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ORIGINAL ARTICLE

A novel N-alkylated prodigiosin analogue induced death in tumour cell through apoptosis or necrosis depending upon the cell type

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Abstract
Purpose To investigate the mechanism of cell death induced by the N-alkylated prodigiosin analogue, 2,2′-[3-methoxy-1′amyl-5′-methyl-4-(1′′-pyrryl)] dipyrryl-methene (MAMPDM) in S-180 and EL-4 tumour cell lines.
Methods Effect of MAMPDM on cell viability was assessed by MTT dye conversion. Induction of apoptosis was assessed by monitoring caspase 3 activity using a fluorogenic substrate, fragmentation of DNA by gel electrophoresis and sub-diploid DNA containing cells by flowcytometry. Necrosis was estimated by flowcytometric analysis of the uptake of propidium iodide.
Results MAMPDM inhibited the proliferation of murine fibrosarcoma, S-180 cells and induced cell death. Investigations into the mechanism of cell death by MAMPDM in S-180 cells showed absence of hallmarks of apoptotic cell death such as activation of caspase 3, DNA fragmentation and presence of cells with sub-diploid DNA content. However, there was a rapid loss of membrane integrity as assessed by uptake of propidium iodide, which is characteristic of necrosis. In contrast to induction of necrosis in S-180 cells, MAMPDM induced apoptotic cell death in EL-4 cells as evident by activation of caspase 3, fragmentation of DNA and sub-diploid DNA containing cells.
Conclusions MAMPDM could induce cell death by either apoptosis or necrosis depending upon the cell type. This would be of advantage in elimination of tumor cells defective in apoptotic pathway and therefore, refractory to the conventional therapies.

Keywords Apoptosis · Necrosis · Prodigiosin · S-180

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Introduction

Apoptosis is distinguished from necrosis or passive cell death by specific biochemical and morphological features such as externalization of phosphatidylserine in the cell membrane, activation of caspases, loss of mitochondrial membrane potential, DNA fragmentation, membrane blebbing and cell shrinkage [1]. The cellular contents of the dying cells are packaged into small apoptotic bodies that are removed by the phagocytic cells. On the other hand, during necrosis there is cell swelling with early loss of plasma membrane integrity and rapid lysis of the cells resulting in the release of cellular contents and inflammation. Apoptosis can be induced by a variety of external agents including cytotoxic drugs. Its significance in cancer and cancer therapy has been emphasized in several reviews [2–4].

Prodigiosins, a family of tripyrrole red pigments isolated from some Gram-negative bacteria, have emerged as a novel group of compounds with diverse biological activities including anti-tumor activity. Prodigiosins and their synthetic derivatives have been reported to inhibit the proliferation and induce apoptosis in tumor derived cell lines [5–8] with no apparent toxicity towards normal cell

lines [5, 6]. The antiproliferative and cytotoxic effects of prodigiosin have also been observed in human primary cancer cells from chronic lymphocytic leukemia patients [9]. Prodigiosins have been reported to induce apoptosis independent of p53 [10], which is frequently mutated in a majority of the tumors. The possible mechanisms involved in the induction of apoptosis by prodigiosins have been reviewed [11]. In vivo too, prodigiosin has been found to suppress the growth of the tumor and its metastasis [5, 12].

A novel red pigment, 2,2′-[3-methoxy-1′ amyl-5′-methyl-4-(1′′-pyrryl)] dipyrryl methene (MAMPDM; Fig. 1) was isolated from a variant of *Serratia marcescens* in our laboratory [13]. Though MAMPDM was structurally similar to prodigiosins, unlike all known prodigiosins, the nitrogen atoms in MAMPDM were in a tertiary state with the amyl chain attached to nitrogen instead of carbon (Fig. 1) [13, 14]. This is the only N-alkylated prodigiosin known in the literature. MAMPDM showed immunosuppressive activity similar to that of the other prodigiosins [14] and selectively inhibited the proliferation tumor derived cell lines of lymphoid [14], myeloid and mesodermal origins as compared to the normal cell lines [15]. Therefore, studies were undertaken to study the mechanism of MAMPDM induced cell death in murine tumor cell lines that could be used for further in vivo investigations.

Materials and methods

Chemicals and reagents

RPMI-1640 cell culture medium, Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and *N*-Acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO) were purchased from Sigma Chemicals Company (St Louis, MO, USA). Fetal calf serum (FCS), penicillin, streptomycin were from GIBCO-BRL (Paisley, UK). Cell proliferation kit was obtained from Roche Applied Science (Penzberg, Germany). All other chemicals were purchased from reputed local suppliers.

