The Thiocarbamate Disulphide Drug, Disulfiram Induces Osteopenia in Rats by Inhibition of Osteoblast Function Due to Suppression of Acetaldehyde Dehydrogenase Activity

Monika Mittal,*† Kainat Khan,* Subhashis Pat,* Konica Porwal,* Shyamsundar Pal China,*† Tarun K. Barbhuyan,* Khemraj S. Baghel,* Tara Rawat,* Sabiyasachi Sanyal,* Smrati Bhadouria,* Vishnu L. Sharma,* and Naibedya Chattopadhyay*†§

*Division of Endocrinology and Center for Research in Anabolic Skeletal Targets in Health and Illness (ASTHI), CSIR-Central Drug Research Institute, Lucknow 226021, India; †AcSIR, CSIR-Central Drug Research Institute, Lucknow 226021, India; ‡Division of Toxicology, CSIR-Central Drug Research Institute, Lucknow 226021, India; §Division of Medicinal and Process Chemistry, CSIR-Central Drug Research Institute, Lucknow 226021, India; and ¶Division of Biochemistry, CSIR-Central Drug Research Institute, Lucknow 226021, India

Received November 14, 2013; accepted January 17, 2014

Dithiocarbamates (DTC), a sulphhydryl group containing compounds, are extensively used by humans that include metam and thiram due to their pesticide properties, and disulfiram (DSF) as an alcohol deterrent. We screened these DTC in an osteoblast via-thiram due to their pesticide properties, and disulfiram (DSF) as pounds, are extensively used by humans that include metam and microla-osteoblasts (ALDH2) inhibition; remodeling; bone microarchitecture; fracture healing.

Bone homeostasis in adult mammalian system is achieved by the remodeling cycle that is regulated by bone resorption by osteoclasts and bone formation by osteoblasts. Dysregulation of this cycle either in favor of increased osteoclast activity or reduced osteoblast function or both result in osteoporosis. Recent reports suggest increased osteoblast apoptosis as the major cause of osteoporosis (Das and Crockett, 2013). On the other hand, in growing animals, bone modeling (also known as modeling drift) contributes to achieving peak bone mass. During bone modeling, bone formation is the dominant event and environmental toxicants that have been reported to cause bone loss in experimental animals include heavy metals such as As, Cd, and Pb, and polychlorinated dioxin such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Heavy metals and TCDD are both reported to induce bone loss via their cytotoxic effect on osteoblasts (Abbas et al., 2013; Iqbal et al., 2013; Ryan et al., 2007). Dithiocarbamates (DTC) are a family of organo sulfur compounds that have applications in agriculture as well as medicine (WHO, 1988). For instance, thiram (tetramethylthiuram) and sodium metam are used as pesticides in fruits and vegetables, whereas the closely related tetraethylthiuram, also known as disulfiram (DSF), is clinically used as a treatment for alcohol
deaddiction (Palatty and Saldanha, 2011). In fish embryos, DTC act as teratogens by causing wave-like deformation of the notochord and cartilage malformation (Streeker et al., 2013; van Boxtel et al., 2010). Thiram has been shown to induce skeletal malformation in mice in the form of cleft palate, wavy and distorted ribs and bones, and micrognathy (Tilton et al., 2006). However, the effect of thiram on growth plate (GP) and endochondral ossification in rats is contentious as one study reported a cytotoxic effect on chondrocytes but another one failed to observe any effect on GP (Zhou et al., 2008). DSF and thiram given in the diet of young broiler chicks have been shown to cause tibial chondrodysplasia and GP abnormalities (Edwards, 1987, 1989). From these reports, it appears that DTC have adverse effect during embryonic and early development by likely affecting chondrocyte function.

DSF acts as alcohol deterrent due to aldehyde dehydrogenase (ALDH) inhibition (Krampe et al., 2011). Mice expressing ALDH2*2, a dominant-negative (SNP R671S) ALDH2 gene, expressed in some Asian population and produces inactive ALDH2 protein, showed osteoporotic phenotype (Hoshi et al., 2012). Furthermore, ALDH2-null mice showed severely compromised bone formation during alcohol-induced osteopenia (Shimizu et al., 2011). From these reports, it appears that DSF and/or DTC could adversely impact osteoblast function.

Both chondrocytes and osteoblasts are derived from mesenchymal stem cells. It is possible that the attributes of skeletal teratogenicity induced by DTC by impairing chondrocyte growth, survival and differentiation could also affect osteoblast function, which has not been studied. Accordingly, we investigated the effect of DTC on osteoblast viability and DSF was found to have the most potent osteoblast cytotoxicity. Consequently, we investigated the mechanism of DSF action in osteoblasts and its effect in growing and adult rat skeleton using bone mineral density (BMD), dynamic and static histomorphometries, and biomechanical strength measurements.

MATERIALS AND METHODS

Materials

Cell culture medium, supplements, and all fine chemicals including DSF, acetaldehyde, and glutathione (GSH) were purchased from Sigma Aldrich (St. Louis, MO). Thiram and metam were synthesized and the purities of the compounds were confirmed by nuclear magnetic resonance analytical methods.

In Vitro Studies

Osteoblast culture. Rat calvarial osteoblasts (RCO) were cultured following our previously published protocol of sequential digestion. Briefly, calvaria from ten to twelve 1–2 day old rat pups were harvested, cleaned, and subjected to five sequential enzymatic digestions (0.1% dispase and 0.1% collagenase P) of 10–15 min each. Cells from second to fifth digestion were collected, centrifuged, resuspended, and grown in α-MEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (complete growth medium) (Abbas et al., 2013).

Cell viability assay. The effect of DSF on RCO viability was determined using well-documented MTT (3- (4, 5-dimethylthiazol-2-y1)-2, 5-diphenyl tetrazolium bromide) reduction assay. Briefly, 2 × 10^3 cells/well were seeded in complete growth medium in 96-well plate and kept in a humidified tissue culture incubator at 37°C with 5% CO₂–95% air. After 24 h, cells were exposed to different concentrations ranged from 10nM to 1µM of metam, thiram, DSF, and 0.001% dimethyl sulfoxide (DMSO) as vehicle in reduced serum (5% FBS) medium for 24 h. The cells were then incubated with 10 µl/well MTT (5 mg/ml) for 4 h and insoluble formazan crystals (formed from reduction of MTT in viable cells) were dissolved by adding DMSO and absorbance at 540 nm wavelength was recorded. The IC₅₀ was calculated using GraphPad Prism 5.00.

