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RESEARCH ARTICLE

The methanolic extract of Shirazi thyme (Zataria Multiflora) and its combination with arsenic trioxide (ATO) changes the expression of onco-miRNAs and TS-miRNAs in acute promyelocytic leukemia cell line (NB4)

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Abstract

Acute promyelocytic leukemia (APL) is responsible for 10–15% of new AML cases. ATO is often used for recurrence after ATRA treatment but may cause complications. MicroRNAs (miRNAs) are crucial in various cancers. Zataria Multiflora (Shirazi thyme) is a medicinal plant that promotes apoptosis. This study investigates the effects of thyme's methanol extract and its combination with ATO on onco-MiRs and TS-MiRs in the NB4 cell line.

Cell viability and metabolic activity of NB4 cells were assessed via trypan blue dye exclusion test and MTT assay. Cell apoptosis rate was evaluated using flow cytometry, and changes in the expression of miRNAs 19a-3p, 23a-5p, 181b-5p, 3156-5p, and 4498 were analyzed through real-time PCR. Finally, docking was performed using MVD and HDOCK software.

Combining ATO 0.25 µM with 20 µg/ml of Shirazi thyme extract significantly reduced cell viability, metabolic activity, and gene expression (except 181b-5p), while increasing apoptosis rates compared to individual treatments. These findings indicate that Zataria Multiflora can act as a synergistic adjuvant with ATO and, in some cases, produce superior effects compared to high doses of ATO alone. Docking results confirmed thymol and carvacrol as the potential compounds in the apoptotic effect of ZME.

Keywords: Zataria Multiflora, arsenic trioxide, microRNA, acute promyelocytic leukemia

Introduction

Acute promyelocytic leukemia (APL) is responsible for about 10–15% of acute myeloid leukemia (AML) cases.[1] It primarily arises from a balanced translocation between chromosomes 15 and 17 that results in the PML-RARA fusion, which produces an oncogenic protein. This oncoprotein impedes the differentiation of myeloid cells at the promyelocytic stage.[2] While the emergence of retinoic acid (RA) and arsenic trioxide (ATO) has transformed APL as a fatal disease to a highly curable one, the risk of APL relapse and complications of these therapies remain significant clinical issues.[3,4] These complications include:

1. Differentiation syndrome (DS): A potentially fatal side effect that might arise from using ATO either alone or in conjunction with ATRA.[5] Unexplained fever, weight gain, pleural effusion, shortness of breath with pulmonary infiltration, renal failure, and hypotension are among the symptoms.[6] This complication is observed in approximately 19% of individuals treated with combined ATO and ATRA.[7]
2. Cardiac conduction abnormalities: seen in 10% of new APL cases and 40% of relapsed and treatment-resistant cases treated with ATO.[8]
3. Liver toxicity: characterized by elevated liver enzymes[9] and observed in 45% of patients treated with the combined regimen of ATO and ATRA.[7]
4. Skin lesions: Chronic exposure to arsenic can lead to lesions such as hypo/hypersegmentation and hypo/hyperkeratosis.[10] These lesions are observed in 33% of relapse cases treated with ATO.[11]
5. Risk of secondary cancers: Due to its toxicity, arsenic can cause secondary cancers such as colon, nasopharyngeal, and salivary gland cancer over time.[12] Considering the numerous side effects and high toxicity of chemotherapy drugs like ATO, new treatment strategies are needed.

Throughout history, humanity has relied on nature to fulfill its basic needs, including medicine supply. Herbal medicines have gained significant popularity for their effectiveness, safety, and reduced side effects compared to synthetic alternatives.[13,14] Zataria multiflora Boiss (ZM), also known as Shirazi thyme in Iran, is a Lamiaceae plant found exclusively in Iran, Pakistan, and Afghanistan.[15] This plant possesses various medicinal properties, including antibacterial,[16] antifungal,[17] and antiprotozoal[18] effects, anti-inflammatory properties,[19] protective effects against radiotherapy and chemotherapy,[20,21] antioxidant properties,[22] liver protective effects,[23] anti-inflammatory properties, and anti-tumoral effects through the regulation and reduction of MDM2 gene expression[24] and induction of cell apoptosis through induction of ROS in the mitochondrial pathway[25] The regulation of apoptosis involves various mechanisms, and microRNAs play an important role in this type of cell death.[26]

MicroRNAs (miRNAs) are single-stranded RNAs with a length of 19-25 nucleotides,[27] constituting a large class of endogenous non-coding RNAs that control the expression of nearly a third of human genes.[28,29] These RNAs bind to complementary regions along the mRNA sequence (mostly in the 3′ region or UTR),[27] causing suppression or reduction of the expression of their target gene. If this connection is incomplete, it will suppress the translation and destroy the mRNA exonucleolytically, and if the connection is complete, it will cause the mRNA to break down endonucleolytically. In the discussion of cancers, miRNAs are seen in two ways: 1) oncogenic miRNAs (onco-miRs) such as MiR181a in acute lymphoblastic leukemia (ALL)[30] and MiR181b in acute myeloid leukemia (AML),[31] which exert their role by targeting tumor suppressor genes (TSGs).[32] 2) tumor suppressive miRNAs (TS-miRs) such as MiR19a, which is part of the MiR17-92 cluster, and MiR23a in chronic myeloid leukemia (CML),[31] which play a role by targeting oncogenes. Studies have demonstrated the intricate communication network between tumor suppressor genes, oncogenes, and miRNAs, highlighting the significant role of miRNAs in cell proliferation, differentiation, survival, apoptosis, tumorigenesis, and targeted therapy in the cancer field.[32,33] Notably, miRNAs have been selected based on their prevalence in myeloid leukemias and their impact on disease pathogenesis, including their effects on histone deacetylase and signaling protein inhibitors.

