic dye and shows up in the basic parts of the cell, i.e. the cytoplasm. For staining, eosin Y is typically used in concentrations of 1 to 5 percent weight by volume, dissolved in water or ethanol. For prevention of mold growth in aqueous solutions, thymol is sometimes added. A small concentration (0.5 percent) of acetic acid usually gives a deeper red stain to the tissue (http://protocolsonline.com/histology/dyes-and-stains/haematoxylin-eosin-he-staining).

**Staining Procedure**

- Stain in Hematoxylin, working solution, for 6 minutes.
- Drain slides.
- Differentiate in 70% Acid-Ethanol, pH 2.5, two changes, three quick dips each.
- Differentiate in 70% Acid-DI, pH 2.5, two changes, three quick dips each.
- Wash in running tap water for 15 minutes. Drain excess water from slides.
- Stain in working Eosin solution for 2 minutes.
Drain slides.

Differentiate and dehydrate in 95% ethanol, two changes, 30 seconds each.

Completely dehydrate in 100% ethanol, three changes, three quick dips each.

Clear in Histoclear, two changes, one minute each.

Put coverslip with mounting medium.

STATISTICAL ANALYSIS

All the experiments were performed three times. Data are expressed as the mean ± standard error of the mean (SEM). A significant difference from the respective controls for each experimental test condition was assessed using Student’s t test for each paired experiment, value of p <0.01 or p <0.05 indicating statistical significance. Graphs were prepared using Graph Pad prism 5 and Microsoft office excel (2007). Densitometry was performed using Image J analysis software (NIH).
REFERENCES