Cell proliferation assay. RCO were seeded as mentioned above for cell viability and treated with vehicle and DSF (0.5µM) for 24 h in complete growth media. The cells were pulsed with bromodeoxyuridine (BrdU) for the last 4 h before termination. Using BrdU ELISA kit from Roche Diagnostics according to the manufacturer’s instructions, cell proliferation was measured at 450 nm with reference wavelength at 690 nm.

Cell cycle analysis. RCO were grown to 70–80% confluence, followed by 6 h serum withdrawal for cell cycle synchronization and treatment with vehicle, DSF, or 0.5% FBS containing media (SF or serum-free) for next 12 h. Both floating and trypsinized adherent cells were collected and fixed with 70% ethanol at room temperature for 1 h. After fixation, cells were washed with PBS and stained with 50 µg/ml propidium iodide (PI) in RNase A containing hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) for 20 min. Stained cells were then analyzed through FL2-H channel using a Becton Dickinson FacsCalibur flow cytometer (Franklin Lakes, NJ).

Apoptosis assay. RCO were treated with vehicle, DSF, serum-starved (0.5% FBS), acetaldehyde (45mM), GSH (2mM), and GSH+DSF for 24 h as indicated in corresponding figure. Then cells were stained with Annexin V-FITC and propidium iodide using the Annexin V-FITC/PI apoptosis detection kit (Sigma Aldrich, St. Louis, MO) according to manufacturer’s instructions and analyzed using flow cytometry.

GSH assay. RCO were seeded in 12-well plate, at 70–80% confluence, cells were treated with either vehicle, DSF, or acetaldehyde. After 24 h, cells were lysed with perchloric acid, neutralized with potassium bicarbonate (Sigma Aldrich) and supernatant was collected. The total GSH content was estimated from fluorescence readings taken at 412 nm for 30 min in GSH reductase enzyme (Calbiochem, Merck, Darmstadt, Germany)
and 5,5-dithiobis (2-nitrobenzoic acid) DTNB (HiMedia laboratories, Mumbai, India) containing buffer.

Reactive oxygen species generation assay. RCO were seeded in six-well plates and treated with vehicle. DSF (0.5μM), acetaldehyde (45mM), GSH (2mM), and GSH+DSF for 6 h in 5% α-MEM, following trypsinization, cells were labeled with 2', 7'-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C in dark, washed with PBS and fluorescence was measured using FacsCalibur flow cytometer (Becton Dickenson).

Quantitative real-time polymerase chain reaction. SYBR Green from Puregene (Genetix Biotech Asia Pvt Ltd) was used to perform quantitative determination of ALDH isoforms, viz. aldehyde dehydrogenase 1α1(ALDH1α1), aldehyde dehydrogenase 2 (ALDH2), and aldehyde dehydrogenase 1β1 (ALDH1β1), compared with housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in osteoblast. Briefly, 70–80% confluent RCO were treated with either vehicle or DSF (0.5μM) in either complete growth media (nondifferentiating—ND) or differentiating media (D) containing ascorbic acid, β-glycerophosphate for 24 h. The cells were collected in trizol to extract RNA, cDNA was synthesized using RevertAid cDNA synthesis kit (Thermo scientific, EU, Lithuania) as per manufacturer’s instructions. The design of sense and antisense primers was done by using the Universal ProbeLibrary, mentioned below (Roche Applied Sciences, Indianapolis, IN). For quantitative polymerase chain reaction (qPCR), the cDNA was amplified using Light Cycler 480 (Roche Molecular Biochemicals, Indianapolis, IN). To investigate the in vivo effect of DSF on osteogenic genes, 1-day old Sprague Dawley (SD) rat pups were injected with DSF at 30 mg/kg as mentioned in ALDH activity assay (see below). On day 4, pups were sacrificed, RNA was extracted from calvaria via pulverization. cDNA was synthesized and amplified with sense and antisense primers of Runx2 and osteocalcin (OCN).

The sequences of primer pairs for each gene were as follows: ALDH2 (NM_032416.1)-5′-ggccctacgcggaagtaagga-3′ and 5′-ggatctgtggtgggttttgg-3′; ALDH1α1 (NM_022407.3)-5′-caacgcgtgctactaatgga-3′ and 5′-ccacccatgctgccctct-3′; ALDH1β1 (NM_001011975.1)-5′-acccgagtttggccgctgagc-3′ and 5′-gacgccccgctgtaataata-3′; Runx2 (NM_053470)-5′-ccacacagctattaagctggattg-3′ and 5′-aacaagtgtttagttagctacaagc-3′; OCN (NM_013414)-5′-ataagctcgcgctacctc-3′ and 5′-ccaggggctgtctgagg-3′; GAPDH (NM_017008)-5′-tttgatgtttagggctgctc-3′ and 5′-agctgtctacaagggaga-3′.

ALDH activity assay. The functional presence of ALDH in osteoblast was determined spectrophotometrically with acetaldehyde as substrate by measuring the reduction of NAD⁺ at 340 nm, both in vitro and in vivo, as previously published protocol with few modifications (Tottmar et al., 1973). Briefly, for in vitro studies, 2 × 10⁴ cells were seeded in 96-well plate and kept in humidified incubator for 24 h in differentiating (D) and nondifferentiating media (Control-ND). Next day, after a basal level reading (Bs), cells were incubated with the assay buffer and substrate containing 50mM sodium pyrophosphate, pH8.8, 0.5mM-NAD⁺, 0.1mM pyrazole, 1mM-acetaldehyde, and 2μM-rotenone. After 1.5 min of incubation with buffer and substrate (B+S), RCO seeded in ND condition were treated (Trt) with either vehicle or DSF (0.5μM) or alda-1(0.5μM), ALDH2 activator at equimolar conc. to DSF and the reaction kinetics was recorded as absorbance at 340 nm for 5 min. For in vivo studies, 1-day old SD rat pups were injected with DSF at 30 mg/kg adopted from equivalent human dose for four consecutive days. At day 4, pups were sacrificed, calvaria was collected in chilled PBS, debris and adherent mesenchymal tissues were removed. After this step, all steps were performed at 4°C. Calvarial tissue was chopped and sonicated via sonic waves with 35% amplitude for 5 min in lysis buffer and centrifuged at 4000 rpm for 10 min. Supernatant was collected, incubated with assay buffer and substrate, and ALDH activity was measured as absorbance at 340 nm after 5 min.