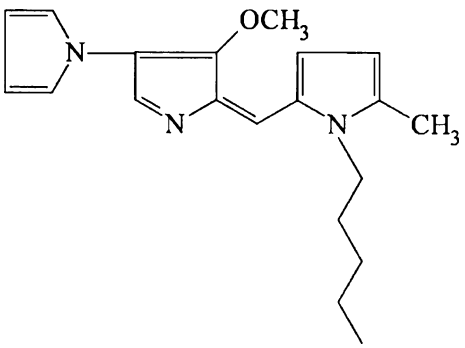

Fig. 1 Structure of MAMPDM. All the nitrogen atoms are in tertiary state with the alkyl side chain attached through the nitrogen atom

Isolation of prodigiosin analogue (MAMPDM)

MAMPDM was isolated by the method of Variyar et al. [13] from a bacterial isolate identified as *Serratia marcenscens* ost3 [15]. Briefly, the cells were harvested from a stationary culture and the pigment was extracted with acetone and *n*-hexane. The red sticky crude pigment was further purified by column chromatography on silica gel (60–120 mesh) followed by preparative thin layer chromatography (silica gel G; type 60) using petroleum ether and acetone as the solvent system. The red band at 0.47 R_f was excised and the red pigment was characterized by ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) in $CDCl_3$ in a Brucker AM 200 (Switzerland). IR spectroscopy was carried out in a Impact 410 Nicolet spectrometer (USA) with spectra referenced to residual solvent signals [13]. The molecular formula of the compound, MAMPDM (Fig. 1), $C_{20}H_{25}N_3O$ was derived by a combination of EI/CI/FAB mass spectrometry (M +, *m/z* 323) and HREI-mass spectrometry (323.19976) as described in our earlier publication [13]. The purified compound (MAMPDM) was dissolved in ethanol at 20 mM and stored at20°C in dark. Working dilutions were prepared from this stock solution prior to use.

Cell lines and culture conditions

Mouse fibrosarcoma (S-180) and T cell lymphoma (EL-4) cells used in these studies were obtained from National Centre for Cell Sciences (Pune, India) and maintained in RPMI 1640 medium containing 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and supplemented with 10% FCS. Cells were routinely subcultured and maintained in a humidified atmosphere with 5% CO_2 at 37°C. S-180 cells have been frequently used for in vivo screening of potential anti-cancer compounds as they can grow in different strains of mice [16] and were, therefore, selected for initial in vitro evaluation of MAMPDM. EL-4 cells are of lymphoid origin and have been reported to be susceptible to MAMPDM [14] and were therefore, used as controls.

Cytotoxicity assay

In vitro cytotoxic activity of MAMPDM against cancer cell lines was assessed by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) dye conversion assay [17]. Briefly, 2×10^5 cells ml^{-1} were cultured in a 96 well plate in absence or presence of different concentrations of MAMPDM. MTT dye was added to the wells after 48 h

of culture and further incubated for 4 h. The blue formazan precipitate was dissolved using solubilization buffer (Cell proliferation kit, Roche) overnight at 37°C. The absorbance at 550 nm was recorded using a scanning plate reader (Bio-Tek Instruments, USA). The results are expressed as the mean absorbance ± SEM for four replicates.

Caspase 3 assay

Cells were cultured with the indicated concentrations of MAMPDM for 16 h, washed two times with phosphate buffered saline (PBS; 10 mM, pH 7.4) and lysed in lysis buffer (10 mM Tris–HCl pH 8.0, 10 mM NaH_2PO4, 130 mM NaCl, 1% Triton X-100, 10 mM Sodium pyrophosphate, 5 mM EDTA, 2 mM PMSF, 5 mM DTT). Activity of caspase 3 in the lysates was estimated by the cleavage of the synthetic substrate, Ac-DEVD-AMC. For the assay, 200 μg of total protein was incubated with 5 μg of substrate in caspase 3 assay buffer (100 mM HEPES, 10% glycerol, 10 mM DTT, 500 μM EDTA; pH 7.2) for 1 h at 37°C. Specificity of the assay was determined by carrying out the assay in presence of specific inhibitor of caspase 3 (Ac-DEVD-CHO). The fluorescence of free AMC released following the cleavage of the peptide substrate by caspase 3 was estimated in a fluorimeter (Perkin-Elmer, USA). The results are expressed as mean fluorescence intensity (arbitrary units) ± SEM for three replicates.

