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Apoptosis of Human Burkitt's Lymphoma Cells Induced by 2-*N,N*-Diethylaminocarbonyloxymethyl-1-diphenylmethyl-4-(3,4,5-trimethoxybenzoyl)piperazine Hydrochloride (PMS-1077)

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Piperazine is one of the heterocycles which are associated with diverse pharmacological activities. 2-*N,N*-Diethylaminocarbonyloxymethyl-1-diphenylmethyl-4-(3,4,5-trimethoxybenzoyl) piperazine hydrochloride (PMS-1077) is a trisubstituted piperazine which contains a trimethoxybenzene ring and a benzhydrylpiperazine fragment, both of which can induce cell proliferation regression by different mechanisms. We have therefore examined the effects of PMS-1077 on Human Burkitt's lymphoma cells (Raji). The viability of Raji cells was determined by MTT assay and also assessed by trypan blue dye exclusion method. The results demonstrate that PMS-1077 can suppress the proliferation of Raji cells in a dose- and time-dependent manner, while inhibit colony formation ability of Raji cells merely in a dose-dependent manner *in vitro*. Meanwhile, morphological changes were observed using fluorescence microscope. Flow cytometric analysis through PI stains showed that PMS-1077 blocked the growth of Raji cells in the G_0/G_1 period, and induced apoptosis of Raji cells after 48 h of incubation. Cell apoptosis induced by PMS-1077 was further confirmed by staining with Annexin-V FITC and PI. Preliminary study by molecular docking suggests that PMS-1077 may inhibit tubulin polymerization. More experiments are in progress in our laboratory to reveal the mode of action of PMS-1077 in the induction of apoptosis of Raji cells.

Key words: 2-N,N-Diethylaminocarbonyloxymethyl-1-diphenylmethyl-4-(3,4,5-trimethoxybenzoyl) piperazine hydrochloride (PMS-1077), Apoptosis, Human Burkitt's lymphoma (Raji), Antiproliferative activity, Tubulin

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INTRODUCTION

Apoptosis is an evolutionarily multistage process and a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage (Ghobrial et al., 2005; Joseph et al., 2000). Defective apoptosis represents a major causative factor in the development and progression of cancer (Kasibhatla and Tseng, 2003; Kiechle and Zhang, 2002; Schmitt and Lowe, 1999; Wilson, 1998). Therefore, developing apoptosis-inducing agent is a new therapeutic approach in cancer research (Makin and Hickman, 2000; Sellers and Fisher, 1999) and promising anticancer drugs could be discovered by studies on apoptosis induction (Bremer et al., 2006; Kerr et al., 1994; Los et al., 2003; Thompson, 1995; Vermeulen et al., 2005; Yuan et al., 2004). On the other hand, Raji cells represent a very reliable cell model to study the anti-tumor property of a compound since they have been previously used for screening chemopreventive drugs with antitumor-promoting activities by inducing cell apoptosis *in vitro* (Lau et al.,

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2004).

Clinical studies have documented an association between inflammation and cancer as well as the cancer developments at the site of inflammation for decades. Many irritants thought to increase cancer development by stimulation of proliferation may also act by aggravating inflammation in the tissue and vice versa (Rosin et al., 1994). So, some anti-inflammatory agents are associated with protection against various tumors (Bellosillo et al., 1998; Ghosh et al., 2006; Mantovani et al., 2007; Samaha et al., 1997; Surh et al., 1999).

Many reports demonstrated that a trimethoxybenzene ring (Fragment **A**) could contribute to block tubulin polymerization by binding to colchicine site of tubulin (Alvarez et al., 2008a; Alvarez et al., 2008b; Lin et al., 1989). Our earlier studies (Miao et al., 2008) also showed that seleno-podophyllotoxin derivatives containing the fragment **A** could induce apoptosis through Bax pathway. On the other hand, benzhydrylpiperazine derivatives present a broad biological activities, such as anticancer (Kumar et al., 2008), anti-PAF or antiplatelet aggregation (Cheng et al., 2007; Serradji et al., 2006), and antihistimines (A Orjales, 1996).

