2-Methylpyridine-1-ium-1-sulfonate as an Inducer of Apoptosis and Cell Cycle Arrest: A comparative in vitro and Computational Study

Article in Nutrition and Cancer · October 2018
DOI: 10.1080/01635581.2018.1506495

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To link to this article: https://doi.org/10.1080/01635581.2018.1506495

Published online: 01 Oct 2018.
2-Methylpyridine-1-ium-1-sulfonate as an Inducer of Apoptosis and Cell Cycle Arrest: A comparative in vitro and Computational Study

Hamid-Reza Mohammadi-Motlagha, Reza Yarani, Mona Sadeghalvada, Elham Adham, Hassan Rasouli, and Ali Mostafaie

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ABSTRACT

“Let food be thy medicine and thy medicine be thy food” was expressed by Hippocrates and the health benefits of medicinal plants and natural products have been considered by humans since historic times. The current study aims to investigate the anti-cancer activity of 2-Methylpyridine-1-ium-1-sulfonate (MPS) isolated from bulbs of Allium hirtifolium. The MPS compound (in a dose-dependent manner) induced arrest the AGS cells in G1 and G2/M phases, and Caco-2 cells in G1 and S phases. These findings were associated with the down-regulation of cyclin D1, CDK4, and up-regulation of p21, p27 and p53. According to the morphological observations and DNA fragmentation assay, the MPS compound induced apoptosis in both cell lines, and also cause a significant increase in the expression of Bax/Bcl-2. In this context, our molecular docking results unveiled that the MPS compound has considerable affinity to interact with the minor groove of ctDNA and also with cell cycle kinases. To approve and find the accurate MPS mode of action against cancer cell lines (especially in gastrointestinal cancer) further studies is highly recommended.

Introduction

Today, cancer and its related complications significantly increase the global concerns about human health throughout the world (1). The major problem with cancer therapy is resistance of malignant cells against conventional drugs. According to the World Health Organization (WHO) report (http://www.who.int/mediacentre/factsheets/fs297/en/), cancer considered for 8.8 million deaths in the entire world in 2015. Lung (1.69 million deaths), liver (788000 deaths), colorectal (774000 deaths), stomach (754000 deaths) and breast (571000 deaths) cancers are the most common causes of cancer death. In this context, search to find new effective drug against cancers have widely received more attention from researchers.

The role of medicinal plants in treatment of human chronic diseases (e.g., cancer) is abundantly investigated over the past decades (2,3). Studies indicated that the main goal of researchers to consider medicinal plants to find new anticancer drugs was the disability of current drugs to control or inhibit cancer. In this regards, plant-based agents including taxol and camptothecin are well known natural compounds discovered to treat different types of cancers (4–6).

Persian shallot (Allium hirtifolium Boiss.), a member of Alliaceae family, is an important oral medicinal plant in Asian countries which exhibited a wide range of biological activities from anticancer to antibacterial properties (7,8). Many studies showed the pharmacological properties of Persian shallot and its constituents, which include anti-proliferative (9), antioxidant (10), hepatoprotective (11) and anti-bacterial (12) effects of different extracts of Persian shallot. In Iranian folk medicine, the bulbs of shallot have used for treatment of different diseases such as rheumatoid, stomach pain, inflammation, arthritis and psoriasis (13).

Previously, our laboratory reported the anti-angiogenic, anti-inflammatory and anticancer activity of hydroalcoholic extract of Persian shallot and its effective fraction(s) using in vitro, ex vivo, and in vivo assays (14–16) and our evaluations showed that the title compound (2-Methylpyridine 1-ium-1-sulfonate (MPS)) is a nontoxic natural product (17). So to
increase our knowledge about the mentioned compound, the current study aims to investigate the possible anticancer activity of MPS compound isolated from ethyl acetate fraction of Persian shallot bulbs against two human adenocarcinoma cancer cell lines (AGS and Caco-2). The generated results from the current study can be considered to design and develop a new drug for treating of cancer.

Material and Methods

Extraction, Isolation, and Identification of the Active Compound

Preparation of the hydroalcoholic extract and ethyl acetate (EA) fraction from Persian shallot bulbs were performed according to our previous reported protocol (14). For isolation of the active compound, the EA fraction was added to a normal-phase silica gel column chromatography. Structure elucidation of the active compound was carried out using NMR and Electron Ionization-MASS (EI-MS) spectroscopic analyses. 1H NMR and 13C NMR spectra were measured on the Bruker (250 and 62.9 MHz) spectrometers using DMSO- ̃d6 as solvent. Chemical shifts were referenced to the residual solvent signal (DMSO: δH 2.48, 3.32; δC 39.9). EI-MS spectra were performed on an Agilent 5975C Network mass selective spectrometer.