Alkaline phosphatase assay. For the measurement of alkaline phosphatase (ALP) activity, cells were seeded in 96-well plate as described above. After 24 h, cells were treated with vehicle, DSF (0.5μM), BMP-2 (100 ng/ml), alda-1 (0.5μM), or cotreatment of DSF and alda-1 in differentiating media. After 48 h, cells were freeze-thawed, followed by incubation in p-nitrophenyl phosphate dissolved in diethanol amine buffer at 2 mg/ml concentration and absorbance was read at 405 nm. ALP activity was also measured from calvaria of rat pups dosed as mentioned in ALDH activity assay.

Mineralization of Bone Marrow cells. For mineralization study, bone marrow cells (BMC) isolated from long bones were cultured for 21 days and treated with either vehicle or DSF (0.5μM) for 7 days at early (0–7 days) and late phase (14–21) of mineralization, to compare the effect of DSF on immature and mature cells. After 21 days, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Alizarin red-S stain was used for staining mineralized nodules, photographed with digital camera, followed by extraction of the stain in 10% cetyl pyridinium chloride (Sisco research laboratories, Mumbai, India), for colorimetric quantification at 595 nm using microplate reader. For ex-vivo mineralization studies, BMC were collected from vehicle or DSF-treated rats and cultured for 21 days with media change at every alternate day, then fixed, photographed, and quantified as mentioned.

In Silico Study

The three-dimensional (3D) structure of ALDH2 was obtained from Protein Data Bank (PDB) with PDB Id 3INJ. Prior subjecting the ALDH2 for docking simulations, it was edited by adding missing residues and by removing hetero atoms includ-
ing water molecules. Further, to obtain stable molecule, energy minimization was performed with GROMOS96, an implementation of Swiss-PdbViewer version 4 (Guex and Peitsch, 1997). The structure of DSF, metam, and thiram was downloaded from PubChem with PubChem Id CID 3117, CID 3001858, and CID 5455, respectively.

Docking with Autodock 4.2 (Morris et al., 1998, 2009) was performed following AutoDock Tools (ADT) routines. Grid map was calculated by AutoGrid with dimensions of 64 A × 64 A × 64 A and spacing of 0.375 A between the grid points so that it can cover the whole active site of the chain2 of ALDH2. Further, the docking simulations were performed with Lamarckian genetic algorithm, the most efficient and reliable method of Autodock. Each docking experiment was performed for 100 runs containing population size of 300 individuals and it was terminated with a maximum number of 2,500,000 energy evaluations or a maximum number of 27,000 generations. Further, for each run the top ranked individual in the population was set to survive into the next generation. Mutation and crossover rates were set at 0.02 and 0.80, respectively. Visualization was performed using UCSF Chimera 1.6.2rc (Pettersen et al., 2004).

In Vivo Studies

All animal experimental procedures were prior approved (Institutional Animal Ethics Committee approval no. CDRI/IAEC/2012/11) and conducted as per the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA 34/1999). Seventy male SD rats were obtained from National Laboratory Animal Center, CDRI and were subjected to 12 h dark-light cycle under controlled temperature and humidity. Study on adult animals: Thirty, 8-weeks old (180 ± 20 gm) male SD rats were equally divided into three groups (n = 10 rats/group) as follows: vehicle (1% Tween-20), DSF at 30 mg/kg, acquired from preferable human dose converted into rats (Aguar et al., 2012; Freireich et al., 1966) and a lower dose of 15 mg/kg. Treatment was given for 4 weeks by oral gavage with ad libitum availability of standard rodent chow diet and water. Study on growing animals: Twenty, 4-weeks old (100 ± 20 gm) male SD rats were treated with either vehicle (n = 10 rats/group) or 30 mg/kg DSF (n = 10 rats/group) for 4 weeks by oral gavage. Study on fractured animals: For drill-hole injury studies, the treatment was given for 12 days post-injury with either vehicle (n = 10 rats/group) or 30 mg/kg DSF (n = 10 rats/group). At the end of the various treatments, all animals were euthanized, femurs and tibias were dissected and separated from adjacent tissue, cleaned, fixed in 70% isopropanol, and stored at room temperature until the measurement of various bone parameters, as described below.

Micro-computed tomographic analysis. The high-resolution X-ray micro-computed tomographic (μCT) for the evaluation of two-dimensional (2D) and 3D geometry of excised bones was carried out using the Sky Scan 1076 μCT scanner (SkyScan, Ltd, Kartuizersweg, Kontich, Belgium) as described in previously published protocols (Sakellariou et al., 2004). Briefly, after scanning the bone samples at a nominal resolution (pixels) of 18 μm, the cross-section reconstruction was made using the SkyScan Nrecon software based on a modified Feldkamp algorithm. To analyze trabecular region, region of interest (ROI) was drawn at a total of 100 slices in the region of secondary spongiosa (SS) situated 1.5 mm away from the distal border of GP excluding all primary spongiosa (PS) and cortical bone. For cortical bone analysis, 350 serial image slides were discarded from GP to exclude the trabecular region, and 200 consecutive image slides were selected using CTAn software and quantification was done by employing Batman software encumbered with trabecular (3D) and cortical (2D) bone programs. Various trabecular parameters and cortical parameters were analyzed by following previously published protocols (Sharan et al., 2011). Using μCT scans, cortical and trabecular BMD of tibia was determined from the volume of interest (VOI) made for cortical and trabecular region, respectively. For calibration, the hydroxyapatite phantom rods of 2 mm of diameter with known BMD (0.25 g/cm3 and 0.75 g/cm3) were employed. In addition, the GP was isolated from the surrounding bone tissue in the μCT images by manual segmentation of 2D slices of sagittal images. The segmented sections were then reconstructed to render 3D images. From these images, GP height was measured using Data Viewer software. To analyze 3D parameters in fractured femurs, whole bone was scanned and the center of the fracture callus was defined manually as the point where the previous organization of the cortical bone in the fracture area was nearly nonexistent. Twenty-five slices of 18 μm were placed above and below from this reference point and analyzed. Furthermore, the representative coronal sectional images for 3D visualization were drawn using SkyScan Data Viewer software.