We have reported in the recent years the synthesis of a great number of tri-substituted piperazine which showed potent dual anti-PAF and anti-HIV-1 activities (Martin et al., 2000; Sallem et al., 2006; Serradji et al., 2000; Serradji et al., 2006; Serradji et al., 2004). One of these compounds (PMS 601) has been demonstrated to be able to cross the blood-brain barrier and considered to be a promising candidates for HIV-associated dementia (HAD) therapy, taking into account the other very interesting properties of this molecule. We found recently that 2-*N,N*-diethylaminocarbonyloxymethyl-1-diphenylmethyl-4-(3,4,5-trimethoxybenzoyl) piperazine hydrochloride (PMS-1077) possesses higher dual anti-PAF and anti-HIV-1 activities than PMS 601. This compound contains both trimethoxybenzene ring (Fig. 1, fragment **A**) and benzhydrylpiperazine moiety (Fig. 1, fragment **B**). This prompted us to evaluate the pro-apoptotic effects of PMS-1077 on Human Burkitt's lymphoma cells (Raji). The results demonstrated that PMS-1077 could suppress the proliferation of Raji cells in a dose- and time-dependent manner, while inhibit colony formation ability of Raji cells merely in a dose-dependent manner *in vitro*. We also found that it induced morphological changes in this cell line, blocked the growth of Raji cells in the G_0/G_1 period, and provoked apoptosis of Raji cells. The latter was further confirmed by staining with Annexin-V FITC and PI. Preliminary study by molecular docking suggests that PMS-1077 may inhibit tubulin polymerization.

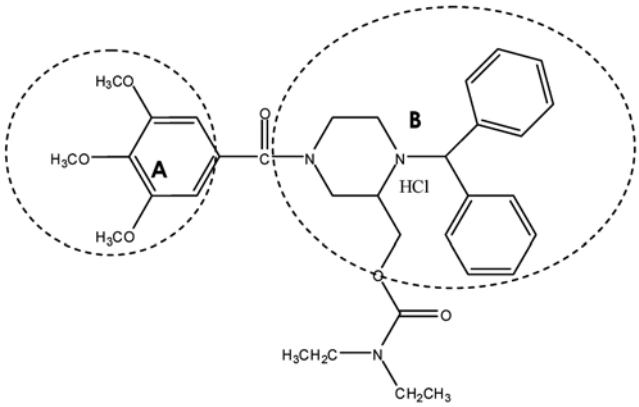

Fig. 1. Chemical structure of PMS-1077. Fragment (**A**) corresponds to trimethoxybenzene group and (**B**) is composed of *N*-benzhydrylpiperazine motif.

MATERIALS AND METHODS

Reagents

PMS-1077 (Fig. 1) was synthesized as previously reported (Serradji, 2006). It was dissolved in dimethylsulphoxide (DMSO) and diluted in culture medium prior to use. The final concentration of DMSO in cultures was always less than 0.4% (v/v). Under this concentration, no cyto-toxicity was observed.

RPMI-1640 medium was purchased from Gibco. Fetal bovine serum (FBS) was provided by Lanzhou Minhai Biotechnology Company.

Cells

Raji cells, obtained from the central lab in the School of Life Science, Lanzhou University, were grown in RPMI-1640 medium, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 g/L $NaHCO_3$, at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell viability (Bernhard et al., 2003)

This was determined by [3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay and also assessed by trypan blue dye exclusion method (Lu et al., 2006).

Raji cells were plated into 96-well plates (1×10^4 cells/well). 24 h later, the cells were treated with various concentrations of PMS-1077 ranging from 10 to 160 μM and maintained in culture for further 24, 48 and 72 h. To the culture medium was then added

MTT to 5 mg/mL. Cells are incubated at 37°C for 4 h and the medium was then removed. The water-insoluble formazan crystals formed, were dissolved by the addition of DMSO (100 μL/well). The optical density of each well was measured with a spectrophotometric microplate reader Bio-Rad 550 at 570 and 630 nm (background). For each concentration tested, wells containing all reagents except for cells served as controls. The absorbance data were corrected by subtracting the corresponding background. Cell survival was presented as an absorbance (A) percentage defined by ($A_{drug}/A_{control} \times 100$) (Ye et al., 2004).

Exponentially growing cells, diluted in culture medium to 1×10^4 cells/mL, were plated in 96-well plates and after 24 h of growth treated with different concentrations (10, 20, 40, 80 and 160 μM) of PMS-1077 or drug solvent (DMSO). Incubation was carried out at 37°C for 24, 48 and 72 h. Cells were finally exposed to 0.4% trypan blue and counted with a hemocytometer.

Soft-agar colony formation test

After treated with PMS-1077 (10, 20, 40, 80 and 160 μM) for 48 h, Raji cells (1×10^3) were cultured on a plate containing 0.5% base agar and 0.17% top agar in total culture medium. After 21 days of incubation at 37°C in a 5% CO_2 humidified atmosphere, the colonies with > 50 cells were counted. Colony-inhibition rate [(1-(number of colonies in experimental groups)/ (control groups)) × 100%] and colony-forming efficiency [1-Colony-inhibition rate] were calculated.

