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Diethyl (6-amino-9H-purin-9-yl) methylphosphonate induces apoptosis and cell cycle arrest in hepatocellular carcinoma BEL-7402 cells: Role of reactive oxygen species

BIN QU, WEI WANG, ZHENYI TAN, DI LI, JUN WAN, JIE SUN, KUN CHENG & HAO LUO

College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao, Shandong, 266042, PR China

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Abstract

The primary purpose of this work was to study the mechanism of the anti-proliferation activity of compound diethyl (6-amino-9H-purin-9-yl) methylphosphonate (DaMP), a novel acyclic nucleoside phosphonate. Using cell survival MTT assay, flow cytometry analysis, DNA laddering, DCF fluorescence detection and caspases assays, this study investigated the effects of this compound on cell apoptosis, cell cycle regulation and reactive oxygen species in human hepatocarcinoma BEL-7402 cell lines. Exposure to DaMP at 80 μM for 24 h, BEL-7402 cells displayed a marked retardation of S-phase progression, leading to a severe perturbation of normal cell cycle. In addition, DaMP also significantly inhibited cell proliferation by inducing apoptosis, disrupting DNA synthesis and increasing the activities of caspase-3 and -9, while the antioxidants could significantly inhibit these effects. This study was the first to demonstrate that DaMP could induce apoptosis and cell cycle arrest by producing reactive oxygen species and activating caspase-3 and -9.

Keywords: *Acyclic nucleoside phosphonate, reactive oxygen species (ROS), oxidative stress, apoptosis, vitamin C*

Introduction

Nucleoside analogues display a wide range of biological activities such as anti-tumour, anti-viral and chemotherapeutic activities [1–4]. Various metabolites of nucleobase analogues, such as 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and 5-fluorouracil (5-FU), are cytotoxic and have been found to have therapeutic uses as anti-neoplastic agents [5,6]. Although being widely used as chemotherapeutic agents in the treatment of cancer, nucleoside analogues do have several drawbacks including rapid degradation, poor oral bioavailability and resistance to chemotherapy [7]. Interestingly, the acyclic analogues of nucleosides appear to pose an attractive solution in cancer therapy because of the absence of a glycosidic bond and the flexibility of the acyclic chain in their structures [8]. We have, therefore, for this study designed and synthesized a novel acyclic nucleoside phosphonate, diethyl (6-amino-9H-purin-9-yl) methylphosphonate (DaMP) (Scheme 1).

Cell cycle regulation has attracted a great deal of attention as a promising target for cancer treatment. Some of the acyclic nucleoside phosphonates are cytotoxic and possess anti-tumour potential through regulating cell cycle and apoptosis [9,10]. For instance, the acyclic 5-FU treatment showed a decrease of the G0/G1-phase cells and a corresponding accumulation of S-phase cells in MCF-7 human breast cancer cell line [9]. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA), the prototype congener of acyclic nucleoside phosphonate, was observed to cause retardation of K562 cells in the S phase of the cell cycle after the drug was converted to an active metabolite PMEApp [11]. The PMEA-induced S-phase arrest of K562 cells was irreversible. This is probably related to the fact that PMEApp, when incorporated into the nascent DNA strand,

Correspondence: Bin Qu, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao, Shandong, 266042, PR China. Tel: 0086 532 8402 2680. Email: danielqu@126.com

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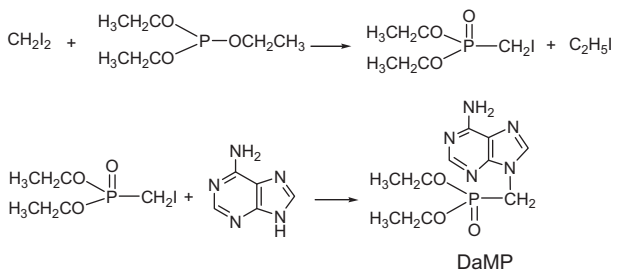

Scheme 1. Synthetic route of compound DaMP.

inevitably causes DNA chain termination due to the lack of the hydroxyl group required for further DNA chain elongation [11].

Apoptosis is an essential phenomenon for maintaining normal cell development and homeostasis and also plays an important role in preventing the development of malignant tumours [2,3,9,10]. Several studies have reported that acyclic nucleoside phosphonates exhibit strong apoptosis-inducing properties in some tumour cells. Campos et al. [12] synthesized a novel class of 5-FU-containing acyclonucleosides that are able to induce myogenic differentiation in rhabdomyosarcoma cells. Cidofovir ((*S*)-HPMPC), an analogue of acyclic nucleoside phosphonates consisting of an acyclic nucleoside moiety to which a phosphonate group has been attached through a stabile P-C linkage, inhibits the growth of endothelium-derived tumours via induction of apoptosis [13]. Although it was argued that PMEA does not induce apoptosis in SK-N-SH neuroblastoma cells, PMEA may block cell proliferation in K562 cells, human myeloid HL-60 and rat choriocarcinoma RCHO cells by directly interfering with DNA synthesis [11,14]. The induction of tumour cells differentiation by PMEA was not universal but appeared to be cell type specific. These findings open new perspectives for the potential application of acyclic nucleoside phosphonate analogues in the anti-cancer field [11–15].