Cell Culture

AGS (human gastric adenocarcinoma) and Caco-2 (human colorectal adenocarcinoma) cell lines obtained from the National Cell Bank, Pasteur Institute (Tehran, Iran). Cells were cultured in DMEM/F12 medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 μg/mL) (Gibco, Grand Island, NY). Cells in all conditioned assays were maintained at 37 °C in a humidified incubator with 5% CO₂.

Cell Growth Assay

Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay. Cells were plated in 96-well culture plates (5 × 10³ cells/well) and treated with vehicle (0.1% DMSO), various concentrations of MPS (1–16 μM) and the positive control cisplatin (10 μg/mL) for 72 h. Thereafter, the MTT solution (5 mg/mL in PBS) was added to each well and plates were incubated at 37 °C for 3 h. The supernatants were discarded and formazan crystals were solubilized by DMSO. The absorbance was measured using a microplate reader (Awareness Stat Fax 2100) at 570 nm. The results expressed as percentage of the control and IC₅₀ (concentration resulting in 50% inhibition) for each cell line was calculated using GraphPad Prism version 6.07.

Cell Cycle Analysis by Flow Cytometry

Cells were plated at a density of 2 × 10⁵ cells/well in six-well plates. After 24 h, medium was removed; cells were washed with PBS and incubated in serum-free DMEM/F12 medium for 24 h for synchronization. The cells were then treated with MPS (2 and 4 μM) in a medium containing 10% FBS. After 24 h, cells were detached by trypsin, washed with PBS, and then fixed in cold 70% ethanol. Fixed cells were resuspended in PBS, treated with RNase A (100 μg/mL, 37 °C and 30 min), and then stained with propidium iodide (50 μg/mL) for 15 min at 4 °C. Cell cycle phase distribution of nuclear DNA was analyzed using a Flow Cytometr (Partec CyFlow space, Germany). The obtained data were analyzed using FloMax software.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

The cells were cultured at a density of 5 × 10⁴ cells/well in 24-well plates for 24 h, then treated with vehicle (0.1% DMSO), MPS (2–8 μM) and the positive control cisplatin (10 μg/mL) for 48 h. TUNEL assay was performed with In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions to determine apoptotic cells. The green fluorescence of apoptotic nuclei was detected by fluorescence microscopy (Nikon, Japan).

Acridine Orange (AO) and Ethidium Bromide (EB) Staining

Cells (5 × 10⁴ cells/well) were plated in 24-well plates. After 24 h, cells were incubated with fresh medium containing 10% FBS, vehicle (0.1% DMSO), different concentrations of MPS (2–8 μM) and the positive control cisplatin (10 μg/mL). After 48 h, cells were collected and stained with 20 μl of AO/EB solution (100 μg/mL of AO and 100 μg/mL of EB in PBS) for 5 min. Photographs were taken by a fluorescent microscope (Nikon, Japan).
**DNA Fragmentation Assay**

Cells were plated at a density of $2 \times 10^5$ cells/well in six-well plates, cultured overnight, and then treated with vehicle (0.1% DMSO) and MPS (4–16 μM) and the positive control cisplatin (10 μg/mL) in 10% FBS medium for 48 h. The cells were washed with PBS and treated with lysis buffer (50 mM Tris–HCl (pH 7.5), 20 mM EDTA, and 0.1% Triton X-100). The obtained supernatants were incubated with 10 mg/mL RNase A at 56°C for 2 h, followed by treatment with proteinase K (10 mg/mL, 37°C and 2 h). DNA was precipitated with 10 M ammonium acetate and cold 70% ethanol at −20°C overnight. The extracted DNA samples were dissolved in 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA. Electrophoresis was performed on 1.5% agarose gel, stained with 0.5 mg/mL EB and visualized under UV transilluminator.

**Measurement of mRNA Expression Levels**

AGS and Caco2 cells were plated in six-well plates at a density of $2 \times 10^5$ cells/well overnight. For synchronization, the cells were washed three times with PBS and incubated in serum free DMEM/F12 medium for 24 h. Cells were then incubated with vehicle (0.1% DMSO) and MPS (2 and 4 μM) in a medium containing 10% FBS. After 24 h starvation, cells were harvested and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). The cDNA was made using a Prime Script RT reagent kit (Takara Bio, Inc., Otsu, Japan). The expression of mRNA encoding Bax, Bcl-2, p21, p27, p53, Cyclin D1, CDK4, and GAPDH was determined by real-time PCR kit (Takara Bio Inc. Otsu, Japan) and the specific primers (Table 1) according to the optimized PCR amplification conditions. The primers were designed using online primer design software including Primer 3 and primer-BLAST. GAPDH mRNA was employed as the endogenous control and analyzed under the identical conditions. The Ct value for the target cDNA was corrected using the Ct value for GAPDH and expressed as ΔCt. Data were expressed as fold changes in the amount of mRNA, which was calculated using the following formula:

$$\text{Fold change} = 2^{(\Delta \text{Ct for untreated cells } - \Delta \text{Ct for treated cells})} = 2^{-\Delta \text{Ct}}.$$  