Cell cycle analysis (FCM) (Wang et al., 2006)

Briefly, cells were treated with PMS-1077 (40, 60, 80 and 100 μM). After 24 and 48 h, cells were prepared as a single cell suspension in cold PBS, fixed with ice-cold 70% ethanol, and maintained at -20°C for more than 24 h. The cells were harvested by centrifugation, re-suspended in PBS supplemented with 0.1% Triton X-100 and RNase A (100 U/mL), incubated at 37°C for 30 min, and stained with 50 μg/mL propidium iodide (PI) in the dark at room temperature for 30 min. The cell cycle distribution and apoptotic cells were detected with FACScan (COULTER Epics XL), and analyzed using a cell cycle analysis software, Multicycle.

Detection of apoptosis

Detection of apoptosis by fluorescence microscopy: Cell morphology was evaluated by fluorescence microscopy following Acridine range (AO)/Ethidium bromide (EB) DNA staining (Shanghai Jitai Technology Co. Ltd.). After incubated in the absence (control cell) or presence of PMS-1077 for 48h, Raji cells were stained with AO (4 μg/mL) and EB (4 μg/mL in PBS, pH7.2). Cell suspension (10 μL) was spotted onto a microscopic slide and covered with a glass coverslip. The slide was examined by fluorescence microscope (Olympas ax80). For each sample 200 cells were counted and recorded as V (viable cells), NVN (non-viable cells with normal nuclei) and NVA (non-viable cells with apoptotic nuclei). The percentage of apoptotic *versus* non-apoptotic dead cells was calculated (% apoptotic cells = 100 × NVA/ (VA + NVN + NVA); % non-apoptotic cells = 100 × NVN/ (VA + NVN + NVA)).

Detection of apoptosis by flowcytometry (Darzynkiewicz et al., 1992; Koopman et al., 1994; McHugh and Turina, 2006; Sgonc and Gruber, 1998): The detection of apoptotic cells was performed using FITC conjugated Annexin-V Flous and PI. Raji cells were treated with 0, 40 and 80 μM PMS-1077 for 48 h and then were pelleted down by centrifugation, washed twice with cold PBS, and then centrifuged at 1000 rpm to collect the cells. Cells were re-suspended in 200 μL of binding buffer, and then 10 μL of Annexin-V FITC, 5 μL of PI and 300 μL of binding buffer were added. Cells were incubated in dark for 30 min and were analyzed by flow cytometry. FACS was performed using 488 nm excitation and band pass filters of 515-545 nm (for Annexin-FITC detection) and 563-607 nm (for PI detection). Data analysis was performed with Cell Quest program.

Molecular modeling

The β-tubulin unit atomic coordinates from tubulin-podophyllotoxin complex were taken from the Brookhaven Protein Data Bank with a resolution of 4.2 Å (PDB ID: 1SA1). The protein was refined with Swiss PDB viewer (Guex and Peitsch, 1997), Pymol (DeLano, 2002) and Chimera (Pettersen et al., 2004) and minimized by Gromos 96 force field for 5,000 steps in Gromacs 4.0 (Van Der Spoel et al., 2005). PMS-1077 was built with Pymol and minimized by Amber 99 force field for 20,000 steps with a step size of 0.002 Å.

Docking simulations were performed with AutoDock 4.0 using a Lamarkian genetic algorithm (Garrett M. Morris, 1998). AutoDocktools (ADT version 1.5.4) was used for protein and ligand preparation and docking calculations. Grid box was constructed with dimensions 48 × 46 × 50 points according to podophyllotoxin binding site. Residues Cys241, Lys254, Asn258, Lys352 and Asn349 were set as flexible residues. All rotations and torsions for PMS-1077 were automatically set in the ADT. The remaining parameters were set as default.

Statistical analysis

All data were expressed as mean ± S.D. from at least three independent experiments.

RESULTS

PMS-1077 reduces Raji cell viability

To determine whether PMS-1077 leads to loss of cell viability, Raji cells were incubated for 24, 48 and 72 h with PMS-1077 and cell viability was determined by MTT assay. As shown in Fig. 2A, the cell survival was inversely correlated with PMS-1077 concentration. Significant loss of viability is detected at 10, 20, 40, 80 and 160 μM PMS-1077 in a dose- and time-dependent manner. The same results were further confirmed by trypan blue dye exclusion method as shown in Fig. 2B.