This study was designed to evaluate the potential of the newly synthesized acyclic nucleoside phosphonate DaMP as an anti-tumour agent through regulation of cell cycle progression and activating apoptosis. To this end, we examined the effects of DaMP on the cell cycle distribution pattern and its anti-proliferation activity in the human hepatocarcinoma BEL-7402 cell line. To gain additional insight into the mechanism of DaMP's anti-tumour activity, we also investigated the properties of the reactive oxygen species (ROS) involved in this action.

Methods

Reagents

RPMI-1640 medium, foetal bovine serum (FBS), dexamethasone, and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Gibco (BRL, Gaithersburg, MD). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), streptomycin sulphate, penicillin G sodium salt, trypan blue, RnaseA, catalase, and Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) were all purchased from Sigma Chemical Co. (St. Louis, MO).

Synthesis of diethyl (6-amino-9H-purin-9-yl) methylphosphonate (DaMP)

Diethyl (6-amino-9H-purin-9-yl) methylphosphonate (DaMP) was synthesized in our lab using procedures modified from references [16–20] (Scheme 1). Diethyl iodomethylphosphonate was obtained in an Arbuzov reactor from the commercial compounds diiodomethane and triethylphosphite [16]. The desired final product DaMP was obtained through the coupling reaction that occurs between phosphonate and adenine in the presence of potassium carbonate in DMF [17–20]. The resulting yellow solid was purified by column chromatography (silica gel, ethyl acetate/methanol, 8:1 v/v) to produce a 45% yield compound DaMP as a white waxy solid.

Elemental analysis was performed using the Perkin-Elmer 240 elemental analyser (Perkin-Elmer, Santa Clara, CA, USA). ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on a 500 MHz NMR instrument (AVANCE 500, Bruker, Karlsruhe, Germany). Chemical shifts are given in δ values (ppm) using tetramethylsilane as an internal standard. Splitting patterns are designated as follows: s = singlet, d = doublet, t = triplet and m = multiplet. High-resolution MS (HRMS) data were obtained on a Micromass Q-TOF mass spectrometer (Waters Micromass, Manchester, UK).

Cell culture

Human hepatocarcinoma BEL-7402 cells were maintained in RPMI-1640 supplemented with the following: 10% (v/v) foetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C [21]. Culture medium was changed three times weekly. DaMP was purified to >95% purity by HPLC and dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%, v:v), and equal amounts of this solvent were included in the control reaction mixtures. In some experiments, z-VAD-fmk (100 μM), catalase (1000 U/ml), vitamin C (1 mM) or MnTBAP (100 μM) were used.

Cell survival assay

The conversion of the dye MTT to formazan crystals by cellular dehydrogenases was used as an index of

cell viability. Cells were seeded overnight at a density of 1.5×10^4 cells per well in flat-bottomed 96-well microplates. After incubating the cells with DaMP dissolved in DMSO under different experimental conditions, MTT was added to cultures at a final concentration of 0.5 mg/ml and incubated for 2 h in the dark. An equal volume of 20% sodium dodecyl sulphate (SDS) in 50% dimethylformamide was then added to each well and the reaction product was left for 12 h and subsequently the results were read as the absorbance at 570 nm [22].

Flow cytometry (FCM) analysis

The flow cytometric evaluation of the cell cycle status was performed according to a method described previously [23]. In brief, 2×10^6 cells were treated with DaMP (80 μM) for 24 h and then washed twice with PBS. The cells were fixed overnight with cold 70% ethanol, following staining with 1 ml of a solution containing 50 μg/ml PI and 100 μg/ml RNase A. After incubation for 30 min at 37°C, cells were analysed by flow cytometry (CyFlow, Partec, Münster, Germany) using Cell Quest software. The percentage of cells in the apoptotic sub-G0/G1 and G1, S-phase and G2-M phase were calculated using Modfit software.

Annexin V staining: phosphatidylserine exposure on the outer layer of the cell membrane was measured using the binding of annexin V–fluorescent isothiocyanate (FITC). BEL-7402 cells were harvested and washed with cold PBS. And then the cells were incubated for 15 min with annexin V–FITC and propidium iodide and were analysed by flow cytometry (CyFlow).

DNA laddering

After treatment with DaMP, the cells were collected and washed three times with PBS. The cells were then disrupted and the nucleoplasm was separated from the high-molecular-weight chromatin. DNA fragments were purified and analysed by conventional electrophoresis in a 1.2% agarose gel containing 0.375 mg/l ethidium bromide as described previously [24–26].

ROS detection

Production of intracellular reactive oxygen species (ROS) was monitored by the fluorescence emission of DCFH-DA within the cell [27]. DCFH-DA was deacetylated intracellularly by non-specific esterase, which was furthered oxidized by ROS resulting in the fluorescent compound 2, 7-dichlorofluorescein (DCF); 10^6 cells under different experimental conditions were incubated with 2 μM DCFH-DA for 30 min at 37°C. The cells were then washed three times with PBS and the fluorescence emission was read at 529 nm with excitation at 503 nm using an F-4500, Hitachi (Japan) fluorescence spectrophotometer. Intracellular levels of reactive oxygen species were also determined by fluorescence microscope image analysis. In some experiments, BEL 7402 cells were treated with z-VAD-fmk (100 μM), catalase (1000 U/ml), vitamin C (1 mM) or MnTBAP (100 μM) for 1–2 h prior to DaMP treatment.