Analysis of DNA fragmentation

Cells (2×10^5 cells ml^{-1}) were cultured in presence of indicated concentrations of MAMPDM for 24 h. The cells were washed two times with PBS and low molecular weight DNA was isolated by incubating the cells in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosine and 0.5 mg ml^{-1} proteinase K) at 50°C for 1 h. The isolated DNA was treated with 5 μl RNAse A (1 mg ml^{-1}) for 1 h and separated in 1% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide in Tris borate EDTA buffer. The DNA ladder pattern was visualized by a UV trans-illuminator and photographed.

Analysis of subdiploid DNA content

Percent apoptotic cells were estimated by staining with propidium iodide (PI) followed by flowcytometric analysis [18]. Briefly, the cells were cultured with or without MAMPDM for 24 h and were labeled with hypotonic PI solution (50 μg ml^{-1} PI in 0.1% sodium citrate and 0.1% Triton-X 100) overnight at 4°C. Three replicates were taken for each group and 20,000 cells were acquired per sample using a FACS Vantage flowcytometer (Becton Dickinson Immunocytometry systems, USA). The cells were analysed using CellQuest™ software and apoptotic cells having subdiploid DNA content (< G0/G1 DNA) were enumerated. The results are expressed as average percent apoptotic cells ± SEM for three replicates.

Determination of necrosis

The cells were cultured with or without MAMPDM for 4 h and washed two times with cold PBS. The cells were resuspended in PI solution (50 μg ml^{-1} in phosphate buffered saline) and were acquired and analyzed by flowcytometry [19]. Under these conditions only the necrotic cells take up PI while the live and apoptotic cells do not take up PI. Three replicates were taken for each sample and 20,000 cells were acquired for each sample.

All the experiments were repeated at least three times. Differences between control and treated samples were considered significant at a value of $P < 0.05$ using Student's *t* test.

Results

Effect of MAMPDM on the proliferation of cancer cell lines

The effect of MAMPDM on the proliferation of two murine tumor cell lines of different origins, EL-4 and S-180 was examined. In presence of increasing concentrations of MAMPDM, a dose dependent decrease in the proliferation of both S-180 and EL-4 cells was observed (Fig. 2). Though S-180 cells (IC_{50} 0.211 ± 0.02 μM) showed slightly higher sensitivity to MAMPDM as compared to EL-4 cells (IC_{50} 0.254 ± 0.01 μM), it was statistically not significant ($P < 0.05$).

DNA fragmentation by MAMPDM

One of the hallmarks of apoptotic cells is the extensive cleavage of genomic DNA at the linker region resulting in oligomers of 180–200 bp. These give a characteristic ladder pattern when analysed by agarose gel electrophoresis. The DNA extracted from S-180 cells treated with either 0.5, 1 or 5 μM of MAMPDM did not show the characteristic ladder pattern (Fig. 3). However, similarly treated (0.5 and 1 μM) EL-4 cells showed fragmentation of DNA and the ladder pattern (Fig. 3).

Analysis of hypodiploid DNA containing cells

Permeabilization of the apoptotic cells results in loss of low molecular weight DNA and these cells appear as sub

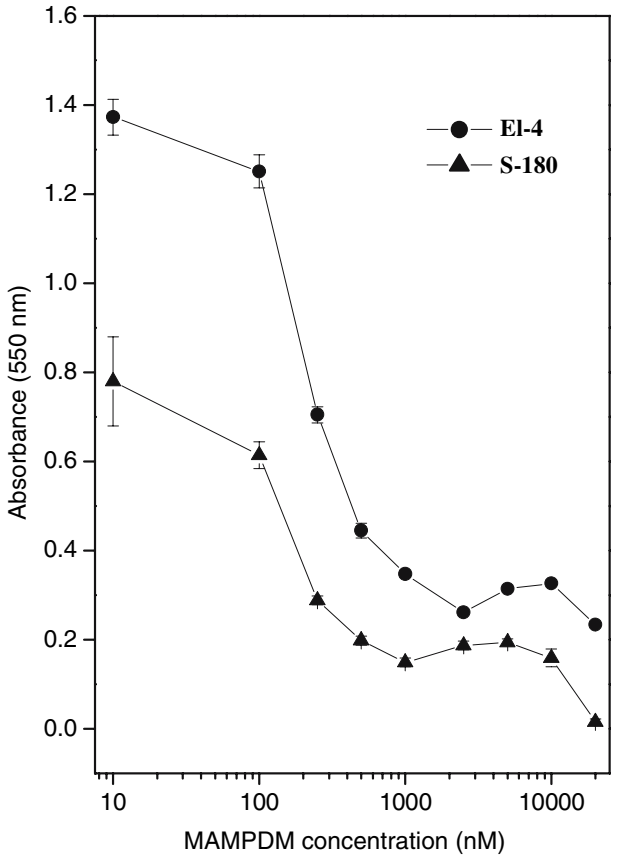

Fig. 2 Effect of MAMPDM on the proliferation of cancer cell lines. Cells (2×10^5 per ml) were incubated in presence or absence of different concentrations of MAMPDM for 48 h and the viability was determined by MTT assay. Each point corresponds to mean of four replicates and the bars show SEM. Representative data from one of the three similar experiments are shown