**Immunoblotting Assay**

To determine if caspase-3 level was affected by the MPS compound, the immunoblotting assay was performed according to a previously described method with some modifications (18). Cells were seeded at $2 \times 10^5$ cells/well in six-well culture plates. After 24-h starvation, cells were washed three times with PBS, and treated with the MPS (2 and 4 μM) and cisplatin (5 μg/mL) in a medium containing 10% FBS for 24 h. Cells were washed twice with ice cold Tris-HCl buffer (10 mM) with 250 mM sucrose and then lysed in 0.5 mL of lysis buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 1% NP-40, 10% glycerol, 1 mM PMSF, 2 mM EDTA and 10 μg/mL aprotinin). After 10 min on ice, cell lysates were collected by centrifugation (14,000 ×g for 15 min at 4°C). The supernatant was boiled in SDS-PAGE sample buffer containing 10% SDS for 10 min and resolved in 12.5% polyacrylamide separating gels at 150 V. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane using tank blotting. The membranes were rinsed 3 times in PBS containing 0.05% (v/v) Tween 20, and then blocked in PBS-BSA 2% (w/v) for 2 h. The membranes were incubated overnight in primary mouse anti-human caspase-3 monoclonal antibody (Santa Cruz Biotechnology) and for 90 min in secondary (human anti-mouse IgG, HRP conjugated) antibodies after washing four times in each step. Finally, the blots were exposed to HRP substrate solution (TMB and H2O2) for detection of target protein bands.

**Molecular Docking Procedure**

**Preparation of Ligand and Receptors**

The possible binding mode of the MPS into several targets involved in cancer was investigated. The selected receptors were circulating tumor DNA (ctDNA) (PDB ID: 1BNA), CDK2 (PDB ID: 1H00)

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**Table 1.** Primer sequences used in reverse transcription real-time PCR. The housekeeping GAPDH gene was used for normalization.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>Product (bp)</th>
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<td>CGGCGGTGGATGGTAGAA</td>
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</tr>
<tr>
<td>p27</td>
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<td>GGTGGCTTGTGGTAGTCA</td>
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<tr>
<td>p53</td>
<td>TAACAGCTTGCTGCAAGGCGGC</td>
<td>AGGCGACGGGACGACCCAC</td>
<td>121</td>
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<td>Cyclin D1</td>
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<td>AGGTCATCACCTAGCTGTCC</td>
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<tr>
<td>CDK4</td>
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<td>CTTGGATCAGAGTTGTGGGCA</td>
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<tr>
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<tr>
<td>Bcl-2</td>
<td>TTGGCCCATCCCTGAGTCTGT</td>
<td>GTGCGGGTCCAGCTGATGCA</td>
<td>114</td>
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<tr>
<td>GAPDH</td>
<td>GCAGGGGGAAGGCAAAGGGGT</td>
<td>TGGTGGCAGTGGTGATGGC</td>
<td>219</td>
</tr>
</tbody>
</table>
and CDK6 (PDB ID: 3NUP). The 3D structures of the selected receptors were taken from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). The unfavorable atoms and water molecules were removed and hydrogen atoms added to selected receptors using SYBYL version 7.2 software (TRIPOS Assoc., Inc., St., Louis, MO, USA). The chemical structure of MPS compound has been drawn using ChemDraw software (based on NMR results). The structure of MPS optimized by semi-empirical method (AM1) and implemented in HyperChem, was used as the input of AutoDock Tool 4.2.6 version, and partial charges of atoms were calculated using Gasteiger–Marsili Method.

**Molecular Docking Method**

The blind docking of MPS compound into selected receptors was performed. All docking calculations were undertaken using AutoDock 4.2.6 tool. First of all, water molecules and other unfavorable atoms were removed from initial structure of receptors, and then missing hydrogens and Gasteiger/Kollman charges were added to the system during preparation of the receptor and ligand structures. AutoDock tool was used for the preparation of coordinate files of ligand and receptor (PDBQT). Afterwards, pre-calculation of grid maps was performed using AutoGrid in order to save a lot of time during the docking. Next, the docking calculation was done by locating a grid map with 60 × 60 × 60 Å points and a grid point spacing of 0.375 Å which was centered on the receptor. The number of independent docking runs performed for each docking simulation was set to 200 with 25 000 000 energy evaluations for each run. The energy cutoff was 0.375 Å which was centered on the receptor. The number of independent docking runs performed for each docking simulation was set to 200 with 25 000 000 energy evaluations for each run. The default values of program were used for other docking parameters. For each result, the generated .pdbqt files converted to .pdb files using PyMOL 1.7 version software. Finally, the lowest binding energy conformer was searched out of 10 different conformers for each docking run. The number of independent docking runs performed for each docking simulation was set to 200 with 25 000 000 energy evaluations for each run. The default values of program were used for other docking parameters. For each result, the generated .pdbqt files converted to .pdb files using PyMOL 1.7 version software. Finally, the lowest binding energy conformer was searched out of 10 different conformers for each docking run. Then, it gave smaller fragments after losing CH3 (m/z 158) and SO3 (m/z 78) groups.