Caspase-3 and caspase-9 assays

Caspase activity was determined by fluorometric assay using the enzyme substrate Ac-DEVD-AFC for caspase-3 and Ac-LEHD-AFC for caspase-9, which are specifically cleaved by the respective enzymes to release the fluorescent group [28]. Briefly, cells were suspended in 20 mM HEPES-KOH buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 7.5), frozen, thawed and sonicated. The homogenate was centrifuged at 12 000 × g, 10 min, 4°C and the supernatant was collected. Aliquots corresponding to 20 μg protein were diluted in caspase buffer (25 mM HEPES, 0.1% CHAPS, 10% sucrose, 10 mM DTT, pH 7.5) and incubated at 37°C for 1 h in the presence of 100 μM Ac-DEVD-AFC or Ac-LEHD-AFC. The fluorescence was read in a PerkinElmer fluorometer at excitation 400 nm and emission 505 nm.

Statistical analysis

All data are reported as means ± SD except where indicated. Comparisons among multiple groups were subjected to a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference *post-hoc* test. The sample *t*-test was used in two group comparisons. In case the data were not normally distributed, a Wilcoxon signed ranks test was used. The 95% confidence limit ($p < 0.05$) was considered statistically significant. All the data presented in the figures were obtained from at least three independent experiments.

Results

Characterization of compound DaMP

The structure of compound DaMP was established on the basis of elemental analysis, NMR and HRMS. Elemental analysis data: Analysis Calculated for C10H16N5O3P: C, 42.11; H, 5.65; N, 24.55; O, 16.83; P, 10.86. Found: C, 41.81; H, 5.52; N, 24.61; O, 16.92; P, 11.14. Spectral data: ^{1}H NMR (DMSO-d_6): 8.17 (s, 1H, H-2); 8.04 (s, 1H, H-3); 7.29 (s, 2H, NH_2); 4.72–4.69 (d, 2H, P-CH_2); 4.06–4.02 (m, 2 × 2H, CH_2 and CH_2'); 1.17–1.14 (t, 2 × 3H, CH_3 and

CH_3') (Scheme 1). ^{13}C NMR (DMSO-d_6): 62.92 (s, 2C, CH_2 and CH_2'); 38.69–37.48 (d, 1C, P-CH_2-N); 16.53 (s, 2C, CH_3 and CH_3'); 156.42, 153.09, 149.86, 141.06, 118.46 (s, purine carbons). ^{31}P NMR (DMSO-d_6): $\delta = 18.95$ ppm. HRMS (TOF MS ES$^+$) m/z: $[M+1]^+ = 286.1$.

DaMP inhibits growth of BEL-7402 cells

To determine whether DaMP has growth inhibitory effects on human hepatocarcinoma BEL-7402 cells, cells were exposed to different concentrations of DaMP (0~120 μM) for 24 h and 48 h. Cell growth was tested by MTT assay [22]. Proliferation of the cells was inhibited significantly upon treatment with DaMP at concentrations above 40 μM (Figure 1). At a concentration of 80 μM , the inhibition rate at 24 h was ~75%.

DaMP caused S phase cell cycle arrest in BEL-7402 cells

The growth of cancer cells is strictly regulated by the cell-cycle progression [29,30]. Using a flow-cytometry technique, we found that exposure of BEL-7402 cells to DaMP at a concentration of 80 μM for a period of 24 h led to cell cycle arrest at S phases (Figures 2A and B). The proportion of cells in S phases rose from 33.9% ± 2.8% for control cells to 60.6% ± 5.3% for DaMP treated cells. The DaMP mediated S phase cell cycle arrest was accompanied by a decrease in G0/G1 cells.

There was also an accumulation of cells in sub G0/G1 for cells treated with DaMP when compared to untreated control cells (Figures 2A–C). The percentage of cells in sub G0/G1 significantly increased from 1.2% ± 0.2% for control cells to 28.8% ± 1.7% for DaMP (80 μM) treated cells. The sub G0/G1 population indicates apoptotic-associated chromatin degradation [29,30].

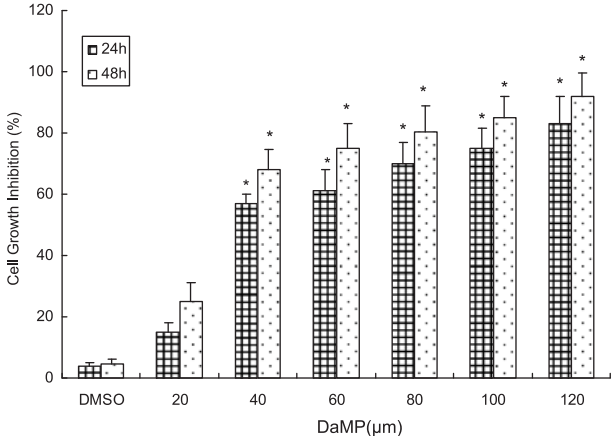

Figure 1. BEL-7402 cells were treated with increasing concentrations of DaMP and harvested at the times indicated. Data are expressed as means ± SD of five or more separate MTT experiments. $^*p < 0.05$ vs DMSO at the indicated times.