PMS-1077 reduces Raji cell's colony formation ability

This assay is designed to evaluate the un-anchored growth ability of each single cell. As shown in Fig. 3, the Raji cell's colony-forming efficiency decreased, when the concentrations of PMS-1077 in the culture medium increased.

PMS-1077 induces an arrest in G0/G1 phase and apoptosis in Raji cells

Cell cycle was analyzed by flow cytometry analysis (Wang et al., 2006). Flow cytometric analysis was performed to evaluate the cell cycle distribution and the proportion of hypodiploid cells. After treatment with PMS-1077, the percentage of cells in G_0/G_1 phase increased from 50.2% to 71.6% (24 h, 80 μM), from 50.2% to 80.1% (48 h, 60 μM). In contrast, the numbers of the cells in G_0/G_1 decreased at higher concentrations, as shown in Fig. 4J. These results suggested

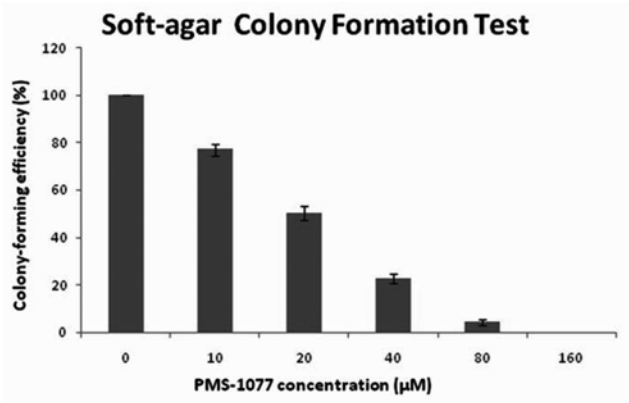

Fig. 3. Raji cell's colony formation ability in the presence of PMS-1077. Cell colonies were counted after Raji cells were treated with different concentrations of PMS-1077 and incubated for 21 days at 37°C in 5% CO_2.

that PMS-1077 inhibited the cellular proliferation of Raji cells via inducing cell cycle arrest at G_0/G_1 phase. The sign of apoptosis was indicated by the accumulation of sub-G_1 population in Raji cells after PMS-1077 treatment. After 48 h incubation with indicated concentrations of PMS-1077, 13.9% (100 μM) of the cells displayed an apoptotic/hypodiploid peaks. As shown in Fig. 4, our result showed that PMS-1077 induced apoptosis in a dose-dependent manner in Raji cells.

Morphological determination of apoptosis by fluorescence microscopy (Krysko et al., 2008; Martelli et al., 2001; Saraste and Pulkki, 2000; Sgonc and Gruber, 1998)

PMS-1077 caused cell death due to induced apoptosis in Raji cells. This phenomenon can be characterized by a series of typical morphological changes, such as chromatin condensation, nuclear shrinkage, plasma membrane blebbing, DNA fragmentation and collapse

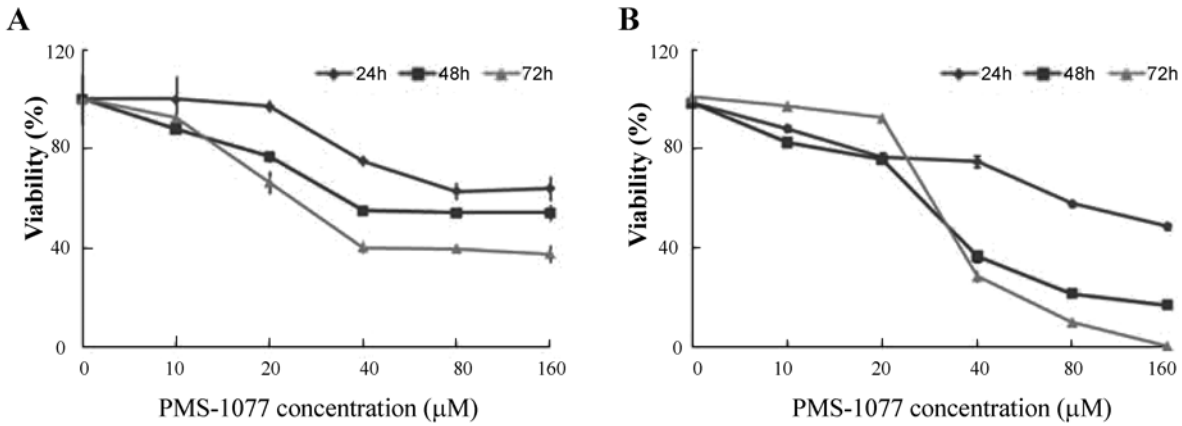

Fig. 2. Effect of PMS-1077 on the viability of Raji cells. Raji cells were treated for 24, 48 and 72 h with PMS-1077 at concentrations from 10 to 160 μM. (**A**) Dose-response curves from MTT assay. (**B**) Dose-response curves obtained by using trypan blue dye exclusion method. Values are means ± S.D. from at least three independent experiments.