**2-methylpyridine-1-ium-1-sulfonate** (MPS): 1H NMR (250 MHz, DMSO): δ H 8.34 (d, J = 6.25, CH), 7.84 (d, J = 8, CH), 7.52–7.46 (m, CH), 7.33–7.28 (m, CH), 2.44 (s, CH3); 13C NMR (62.9 MHz, DMSO): δ C 150.0, 138.9, 126.9, 123.2, 121.8, 21.7. It was in agreement with the presence of four signals in the aromatic region, δ 8.34 (d, J = 6.25, CH), 7.84 (d, J = 8, CH), 7.52–7.46 (m, CH), 7.33–7.28 (m, CH) in the 1H NMR spectra with one methyl group at δ 2.44. The structure of isolated compound is shown in Fig. 1A.

Regarding mass spectrum, the molecular ion was appeared at m/z 173 (C6H6O3SN). Then, it gave smaller fragments after losing CH3 (m/z 158) and SO3 (m/z 78) groups.

**Post Docking Analysis**

The 3D structures of the selected receptors were taken from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). The unfavorable atoms and water molecules were removed and hydrogen atoms added to selected receptors using SYBYL version 7.2 software (TRIPOS Assoc., Inc., St., Louis, MO, USA). The chemical structure of MPS compound has been drawn using ChemDraw software (based on NMR results). The structure of MPS optimized by semi-empirical method (AM1) and implemented in HyperChem, was used as the input of AutoDock Tool 2.4.6 version, and partial charges of atoms were calculated using Gasteiger–Marsili Method.

**Statistical Analysis**

All data represent three independent experiments and are expressed as the mean ± standard deviation (SD). A one-way ANOVA was employed to determine statistical significance of the results. Where the differences were significant, a post-hoc (Tukey) test was applied to determine the place in which difference was occurred. Value of P < 0.05 was considered to show significant differences between groups. The Prism software version 6.07 was used for statistical analysis.

**Results**

**Identification of the Active Compound**

The 1H and 13C NMR data confirmed the presence of a substituted pyridine ring (δ 150.0, 138.9, 126.9, 123.2, and 121.8) with one methyl group at δ 21.7. It was in agreement with the presence of four signals in the aromatic region, δ 8.34 (d, J = 6.25, CH), 7.84 (d, J = 8, CH), 7.52–7.46 (m, CH), 7.33–7.28 (m, CH) in the 1H NMR spectra with one methyl group at δ 2.44. The structure of isolated compound is shown in Fig. 1A.

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displayed that the AGS cells were more sensitive to MPS compound than another cell line (Fig. 1B). It is noteworthy to mention that the results from MTT and Trypan blue assays showed that the same concentrations of MPS which inhibited growth of these cancer cells were not toxic on Human Umbilical Vein Endothelial (HUVEC) cells, as a normal cell (IC50: 16.47 \text{\mu M}). This finding confirmed that the anti-growth effect of MPS was selective and more affected the cancer cells.

**Effect of MPS on Cancer Cell Cycle Distribution**

The possible inhibitory activity of MPS compound on growth of AGS and Caco-2 cells was investigated using flow cytometry. The generated results of cell cycle distributions in different phases at 24 h treatment with MPS (2 and 4 \text{\mu M}) are presented in Fig. 2. As depicted, the MPS treatment led to a remarkable inhibition in cell cycle progression, through increasing number of the AGS cells in G0/G1 phase (control:
63.1%, 2 μM: 70.2%, 4 μM: 71.2%; P < 0.05) and decreasing number of the cells in G2/M phase (control: 14.0%, 2 μM: 7.4%, 4 μM: 5.1%; P < 0.05). Also, the significant changes in S phase was not observed (control: 23.9%, 2 μM: 22.9%, 4 μM: 24.1%). The generated results for treatment of Caco-2 cells with MPS for 24 h showed a significant increase in accumulation of the cells in S phase (control: 22.5%, 2 μM: 36.5%, 4 μM: 36.5%; P < 0.05) and a remarkable reduction in G0/G1 population (control: 62.7%, 2 μM: 50.4%, 4 μM: 49.4%; P < 0.05). The significant changes in G2/M phase was not observed (control: 15.1%, 2 μM: 13.8%, 4 μM: 15.6%) (Fig. 2). These results suggested that the observed apoptosis and growth inhibition by MPS in AGS and Caco-2 cancer cells may be mediated through cell cycle arrest in G1-S and S-G2 transitions, respectively.