DaMP induces apoptosis and activates caspase-9 and -3 in BEL-7402 cells

To confirm the cell death was attributed to apoptosis, cells were subjected to annexin V staining. Cytometric analyses of annexin V-positive cells demonstrated a 43.70% ± 4.6% increase in cells treated with DaMP (80 μM), as shown in right lower and upper quadrants (Figures 3A and B). To further confirm apoptosis, a DNA ladder experiment was conducted by agarose gel electrophoresis assay since one of the major properties of apoptosis is the formation of fragmented DNA [25,26]. As shown in Figure 3C, DaMP-mediated formation of DNA ladders appeared after the cells were exposed to 80 μM DaMP for 24 h (lane 4). In contrast, untreated control cells showed no DNA laddering (lane 2). Antioxidant vitamin C could partly prevent the formation of DNA laddering in DaMP treated cells (lane 3). These results indicate DaMP-induced cell death is mainly due to apoptosis.

Activation of the family of caspase was known as a crucial mechanism for induction of death signals of apoptosis [28,31]. Among the caspase family, caspase-9 is the initiator caspase of the apoptotic pathway, which ultimately activates caspase-3. To determine whether caspase play a role in DaMP

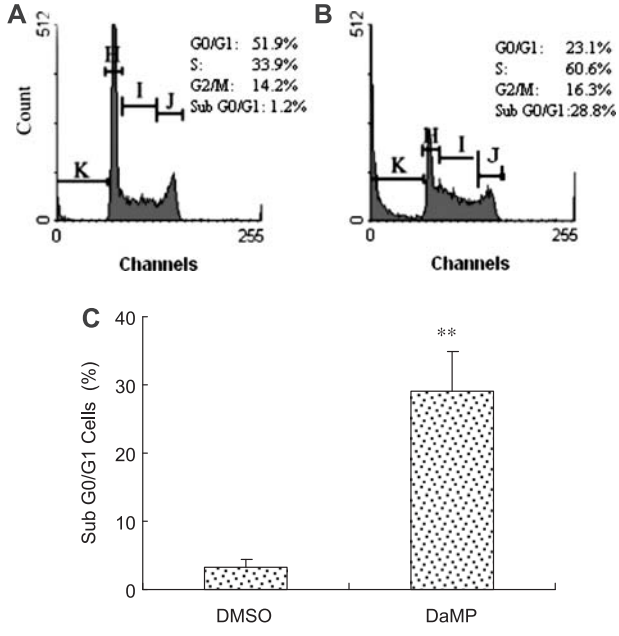

Figure 2. Effect of DaMP on cell cycle assessed by flow cytometric analysis. 2×10^6 cells were analysed in the absence (A) or presence of 80 μM DaMP (B) and the results presented as a percentage of cells in each phase of the cell cycle. H, I, J and K represented G0/G1, S, G2/M and Sub-G0/G1, respectively. Percentage of apoptosis cells (C) was estimated by the extent of K region shown in (A) and (B). Data are means ± SD of three independent experiments performed in duplicate. $^{**}p < 0.01$ vs DMSO.

