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PRECLINICAL STUDIES

A novel structural derivative of natural alkaloid ellipticine, MDPSQ, induces necrosis in leukemic cells

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Summary DNA intercalating molecules are promising chemotherapeutic agents. In the present study, a novel DNA intercalating compound of pyrimido[4′,5′:4,5]selenolo(2,3-b) quinoline series having 8-methyl-4-(3 diethylaminopropylamino) side chain is studied for its chemotherapeutic properties. Our results showed that 8-methyl-4-(3 diethylaminopropylamino) pyrimido [4′,5′:4,5] selenolo(2,3-b)quinoline (MDPSQ) induces cytotoxicity in a time- and concentration-dependent manner on leukemic cell lines. Both cell cycle analysis and tritiated thymidine assays revealed that MDPSQ affects DNA replication. Treatment with MDPSQ resulted in both elevated levels of DNA strand breaks and repair proteins, further indicating its cytotoxic effects. Besides, Annexin V/PI staining revealed that MDPSQ induces cell death by triggering necrosis rather than apoptosis.

Keywords Chemotherapy · Double-strand breaks · Cytotoxicity · DNA damage · Anti cancer drugs

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Introduction

Cancer is one of the major causes of death worldwide. Haematopoietic malignancies like leukemia and lymphoma account for approximately 8% of total human cancers [1]. These cancers are characterized by the presence of genetic abnormalities like chromosomal translocations, which play a key role in their initiation [1–4]. These abnormalities may result in upregulation of proto-oncogenes, downregulation of tumor suppressor genes or fusion of genes [5]. Even though there have been tremendous progress in understanding the underlying mechanisms that cause these cancers, finding a complete cure has remained a major challenge and an important research area [1, 5]. Chemotherapy is one of the methods used for the treatment of neoplasia and there has been an increasing need for the development of new and more potent cytotoxic agents to combat cancer.

Development of structural analogs of known chemotherapeutic compounds has been of great significance in the past. Ellipticine is one such successful drug, whose derivatives are used to treat breast cancers and other tumors. 5,11-dimethyl-6H-pyrido[3,2-b]carbazoles are structural derivatives of ellipticine which have shown antitumour activity against leukemia, melanoma and sarcoma cell lines [6]. Since ellipticine has been shown to have an effect on leukemic cells, we synthesized compounds related to ellipticine/pyridocarbozoles which exhibit antitumour activity by DNA intercalation [7–11].

We have reported the activities of pyrimidothieno/selenoloquinolines with methoxy, morpholino, oxo-chloro, anilino, butylamino and piperazino substitutions earlier and observed that the length and position of the side chain is crucial for their cytotoxicity [12–16]. Substitution of positively charged butylamino group with hydrophilic alkyl side chain, was able to arrest cell cycle at S phase and alter

the proapoptotic to antiapoptotic protein ratio in leukemic cells [13]. This was possible as the linear side chain could bury itself in the DNA, thereby having more retention time [13]. On the same chemical skeleton, replacement of butylamino group with hydrophobic aryl piperazino side chain drastically reduced the cytotoxic potency as the aryl nature of piperazino substitution prevented it from interacting with DNA [13]. Considering the consequences of butylamino side chain substitution and in search of a more potent cytotoxic molecule, we screened a compound having positively charged bulky alkyl side chain, 8-methyl-4-(3 diethylaminopropylamino) pyrimido [4′,5′:4,5] selenolo (2,3-b)quinoline (MDPSQ). We have previously characterized another compound, MDPTQ, carrying diethylaminopropylamino, a bulky chain of positive charge and reported its DNA binding ability and cytotoxicity [12, 17, 18].

In this study, we report for the first time the anti-cancer property of the novel compound, 8-methyl-4-(3 diethylaminopropylamino) pyrimido [4′,5′:4,5] selenolo(2,3-b)quinoline, a structural derivative of the natural alkaloid ellipticine. We find that MDPSQ exhibits a time- and dose-dependent cytotoxic activity on leukemic cells by causing DNA damage, which leads to upregulation of DNA double-strand break repair proteins. Finally, we show that the cell death by MDPSQ occurs through necrosis rather than apoptosis.

Materials and methods

Chemicals and reagents

Unless otherwise mentioned, all the chemicals used were from Sigma-Aldrich, USA. Tritiated thymidine was purchased from BRIT, India. Annexin V-FITC and antibodies were purchased from Santa Cruz Biotechnology, USA.

Cell lines and culture

Human cell lines, K562 (chronic myelogenous leukemia) and CEM (T-cell leukemia) were purchased from National Centre for Cell Science, Pune, India. B-cell leukemic cell lines, REH and NALM6, were obtained from Prof. Michael Lieber, University of Southern California, USA. In all the cases, cells were grown in RPMI 1640 containing 10% FBS, 100 U of Penicillin G/ml and 100 μg of streptomycin/ml at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of compounds

The synthesis and characterization of the compound used in the present study was reported earlier [19, 20]. In brief, a mixture of 4-chloropyrimido[4′,5′:4,5]- selenolo(2,3-b) quinoline (0.4 g, 0.0013 M) in ethyl alcohol (80 ml) and N, N′-diethylamine (0.228 g, 0.003 M) was refluxed with stirring for 6 h. The reaction mix was cooled & poured into ice-cold water. The resultant precipitate was filtered & recrystalized in aqueous alcohol. NMR (ppm): ($CdCl_3$: ∂,1.4 (6H), t, $2CH_3$, 3.75 (4H) q, $2CH_2$, 7.2–8.15 (4H,m, H-7,H-8,H-9,H-10), 8.65 (1H,s,H-11),9.35 (1H,s,H-2). Stock solution of MDPSQ (Fig. 1a) was prepared by dissolving in DMSO, followed by addition of PBS.

Trypan blue exclusion assay

Trypan blue exclusion assay was performed to assess the effect of MDPSQ on the viability of cells [13]. Approximately 1×10^5 cells/ml were cultured in six well plates for 24 h and MDPSQ was added at concentrations of 10, 50,100 and 250 μM. After treatment, cells were collected every 24 h. The viable cells were counted using haemocytometer under a microscope. Each experiment was done a minimum of three times with very good agreement.