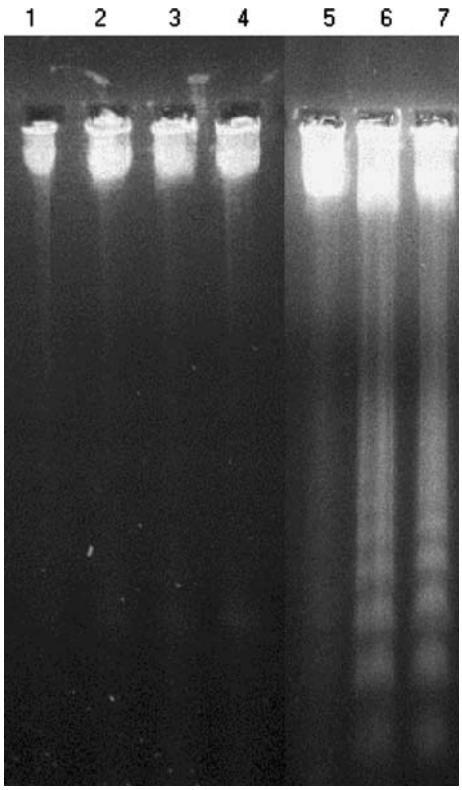

Fig. 3 Effect of MAMPDM on DNA fragmentation in S-180 cells (lanes *1–4*) and EL-4 cells (lanes *5–7*). Cells were treated with the indicated concentrations of MAMPDM for 24 h and low molecular weight DNA was extracted. The agarose gel electrophoresis was carried out as described in methods. Lanes- *1* Untreated S-180 control cells; *2* treated with 0.5 μM MAMPDM; *3* treated with 1 μM MAMPDM. *4* treated with 5 μM MAMPDM; *5* Untreated EL-4 control cells; *6* treated with 0.5 μM MAMPDM; *7* treated with 1 μM MAMPDM. Representative data from one the three similar experiments are shown

G1 peak in DNA analysis by flowcytometry. In S-180 cells treated with MAMPDM for 24 h, only a marginal increase, from $2.43 \pm 0.08\%$ in untreated cells to $6.45 \pm 0.73\%$ and $6.81 \pm 0.52\%$ was observed in cells treated with 0.5 and 1 μM MAMPDM, respectively (Fig. 4). In contrast, the percentage of the EL-4 cells with hypodiploid DNA content increased from $3.06 \pm 0.12\%$ in control cells to $35.70 \pm 0.36\%$ and $50.95 \pm 1.26\%$ in presence of 0.5 and 1 μM MAMPDM, respectively (Fig. 4).

Effect of MAMPDM on caspase 3 activation

Activation of caspase 3 is one of the most important events during apoptotic cell death. Lysates of S-180 cells treated with MAMPDM did not show any significant increase in the caspase 3 activity (Fig. 5). Even at 5 μM MAMPDM, which showed almost 90% cytotoxicity, there was no significant increase in caspase 3 activity (data not shown). However, lysates of EL-4 cells treated with 0.5 and 1 μM MAMPDM for 16 h showed an 8 to 9-fold increase in the fluorescence intensity due to the cleavage of synthetic caspase substrate as compared to the untreated cells (Fig. 5) indicating the presence of active caspase 3 in these cells. The specificity of substrate cleavage by caspase 3 was confirmed by the negligible fluorescence in presence of a specific inhibitor of caspase 3(Ac-DEVD-CHO).

Effect of MAMPDM on membrane permeability

The necrotic cells show an early loss of cell membrane integrity while in apoptotic cells the membrane integrity is maintained but the asymmetrical distribution of phosphatidyl serine is lost. This property has been used to discriminate apoptotic cells from the necrotic cells using Annexin V and propidium iodide. Since, MAMPDM interfered with Annexin V-FITC fluorescence (data not shown), only