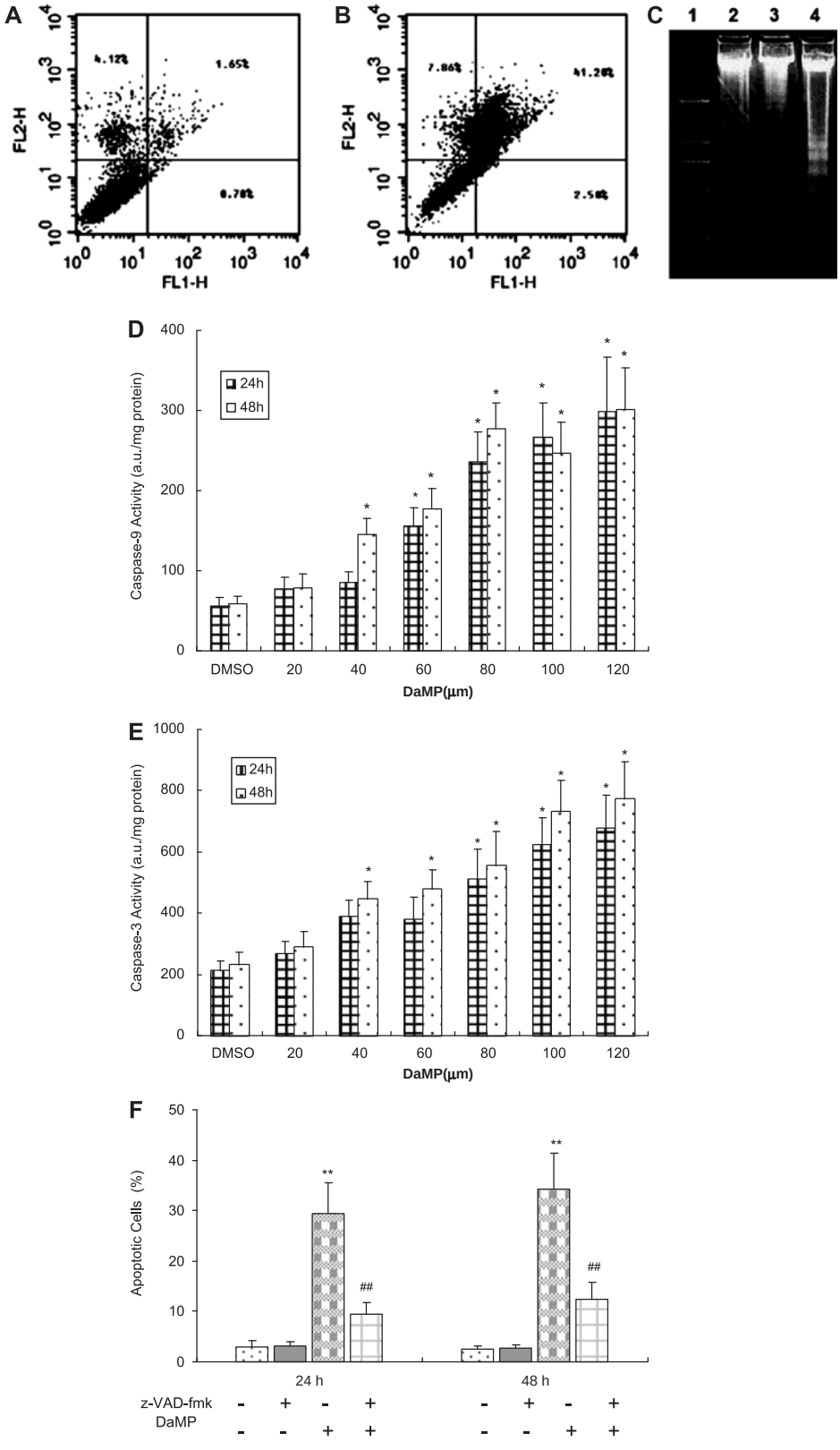

Figure 3. Effect of DaMP on apoptotic cell death. Cells exposed to DMSO (A) or 80 μM of DaMP (B) for 24 h were stained with FITC-conjugated annexin and quantified by flow cytometric analysis. Numbers indicate the percentage of cells in each quadrant. Internucleosomal DNA fragmentation in BEL-7402 induced by DaMP (C). (lane 1: marker; lane 2: DMSO; lane 3: 80 μM DaMP + 1 mM Vitamin C; lane 4: DaMP 80 μM. From top to bottom for marker: 2000, 1000, 750, 500, 250, 100 bp). Induction of caspase-9 (D) and -3 (E) activities in DaMP treated cells. The lysates were assayed from cells treated with 80 μM DaMP for 24 h. Data are represented as mean ± SD of four replicates in each case. $^{*}p < 0.05$ vs DMSO. Cells were treated with z-VAD-fmk (100 μM) for 2 h prior to DaMP treatment for 24 h or 48 h (F). Data represented as means ± SD of at least three independent experiments performed in triplicate; $^{**}p < 0.01$ vs DMSO ; $^{\#\#}p < 0.01$ vs DaMP.

mediated apoptosis, we measured caspase-9 and caspase-3 activities of BEL-7402 cells treated with DaMP. After BEL-7402 cells were exposed to DaMP at different concentrations, there was an obvious increase of caspase-9 activity by fluorometric assay (Figure 3D). Caspase 3 activity also increased when DaMP's concentration was over 40 μM (Figure 3E). The results show that both 24 h and 48 h treatment significantly increase the activities of caspase-9 and caspase-3 in a dose-dependent manner, but the effect of 48 h treatment is more pronounced.

To further elucidate the death signalling of DaMP-induced apoptosis, cells were pre-treated with pan-caspase inhibitor z-VAD-fmk for 2 h prior to apoptosis analysis. The DaMP-induced apoptosis cells were prevented significantly by pre-treatment with z-VAD-fmk, suggesting that caspase activation were crucial for DaMP-induced apoptosis (Figure 3F).

DaMP increases intracellular reactive oxygen species generation

Apoptosis by a number of stimuli are frequently accompanied by the generation of reactive oxygen species (ROS), which may act as important mediators of cell death [27]. Excessive production of ROS is one potential explanation for apoptosis. To understand the mechanisms underlying DaMP-induced growth inhibition, oxidative stress was examined by using fluorescent probe DCFH-DA to quantify the generation of net intracellular reactive oxygen species [32,33]. As shown in Figure 4A, an increase in reactive oxygen species levels was observed after cells were incubated with increasing concentrations of DaMP for indicated times, as evidenced by the shift in DCF fluorescence. DaMP-induced intracellular oxidative stress was confirmed by fluorescence images of DCF staining (Figures 4B–D). This effect was almost complete inhibited by the addition of catalase (H_2O_2 scavenger), indicating the specificity of peroxide detection in the test system. Likewise, the general antioxidant vitamin C strongly inhibited the fluorescence signal, whereas the SOD mimetic MnTBAP (superoxide anion scavenger) showed minimal effect. These suggest that DaMP-induced oxidative stress mainly through the reaction of intracellular hydroperoxide (Figure 4E). DaMP-induced ROS generation was partially inhibited in the presence of z-VAD-fmk pre-treatment, suggesting caspase activation contributes to sustaining ROS levels (Figure 4F).