MTT assay

MTT assay was performed as described earlier [21, 22]. Briefly, cells in exponential growth phase were cultured in triplicates in 96 well plates at a density of 1×10^4 cells/ well in a volume of 100 μl. MDPSQ was added at concentrations of 10, 50,100 and 250 μM and studied after 48, 72 and 96 h of culture. Cells grown in culture media alone or with appropriate concentrations of DMSO were used as controls. The cytotoxicity caused by the compound was expressed as % cell proliferation.

[^{3}H] thymidine incorporation assay

The [^{3}H] thymidine assay was performed as described earlier [23]. Cells were cultured in duplicates in 96 well plates at a density of 1.25×10^4 cells/well and treated with different concentrations of MDPSQ. The [^{3}H] thymidine (1 μCi) diluted in RPMI 1640 was added and after 48 and 96 h of incubation, the cells were harvested, processed and subjected to scintillation counting. Radioactivity of the samples were expressed as counts per minute.

Flow cytometry

Cells were cultured and treated with various concentrations of the compound as described above. After 72 and 96 h of treatment, cells were harvested, washed in ice cold PBS and permeabilized using 70% ethanol overnight. The fixed cells were centrifuged, washed and

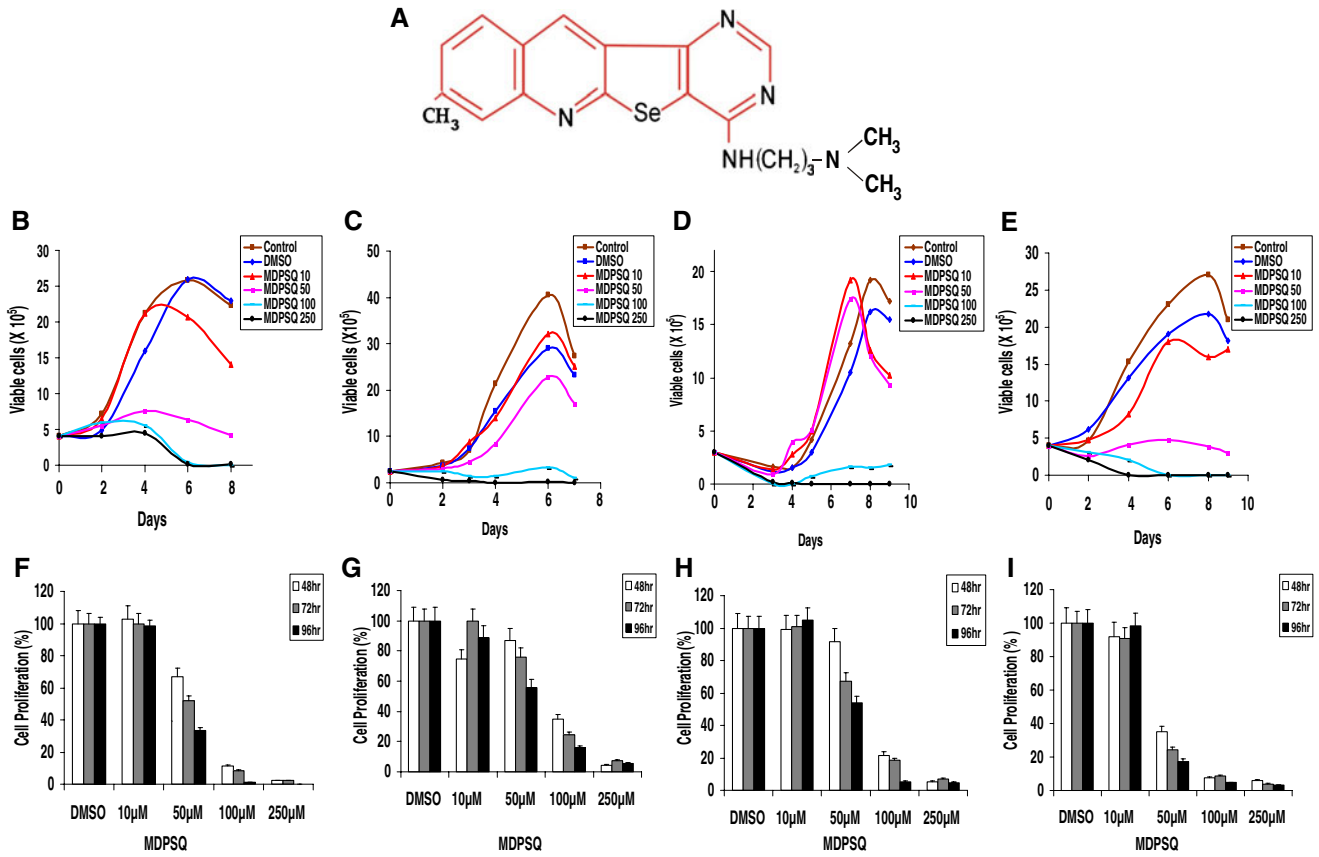

Fig. 1 Structure of MDPSQ and its cytotoxicity in leukemic cells. **a**. Structure of 8-methyl-4-(3 diethylaminopropylamino) pyrimido [4′,5′:4,5] selenolo(2,3-b)quinoline [MDPSQ]. **b–e.** Trypan blue assay was used to determine the cell viability. 1×10^5 cells/ml of K562 (**b**), REH (**c**), CEM (**d**) and NALM6 (**e**) were cultured. MDPSQ (10, 50, 100 and 250 μM) was added after 24 h and cells were counted every 24 h, till the control cells attained stationary phase. Data is represented as graphs. Cells alone and cells treated with DMSO were used as the controls. **f–i**. MTT assay showing effect of MDPSQ on cell survival. K562 (**f**), REH (**g**), CEM (**h**) and NALM6 (**i**) (1×10^5 cells/ml) were cultured in the absence (control) or presence of 10, 50, 100 and 250 μM of MDPSQ for 48, 72 and 96 h. MTT reagent was added and cells were processed. Mitochondrial reduction of MTT was determined by measuring the absorbance at 570 nm using microplate reader. Each of these experiments have been performed a minimum number of three times with very good agreement. Data is presented as histograms and the error bars are indicated

resuspended in ice cold PBS. RNase (100 μg/ml) was added and incubated overnight at 37°C. Propidium iodide (PI, 50 μg/ml) was added half an hour before acquiring the flow cytometric reading (FACScan, BD Biosciences, USA). A minimum of 10,000 cells were acquired per sample and histograms were analyzed by using WinMIDI 2.8 software.