Drill-hole injury in femur. A drill-hole injury was created by inserting a drill bit with a diameter of 0.8 mm in the anterior portion of the diaphysis of the bilateral femurs, 2 cm above the knee joint (Abbas et al., 2013). One day post-surgery, half of the animals were treated with vehicle and remaining half with DSF (30 mg/kg) for 12 days. Each animal was administered calcine (20 mg/kg, i.p.) 24 h prior to sacrifice. On the twelfth day, the rats were anesthetized and sacrificed to collect their femur for the measurement of bone microarchitectural parameters in the drill hole. The bones were kept in 70% isopropanol and embedded in an acrylic material. Then, 60 μm sections were made from the bones using Isomet-Slow Speed Bone Cutter (Buehler, Lake Bluff, IL), followed by photography using confocal microscope (LSM 510 Meta, Carl Zeiss, Inc., Thornwood, NY) with appropriate filters. The intensity of calcein binding was calculated using Carl Zeiss AM 4.2 image-analysis software.

Alizarin/alcan costaining. Undecalcified upper tibial GPs and subjacent metaphyseal bone from vehicle and DSF-treated rats were fixed in 4% paraformaldehyde and then embedded
Data represent mean ± SE of three independent experiments in triplicate.

in methyl methacrylate. Then, 60 μm sections were made and costained with alizarin red and alcian blue following a previously reported protocol (Abbas et al., 2013). Standard light microscopy was then performed.

Compression test. Bone mechanical strength was examined by compression test of tibial head and three-point bending of femoral diaphysis with bone strength tester model TK 252C (Muromachi Kikai Co. Ltd, Tokyo, Japan), according to our previously published protocols (Khan et al., 2013).

Histomorphometry of bone. For dynamic histomorphometric measurements, double calcein labeling was done with an interval of 15 days between two calcein injections, first at 16d and second at 1d prior to sacrifice. Bone formation rate/total bone surface (BFR/BS) and mineral apposition rate (MAR) were calculated as previously published protocols (Khan et al., 2013).

Statistical Analysis. Data are represented as mean ± SE of an indicated number of experiments or animals. Statistical analysis was carried out using GraphPad Prism 5.00 software. Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc or Newman-Keuls Multiple comparison test.

RESULTS

Evaluation of DTC on Osteoblast Viability

RCO with 60–70% confluency were treated with common DTC to which humans are exposed, i.e., thiram, metam, and DSF for 24 h at concentrations that ranged from 10nM to 1μM. DSF was the most cytotoxic of all with IC₅₀ of 488nM followed by thiram (541nM) and metam (> 1μM) (Fig. 1).

Because, amongst these DTC, DSF had the most potent cytotoxic effect on osteoblasts, we subsequently made detailed studies of this compound. The IC₅₀ of 488nM for DSF was rounded off to 500nM (0.5 μM) in subsequent experiments unless stated otherwise.

DSF Inhibits Osteoblast Proliferation and Induces Apoptosis

In comparison to the control RCO (receiving vehicle), DSF treatment reduced BrdU incorporation by > 50% (Fig. 2A). To analyze the growth inhibitory effect of DSF, we studied cell cycle phase distribution and apoptosis. In complete growth medium (10% FBS, control), 37.62% cells were in G1 and 44.33% in S phases; and SF medium (positive control for cell cycle arrest mediated apoptosis) exposure to RCO for 12 h yielded 92.30% cells in G1 and 4.79% in S phases. DSF treatment in growth medium for 12 h resulted in 45.56% cells in G1 and 30.59% in S phases (Fig. 2B). Because DSF caused G1 arrest of RCO, we next studied its effect on osteoblast apoptosis by annexin V-FITC/PI staining followed by flow cytometry detection. In comparison to control, DSF treatment resulted in 8.15- and 1.11-fold increases in early and late apoptotic cells, respectively, whereas the corresponding values in SF medium were 4.31- and 1.88-folds higher than the control (Fig. 2C).

Early apoptosis induced by DSF was ~90% higher than that by SF medium.

DSF Induces Apoptosis in Osteoblast by GSH Depletion and Reactive Oxygen Species Production

Because DSF contains disulphide bond, we speculated that DSF might oxidize the cellular antioxidant, GSH and thereby increase oxidative stress (Grosicka-Maciag et al., 2010). Consistent with our hypothesis, we found that DSF and acetaldehyde (produced by DSF action on cells and reduces GSH) significantly decreased total GSH content in osteoblast (Fig. 3A). Next, the effect of DSF on reactive oxygen species (ROS) production in osteoblast was studied. Figure 3(B) showed that DSF treatment to RCO for 6 h caused 80% increase in ROS generation compared with control, however, acetaldehyde had a far greater increase. Presence of trolox (an antioxidant) had no effect on DSF-induced ROS generation (data not shown) indicating that GSH depletion by DSF could be responsible for ROS production. Hence, we treated RCO with DSF along with exogenous GSH and observed a substantial fall in the DSF-induced ROS production (Fig. 3B), which suggested that DSF induced oxidative stress in osteoblasts by depleting GSH.

Subsequently, we investigated the effect of GSH on DSF-induced osteoblast apoptosis. As expected, DSF treatment strongly induced RCO apoptosis (77% apoptotic cells in DSF group vs. 30% in control) whereas GSH cotreatment markedly abolished the DSF-induced apoptosis to 22% (Fig. 3C), suggesting that depletion of GSH content by DSF caused osteoblast apoptosis.