ROS scavenging abrogates DaMP-induced S phase arrest and apoptosis

To elucidate whether DaMP induced cell cycle arrest and apoptosis are associated with its pro-oxidant activity, exponentially growing BEL-7402 cells were incubated with DaMP (80 μM) for 24 h in the presence or absence of antioxidants, including catalase, vitamin C and MnTBAP. Treatment with vitamin C reversed DaMP-induced S phase arrest with the percentage of S phase decreasing from 60.5% ± 5.3% in DaMP-treated cells to 44.7% ± 5.8% in DaMP and vitamin C-treated cells (Figures 5A and B). Catalase and MnTBAP also have a similar effect on S phase arrest (data not show).

Figure 5C shows that all the tested antioxidants were able to inhibit apoptosis, indicating that multiple ROS are involved in the apoptotic process. The inhibitory effects of catalase and MnTBAP further indicate that H_2O_2 and superoxide anion play an important role in the process. Similar results were obtained for the inhibition of caspase-3 activation by these antioxidants (Figure 5D). These results suggest that DaMP induce apoptosis through a ROS-dependent mechanism.

Discussion

Acyclic nucleoside phosphonates are structural analogues of nucleotides consisting of an acyclic nucleoside moiety to which a phosphonate group has been attached through a stabile P-C linkage [13]. Several closely structurally related acyclic phosphonate nucleosides are reported to display significant anti-tumour activity in a number of *in vitro* tumour cell lines and *in vivo* animal models [11,13,14]. This anti-proliferation effect benefited from the unique structure of acyclic nucleoside phosphonates in that the phosphorous atom is attached to the alkyl side chain of the purine base via an O–C–P bond which is resistant to enzymatic degradation when compared with the usual C–O–P bond [34]. The flexibility of the acyclic chain also allows adopting a suitable conformation for interaction with an active site of an enzyme or with a receptor [35,36]. Diethyl (6-amino-9H-purin-9-yl) methylphosphonate (DaMP), a novel acyclic nucleoside phosphonate, was synthesized in our lab. The present study was a detailed investigation of the mechanism of the action of DaMP as a potential apoptosis inducing and cell cycle regulation agent in hepatocarcinoma BEL-7402 cells. This appears to be the first report of the synthesis of an acyclic nucleoside phosphonate which has anti-proliferative effects on hepatocarcinoma cells.

Disturbance of the cancer cell cycle is one of the therapeutic targets for development of new anti-cancer drugs [37,38]. Accumulated evidence has shown that cell cycle arrest might result in apoptosis due to the existence of cell cycle checkpoint and feedback control [39]. Although evidence has suggested that some anti-cancer drug induced apoptosis may occur via a signalling pathway independent of cell cycle arrest [40], our result showed that DaMP could induce cell cycle arrest in S phase. This is consistent