**Effect of MPS on Cancer Nuclear DNA**

To examine the induction of apoptosis by MPS compound, TUNEL assay was performed. The induced apoptotic cells by MPS were identified using the appearance of specific nuclear staining and condensed nucleus (Fig. 3A). The number of apoptotic cells was expressed as a percent of total cells counted in three random microscopic fields (Fig. 3D). Summing up, treatment of AGS cells with the MPS compound (4 and 8 μM) caused a significant increase in apoptotic cells (31 ± 3.4%; P < 0.05 and 95 ± 2.8%; P < 0.01, respectively). Indeed, the presence of apoptotic Caco-2 cells after treatment with the MPS compound (4 and 8 μM) were 23 ± 3.7% (P < 0.05) and 91 ± 3.5% (P < 0.01), respectively. These results indicated that the MPS significantly triggered apoptosis in AGS and Caco-2 cells in a dose-dependent manner.

**DNA Fragmentation Induction by MPS**

To determine the apoptotic activity of the MPS compound, the DNA fragmentation assay was also performed. As depicted in Fig. 3C, the non-degraded high molecular weight DNA was observed in control samples, and the cells treated with 4 μM concentration of MPS. Meanwhile, the fragmented DNA was detected in AGS and Caco-2 cells upon 48 h treatment with MPS at 8 and 16 μM, respectively.

**Effect of MPS on Gene Expression Profile in the Cancer Cells**

After 24-h treatment with the MPS, the expression of genes involved in cell cycle and apoptosis was analyzed by reverse transcription real-time PCR in the AGS and Caco-2 cells. As detailed in Table 2, treatment with 2 and 4 μM of MPS had no effect on the mRNA levels in cyclin D1 and CDK4 in the Caco-2 cells significantly (P > 0.05). In contrast to this result, the mRNA levels of cyclin D1 and CDK4 in AGS cells were decreased with the same concentrations of MPS (P < 0.05). Nonetheless, the p21, p27 and p53 mRNA levels in both AGS and Caco-2 cells were significantly increased upon treatment with MPS in a dose-dependent manner (P < 0.05 and P < 0.01) (Table 2).

Our results also indicated that the expression of pro-apoptotic Bax gene significantly increased in both AGS and Caco-2 cells in the presence of 2 and/or 4 μM MPS (P < 0.05) (Table 2). Mutually, the expression of anti-apoptotic Bcl-2 gene was significantly reduced in the AGS and Caco-2 cells treated with MPS (P < 0.05). Consequently, the Bax/Bcl-2 ratio at mRNA level was significantly increased in a concentration-dependent manner of MPS (P < 0.05). These data indicated that the up-regulation of Bax/Bcl-2 ratio may be one of the major contributing key in the induction of apoptosis in AGS and Caco-2 cells using the MPS compound.

**Effect of MPS on Caspase 3 Expression in the Cancer Cells**

To determine whether MPS induces apoptosis in AGS and Caco-2 cells, the caspase-3 expression level in the cancer cells treated with MPS was measured using western blotting. Our results showed that the level of the pro-caspase-3 form was significantly reduced by MPS at a dose-dependent manner (Fig. 4). However, this reduction was more obvious in case of AGS cells at the MPS concentrations used.

**Effect of MPS on Cancer Cells Morphology**

Morphological changes of cells were observed under fluorescent microscope using AO/EB staining. Acidine orange stains both live and dead cells while Ethidium bromide stains only dead cells. Live cells and also early apoptotic cells (contain highly condensed chromatin and fragmented nuclei) will show green fluorescence whereas late apoptotic cells and necrotic cells show orange color. As shown in Fig. 3B, a dose-dependent increase in apoptosis induction was observed for both cell lines, which is also in accordance with the results of the growth assay. According to these results, the compound has ability to induce apoptosis in the studied cancer cell lines.
Figure 3. (A) TUNEL staining of AGS and Caco-2 cells after 48 h exposure to 2–8 μM of 2-Methylpyridine-1-ium-1-sulfonate (MPS) and the positive control cisplatin (10 μg/mL). Magnification: 100X. (B) Morphological changes in AGS and Caco-2 cells after 48 h treatment with MPS (2–8 μM) and the positive control cisplatin (10 μg/mL), and staining with acridine orange/ethidium bromide (AO/EB). White arrows: apoptosis blebbing, white dashed arrows: late apoptosis or necrosis. The images were taken at 545 nm using fluorescence microscopy at 100X. (C) Induction of DNA fragmentation by MPS (4–16 μM) in AGS and Caco-2 cells. DNA was extracted and resolved using 1.5% agarose gel electrophoresis. Marker (100 bp), Control (0.1% DMSO), Csp: cisplatin (10 μg/mL). (D) Quantification of the apoptotic cells against control in TUNEL assay which was expressed as a percent of total cells counted in three random microscopic fields. Results were expressed as means ± SD. *P < 0.05 and ***P < 0.001.
Computational Results