Annexin V-FITC/ propidium iodide staining assay

Flow cytometric detection of phosphatidyl serine exclusion on K562 cells treated with MDPSQ using FITC conjugated Annexin V was carried out as described earlier [13, 24]. Briefly, treated cells were washed in PBS and resuspended in binding buffer. Annexin V- FITC (0.2 μg/μl) and PI (0.05 μg/μl) were added and cells were subjected to FACS analysis. At least 10,000 events were recorded and represented as dot plots.

Confocal microscopy

Confocal microscopy was performed to visualize the early and late apoptotic cells as previously described [25]. MDPSQ treated cells were resuspended in the binding buffer. Annexin V- FITC and PI (0.05 μg/μl) were added to this mix and the cells were spread over a slide and observed under an inverted confocal laser scanning microscope (Ziess LSM 510^{MK4}, Germany).

LDH release assay

Release of lactate dehydrogenase (LDH) is an indicator of loss of membrane integrity and hence cell injury. LDH assay was performed to assess its release into the medium following MDPSQ treatment (100 μM) on K562 cells for 12, 24 and 48 h using standard protocols [23]. The percentage of LDH released was calculated

as: (LDH activity in media) / (LDH activity in media + intracellular LDH activity) × 100.

Comet assay

Comet assay was performed based on the studies described earlier [22]. Treated K562 cells were washed and mixed with low melting agarose (0.5%) and spread on agarose (1.5%) precoated slides. The slides were placed on ice for 10 min, submerged in lysis buffer for more than one hour, equilibrated in alkaline buffer and electrophoresed at 0.86 V/cm. The slides containing cells were then neutralized in 0.4 M TBE, washed and dried. Slides were stained with PI (2.5 μg/ml), destained in distilled water and observed under fluorescence microscope (Olympus BX51 Upright fluorescence Microscope, USA).

Western blotting

Following 12, 24, 48 or 72 h of MDPSQ treatment (100 μM), K562 cells were harvested, washed with PBS, resuspended in RIPA buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS; 1 ml of RIPA buffer for 5×10^6 cells) with protease inhibitors and incubated on ice for 1–2 h. The pellets were sonicated, supernatant was collected and protein concentration was estimated by Bradford method.

For immunoblot analysis, ~40 μg of proteins was loaded over a SDS-PAGE (8–10%). After electrophoresis, the protein was transferred to PVDF membrane (Millipore, USA) using standard protocol [22]. After incubating in blocking solution (5% skimmed milk) for 2 h at room temperature, the membrane was probed with primary antibodies against Ku80, DNAPKcs, MRE11, RAD50, NBS1, PARP, PCNA, Caspase3, Caspase9, BCL2, FAS, p73 or p53, overnight at 4°C. Following washing (0.1% Tween 20 in PBS), the membrane was incubated (2 h at 4°C) with appropriate HRP-conjugated secondary antibodies, which were either monoclonal anti-mouse, polyclonal anti-rabbit or anti-goat. The blot was developed using chemiluminescent solution (Immobilon™ western, Millipore, India) and scanned by gel documentation system (LAS 3000, FUJI, JAPAN). Blots were stripped subsequently as per standard protocol and reprobed with anti-tubulin.

Results

MDPSQ, a compound with methyl diethylaminopropylamino side chain reduces cell viability

We have used trypan blue and MTT assay to determine the cytotoxic effect of MDPSQ on leukemic cells K562, REH, CEM and NALM6. Cells growing in log phase were treated with 10, 50, 100 or 250 μM concentrations of MDPSQ. Since the compound was dissolved in DMSO, the cells with DMSO (equivalent to DMSO used in 250 μM concentration) were used as vehicle control. The viability of the cells was determined using trypan blue, at intervals of 24 h till the control cells attained stationary phase (Fig. 1b–e). Results showed that addition of MDPSQ affected the viability of the cells significantly in all four leukemic cell lines (Fig. 1b–e). The effect was pronounced when 50 μM MDPSQ was used in the case of K562 and NALM6 as early as 48 h (Fig. 1b, e). However, concentrations of 100 and 250 μM resulted in significant cell death. In the case of REH and CEM, MDPSQ suppressed cell growth only at 100 and 250 μM (Fig. 1c, d), suggesting that the effect of MDPSQ differs between cell types. In all the panels, growth of control cells and DMSO treated cells were comparable (Fig. 1b–e). These results suggest that MDPSQ induces cytotoxicity on human leukemic cells.

The cytotoxicity was further verified by using MTT assay. K562, REH, CEM and NALM6 cells were treated with 10, 50, 100 and 250 μM of MDPSQ, harvested after 48, 72 and 96 h and were subjected to MTT assay (Fig. 1f–i). Results showed that in the case of K562 and NALM6, cell death was prominent at 50 μM concentration itself, while viability was decreased more than 90% at 100 and 250 μM (Fig. 1f, i). However, in the case of REH and CEM MDPSQ treatment led to reduced cytotoxicity (Fig. 1g, h). These results further suggest that MDPSQ affects the viability of the cells in a time- and dose-dependent manner which varied among different leukemic cells.

MDPSQ affects cell proliferation by inhibiting DNA replication

Since trypan blue and MTT results showed that MDPSQ affects the viability of the cells, we were interested to test whether it affects the cell division. To study this, we cultured K562, REH, CEM and NALM6 cells in the presence of [^{3}H] thymidine after the addition of MDPSQ as described in "Materials and methods". Results showed that MDPSQ affected the incorporation of [^{3}H] thymidine significantly in K562, REH and NALM6 at 50,100 and 250 μM (Fig. 2a, b, d) but to a lesser extent in CEM cells (Fig. 2c). These results indicate that MDPSQ affects the cell proliferation by inhibiting cell division, possibly by abrogation of DNA replication.