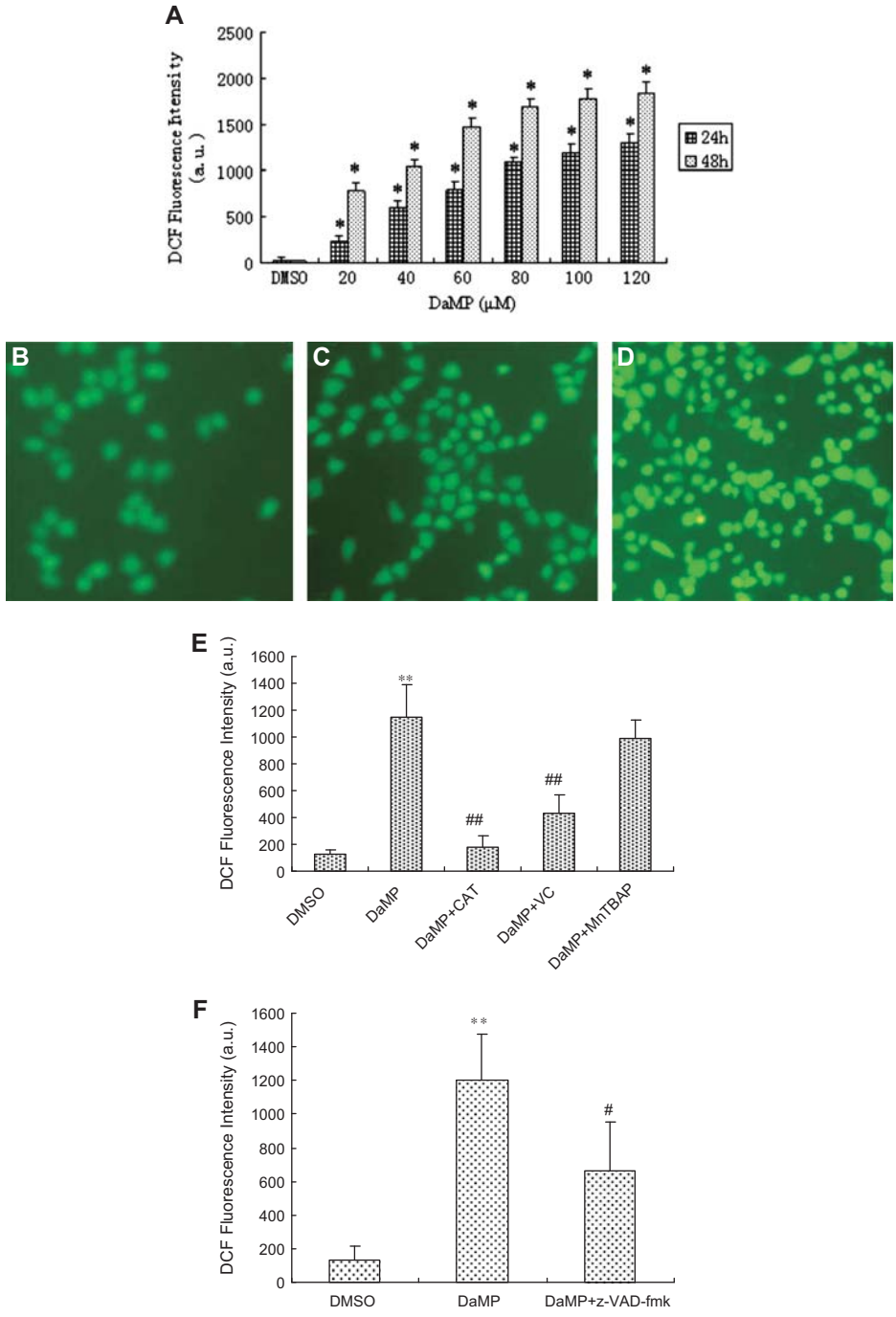

Figure 4. DaMP-induced production of ROS in BEL-7402 cells. (A) Intracellular ROS production after the BEL-7402 cells were treated with different concentrations of DaMP for indicated times. Data represent means ± SD for six independent samples. $^{*}p < 0.05$ vs DMSO. (B–D) Intracellular ROS levels after the cells were treated without or with DaMP for 24 h, observed by fluorescence microscope. (B) control; (C) 80 μM DaMP + 1 mM Vitamin C; (D) 80 μM DaMP. Pictures are representative of three independent experiments. At least five fields were viewed in each of the experiments. (E) Cells were either left untreated or pre-treated with catalase (1000 U/ml), vitamin C (1 mM) or MnTBAP (100 μM) for 1 h. The cells were then treated with 80 μM DaMP for 24 h. Data represent means ± SD for six independent samples. $^{**}p < 0.01$ vs DMSO. $^{\#\#}p < 0.01$ vs DaMP. (F) Cells were treated with z-VAD-fmk (100 μM) for 2 h prior to DaMP treatment for 24 h. Data represented as means ± SD of at least three independent experiments performed in triplicate; $^{**}p < 0.01$ vs DMSO ; $^{\#\#}p < 0.01$ vs DaMP.

with Hatse et al. [11], who examined the effects of PMEA on the cell cycle distribution pattern in K562 and THP-1 cell lines and concluded that cell differentiation and apoptotic cell death are two cell-specific alternative responses to PMEA-induced cell cycle arrest in S phase [11].

Apoptosis has been studied in terms of cancer development and treatment with attempts made to identify its role in chemotherapeutic agent-induced cytotoxicity. Apoptosis is a form of cell death including the following properties: cell shrinkage, loss of cell–cell contact, chromatin condensation and

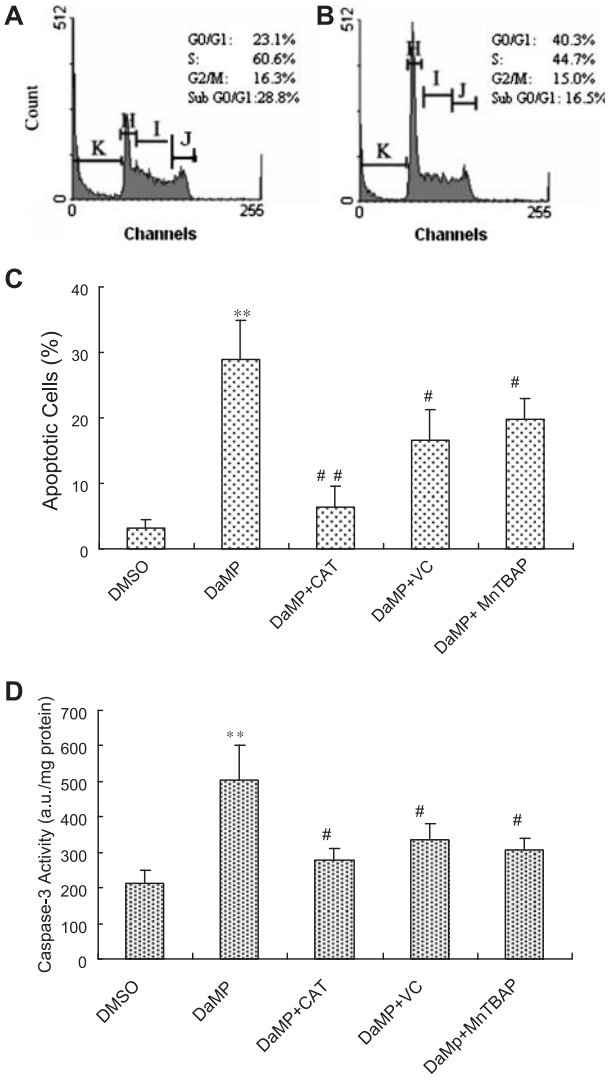

Figure 5. The effect of antioxidants on apoptosis and caspase activity. Cells were treated with 80 μM DaMP (A) or 80 μM DaMP + 1 mM Vitamin C (B). Flow cytometric analysis as described in Percentage of apoptotic cells (C) were quantified by the percentage of cells in sub G0/G1 population. Caspase 3 (D) activity was determined as described in Materials and methods. Cells were either left untreated or pre-treated with catalase (1000 U/ml), vitamin C (1 mM) or MnTBAP (100 μM) for 1 h. The cells were then treated with 80 μM DaMP for 24 h. Data represent means ± SD for six independent samples. $^{**}p < 0.01$ vs DMSO. $^{\#}p < 0.05$ vs DaMP. $^{\#\#}p < 0.01$ vs DaMP.