As depicted in Fig. 5, the MPS compound bound to the critical residues of CDK2 active site. As shown, the MPS compound constructed a significant hydrogen bond with Glu81 residue (Fig. 5A,B). The C3 atom from the MPS compound and oxygen atom from Glu amino acid caused this type of interaction (Fig. 5C). The CDK2 in association with cyclin A/E has a central role in S phase of cell cycle (19). The results showed that the MPS compound has good affinity to interact with the critical residues of CDK2 receptor. Its docking energy was \(-9.9\) kcal/mol.

Also this compound showed affinity to interact with the active site of CDK6 (Fig. 6A,B), its binding energy for interaction with this receptor was \(-10.1\) kcal/mol, respectively. There are several critical residues in the active site of CDK6 that can significantly regulate the activation or/inhibition of this kinase. These include Lys43, Phe98, His100, Asp104 and Thr107 residues (20). The molecular docking results for interaction between the MPS compound and CDK6 showed that about three hydrogen bonds have constructed during this interaction (Fig. 6C). Glu99, His100 and Val101 are suspected residues to create hydrogen bonds with the MPS compound. The C6 atom of the MPS compound makes a bond with the oxygen atom from Glu99. Also, the O9 from the MPS bound to the CA atom from His100 and N atom from Val101. This stable binding mode of the MPS compound into the CDK6 active site exhibits the possible toxicity effect of this compound against this cell cycle kinase.

Studies speculated that DNA minor groove binding agents exert their antitumor effects by covalent or/non-covalent binding to the cleft of minor groove in DNA (21). Fig. 7 presents the binding mode of the MPS compound into the minor groove of ctDNA. As depicted, the MPS compound can interact with ctDNA significantly. Herein, our results are fully in accordance with literature and the MPS compound has interacted with A:T regions in the cleft of ctDNA minor groove. The calculated binding free energy for this interaction was \(-8.9\) kcal/mol.

Discussion

Meta-analysis and epidemiological studies have showed that there is a close inverse relationship between consumption of Allium plants and human gastrointestinal cancers risk (22,23). Studies showed that the main reason for anticancer activity of Allium species is due to the presence of organosulfur compounds (24,25). Generally, these compounds can

Table 2. Effect of 2-methylpyridine-1-ium-1-sulfonate (MPS) on mRNA level of the genes involved in cell cycle and apoptosis in AGS and Caco-2 cells. The cells were treated with 2 and 4 μM of MPS for 24 h. Data presented as relative gene expression changes in the treated cells compared to the controls. *P < 0.05 and **P < 0.01.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene expression (fold change)a AGS</th>
<th>Gene expression (fold change)a Caco-2</th>
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</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>0.70 ± 0.08*</td>
<td>0.94 ± 0.18</td>
</tr>
<tr>
<td>CDK4</td>
<td>0.89 ± 0.11*</td>
<td>0.89 ± 0.24</td>
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<td>p21</td>
<td>2.07 ± 0.31***</td>
<td>2.12 ± 0.8***</td>
</tr>
<tr>
<td>p27</td>
<td>1.38 ± 0.17*</td>
<td>2.65 ± 0.1***</td>
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<tr>
<td>p53</td>
<td>1.89 ± 0.16*</td>
<td>3.17 ± 0.13***</td>
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<td>0.86 ± 0.12*</td>
<td>0.77 ± 0.19*</td>
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<tr>
<td>Bax</td>
<td>0.93 ± 0.09</td>
<td>2.18 ± 0.18*</td>
</tr>
<tr>
<td>Bax/Bcl-2 ratio</td>
<td>1.08 ± 0.07</td>
<td>2.83 ± 0.11*</td>
</tr>
</tbody>
</table>

*aFold change: values are presented as fold change = 2 \(^{-\Delta\Delta Ct}\) for genes in treated relative to control samples.
Figure 5. The interaction between the MPS compound and CDK2. (A) The critical residues of CDK2 in complex with anilino pyrimidine. (B) The binding mode of MPS into the active site of CDK2. (C) The 2D illustration of the interaction between critical residues of CDK2 and the MPS compound.