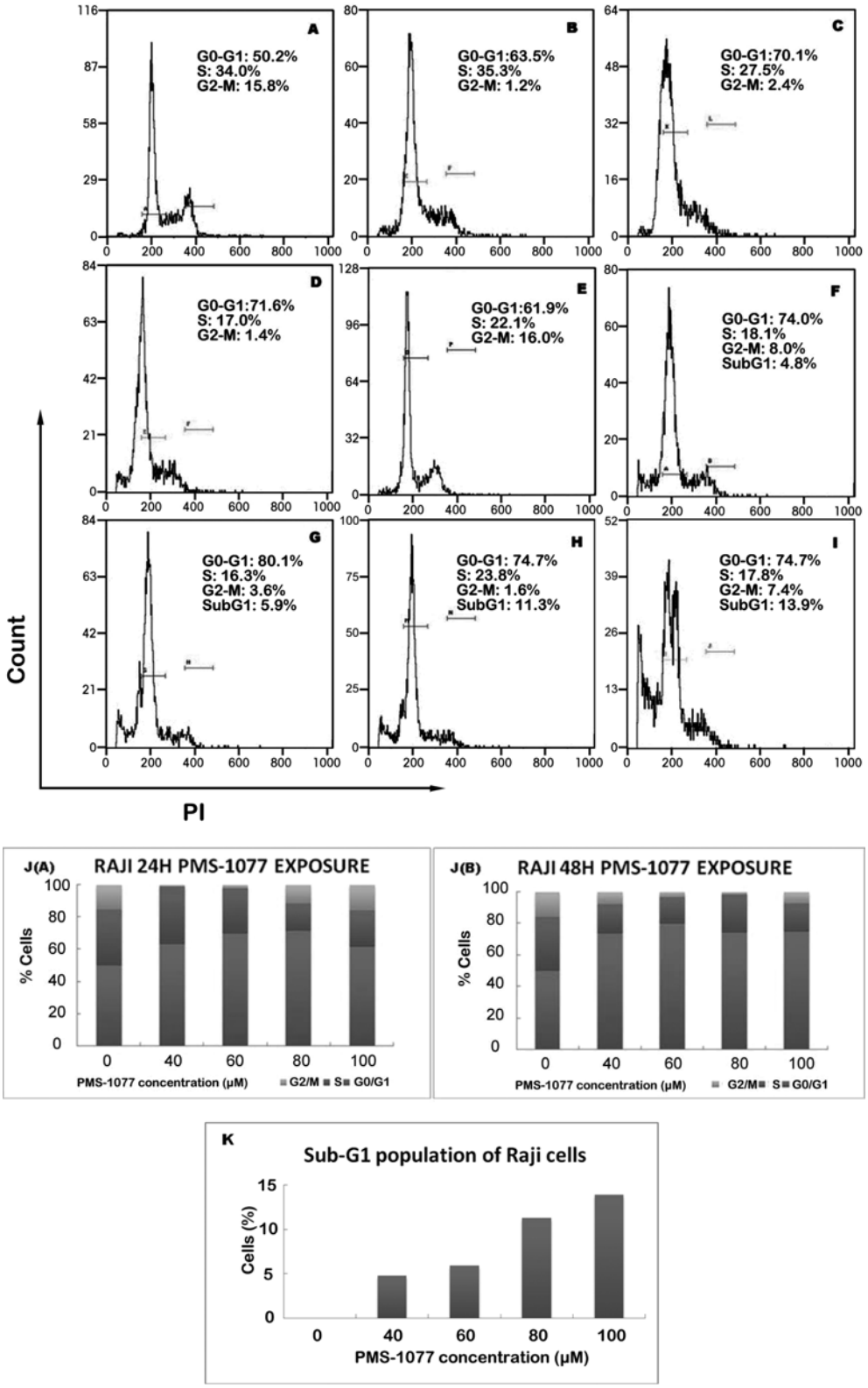

Fig. 4. Flow cytometric analysis of PMS-1077-treated Raji cells. Cells stained with propidium iodide were subjected to flow cytometric analysis for cell distribution at each phase of cell cycle. Cells were cultured for 24 h (**A**-**E**) or 48 h (**F**-**I**) in the absence (**A**) or in the presence of 40 μM (**B**, **F**), 60 μM (**C**, **G**), 80 μM (**D**, **H**), 100 μM (**E**, **I**) of PMS-1077. (**J**) Cell cycle arrest induced by PMS-1077. Cells were treated with 40, 60, 80, 100 μM of PMS-1077 for either 24 h (**A**) or 48 h (**B**). (**K**) Sub-G_1 population of Raji cells treated with indicated concentrations of PMS-1077 for 48 h.