intra-nucleosomal degradation of DNA. These properties are apparently different from necrosis [9,37]. In our work, cytometric analyses of annexin V-positive cells demonstrated the apoptosis in cells treated with DaMP (Figures 3A and B). DNA from DaMP-treated cells also exhibited a characteristic banding pattern when analysed by electrophoresis (Figure 3C). Furthermore, DaMP treatment produced increased DNA content at the sub-G0/G1 fragment, indicating apoptosis occurred (Figure 2C). These data suggest that DaMP treatment could induce apoptosis. On the other hand, cell growth study shows BEL-7402 cells were inhibited significantly if the concentration of DaMP reached 40 μM, but the inhibition rate increased slightly above 40 μM (Figure 1). However, the generation of ROS by DaMP was dose-dependent, as well as DaMP-induced caspase activity (Figures 3 and 4). This may indicate that cell growth inhibition is not proportional to the oxidative stress or caspase activation if cell growth inhibition rate is higher than 75%.

It is known that one of the important steps in the induction of apoptosis is the activation of caspases, a family of cysteine proteases that cleave their substrates at specific aspartate residues [31,41]. Among the caspases, caspase-3 is one of the key executors of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many essential proteins, such as the nuclear enzyme, poly(ADP-ribose) polymerase [42–44]. Our study shows that caspase-3 and -9 levels were increased significantly in DaMP-treated cells, which is consistent with Marzo et al. [10], who reported that nucleoside analogue 2-chloro-2′-deoxyadenosine (2CdA or cladribine) could induce apoptosis in human leukaemia cells in a caspase-dependent way [10]. These results suggest that BEL-7402 cells treated with DaMP undergo apoptosis through a caspase involved mechanism. It is also supported by the fact that induction of apoptosis can be inhibited by pan-caspase inhibitor z-VAD-fmk (Figure 3F).

Oxidative stress is implicated in a number of cellular processes including apoptosis and many chemotherapeutic agents are known to induce cytotoxic effects in tumour cells by a ROS mediated mechanism. It is suggested that ROS generated during the acyclic nucleoside phosphonates treatment might contribute to the increased p53 expression [45]. Hwang et al. [46] also demonstrates that 5-FU induced ROS production was stimulated through reactive oxygen species modulator 1(Romo1) induction. In our work, the generation of ROS induced by DaMP seems to have occurred from caspase activation since z-VAD-fmk effectively inhibited this generation. There is evidence which suggests that ROS may act as a signal to molecules to initiate and execute an apoptotic cell death programme [27,47,48]. In this study, ROS level was proportional to DaMP exposure concentration in BEl-7402 cells (Figure 4A). Meanwhile, inhibition of ROS generation by antioxidants effectively inhibited apoptosis and caspase activity, indicating the role of ROS in the process (Figures 4E, 5C and D). The sustaining ROS level was also the result of caspase activation since z-VAD-fmk could significantly inhibit ROS (Figure 4F). After the onset of apoptosis, the fragments of P53 will be generated by caspase activation, which will further augment mitochondrial membrane depolarization and ROS generation [49]. One of the consequences of membrane potential disruption is the uncoupling of oxidative phosphorylation [50] and the accelerated

generation of superoxide anion from the uncoupled respiratory chain [51]. In addition, after the decrease of $\Delta\Psi_m$ and caspase activation, a steady increase of mitochondrial Ca^{2+} was observed, while increased intracellular Ca^{2+} is known to be critical for increasing O_2^- generation [52]. It seems that DaMP was able to induce peroxide generation in BEL-7402 cells since catalase could inhibit ROS completely, whereas the SOD mimetic MnTBAP showed minimal effect (Figure 4E). It is interesting to note that MnTBAP could not effectively inhibit the production of DaMP-induced ROS, although it can inhibit DaMP-induced apoptosis (Figures 4E and 5C). This may suggest that another source of superoxide anion is involved in the process of apoptosis. All these results suggest that DaMP induced-apoptosis in BEL 7402 cells appears to occur, at least in part, due to increased intracellular ROS.

In conclusion, we have demonstrated that the novel acyclic nucleoside phosphonate DaMP under certain conditions can result in anti-tumour activity in BEL 7402 cells. DaMP exhibited a strong inhibitory effect on the proliferation of BEL 7402 cells *in vitro*, probably related to apoptosis causing cell cycle arrest in S phase and caspase activation. Antioxidants attenuate the anti-proliferative effect of DaMP, suggesting that ROS production is also involved in the anti-cancer activity of DaMP. These experimental findings provide evidence of specific anti-cancer activity of the new acyclic nucleoside phosphonate and warrant further evaluation using *in vivo* subjects.

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