Cell cycle analysis after MDPSQ treatment

FACS analysis was performed to study the effect of MDPSQ on cell cycle progression. K562 and CEM cells were harvested after 72 and 96 h of treatment with the

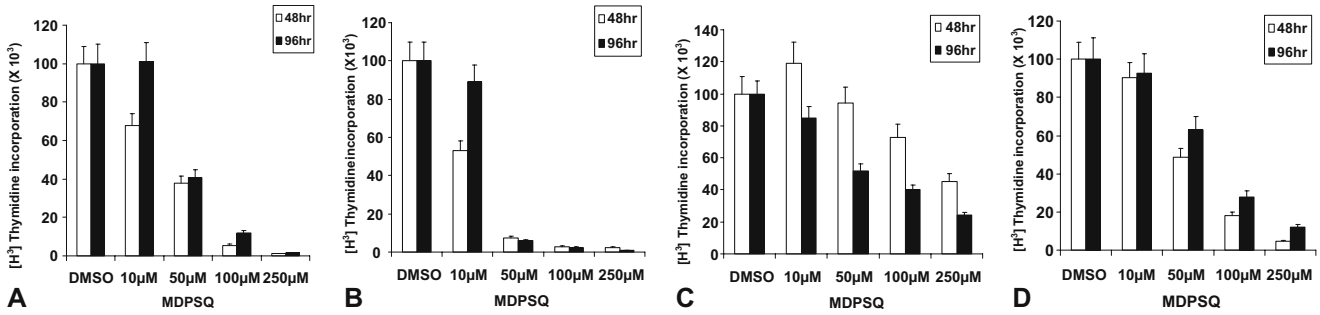

Fig. 2 Effect of MDPSQ on DNA synthesis as measured by ^{3}[H]-thymidine incorporation assay. 10, 50,100 or 250 μM of MDPSQ was added to K562 (**a**), REH (**b**), CEM (**c**) and NALM6 (**d**) cells (1×10^{5}/ml) after 24 h of culture. Upon 8 h of addition of MDPSQ, tritiated thymidine was added, and incorporation was measured after 48 and 96 h by liquid scintillation counter. The experiment was done atleast twice with good agreement

compound. The cells were stained with PI and subjected to flow cytometry. The histogram of control and DMSO treated cells showed a standard cell cycle pattern, in which G1 and G2 peaks were separated by S phase (Fig. 3a–d). Sub G1 peak (mostly dead cells) was either absent or not prominent. Interestingly upon addition of MDPSQ to K562 and CEM cells, a concentration- and time-dependent change was observed in the cell cycle pattern. Although we could not find any prominent cell cycle arrest, we did observe a distinguishable increase in S phase at low concentration of MDPSQ, both in the case of K562 and CEM cells (Suppl. Fig. 1A, B, D, E). The effect was more prominent at 72 and 96 h suggesting that MDPSQ could induce cell cycle arrest at S phase. Interestingly, 100 and 250 μM concentration at 72 h caused an increase in the accumulation of cells in sub G0/G1 peak and decline in both G1 and G2/M peaks indicating that the treatment led to cell death (Fig. 3a, c). Comparable results were obtained when flow cytometric analysis was carried out at 96 h (Fig. 3b, d).

The observed cell cycle arrest was further tested using cell cycle specific antibodies. Cell lysate was prepared from K562 cells after treating with MDPSQ for different time points (12, 24, 48 and 72 h). We probed for the expression of PCNA, a protein required for replication and found that its level reduced in a time dependent manner, upon treatment with MDPSQ (Suppl. Fig. 1C). Further studies are required to understand the exact mechanism of cell cycle arrest by MDPSQ.

MDPSQ treatment induces translocation of phosphatidyl serine to the outer membrane

In order to test whether MDPSQ induces cell death by apoptosis or necrosis, we used Annexin V-FITC/PI double staining. K562 cells were harvested after 48 and 72 h of treatment with MDPSQ (10, 50,100 and 250 μM) and subjected to double-staining followed by FACS analysis. Dot plots of MDPSQ treated K562 cells showed that at a concentration of 100 μM, about 18.9% of the cells were in early apoptotic stage (stained only by Annexin V-FITC), while about 8.9% were in late apoptotic stage (stained by both Annexin V-FITC and PI) (Fig. 4A(d)). Interestingly, at the highest concentration (250 μM), we could observe 41.3% of the cells as PI positive, whereas 32.6% were in the late apoptotic stage. When DMSO treated cells were subjected to similar analysis, only about 3.6% of the cells were apoptotic (Fig. 4A(a)). These results showed that in majority of cells, MDPSQ treatment led to nuclear staining (stained by PI) suggesting necrosis due to complete disruption of cell membrane. These conclusions were further verified by observing the above stained cells under a confocal microscope. The results showed that most of the K562 control cells were negative for Annexin V-FITC and PI (Fig. 4C(a)). Upon treatment with MDPSQ we could see cells stained by both Annexin V-FITC (green color) and PI (red color) (Fig.4C(b, d, e)) and PI alone, suggesting severe damage resulting in necrosis (Fig. 4C(c)).

In addition, we measured LDH release due to cellular damage upon MDPSQ treatment of K562 cells. We observed a time dependent increase in the release of LDH into the surrounding media upon treatment with MDPSQ (Fig. 4D). Intracellular release of LDH was also measured and taken into consideration when determining the percentage of LDH release as described in "Materials and methods". This result further confirms the potential of MDPSQ to destroy integrity of cell membrane.