There are reports showing cytotoxic effect of DSF on breast cancer cells by copper chelation and copper-DSF complex synergistically enhanced the cytotoxic effect of the drug (Cen et al., 2004). We investigated the effect of DSF, CuCl₂, and DSF-copper complex on osteoblast viability and found that all three
FIG. 2. DSF diminished osteoblast proliferation and induced apoptosis. (A) RCO were exposed to vehicle (DMSO) or DSF (0.5 μM) for 24 h and proliferation rate was measured by BrdU incorporation by ELISA. (B) For cell cycle analysis, RCO were treated with either vehicle or DSF or 0.5% FBS (SF, “serum free” as positive control) for 12 h. Fixed cells were analyzed using PI labeling by flow cytometry. Data represent percent of total cells. (C) Annexin V-FITC/PI labeled RCO were detected by flow cytometry to measure osteoblast apoptosis. Shown are representative dot plots (left panel) and quantified data as percent of total cells (right panel). Data represent mean ± SE of three independent experiments in triplicates; *p < 0.05, **p < 0.01, ***p < 0.001 versus control (receiving vehicle) or as indicated.

FIG. 3. DSF reduces GSH in osteoblasts, leading to oxidative stress and apoptosis. (A) Total GSH content was analyzed after treating RCO for 24 h with vehicle, DSF (0.5 μM) or acetaldehyde (45 mM) and absorbance was measured at 412 nm. (B) ROS generation was determined by flow cytometry using DCFH-DA dye following a 6 h treatment of RCO with vehicle, DSF (0.5 μM), acetaldehyde (45 mM), GSH (2 mM), and DSF+GSH. (C) Apoptosis of RCO was measured as described in Figure 2C following a 24 h treatment with various agents as indicated. Data represent mean ± SE of three independent experiments in triplicates; *p < 0.05, **p < 0.01, ***p < 0.001 versus control or as indicated.
had cytotoxic effect on osteoblasts and DSF-copper complex had additive effect (Supplementary fig. 1).

**DSF Inhibits ALDH Activity and Osteoblast Differentiation Phenotype In Vitro and In Vivo**

DSF has been reported to inhibit ALDH activity (Kitson, 1975) and ALDH2 has been shown to regulate osteoblast function. We screened for the expression of different ALDH isozymes, including ALDH1a1 (retinaldehyde dehydrogenase, cytosolic), ALDH1b1 (cytosolic), and ALDH2 (mitochondrial) in osteoblast at the transcript level by qPCR. Only ALDH2 mRNA was expressed in osteoblasts, and other two were undetectable (ct value for ALDH2 = 30; ct values for ALDH1a1 and ALDH1b1 were > 35). Induction of differentiation in osteoblasts using β-glycerophosphate and L-ascorbate in the culture medium resulted in a significant increase in ALDH2 in comparison to undifferentiated cultures and DSF treatment for 24 h downregulated ALDH2 mRNA levels at both culture conditions (Fig. 4A).

Next, we assessed ALDH activity in osteoblasts, and data showed that compared with control RCO, DSF treatment at 0.5μM resulted in a sharp drop in enzyme activity after 1 min but increased for the next 4 min however remained significantly lower than the control at 5.5 min (taken as endpoint as activity remained unchanged beyond this time). Alda-1 is a specific agonist of ALDH2. At 0.5μM, Alda-1 time-dependently increased the enzyme activity and the activity at 5.5 min was significantly higher than the control, suggesting the functional expression of ALDH2 in osteoblasts. In addition, the enzyme activity in differentiated osteoblasts was significantly higher than nondifferentiated cells, which correlated with the transcript data of ALDH2 (Fig. 4B).

We next employed docking simulations to determine whether DSF influenced the activity of ALDH2. Alda-1 has been shown to interact with ALDH2 at the substrate-binding tunnel and kinetically increase the rate of catalysis by reducing the probability of nonproductive substrate hydrolysis (Perez-Miller et al., 2010). We studied DSF, thiram, and metam in the docking experiment along with Alda-1 as a positive control. Total binding energy, as an estimate of molecular complex formation was studied. For clarity, the docked conformation of the compounds with protein crystal is shown without protein backbone (Fig. 4D).

Alda-1 exhibited the least binding energy (−7.07 kcal/mol) followed by DSF (−5.97 kcal/mol), thiram (−4.87 kcal/mol), and metam (−4.16 kcal/mol). Metam being structurally different binds distant from catalytic site of ALDH2. Lower binding energy corresponds to higher binding affinity and thus, DSF has the higher affinity to ALDH2 over the other two DTC and correlates well with the cytotoxicity data which shows that DSF having the lowest IC₅₀ of the three (as shown in Fig. 1).

Both DSF and thiram are bound to the aromatic and hydrophobic conduit comprising residues ASN169, PHE170, LEU173, MET174, TRP177, THR244, GLU268, PHE296, CYS301, CYS302, CYS303, ASP457 PHE459, PHE465 via the hydrophobic interactions (Figs. 4E, F). Although, DSF and thiram occupied the same substrate binding tunnel as Alda-1 however, unlike Alda-1, the two DTC compounds stretched further into catalytic domain in substrate binding tunnel thereby shielding GLU268, CYS301, CYS302, and CYS303 to prevent the catalytic activity of ALDH2.

Based on the preceding data, it appeared that DSF may influence osteoblast differentiation. ALP activity serves as a well-recognized early marker of osteoblastic differentiation. When cultured in differentiation induction medium (as in Figs. 4A, B), DSF diminished ALP activity of RCO by 60%, whilst BMP-2 (positive control) and Alda-1 increased it by 26% and 40%, respectively. Presence of DSF significantly attenuated Alda-1-induced ALP production, suggesting that DSF suppressed osteoblast differentiation by inhibiting ALDH2 activity (Fig. 4G).

To confirm that the observed suppression of osteoblast differentiation by DSF was not due to its apoptosis inducing effect, DSF treatment was given to BMC (osteoprogenitor cells) at the early and late stages of differentiation. DSF treatment concurrent to differentiation induction failed to show any mineralized nodules, indicating that the effect was largely due to osteoblast apoptosis. However, addition of DSF at the later stage, i.e., when cells had already differentiated, resulted in reduced formation of mineralized nodules than the control, suggesting that DSF inhibited differentiation of BMC (Fig. 4H).

In addition, DSF treatments given to one-day-old rats decreased both ALDH and ALP activity in the calvarial tissue compared with the corresponding vehicle treated control, suggesting that DSF indeed suppressed the enzyme activity and osteoblast differentiation *in vivo* (Fig. 4I, upper panel). However, Runx2 and osteocalcin (OCN) mRNA levels were increased in the DSF group compared with control (Fig. 4I, lower panel).