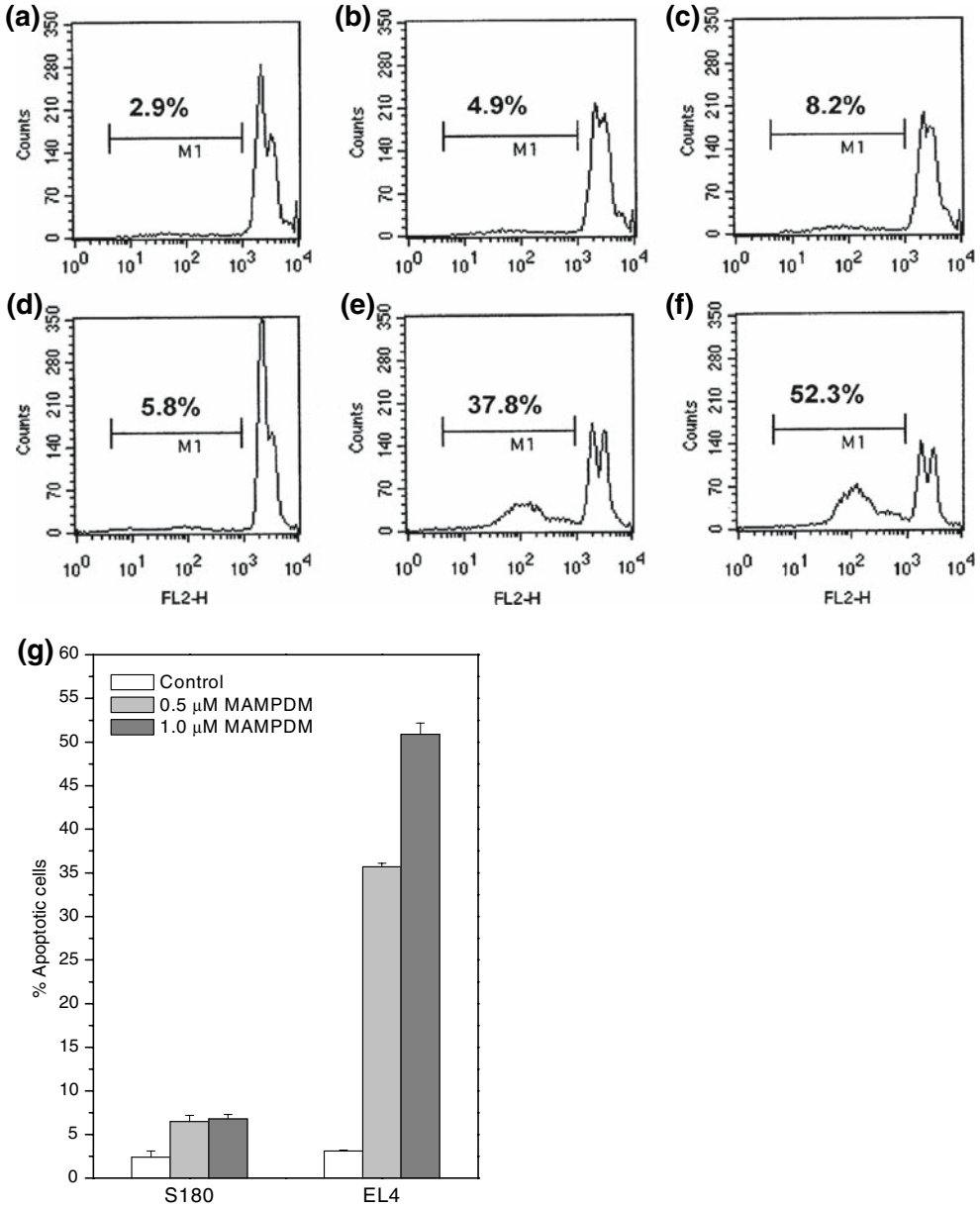

Fig. 4 Flowcytometric profiles of propidium iodide labeled S-180 cells (**a**–**c**) and EL-4 cells (**d**–**f**). S-180 and EL-4 cells were treated with vehicle (**a** ,**d**), 0.5 μM MAMPDM (**b** , **e**) or 1 μM MAMPDM (**c** , **f**) respectively, for 24 h and labeled with hypotonic propidium iodide solution. The cells were acquired and analyzed in a flowcytometer. The fraction of cells (M1) with sub-G1 DNA content (apoptotic cells) is indicated in each profile. (**g**) The data points indicate the mean percent apoptotic cells ± SEM for three replicates. Representative histograms from one of the three similar experiments are shown

uptake of PI was estimated. As seen from Fig. 6, in S-180 cells treated with 0.25 μM and 0.5 μM MAMPDM for 4 h, the percent PI positive cells increased to 25.83 ± 0.55% and 73.78 ± 0.84%, respectively, as compared to 4.54 ± 0.60% in untreated cells (Fig. 6a–c). In contrast, in EL-4 cells similarly treated with 0.25 and 0.5 μM MAMPDM for 4 h the percentage of PI positive cells was 6.72 ± 0.19% and 25.07 ± 2.49% respectively, as compared to 9.20 ± 0.69% in control (Fig. 6d–f).