DNA fragmentation and strand breakage induced by MDPSQ

The nuclear condensation and chromosomal DNA fragmentation were the parameters used to assess DNA damage upon treatment with MDPSQ. K562 cells treated with 50 or 100 μM of MDPSQ were harvested after 72 h and subjected to single cell gel electrophoresis. Around 7–

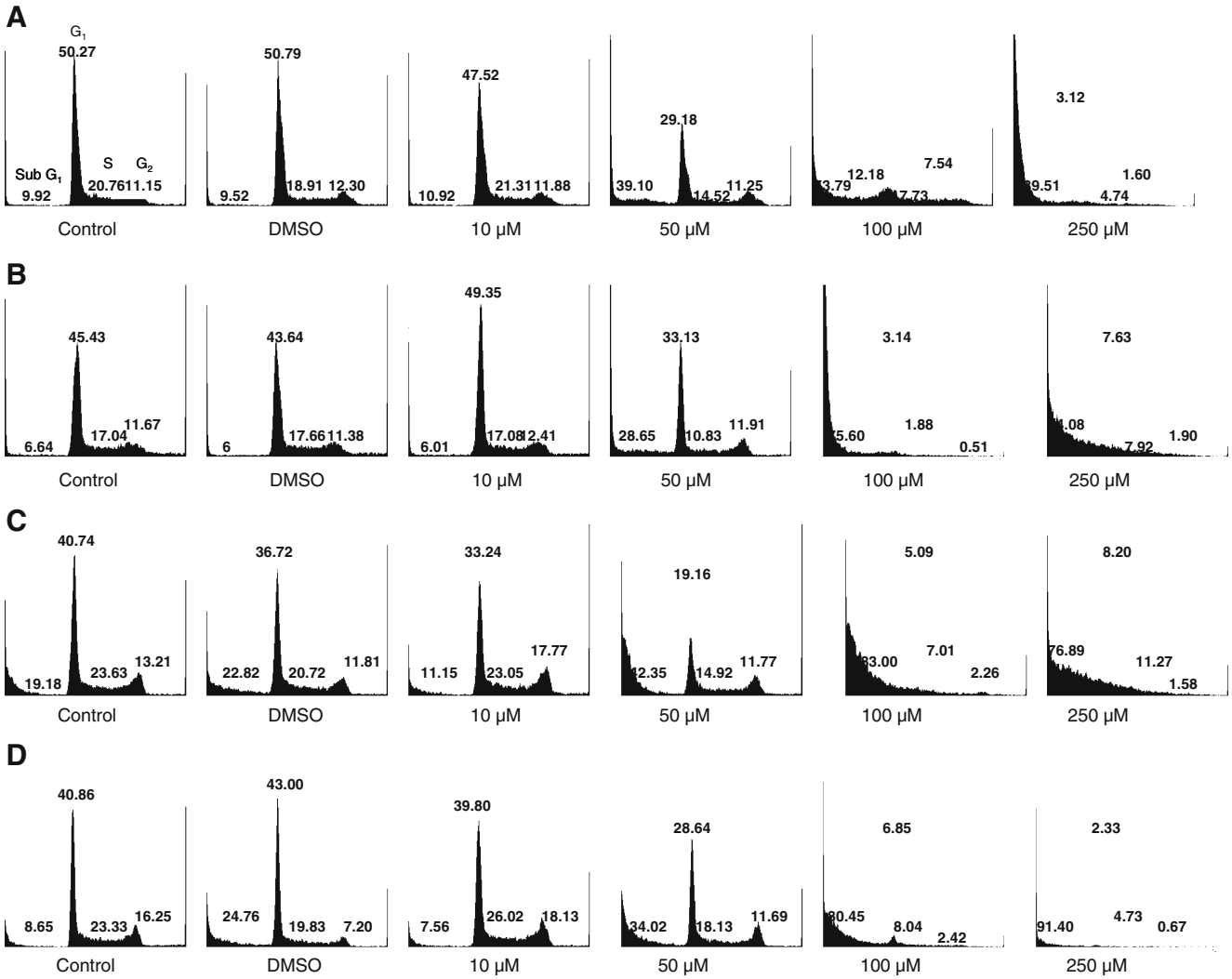

Fig. 3 Cell cycle analysis of K562 and CEM cells following treatment with MDPSQ. K562 (**a,b**) and CEM (**c,d**) cells were treated with increasing concentrations of MDPSQ for 72 (**a**, **c**) and 96 (**b**, **d**) hr. Cells with or without DMSO (1.25%) served as controls. DNA content of at least 10,000 cells per sample stained with propidium iodide was assessed using flow cytometer. The distribution of the population of cells in different cell cycle phases is shown. The number of cells in each phase is indicated as percentage in respective peaks

8 slides were observed (a minimum of 100 cells per slide) from both normal and treated samples (Fig. 5a–f). Results showed appearance of small comets at 50 μM (Fig. 5b, c), whereas 100 μM of MDPSQ induced the formation of comets with longer tails which is also a marker for nuclear damage (Fig. 5d–f). These results in conjunction with Annexin V-FITC staining further suggest that in addition to cell cycle arrest, MDPSQ induces severe damage by DNA fragmentation leading to cell death.

MDPSQ induces changes in the expression levels of DNA repair proteins

Since we have seen evidence for induction of severe DNA damage by MDPSQ in the above experiments, we tested for the expression of proteins specific to the DNA double-strand break repair pathways by immunoblot analysis. The cell lysate was prepared from K562 cells after treating with 100 μM MDPSQ for 12, 24, 48 or 72 h as described in the "Materials and methods". Proteins were resolved on a denaturing PAGE, transferred to PVDF membrane and probed with antibodies of interest.