**DSF Treatment to Adult Rats Causes Trabecular Osteopenia and Reduces Bone Regeneration After Fracture**

Because trabecular bones are metabolically more active than the cortical bones and that these bones are more readily lost under various osteopenic insults, we next studied the effects of DSF on proximal tibia metaphysis (comprising of trabecular bones). Representative μCT images showed that compared with control (rats receiving vehicle), the DSF groups had significant depletion of PS (the ossification zone consisting of calcified cartilage core with rapidly mineralizing osteoid) and SS (the remodeled lamellar bone) (Fig. 5A). Trabecular response to DSF treatment was quantified using 3D reconstruction images, which showed that compared with control group, rats treated with DSF at 30 mg/kg dose but not the lower dose (15 mg/kg) had reduced bone volume/trabecular volume (BV/TV), trabecular...
FIG. 4. DSF inhibits osteoblast differentiation via ALDH2 inhibition both in vitro and in vivo. (A) RCO were seeded in either ND or differentiating medium (D) and then treated with vehicle or DSF (0.5 μM). ALDH2 mRNA levels were analyzed by real-time PCR. (B) RCO were seeded in either ND medium or D medium, incubated with substrate (acetaldehyde) for 1 min and treated with vehicle, DSF (0.5 μM) or alda-1 (0.5 μM). ALDH activity kinetics was followed for 5 min at 340 nm. (C) Schematic drawing of predicted binding pocket and interaction of ALDH-2 with different ligand molecules; Alda-1 (blue), DSF (red), thiram (green), and metam (orange). (D) Superimposed docking structures of alda-1, DSF, and thiram without protein backbone. 3D presentation of interacting residues of ALDH2 with DSF (E) and thiram (F). (G) RCO were exposed to vehicle, DSF (0.5 μM), BMP-2 (100 ng/ml), Alda-1 (0.5 μM), or DSF+Alda-1 to evaluate osteoblast differentiation using ALP assay. (H) BMC were treated with vehicle or DSF (0.5 μM). Cells were treated with DSF either from 0 to 7 days followed by its removal or from 14 to 21 days. All cultures were terminated on the 21st day and Alizarin red-S staining was performed. Shown are the representative photographs of Alizarin red-S stain of the fixed cultures as mineralized nodules (upper panel) and quantification data obtained following dye extraction (lower panel). Data represent mean ± SE of three independent experiments in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001 versus control or as indicated. (I) New-born pups were given vehicle (20% ethanol) as control or DSF (30 mg/kg) for 4 days and calvarial protein and RNA were extracted to determine the activity of enzymes (ALDH and ALP) and mRNA levels of Runx-2 and OCN. All values are expressed as mean ± SEM (n = 5 rats/group); *p < 0.05, **p < 0.01, ***p < 0.001 versus control.
FIG. 5. DSF caused trabecular osteopenia and impaired bone regeneration at the fracture site. Adult male rats were administered with DSF at 15 mg/kg and 30 mg/kg or 1% Tween-20 as vehicle for 4 weeks. (A) Representative μCT images are showing depletion of trabecular region comprising of GP, PS, and SS in DSF-treated groups, particularly at 30 mg/kg dose (dose chosen for subsequent experiments). (B) Quantification of trabecular bone parameters (3D μCT) revealed osteopenia in rats exposed to DSF. (C) Shown are the representative photographs (upper panel) and quantified Alizarin red-S staining (lower panel) of BMC harvested from various groups of rats. (D) Representative confocal images (100×) of calcein (binds to nascent bone) labeling are shown in the callus of drill hole of the two groups after 2 weeks of treatments. Quantification of the mean intensity of calcein label per pixel showed reduced mineral deposition in the DSF group compared with control. (E) Microarchitectural assessment of the callus by μCT revealed inferior quality of the newly formed bone at the fracture site of the DSF group. All values are expressed as mean ± SEM (n = 10 rats/group); *p < 0.05, **p < 0.01, ***p < 0.001 versus control. BV/TV—trabecular bone volume, Tb.N.—trabecular number, Conn.D.—connection density, Tb.Sp.—trabecular separation.

ular number (Tb.N), and connection density (conn.D). Trabecular spacing (Tb.Sp) was dose-dependently increased by DSF compared with control, suggesting increased nonosseous area caused by this treatment (Fig. 5B).

We next investigated the impact of DSF treatment on osteoblast differentiation in vivo. At the end of the 4-week treatment, BMC harvested from the long bones show reduced mineralized nodules in the DSF (30 mg/kg) treated rats compared with the control or DSF at its lower dose group (Fig. 5C, upper panel showing representative photomicrograph of alizarin red-S stained cells). Quantification of the extracted alizarin stain showed that DSF treatment significantly inhibited mineralization at 30 mg/kg DSF dose compared with control or the lower dose of DSF (Fig. 5C, lower panel). Hence, 30 mg/kg dose of DSF was chosen for the subsequent studies.

Because appropriate bone regeneration at the fracture site is dependent on osteoblast function, we investigated the fracture healing ability of DSF-treated rats compared with control. The bone deposition at the fracture site was reduced significantly in the DSF group (measured from the intensity of calcein labeling in the callus), suggesting reduced osteoblast function (Fig. 5D). The 3D μCT assessment of the callus microarchitecture revealed a reduction in the BV/TV, Tb.N, and conn.D and an increase in Tb.Sp in DSF group compared with control (Fig. 5E).

**DSF Diminishes Parameters of Peak Bone Achievement**

Skeletal growth involves the formation of calcified cartilage in the GP, which is first converted into woven spongy bone (made up of bony trabeculae of mineralized cartilage cores) and subsequently remodeled into lamellar trabecular bone. In the metaphysis, the μCT of 3D coronal sections showed decreased PS and dramatically reduced SS as well as decreased GP height in DSF rats compared with control (Fig. 6A). μCT quantification showed ~20% decrease in the GP height of DSF rats compared with control. In addition, DSF group had reduced BMD, BV/TV, Tb.Th, Tb.N, and conn.D; and increased Tb.Sp and structure model index (SMI) compared with control (Fig. 6B).