Effect of staurosproine and actinomycin D on S-180 cells

Since our observations showed that MAMPDM induced necrosis in S-180 cells, we evaluated the effect of other known anti-tumor compounds and inducers of apoptosis on S-180 cells to see whether these cells were defective in apoptotic mechanisms. Our results showed that though staurosporine and actinomycin D induced concentration dependent decrease in the viability of S-180 cells (Fig. 7a),

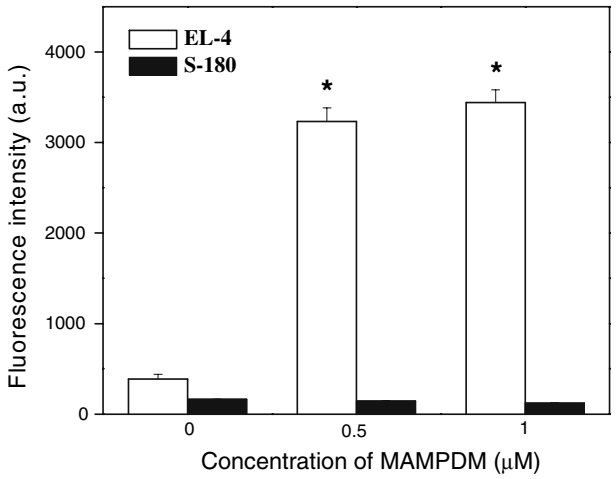

Fig. 5 Effect of MAMPDM on activity of caspase 3 in tumor cells. Lysates of the treated cells were incubated with the fluorogenic substrate, DEVD-AMC in presence or absence of specific inhibitor (DEVD-CHO). The data points indicate the mean of three replicates and the bars indicate SEM. Representative data from one of the three similar experiments are shown

DNA fragmentation characteristic of apoptosis was not observed in the treated S-180 cells (Fig. 7b).

Discussion

Most of the cytotoxic agents used in cancer chemotherapy have been reported to exert their effect by inducing the apoptotic program in the tumor cells [20, 21]. The proposed potential of prodigiosins as an anti-cancer agent is based on their ability to selectively inhibit the proliferation and activate apoptotic death program in cancer cells. However, a major issue in cancer chemotherapy is the resistance of the tumor cells to undergo apoptosis as a consequence of accumulated mutations [20]. Although many cancer therapeutics induce classical apoptosis, potential drugs engaging other death routes are emerging [22, 23].

MAMPDM has earlier been reported to induce apoptotic cell death in mitogen stimulated lymphocytes [14]. However, tumor cells often differ in the death signaling pathways from the normal cells. Therefore, studies were carried out to investigate the anti-tumor activity of MAMPDM. It inhibited the proliferation of S-180 cells in vitro (Fig. 2). Though there was a reduction in the viability of S-180 cells in presence of MAMPDM, it was not due to induction of apoptosis. The important hallmarks of classical apoptotic death such as activation of caspase 3 (Fig. 5), fragmentation of DNA (Fig. 3) and presence of cells with hypodiploid DNA content (Fig. 4) were absent in MAMPDM treated S-180 cells. These cells also showed an early loss of cell permeability (Fig. 6), which is considered characteristic of necrotic cell death rather than apoptotic cell death [19]. Differences between prodigiosin analogues in their ability to induce apoptotic cell death in lymphocytes have been reported [24]. Therefore, the ability of MAMPDM to induce apoptosis was assessed in another tumor cell line, EL-4 . In contrast to S-180 cells, EL-4 cells showed increase in the activity of caspase 3 (Fig. 5), fragmentation of DNA giving characteristic ladder pattern (Fig. 3), presence of hypodiploid DNA containing cells (Fig. 4) and retention of membrane integrity (Fig. 6d–f) when treated with similar concentrations of MAMPDM. These results showed that MAMPDM could induce apoptosis in tumor cells but the mechanism might not be the same in different cell lines. Similar cell type related differences in mode of induction of cell death (apoptosis or necrosis) have been reported for TNF-α [25].

