Biological activity studies

Titanium, vanadium and palladium containing aminophosphates were evaluated for their biological activity such as antimicrobial, nematicidal, DNA cleavage and anticancer activities.

Antimicrobial activity

The antimicrobial cell susceptibility test was performed by agar disc-diffusion technique according to literature procedure against two gram-positive bacteria *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*) and two gram-negative bacteria *Pseudomonas fluorescens* (*P. fluorescens*), *Escherichia coli* (*E. coli*), *Proteus vulgaris* (*P. vulgaris*) and also against fungi *Candida albicans* (*C. albicans*) [18]. Standard inoculum of $1 - 2 \times 10^7$ cfu/mL 0.5 Mc Farland standards were introduced onto the surface of sterile nutrient agar plate and evenly spread by using a sterile glass spreader. Sterile antibiotic discs (6 mm in diameter, prepared using Whatmann No. 1 paper) were placed over the medium. To determine the antimicrobial activity, 100 μg of the compounds initially dissolved in dimethyl sulphoxide (DMSO) were transferred to each disc with the help of a micropipette, simultaneously maintaining a standard ampicillin (30 μg/disc) against bacteria and clotrimazole (10 μg/disc) for fungi. After overnight incubation at 37 °C for bacteria and 25 °C for fungi, the zone of inhibition is measured in 'millimeters' and compared with standard antibiotics. DMSO (which has no activity) and standard antibiotics were used as negative and positive controls for antimicrobial activity studies. The activity results are calculated as a mean of triplicates.

Minimum inhibitory concentrations (MIC) of the compounds which showed antimicrobial activity were determined using literature method [19]. Diluted compounds ranging from 100 – 0.1 $\mu g\ mL^{-1}$ were mixed in nutrient broth and 0.1 mL of active inoculums was added to each tube. The tubes were incubated aerobically at 37 °C for bacteria and 25 °C for fungi for 24 h. The lowest

concentration of the compound that completely inhibited bacterial growth (no turbidity) in comparison to control was regarded as MIC.

Nematicidal activity

Culture preparation

Fresh egg masses of *Meloidogyne incognita* were collected from the stock culture maintained on tomato (*Lycopersicon esculentum*) root tissues and was kept in water for egg hatching. The eggs suspension was poured on a cotton-wool filter paper and incubated at 28 ± 2 °C to obtain freshly hatched juveniles (J2). Juveniles collected within 48 h were used for screening nematicidal activity of the compounds.

Mortality test

All the synthesized compounds were initially dissolved in dimethyl sulfoxide (DMSO) and then in distilled water to make dilutions of 250, 150 and 50 μg mL^{-1}. Experiments were performed under laboratory conditions at 28 ± 2 °C. About 100 freshly hatched second stage juveniles were suspended in 5 mL of each diluted compound and incubated. Distilled water with nematode larvae was taken as control. The dead nematodes were observed under an inverted binocular microscope after incubation of 24 and 48 h and percentage of mortality was calculated. Nematodes were considered dead if they did not move when probed with a fine needle [20].

DNA cleavage studies

Agarose gel electrophoresis

2 μL of each sample (for - ve control no sample is added, for +ve control 2 μL $FeSO_4$ and metal aminophosphates) was taken separately in eppendorf tubes and were added to the λDNA (5 μL). The contents were incubated at 37 °C for 1 h and then 10 μL of enzyme buffer, 1 μL of Hind-III enzyme and 13μL of deionized water were added and incubated at 37 °C for 3 h. The contents

of the wells were mixed with bromophenol blue dye (1:1) and were loaded carefully into the electrophoresis chamber wells along with standard DNA marker containing TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1 L) and finally loaded on agarose gel and passed 50 V of current for around 2 h. The gel was removed and stained with 10 μg mL^{-1} ethidium bromide for 10 - 15 min and the bands observed under UV transilluminator and photographed to determine the extent of DNA cleavage. The results were compared with standard DNA marker. **It was observed that in the standard 2** μL **$FeSO_4$ the complete DNA cleavage was observed.**

Anticancer activity

Preparation of media and other reagents required for cell culture

DMSO has shown good solubility for all the compounds. 80, 40, 20 and 10 μg mL^{-1} doses were prepared for each compound by using growth medium.

Cell culture and drug treatment

HL60 cell line (human cervix cancer cell line), HeLa (human leukemia cancer cell line) and MCF7 (human breast carcinoma cell line) were used. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) in a humidified atmosphere containing 5% CO_2. About 5×10^3 cells/well was seeded in 96-well micro titer plate using culture medium. After 24 h, the new medium with compounds in the concentration of 80, 40, 20 and 10 μg mL^{-1} were added at respective wells and kept in incubator for 48 h. After incubation the sulforhodamine B assay was performed.

Sulforhodamine B (SRB) assay

Each compound was tested at four different concentrations against three different cancer cell lines such as Human Leukemia Cancer Cell Line (HL60), Human Breast Cancer Cell Line (MCF7) and Human Cervix Cancer Cell Line (HeLa). The cells were inoculated into 96-well micro titer

plates in 90 µL at plating density of 5×10^3 cells per well depending on the doubling time of individual cell lines. After cell inoculation, the micro titer plates were incubated for 24 h prior to addition of standard. After 24 h, one plate of each cell line was fixed *in situ* with trichloroaceticacid (TCA) to represent a measurement of the cell population for each cell line at the time of drug addition. Experimental drugs were solubilized in appropriate solvent at 400-fold of the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 10^{-3}. Additional three, 10-fold serial dilutions were made to provide a total of four drug concentrations plus control. Aliquots of 10 µL of these different drug dilutions were added to the appropriate micro titer wells already containing 90 µL of medium, resulting in the required final drug concentration.

After the addition of compounds, plates were incubated for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µL of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. Sulforhodamine B (SRB) solution (50 µL) at 0.4% (w/v) in 1% acetic acid has been added to each well and the plates were incubated for 20 min at room temperature. Bound stain was subsequently eluted with 10 mM trizma base and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percentage growth was calculated on a plate by plate basis for test wells relative to control wells. Percentage growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells × 100. Using six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels.

Percentage growth inhibition was calculated as:

[(Ti-Tz)/(C-Tz)] × 100 for concentrations for which Ti>/=Tz (Ti-Tz) positive or zero.

[(Ti-Tz)/Tz] × 100 for concentrations for which Ti<Tz. (Ti-Tz) negative.

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI50) was calculated from [(Ti-Tz)/(C-Tz)] × 100 = 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti-Tz)/Tz] × 100 = -50. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. Among the series, nine compounds were evaluated for anticancer activity on three different human cancer cell lines with standard drug doxorubicin for comparison. All experiments were repeated for a minimum of three times with each experiment done in four replicates.