To examine the cartilage-to-bone transition, sections through...
FIG. 6. DSF negatively impacts trabecular bones of growing rats. Recently, weaned male rats were treated with 30 mg/kg/p.o. DSF for 4 weeks. (A) Trabecular response was evaluated using 3D μCT. Representative coronal section images exhibited deteriorated trabeculae in DSF group compared with control. (B) GP height, BMD, and trabecular bone volume fraction (BV/TV) were reduced upon DSF exposure. Trabecular connectivity parameters viz. Tb.Th, Tb.N, and Conn.D reduced, whereas geometric parameters viz. Tb.Sp and SMI increased in DSF-treated rats. (C) Histological examination (4×) of femoral GP and PS in undecalcified bone sections costained with Alizarin red-S for calcium and alcian blue for glycosaminoglycans, showing hypomineralized osteoid in DSF group evident from the lack of Alizarin red staining. (D) The trabecular strength parameters were calculated using compression test where DSF group had reduced maximum energy to load capacity but stiffness was increased. All values are expressed as mean ± SEM (n = 10 rats/group); *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

We studied the effect of DTC on osteoblast viability. The major findings include, (1) DSF has the most potent cytotoxic effect on osteoblasts amongst various DTC due to the presence of DTC core which covalently interacts with thiol groups of ALDH2 (Kitson, 1978), (2) DSF diminishes proliferation and induces G1 arrest to cause apoptosis and the later event is caused by ROS production due to diminished GSH content, (3) ALDH2 is the only ALDH isozyme expressed in osteoblasts and its ex-
TABLE 1
Static and Dynamic Cortical Bone Histomorphometric Measurements in Growing Rats Administered With Either Vehicle or DSF (30 mg/kg) for 4 Weeks

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (1% Tween-20)</th>
<th>DSF (30 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Static cortical bone histomorphometric measurements at femur diaphysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g HA/cm³)</td>
<td>0.94</td>
<td>1.04***</td>
</tr>
<tr>
<td>B.Ar. (mm²)</td>
<td>3.66</td>
<td>2.50***</td>
</tr>
<tr>
<td>T. Ar. (mm²)</td>
<td>10.49</td>
<td>8.11***</td>
</tr>
<tr>
<td>T.Pm. (mm)</td>
<td>13.63</td>
<td>12.11***</td>
</tr>
<tr>
<td>B.Pm. (mm)</td>
<td>45.85</td>
<td>23.58***</td>
</tr>
<tr>
<td>B.Pm.-T.Ar. (mm)</td>
<td>35.36</td>
<td>15.47***</td>
</tr>
<tr>
<td>Cs.Th. (mm)</td>
<td>0.17</td>
<td>0.21**</td>
</tr>
<tr>
<td>Ps (cl) (%)</td>
<td>28.06</td>
<td>63.11***</td>
</tr>
<tr>
<td><strong>Biomechanical strength of femur</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max energy (mJ)</td>
<td>31.12</td>
<td>27.85</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>108.51</td>
<td>89.23</td>
</tr>
<tr>
<td><strong>Dynamic histomorphometric measurements at femoral diaphysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMAR (µm/day)</td>
<td>2.51</td>
<td>1.81*</td>
</tr>
<tr>
<td>pBFR/BS (µm³/µm²/year)</td>
<td>9.33</td>
<td>2.67**</td>
</tr>
<tr>
<td>eMAR (µm/day)</td>
<td>1.63</td>
<td>ND</td>
</tr>
<tr>
<td>eBFR/BS (µm³/µm²/year)</td>
<td>1.71</td>
<td>ND</td>
</tr>
</tbody>
</table>

Notes. BMD, bone mineral density; B.Ar., bone area; T.Ar., periosteal area; T.Pm., periosteal perimeter; B.Pm., cortical bone perimeter; B.Pm.-T.Ar., endosteal perimeter; Cs.Th., cortical thickness; Ps (cl), cortical porosity; MAR, mineral apposition rate; BFR/BS, bone formation rate to total bone surface (p, periosteal; e, endosteal). ND is not detected.

*p < 0.05, **p < 0.01, ***p < 0.001 versus control (receiving vehicle).

pression is increased by the induction of differentiation. (4) DSF suppresses ALDH2 expression and activity in osteoblast to inhibit differentiation both in vitro and in vivo, and (5) at a human-equivalent dose, DSF induces trabecular osteopenia and reduces bone regeneration at the fracture site in adult rats, and decreases parameters of peak bone gain in growing rats by inhibiting osteoblast function.

Among the DTC that are in human use, we found that DSF had the most potent cytotoxic effect on osteoblasts. Docking study has suggested that an N,N-diethyl group (DSF) in N,N-dialkyldithiocarbamates class of compounds had optimal interaction with sulfhydryl (SH) groups of ALDH2 and replacement of diethyl group with a smaller group (N,N-dimethyl group as in thiram) resulted in diminished interaction with sulphhydryls of ALDH2. Metam being structurally different did not interact with ALDH2.

DSF reduced osteoblast proliferation by half and strongly induced apoptosis by G1 arrest. DSF is known to stimulate cellular accumulation of acetaldehyde. Indeed, ALDH suppression has been shown to increase acetaldehyde (Hoshi et al., 2012) and our data showed that exogenous addition of acetaldehyde induced osteoblast apoptosis. Exposure to aldehydes has been reported to deplete cellular GSH (Towell and Wang, 1987), the most abundant cellular antioxidant thiol. We observed that DSF reduced GSH content and cotreatment with GSH but not trolox completely reversed the apoptotic impact of DSF in osteoblasts, suggesting that the thiol group was required for DSF effect. Taken together, data suggest that inhibition of ALDH2 function results in GSH depletion thus increasing oxidative stress, and leading to cell cycle arrest and increased apoptosis (Lean et al., 2003).

Besides being an irreversible inhibitor of ALDH, DSF can also complex metals and react with sulphhydryl groups. The ability of DSF to complex with metals, particularly copper has been shown to induce apoptosis of breast cancer but not normal breast epithelial cells (Chen et al., 2006). However, unlike breast cancer cells, where DSF or DSF-copper complex but not copper alone induced cytotoxicity, all three had cytotoxic effect on osteoblasts. In addition, DSF had no effect on the viability of U2OS human osteosarcoma cells (data not shown). Thus, it appears that the response of osteoblastic cells (normal or malignant) to DSF is quite different from the breast epithelial cells. Furthermore, Cu²⁺ is known to inhibit osteoblast function (Kozuka, 1995) and our data showed that it significantly inhibited osteoblast viability albeit less potent than DSF. Cytotoxic effect of DSF-copper complex on osteoblast was additive and not synergistic as opposed to that reported with breast cancer cells. These data suggested that the cytotoxic effect of DSF in osteoblast was independent of copper-thiolate chelation mechanism and likely mediated by ALDH inhibition.