Absence of caspase 3 activity in S-180 cell could explain the absence of DNA fragmentation as it has been reported to be essential for DNA fragmentation [26]. However, prodigiosin induces classical features of apoptotic cell death in MCF-7 cells that do not have caspase 3 through activation of alternate executioner caspase 7 [27]. So it is likely that in S-180 cells the activation of the executioner caspases does not take place. Inhibition of caspases by specific inhibitors has been reported to switch the mode of death from apoptosis to necrosis rather than promote cell survival [28] indicating that caspases are not responsible for active cell death per se, but for its characteristic features. This was also reflected in the lack of apoptotic features in S-180 cells upon treatment with cytotoxic concentrations of MAMPDM. It would have been interesting to compare the effect of MAMPDM with some of the other prodigiosins, however, due to non-availability of prodigiosins commercially such comparisons could not be carried out. Our results suggest that S-180 cells are possibly deficient in the apoptotic pathway as other well known anti-tumor compounds like actinomycin D and staurosporine also did not show any of the classical hallmarks of apoptosis in S-180 cells though there was a reduction in cell viability (Fig. 7). These results suggest that S-180 cells do not undergo apoptotic cell death. However, there are a few reports of some features of apoptosis in S-180 cells [29, 30].

It is known that different cell death pathways coexist in mammalian cells and their activation depends upon the type and intensity of the stimulus [22]. A lot of importance has been given to the apoptotic cell death in anti-tumor therapy but the physiologic importance of necrotic cell death in cancer therapy has not been explored. Necrotic cell death leads to release of the cellular contents in the environment thereby inducing inflammation, which has been reported to contribute to the process of carcinogenesis [31, 32]. However, there are also some reports suggesting that necrotic cell death promotes anti-tumour immunity. It has been reported that macrophages co-cultured with necrotic tumor