We first probed for the expression of two of the proteins involved in non-homologous end joining pathway (NHEJ), which is the major double-strand break (DSB) repair pathway in higher eukaryotes. Ku80 is a DNA end binding protein, which recognizes the DSBs. We found a time dependent increase in the expression of Ku80 upon treatment with MDPSQ (Fig. 6a). Another major protein, DNAPKcs is a protein kinase essential for activation of

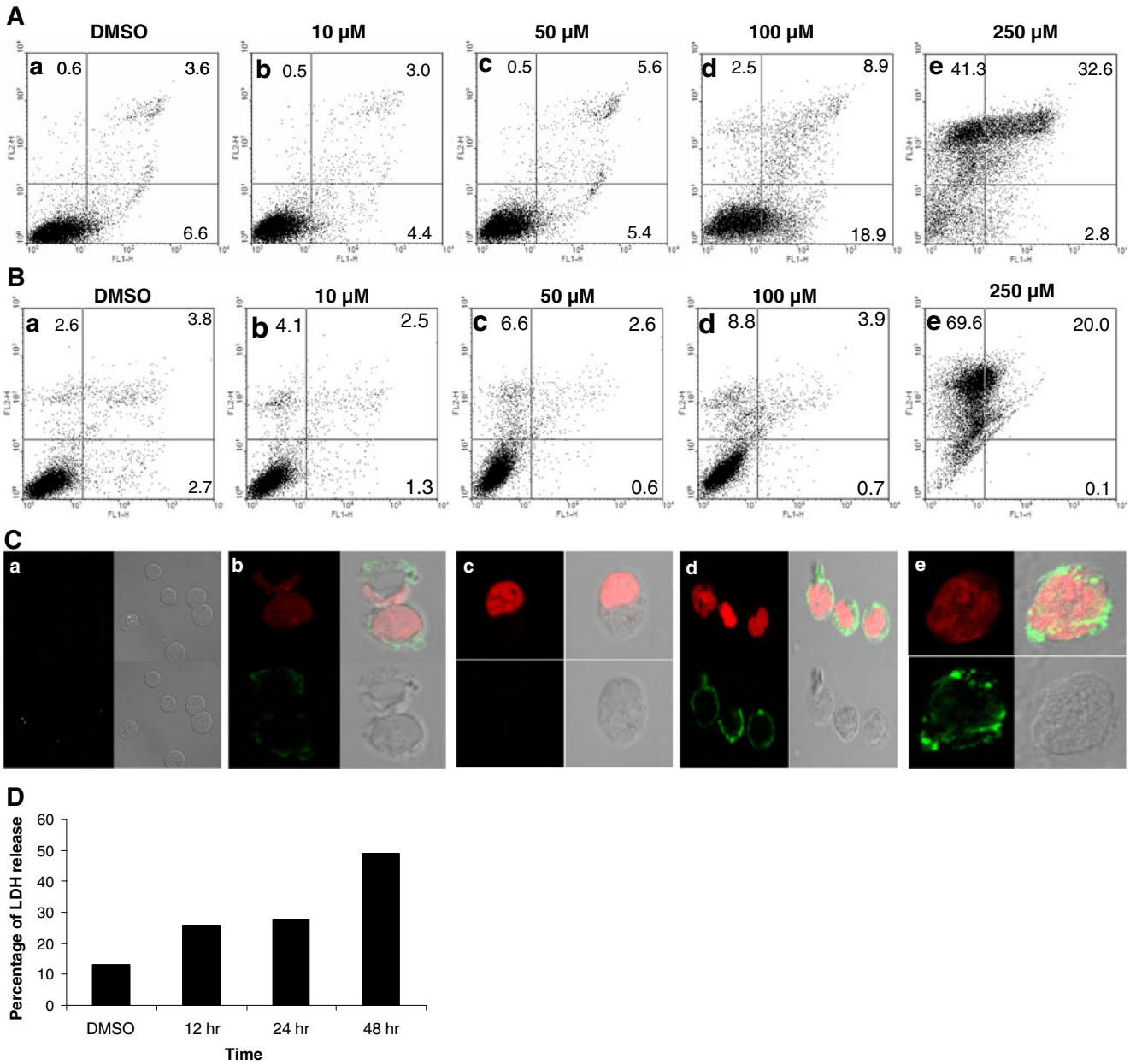

Fig. 4 Annexin V/PI dual staining to quantitate necrosis and apoptosis induced by MDPSQ. K562 cells were cultured for 48 and 72 h after treatment with 10, 50,100 and 250 μM of MDPSQ and processed for Annexin V-FITC dual staining. Among the four quadrants, lower left quadrant shows native cells negative for both Annexin V-FITC and PI, lower right shows cells in early stage of apoptosis positive for Annexin V. PI positive necrotic cells are in upper left quadrant, whereas the upper right quadrant shows cells in late apoptosis as well as early necrosis which are positive for both Annexin V and PI. The values mentioned in the quadrants show the percentage of cells positive for respective staining. Panels shown are **A**. (a) negative control K562 cells (b) 10 μM (c) 50 μM (d) 100 μM and (e) 250 μM MDPSQ treated K562 cells for 48 h. **B**. (a) negative control, (b) 10 μM, (c) 50 (d) 100 μM and (e) 250 μM MDPSQ treated K562 cells for 72 h. **C**. Confocal images of the Annexin V/PI stained control cells (a) and 100 μM MDPSQ treated K562 cells (b, c, d, e). **D**. LDH release from 100 μM MDPSQ treated K562 cells at different time points. The LDH release was measured at an absorbance of 490 nm. The percentage of LDH release was calculated as: (LDH activity in media) / (LDH activity in media + intracellular LDH activity) × 100

many downstream proteins involved in NHEJ. Similar to Ku80, we could find an increase in the expression of DNAPKcs upon MDPSQ treatment (Fig. 6a). This suggests activation of NHEJ upon treatment with MDPSQ. We further checked the expression levels of MRE11, RAD50 and NBS1, which are known to play a major role in the homologous recombination (HR) pathway and have been shown to act in NHEJ as well. Results showed an