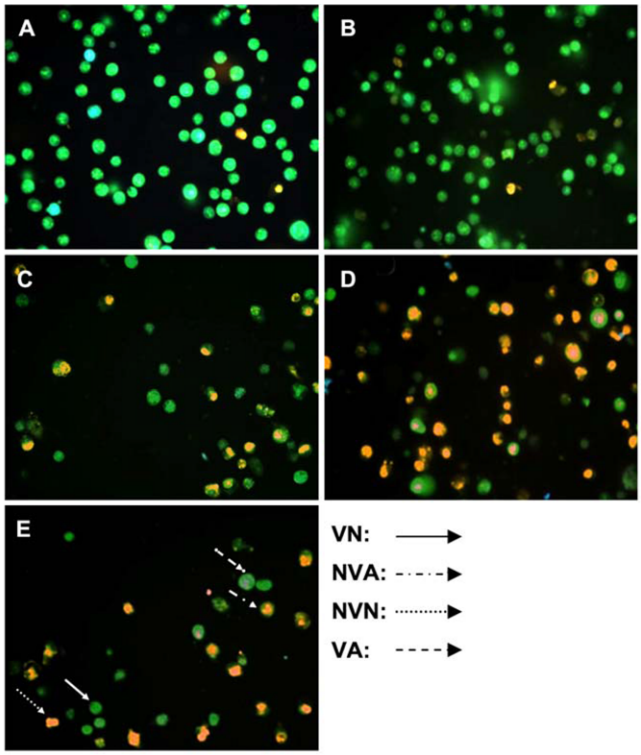

Fig. 5. Fluorescence microscope images showing apoptosis-inducing activity of PMS-1077 in Raji cells. The concentrations of PMS-1077 used are as follows: 0 μM (**A**, control), 40 μM (**B**), 60 μM (**C**), 80 μM (**D**), 100 μM (**E**) and the cells were stained for 48 h using nuclear staining dyes, AO (green fluorescence) and EB (orange fluorescence). Early apoptotic cells (VA), Late apoptotic cells (NVA), Viable cells (VN), Necrotic cells (NVN).

of the cell into small intact fragments (apoptotic bodies).

The morphology of Raji cells stained with AO and EB was observed under fluorescence microscope, after they were treated with indicated concentrations of

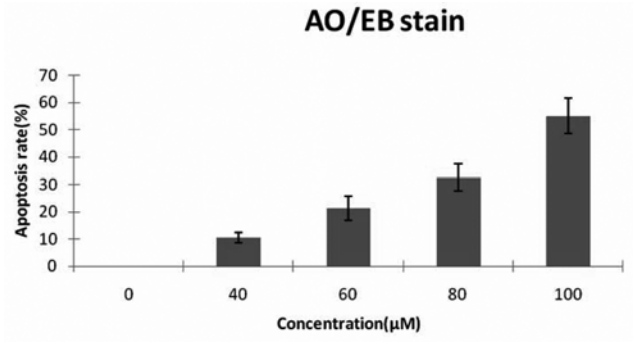

Fig. 6. Dose-dependent apoptosis induction of Raji cells by PMS-1077. Values are means ± S.D. from at least three independent experiments.

PMS-1077 for 48 h (Fig. 5). Living cells appeared uniformly green, while early apoptotic cells stain green and contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells can be easily distinguished from necrotic cells. As the latter, late apoptotic cells also incorporate ethidium bromide and therefore stain orange, but they show condensed and often fragmented nuclei as well. By contrast, necrotic cells possess a nuclear morphology resembling that of living cells, with no condensed chromatin.

The apoptotic cell and the total cell numbers were counted for each concentration of PMS-1077 and the ratios between these numbers were plotted against the concentration in Fig. 6. It can be observed that PMS-1077 induces the apoptosis of Raji cells in a dose-dependent manner.