Figure 6. The interaction between the MPS compound and CDK6. (A) The critical residues of CDK6 in complex with 4-(pyrazol-4-yl)-pyrimidine. (B) The binding mode of MPS into the active site of CDK6. (C) The 2D illustration of the interaction between critical residues of CDK6 and the MPS compound.
suppress growth of various cancer cell lines through different mechanisms including inhibition of tumor cell proliferation and angiogenesis as well as induction of apoptosis (26). According to the review of literature, organosulfur compounds inhibit growth of various cancer cells through targeting multiple signaling pathways leading to cell cycle arrest and apoptosis induction (27). Targeting specific aspects of the tumor growth through induction of apoptosis using therapeutic agents may be an important approach in cancer prevention and therapy (28–30).

In the present study, our results showed that the MPS compound significantly inhibited the growth of both human adenocarcinoma cancer cells. Significant growth inhibition was occurred at lower concentrations of MPS for AGS cells (IC$_{50}$: 6.34 μM) in comparison to Caco-2 cells (IC$_{50}$: 7.25 μM).

Based on our findings, we speculated that the MPS-mediated inhibition of cell growth is related to the induction of the apoptosis and cell cycle arrest. Therefore, to evaluate the induction of apoptosis mediated by MPS, several assays such as TUNEL, acridine orange/ethidium bromide staining and DNA fragmentation tests were performed. The result of TUNEL assay showed that the higher levels of MPS caused a significant apoptosis. This result has confirmed using the obtained results from acridine orange/ethidium bromide staining and DNA fragmentation assays. In an interesting study, Sundaram and Milner reported that the growth inhibitory induced by

\[ \text{Figure 7. The binding mode of the MPS compound into the minor groove of ctDNA.} \]
organosulfur compounds in human colon tumor cells was associated with the induction of apoptosis (31). According to their results, treatment of human colon cancer cells with diallyl disulfide (DADS) caused DNA fragmentation and other morphological changes (31). From this result, it can be concluded that our results are in line with their observations for treating colon cancer using DADS.

Cell cycle dysregulation is an important feature of cancer cells. During cell cycle progression, cyclin-CDK complexes play a critical role in transition of cells from one phase to another. The functions of these complexes are negatively regulated by CDK inhibitors including p16, p21, and p27 (32). Thus, modulation of these elements in cell cycle progression is an important strategy for inhibition of cancer cell growth. The obtained data from flow cytometry revealed that the MPS compound induced cell cycle arrest at G0/G1 phase in AGS cells after 24 h. Similarly, treatment of Caco-2 cells with MPS caused a significant prolonged S phase. These findings suggest that in addition to apoptosis, cell cycle arrest is another effect of the MPS that exhibits inhibitory activity against cell growth. To evaluate the possible molecular mechanism which followed by MPS, the gene expression analysis was performed. As detailed in Table 2, treatment with MPS caused remarkable changes in expression levels of the studied genes which are related to the cell cycle arrest and apoptosis induction. RT-qPCR analysis revealed that treatment with MPS is associated with a significant increase in the expression of CIP/KIP cyclin-kinase inhibitors (i.e., p21 and p27) in both cell lines and down-regulation of CDK4 and cyclin D1 in AGS cells, in a concentration-dependent manner. Therefore, these findings provided a strong evidence to confirm the role and mechanism of p21 and p27 up-regulation in MPS-induced G0/G1 and S arrest in AGS and Caco-2 cancer cells, respectively.

The induction of pro-apoptotic elements by anticancer drugs is associated with the inhibition of anti-apoptotic genes which can be considered as a valuable policy in cancer therapy and chemoprevention. The encoded proteins by Bcl-2 gene families (e.g., bax and bcl-2) regulate the apoptosis signaling pathways. The molecular mechanisms which followed by the generated products from the expression of Bcl-2 family caused a significant controlled apoptosis in the cells (33,34). The regulation of pro- and anti-apoptotic members balance determines the induction and/or inhibition of apoptosis in cells (33,34). As mentioned earlier, the MPS compound caused a notable increase in the expression of pro-apoptotic Bax. Also, a considerable decrease of anti-apoptotic Bcl-2 at mRNA level was observed. Taken together, this event is associated with the increase of Bax/Bcl-2 ratio. These results are fully in agreement with previous reports for organosulfur compounds (i.e., DAS and DADS) that their potentials to induce the apoptosis in non-small cell lung cancer cell lines (35) and neuroblastoma SH-SY5Y cells (36) through increasing of the ratio of Bax/Bcl-2 have been well documented. p53 as a tumor suppressor gene has an important role in cell cycle arrest and cell death. The p53 gene encodes a transcription factor that regulates the cell cycle as well as apoptosis through activation of its downstream genes. In addition, p53 induces the apoptosis through direct interaction with members of the Bcl-2 family (34,37,38). Herein, after 24-h treatment of both AGS and Caco-2 cells with the MPS compound, the mRNA level of p53 was significantly increased in a dose-dependent manner.