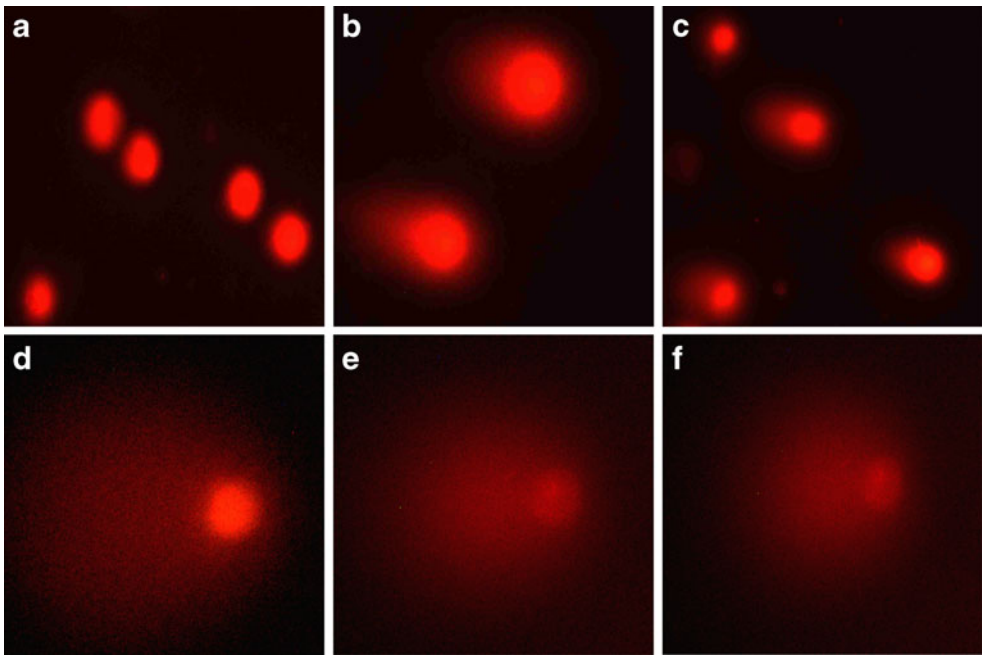

Fig. 5 DNA strand breakage analysis by comet assay. K562 cells were treated with 50 and 100 μM of MDPSQ for 72 h. Normal and treated cells were then layered over a mini-agarose gel, lysed, electrophoresed in alkaline buffer, and stained with propidium iodide. **a**. K562 cells incubated in DMSO. **b, c**. K562 cells treated with 50 μM of MDPSQ. **d–f**. K562 cells treated with 100 μM of MDPSQ

upregulation in each of these proteins, upon treatment with MDPSQ (Fig. 6a). Together, these results confirm our earlier findings, that MDPSQ treatment results in DNA damage and fragmentation, which subsequently leads to the activation of DNA DSB repair pathways in the cells.

Since, treatment with MDPSQ leads to DNA damage in the cells and Annexin V/PI staining indicated activation of mostly necrosis, we were interested to check for the induction of different proteins related to apoptosis and necrosis by immunoblotting. Poly (ADP-ribosyl) polymerase (PARP) is a single strand break repair enzyme (116 kDa). It is known that upon activation of apoptotic pathway, the caspases cleave PARP into 89 and 27 kDa polypeptides [26]. By immunoblotting using anti-PARP, we found that addition of MDPSQ (from 12 h onwards) led to a detectable PARP cleavage, resulting in accumulation of 89 kDa product, which was absent in the DMSO treated control (Fig. 6b). A point to be noted is that though the cleavage was detectable, it was quite weak. We further checked the expression of apoptotic proteins like caspase 3, 9, FAS and BCL2. Results did not show any cleavage of caspase 3 or 9 (Fig. 6b). On the contrary, we found a slight upregulation in the levels of both the proteins. The levels of BCL2, an anti-apoptotic protein, did not change significantly upon treatment, albeit increased slightly at around 72 h (Fig. 6b). We also found a time dependent down regulation in the expression of FAS ligand, which is involved in the induction of extrinsic pathway of apoptosis (Fig. 6b). This suggests that MDPSQ may not be able to induce either the intrinsic or extrinsic pathway of apoptosis, which also corroborates the Annexin V/PI result where we observed only a relatively smaller population of cells in the late apoptosis stage, at lower concentrations of the compounds.

We had previously observed induction of significant DNA damage after treatment with MDPSQ. We, therefore tested the status of DNA damage checkpoint proteins like p53 and p73, which act as DNA damage sensors and lead the cells to an apoptotic pathway. Interestingly, we find a complete down regulation of both of these proteins, with increase in time (Fig. 6c). In conclusion, various lines of experimental evidences suggest that MDPSQ activates necrosis to induce cytotoxicity in K562 and CEM cells.

Discussion

Polycyclic aromatic molecules can intercalate into double-stranded DNA. The consequences of DNA intercalation by such molecules have gained significant interest in medicinal chemistry, as it leads to considerable modification of the DNA structure [27, 28], which can interfere with DNA replication and gene transcription [29]. Ellipticine is one such natural alkaloid which is a known DNA intercalator and inhibits topoisomerase II activity [30]. It is a well known, commercialized chemotherapeutic drug and has been used for the treatment of a wide variety of cancers.

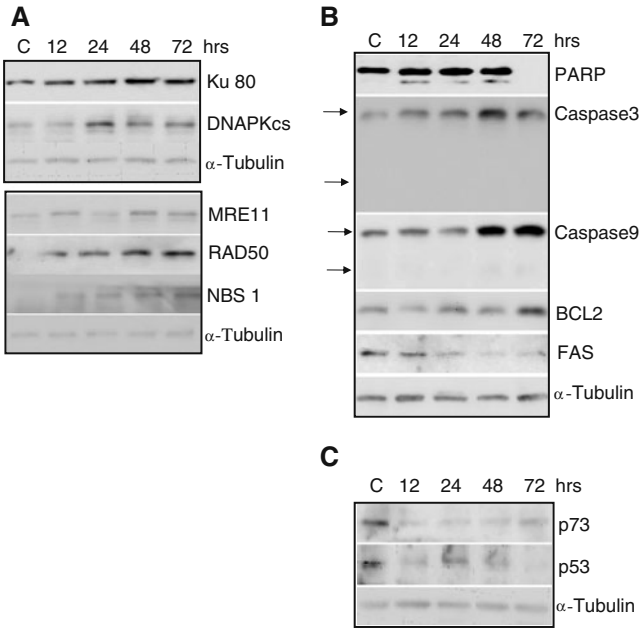

Fig. 6 Effect of MDPSQ on the expression of DNA repair and other proteins controlling genomic stability. K562 cells (0.75×0^5/ml) were cultured with the vehicle (DMSO, 0.24%) or 100 μM of MDPSQ for 12, 24, 48 and 72 h. After specified time points the cell lysate was prepared. Equal amounts of cellular proteins (~40 μg) were resolved by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was probed for the expression of (**a**) Ku 80, DNAPKcs, MRE11, RAD50 and NBS1 (**b**) PARP, Caspase 3, Caspase 9, BCL2 and FAS and (**c**) p73 and p53 with specific primary and secondary antibodies. In all the panels, "C" is DMSO control, 12, 24, 48 and 72 h are the cells harvested after respective time points of MDPSQ treatment. Each experiment was done a minimum of three times with good agreement