Flow cytometric detection of apoptosis in Raji cell line

Apoptosis, as revealed through annexin-V FITC and Propidium iodide (PI) staining in Raji cells treated

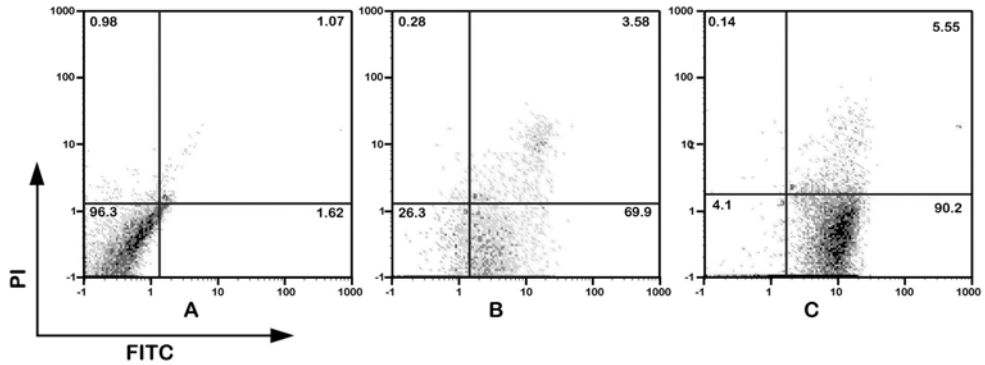

Fig. 7. Flow-cytometric analysis of Raji cells by double labeling with Annexin-V FITC and PI. Dual parameter dot plot of FITC-fluorescence (*x*-axis) vs. PI-fluorescence (*y*-axis) shows logarithmic intensity. Quadrants: lower left-live cells; lower right-apoptotic cells and upper right-necrotic cells. Percentage of apoptotic nuclei are: (**A**) control cells: 1.62%, (**B**) PMS-1077 (40 μM, 48 h): 69.9%, (**C**) PMS-1077 (80 μM, 48 h): 90.2%.

with PMS-1077, is shown in Fig. 7. Cells treated with 40 μM and 80 μM of PMS-1077 for 48 h showed enhancement of apoptosis by 43.1- and 55.7-folds, respectively, when compared to control.

PMS-1077 docked into podophyllotoxin binding pocket of tubulin-beta

In order to understand how PMS-1077 can induce apoptosis of Raji cells, a preliminary study of docking was performed using β-tubulin as target and PMS-1077 as ligand, which presents a certain analogy to podophyllotoxin, a small tubulin-binding compound. The results showed that PMS-1077 bound to a hydrophobic pocket where podophyllotoxin docks to tubulin. The binding free energy of podophyllotoxin was found to be -7.0 kcal/mol, while that of PMS1077 -6.1 kcal/mol.

DISCUSSION

We have reported that PMS-1077 showed interesting dual anti-HIV-1 and anti-PAF activities (Serradji et al., 2000). In present study, we investigated the anti-proliferative and apoptosis-inducing effects of PMS-1077 on Raji cells. The loss of cell viability was detected in a dose- and time-dependent manner by MTT assay and these results were confirmed by using trypan blue dye exclusion method. In the presence of PMS-1077, Raji cells become less suitable for colony formation and this decrease was dose-dependent. Moreover, fluorescence microscope showed a dose-dependent increase in the proportion of Raji cells (Fig. 6) having the morphological features characteristic of cell death when treated with 40, 60, 80 and 100 μM of PMS-1077, respectively, for 48 h. The results of flow cytometry showed that PMS-1077 induced cell cycle arrest at the G_0/G_1 phase in Raji cells (Fig. 4). After cell cycle arrested at G_0/G_1 phase, PMS-1077-treated cells underwent apoptosis. The morphology of PMS-1077-treated Raji cells showed some pattern of apoptosis as observed by fluorescence microscopy (Fig. 5).

Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of apoptosis (Bellosillo et al., 1998; Ghosh et al., 2006; Mantovani et al., 2007; Samaha et al., 1997; Surh et al., 1999). In addition, the induction of apoptosis is thought to be one of the most interesting therapeutic strategies, targeting specifically cancer cells (Bremer et al., 2006; Kerr et al., 1994; Los et al., 2003; Thompson, 1995; Vermeulen et al., 2005; Yuan et al., 2004).