We found that osteoblasts expressed ALDH2 but not ALDH1. The importance of ALDH2 in osteoblast function has recently been demonstrated by “knocking in” a human dominant-negative ALDH2 gene in mice that manifested osteopenia due to impaired osteoblastogenesis (Shimizu et al., 2011). We showed that Alda-1, a specific activator of ALDH2, increased osteoblast differentiation greater than BMP-2, which provided pharmacological support to differentiation promoting
action of ALDH2. Abrogation of Alda1-induced osteoblast differentiation by DSF provided additional support in favor of the functional role of ALDH2 in osteoblasts.

In adult rats, 30 mg/kg dose of DSF given for 4 weeks decreased the formation of mineralized nodules by BMC, further confirming impaired osteoblast function in vivo. In a more acute experiment, where DSF (30 mg/kg) was injected to new born pups for 4 days suppressed ALDH activity and ALP production in calvarial bone (composed of proliferating osteoblasts), confirming that DSF impaired osteoblast function. Inhibition of osteoblast differentiation by DSF appeared to have contributed to the development of osteopenia evident from significant trabecular loss. Additional evidence of impaired osteoblast function has been provided by assessing bone regeneration at the fracture site in rats treated with DSF. Newly formed bone at the fracture site could be measured by the amount of calcein (a bone seeking dye) deposition. Calcein labeling was diminished in rats that received DSF for 12 days compared with the control, suggesting reduced bone formation at the fracture site. In addition, newly formed bones in the DSF group had reduced compactness evident from decreased bone volume fraction and connectivity and increased space comparing with control. Together, these data suggested that impaired osteoblast function in adult rats induced osteopenia and diminished the ability to heal fracture. Because alcoholism is an independent risk factor for the development of osteoporosis (Giuliani et al., 1999; Maurel et al., 2011), the antialcoholism drug, DSF inhibiting osteoblast function and inducing bone loss might exacerbate fracture risk and/or delay fracture healing in humans.

Inhibition of osteoblast viability and differentiation could dampen peak bone accrual, an event that takes place during growth and adolescence. In the growing rats, bone mass is increased chiefly due to longitudinal bone growth and modeling drift. Unlike remodeling which is a coupled sequential process of the osteoclast and osteoblast activity on the same surface seen in adult endochondral bones, modeling drift is an uncoupled sequence with bone formation and resorption surfaces being different. Through modeling, the shape and the size of the rat bone is altered during growth, similar to that seen in humans, and thus growing rat is a suitable model for investigating the effect of drugs on bone growth. In the epiphyseal sections of the DSF group, alizarin red (stains calcium) in PS and SS was undetectable. Alcian blue that stained collagen however was readily observed in the same sections of DSF-treated rats, further attested to impaired ability of osteoblasts to mineralize in vivo. In keeping with reduced mineralization, BMD was nearly half of the control value in the DSF group. SS, which develops into trabecular bone, was severely depleted in the DSF group resulting in fewer trabeculae. Higher proportion of rod-shaped trabeculae (increased SMI) in the DSF group over control suggested poor trabecular geometry. Low BMD and microarchitectural deterioration of the trabecular bone of DSF group resulted in decreased energy to compression but stiffness was increased likely due to osteoid hypomineralization. These data suggest that DSF decreases trabecular bone accrual during skeletal growth and modeling. Whether DSF modulated osteoclast function would be important to address in future investigation.

Rapid gain in cortical bones is the hallmark of skeletal growth. DSF treatment decreased cross-sectional area but increased cortical thickness and BMD of tibia diaphysis. These histomorphometric features are similar to that reported in ALDH2 null mice (Tsuchiya et al., 2013). Cortical porosity was dramatically increased in the DSF group compared with the control but femur bending strength was comparable between the groups. No differences in femur mechanical parameters between ALDH2-null and wild-type mice have been reported. Intriguingly, despite decreases in ALP production and mineralized nodule formation by osteoblasts of ALDH-2 null mice, there was anomalous increase in the expression of osteogenic genes including Runx2 and OCN. We also observed that DSF exposure upregulated the expression of Runx-2 and OCN even when DSF inhibited the differentiation phenotypes of osteoblast. Given that the cortical bone response to ALDH2 gene deletion has remarkable similarity with DSF-treated growing rats, it is reasonable to conclude that the skeletal effect of DSF is caused by the inhibition of ALDH2 in osteoblasts. Periosteal MAR and BFR/BS were reduced in DSF group compared with control. BFR/BS represents formation phase of bone modeling/remodeling. Surface lining cells significantly contribute to bone formation. One striking observation was that endosteal MAR and BFR/BS were undetectable in DSF group. A more severe impact of DSF on bone formation at the endosteal over periosteal surface showing the striking similarity to the ALDH2-null mice confirming the effect of DSF on bone formation is due to ALDH inhibition.

Taken together, our study shows that DSF is the most potent among all the DTC in inhibiting osteoblast function. DSF diminishes GSH content to increase ROS production resulting in osteoblast apoptosis and inhibits ALDH2 synthesis and activity to reduce osteoblast differentiation. At a human-equivalent dose, DSF induced osteopenia in both adult and growing rats and diminished bone regeneration at the fracture site due to reduced osteoblast function. Substantial bone loss is caused by DSF in adult and growing rats in 30 days. An even greater cause of concern is the observation that fracture healing in adult rats is impaired by only 12 days treatment of DSF. These characteristics may include DSF under the category of potent osteopenia inducing drugs. DSF also has the potential to worsen the fracture risk in existing osteoporosis, e.g., in post-menopausal women and patients receiving synthetic glucocorticoids.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.
The authors are thankful for the technical assistance provided by Kavita Singh at the confocal facility of the Electron Microscopy Unit, SAIF division, CSIR-CDRI, Lucknow, India.

REFERENCES