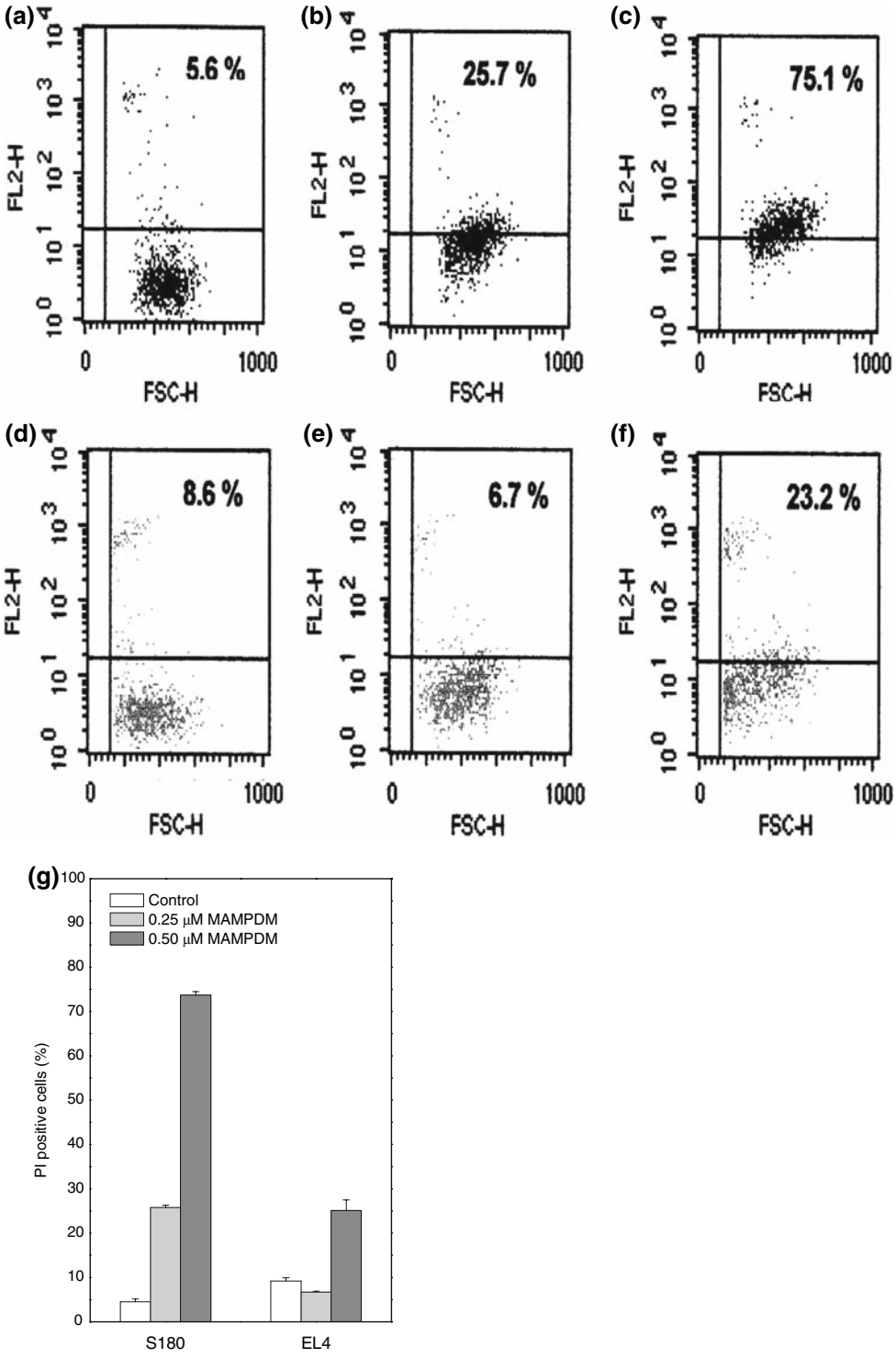

Fig. 6 Loss of membrane permeability as measured by propidium iodide uptake in S-180 (**a**–**c**) cells and EL-4 cells (**d**–**f**). S-180 and EL-4 cells were treated with vehicle (**a** ,**d**), 0.25 μM MAMPDM (**b** , **e**) or 0.5 μM MAMPDM (**c** ,**f**), respectively, for 4 h and were labeled with PI. Upper right quadrant indicates cells that are positive for PI (**g**) The data points indicate the mean per cent necrotic cells ± SEM for three replicates. Representative data from one of the three similar experiments are shown

cells developed enhanced competence to get rid of tumor cells, while those co-cultured with apoptotic cells showed not only decreased clearance, but also promoted the growth of tumor cells [33, 34].

Necrotic cell death is believed to be accidental and its regulation is not well understood. However, several reports have shown that like apoptosis necrosis is also regulated by intrinsic death programs [28, 35, 36]. Activation of the necrotic program by MAMPDM as observed in S-180 cells would, therefore, be complementary to the apoptosis inducing activities. In addition, a model system for necrotic cell death would aid in better understanding and manipulation of this form of cell death. Thus, MAMPDM induced necrotic cell death in a sarcoma cell line and apoptosis in a

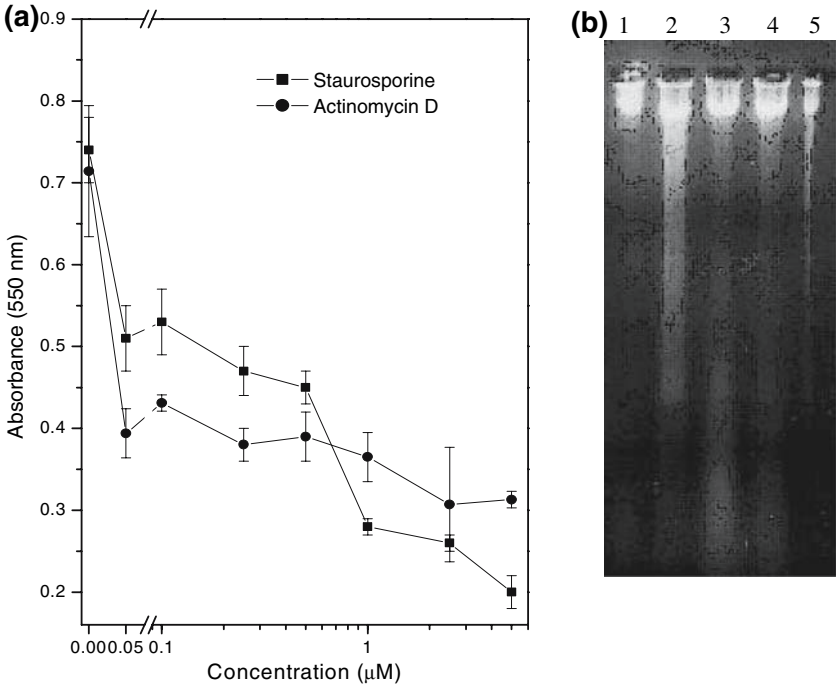

Fig. 7 **a** Effect of actinomycin D and staurosporine on the viability of S-180 cells. The cells (2×10^5 cells per ml) were cultured in presence of different concentrations of either actinomycin D or staurosporine for 48 h and the cell viability was assessed by MTT assay. Each data point represents the mean of four replicates and the bars indicate SEM. **b**)Effect of actinomycin D and staurosporine on DNA fragmentation in S-180 cells. Different treatment groups were lane *1* vehicle, *2* 1 μM staurosprine, *3* 2 μM staurosporine, *4* 1 μM actinomycin D and *5* 2 μM actinomycin D. Low molecular weight DNA was isolated after 24 h and was subjected to electrophoresis on agarose gel. Representative data from one of the two similar experiments are shown

lymphoid cell line reported here shows that the response of tumor cells to this cytotoxic agent is cell type specific.

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