Many analogs of ellipticine have been synthesized in order to improve the anticancer properties, such as 2-N-methyl-9-hydroxyellipticinium (NMHE), which has been used in the treatment of breast cancer, and 9-methoxyellipticine which is an established chemotherapeutic agent [31, 32]. We have reported the synthesis of compounds containing a novel tetracyclic condensed quinoline system, pyrimido[4′,5′:4,5] thieno(2,3-b)quinoline, which is a structural derivative of ellipticine [19]. Previously we have shown that some of these molecules have potent anticancer properties against leukemic cells [13]. In the present study, we have tested the anti-cancer property of one such derivative, MDPSQ against leukemic cells. We find that MDPSQ is cytotoxic against many leukemic cells, with an IC_{50} value of approximately 50 μM. Reduced tritiated thymidine incorporation suggests that MDPSQ may be exhibiting its toxicity by inhibiting DNA replication. This is understandable as these classes of compounds intercalate with DNA, thereby affecting its physiological functions [33].

Further, we checked the cell cycle profile by FACS analysis and found that upon treatment with MDPSQ there was no major cell cycle arrest. However, a detectable S phase arrest was noted at the lowest concentration of MDPSQ studied (10 μM) and that was consistent in both cell lines, K562 and CEM. Interestingly the cell cycle analysis also showed an appreciable population of cells at sub-G1 phase, which consists of cells either in apoptotic or necrotic population, which further increased with an increase in the concentration of the compound. Since, MDPSQ forms an adduct with DNA [34, 35], we attempted to determine the ability of MDPSQ to induce DNA damage in the cells. For this, we performed comet assay which showed the extent of DNA damage and fragmentation occurring in the genome. We observed formation of good tailed comets in K562 cells upon treatment. This suggests that MDPSQ can induce significant DNA damage and subsequently induce cell death.

In order to understand the molecular mechanism of cell death by MDPSQ in leukemic cells, we performed a series of experiments. To distinguish the apoptotic and necrotic cells, we performed Annexin V/PI dual staining and found that, only a small population of cells entered into apoptosis. On the contrary, we found a considerable population was PI stained after both 48 and 72 h of MDPSQ treatment. This was also qualitatively assessed by confocal microscopy. This indicates that MDPSQ may not be inducing apoptosis for promoting cell death, but may be utilizing an alternate pathway like necrosis. We further determined the expression of some of the apoptosis related proteins such as caspases, PARP and BCL2. Interestingly we could not find activation of any of the caspases. PARP cleavage, although observed from 12 h onwards, was very weak and surprisingly, at 72 h we could not observe the presence of even the uncleaved PARP protein. This could be due to the fact that at 72 h, cell death was too high and there were only few cells left viable. The expression of FAS ligand also decreased with increasing time upon treatment, thus ruling out the role of the extrinsic apoptotic pathway [36]. An interesting point to be noted here is that a similar compound, belonging to the same family, BPSQ, has been previously shown by us to induce the extrinsic pathway of apoptosis in leukemic cells [13]. What makes the different side chains to trigger variant responses in cells is a question that could be addressed in the future. An unexpected observation was the consistent upregulation of BCL2, the anti-apoptotic protein. This could further explain why we couldn't see the induction of the apoptotic machinery upon MDPSQ treatment. Previous studies have shown that in a background of higher BCL2 expression, the cells undertake necrotic pathway for undergoing cell death [37].

Since, MDPSQ induces significant DNA damage to the treated cells, we checked for the expression of some of the DNA repair proteins upon treatment. We indeed found a time dependent upregulation of proteins belonging to both

the NHEJ and HR pathways, like Ku 80, DNAPKcs and the MRN complex [38]. This further confirms our earlier results and indicates that MDPSQ can trigger DNA damage, which subsequently results in the activation of the repair pathways in the cells. However, the prolonged treatment of MDPSQ will result in exhaustion of the repair machinery finally leading to cell death. In addition to the upregulation of the DNA repair proteins, we wondered whether the DNA damage sensor proteins like p53 get affected upon MDPSQ treatment. To our surprise, we found a total down regulation of p53 and p73, upon treatment with MDPSQ. All this clearly suggests that MDPSQ may not be inducing the apoptotic pathway, but would push the cells towards a necrotic pathway of cell death. This accounts for the total reduction in the levels of some of the key proteins essential for survival [39].

Although it may be a well accepted fact that apoptosis is a preferred pathway of cell death, resulting in no inflammatory response, there are still many commonly used anticancer compounds, which induce necrosis [40]. This becomes especially important in tumors resistant to apoptosis induced by drugs or those having mutations in the apoptotic protein machinery, where necrosis can serve as an alternative mechanism [37]. Some studies also suggest that necrosis, as a mechanism for cell death, has more broad range of action as it is not dependent on the fate of a few apoptotic proteins [41]. In addition, studies indicate that exposure of necrotic cells to macrophages provides enhanced activation of macrophage antitumor activity and results in rapid tumor clearance [42, 43]. It has also been shown that necrosis is major mechanism of tumor clearance upon radiation treatment [44]. Therefore, our findings on MDPSQ triggering necrosis over apoptosis is significant and provides an extra advantage for this family of molecules in their use as chemotherapeutic agents.

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