Studies reported that p21 as a cyclin-dependent kinase inhibitor, is activated through both p53-dependent and –independent pathways. Therefore, p21 causes G1, G2 and S-phase arrest in cells in response to various stimuli (34,39). On the other hand, p53 induces a G1 arrest by inducing expression of p21 and the consequent inhibition of cyclin D/CDKs. Studies demonstrated that p53 can modify the balance between Bax and Bcl-2 genes during apoptosis (40). According to the generated results from gene expression analysis, it can be concluded that p53 can play a central role in MPS-induced cell cycle arrest and apoptosis in both cancer cells. However, further investigations will be needed to confirm this hypothesis.
In this study, we also performed western blotting for caspase-3 for both cell lines. Caspase-3 plays a central role in apoptotic DNA fragmentation during apoptosis (34). Our results showed that the MPS significantly reduces the pro-caspase-3 level in a dose-dependent manner. Thus, it can be concluded that one of the possible mechanisms by which MPS induces apoptosis in the cancer cells is through modulating the activity and expression of caspase-3.

Recently, DNA minor groove binding agents have received more attention because of their biological functions to prevention of human diseases especially cancer (21). Circulating tumor DNA (ctDNA) is a small fragment of DNA that found in blood circulation of cancerous patients (41). Studies speculated that this tumor-based DNA fragment can act as a leader for migration of tumor cells to other parts of body (41). In a possible hypothesis, it is suggested that ctDNA minor groove binding agents may help to inhibition of tumor migration (42). Our theoretical result for interaction between the MPS compound and ctDNA unveiled that this compound can significantly bind to the minor groove of this tumor-derived fragment.

According to the generated results from the experimental section, the MPS compound is able to inhibit of the cell cycle and induces of apoptosis in the studied cancer cell lines in a dose-dependent manner. However, our observations showed that the MPS compound induced cell cycle arrest in G0/G1 (for AGS cells) and S phase (for Caco-2 cells) (Fig. 8). In a possible way, we assumed that this compound can directly interact with cyclin-dependent kinases (CDKs). Therefore, the CDK2 and 6 were selected as favorable receptors for molecular docking. The review of literature showed that a number of natural and synthetic compounds have identified as CDK inhibitors. In this study, we described a novel potent inhibitor for cell cycle kinases. The generated results from molecular docking confirmed our hypothesis and the interaction of the MPS compound and the critical residues of selected receptors were observed. Summing up, our results showed that the MPS compound is a potent inhibitor for cell cycle kinases that can act selectively in different cell lines.

Our computational results confirmed the experimental sections and showed that the MPS compound can be considered as potent inhibitor to control different targets in cell cycle signaling pathway. Generally, natural compounds isolated from oral plants are not toxic and the oral administration of these compounds can show no side effects on gastrointestinal system (43,44). Mainly, the reported toxicity effects for plant-based compounds is very low and only some classes of plant secondary metabolites (e.g., poisonous alkaloids) can be dangerous for human and animal (45). The optimum doses of these compounds not only eliminate the growth of tumor cells, but also can decrease the resistance of cancer cells to the administered drugs. From MTT assay, we found that the title compound was not toxic for normal cells and therefore its consumption cannot provide toxicity for human body. More importantly, the mentioned plant that used to isolate the MPS compound is an oral medicinal plant in Iran and other countries and it is an important part of people diet throughout the world. However, the tale of cancer and its treatment is continues story, and one day plant-based secondary metabolites (especially those are isolated from oral plants) can defeat this chronic disease to provide a safe world for people.

Conclusions
As a first study, our results clearly displayed that the MPS compound isolated from bulbs of Allium hirtifoliuim, inhibits growth of AGS and Caco-2 cell lines through G0/G1 and S phases arrest, respectively, as well as apoptosis induction. The mentioned compound selectively exhibited inhibitory activity against different molecular targets and the presented agreement between computational and experimental assays proved this fact. Our results provided valuable reasons to confirm the drug like activity of MPS to consider its structure for further investigations. Although natural products are not real drugs for the treatment of the cancer, nonetheless they are good targets for pre-management of cancer to improve the health condition of affected patients. To validate the accurate anticancer activity of the isolated compounds further in vivo studies is highly recommended.

Acknowledgments
We extend special thanks to Dr Kamran Mansouri (Medical Biology Research Center, Kermanshah University of Medical Sciences) for his comments and technical assistance. The instructive comments and invaluable help provided by the anonymous reviewers are also greatly appreciated.

Disclosure Statement
The authors declare that they have no conflicts of interest.
Funding
The results presented here were financially supported by a research fund (No. 91204) of the Vice-Chancellorcy for Research and Technology, Kermanshah University of Medical Sciences.

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