Then, we subsequently assessed that the cell proliferation inhibition is mediated via an apoptotic mechanism. In the first stage of apoptosis, the changes in the membrane composition lead to extracellular exposure to phosphatidyl serine (PS) residues which bind Annexin-V. Membrane changes leading to PS exposure occur rapidly in apoptotic cells, while the cells lose membrane integrity later in the apoptotic process and expose DNA. So using a DNA binding dye PI in tandem with fluorochrome-conjugated Annexin-V, apoptotic cells and necrotic cells can be discriminated by flow cytometry (Darzynkiewicz et al., 1992; McHugh and Turina, 2006; Surh et al., 1999). So flow cytometry by this double staining provides clear detection of three populations of cells (viable, apoptotic and necrotic). Thus, the early apoptotic cells bind only to Annexin-V FITC and late apoptotic cells with both Annexin-V FITC and PI and viable cells do not take any of the dye. In our study, the Annexin-V/PI assay confirms further apoptosis induced by PMS-1077 in Raji cell (Fig. 7).

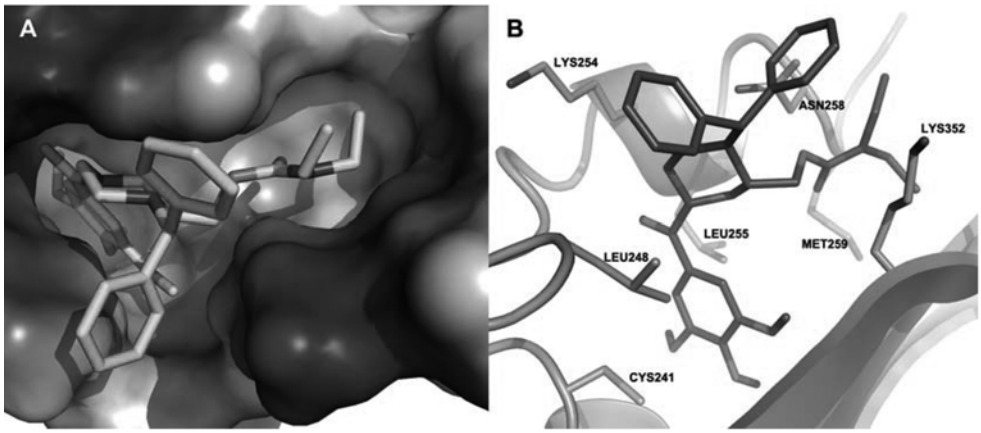

Fig. 8. Models of PMS-1077 bound to β-tubulin. (**A**) PMS-1077 binds to the pocket of podophyllotoxin binding site in tubulin. The pocket is shown in electrostatic potentials. (**B**) Orientation of PMS-1077 in the podophyllotoxin binding site showing the main residues.

Tubulin has been recently described in the nucleus of cells and in mitochondria. Downstream events from tubulin binding are believed to be critical events for the generation of apoptosis in the malignant cell. Trimethoxybenzene derivatives are shown to exhibit powerful effects in blocking tubulin polymerization and inhibiting cell proliferation in many reports (Alvarez et al., 2008a; Alvarez et al., 2008b; Lin et al., 1989). Tubulin-disruption induces apoptosis through phosphorylation of Bcl-2 and activating Bax (Doudican et al., 2008; Jiang et al., 1998). Our earlier work has shown that seleno-podophyllotoxin (a trimethoxybenzene derivative) induced apoptosis through Bax pathway (Miao et al., 2008). PMS-1077 may also inhibit tubulin polymerization. In general, anti-tubulin agents provoke anti-proliferation through cell cycle arrest at G_2/M phase when tubulin plays a role in mitosis, but the research (Hoessel et al., 1999) showed that indirubin-3'-monoxime had antiproliferative activity, leading to a G_2/M arrest in almost all cell types studied and to G_1/S arrest in Jurkat cells. The study indicated the phase which the cell arrested was related to cell line. Another research (Owa et al., 2000) revealed that there were two different classes of potent cell cycle inhibitors; one arrested mitosis and the other caused a cellular accumulation in G_1 phase. The mitotic arrest observed was shown to result from the inhibition of tubulin polymerization, whereas the precise mechanism of the G_1 increase was still unclear, but the G_1 increase caused by inhibition of tubulin polymerization could not be excluded. In this study, PMS-1077 was well docked into a hydrophobic pocket in tubulin by flexible molecular docking. PMS-1077 may inhibit tubulin polymerization and activate Bax pathway by which it induces apoptosis.

In conclusion, our results show that PMS-1077 has a certain antiproliferation effect on Raji cells. At the same time, flow cytometry also shows that PMS-1077 can arrest cell cycle progression in G_0/G_1 phase. Moreover, PMS-1077 induces apoptosis in Raji cells, as judged by morphologic criteria and by flow cytometry analysis. Despite these findings, the molecular mechanisms by which PMS-1077 can exert its anti-proliferative effect on Raji cells remain a subject to be further elucidated.

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