Due to need of combination therapy in case of lowering the complications of chemotherapy and improving the treatment outcomes,[34] and based on previous research demonstrating the anti-tumor properties of Shirazi thyme through the stimulation of cell apoptosis,[35-37] our study aims to investigate the effects of methanol extract of thyme and its combination with the ATO drug on the expression of onco-miRs and TS-miRs in the NB4 cell line associated with acute promyelocytic leukemia (APL). Moreover, this study also aims to analyze the interaction between RAF1 protein (as a key role downstream protein in MAPK signaling pathway[38] and main elements of ZME (carvacrol and thymol), as ligands, via docking simulation.

Materials and Methods

Ethical committee of Kerman University of Medical Sciences with the code IR.KMU.REC.1401.015 approved this study with Reg. No. 400000607.

2.1. Preparation of treatment solutions

2.1.1. ZM methanolic extract preparation.

The flowering branches of the Shirazi thyme plant were collected from the mountainous regions of Fars province

and approved by a botanist. A herbarium specimen was prepared and kept in the herbarium of the Faculty of Pharmacy in Kerman under the number (THE 6729).

To make the metabolic extract from this plant, we cut and dried the fresh leaves and stems of Shirazi thyme, then crushed them into powder. Then, we separated the substances using petroleum ether, chloroform, and methanol one after another. First, the liquid is removed using a machine that spins it around at 4 °C. Then, the powder is dried in a refrigerator at a temperature of 4 °C. For the extract from this preparation, 0.5 grams of ZM powder was weighed and dissolved in 0.5 ml of DMSO (Merck, CAS 67-68-5). The volume was then adjusted to 5 ml using RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) medium. The resulting medicinal solution was sterilized using 0.22-micrometer filters under the cell culture hood. The final stock concentration was 100,000 micrograms/milliliter (µg/ml), from which a 1:100 dilution was prepared for use in tests. It was stored at a temperature of 4 °C.

2.1.2. ATO preparation

The drug was prepared as a solution from the pharmacy and stored at ambient temperature. Then, 10 µl of the stock solution was mixed with 490 µl of RPMI 1640 medium to prepare 500 ml of working solution with a concentration of 100 µM.

2.1.3. Primer preparation

The primers arrived in lyophilized form. According to the catalog, a suitable volume of sterile TE buffer (93283 product number, Sigma-Aldrich, USA) was mixed to create 100 µM stocks, and then a diluted 10 µM stock was prepared. This diluted source was divided into appropriate volumes and stored at -20°C to prevent repeated freezing and thawing.

2.2. Cell culture

Acute promyelocytic leukemia cell line (NB4) was purchased from the Pasteur Institute, Tehran, Iran, and was cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 incubator at 37 °C.

2.3. Viability Assay

Cell viability was examined using the trypan blue dye exclusion test (Sigma-Aldrich). When the cell density reached over 85% with over 95% viability, NB4 cells were treated with different concentrations of ATO (0.25, 0.5, and 1 µM) and Shirazi thyme methanol extract (20, 40, and 80 µg/ml), as well as 4 combined concentrations of these two (0.5 and 0.25 µM ATO and 40 and 20 µg/ml ZM). After 24 and 48 hours of incubation, the treated cells were exposed to trypan blue 0.04% in a 1:1 proportion and incubated for 2 minutes at room temperature. The mixture was then examined on a neobar slide under a microscope. Live (transparent) and dead (blue) cells were counted, and survival was calculated using the formula: "Viability percentage = (number of living cells / total number of cells) x 100".

2.4. Metabolic activity assay

Treated NB4 cells were transferred to two 96-well plates (6 x 10^3 cells in each well), with a final volume of 100 µL in each well. After 24 and 48 hours, the plates were centrifuged at 200 g for 10 minutes, and the perimeter of the wells was removed. Then, 100 microliters of MTT solution (0.5 mg/mL in filtered PBS) were added to each well, and the cell pellet was slowly dissolved in the MTT solution. The 96-well plate was incubated for 4 hours at 37°C. After incubation, 100 µL of DMSO solution was added to each well to dissolve the purple formazon crystals. The plate was covered with aluminum foil and mixed on a shaker for 15 minutes to allow the color to appear. The amount of optical absorption was measured at a wavelength of 570 nm using an ELISA reader. Finally, the amount of metabolic activity was calculated using the formula: "cellular metabolic activity percentage (light absorbance of test / light absorbance of control) x 100".

2.5. Evaluating the effect of Zataria multiflora extract on normal PBMCs

To investigate the cytotoxicity of the plant extract on normal cells, normal peripheral blood smear cells (PBMC) were isolated using Ficoll Lymphodex (Innotrain, Germany). Then, metabolic activity was determined after cell-treating with ZM 20 µg/ml, ATO 0.25 µM, and their combination for 24 h.

2.6. Flow cytometry

The flow cytometry technique was implemented using annexin V-propidium iodide (PI) staining to evaluate the effect of ZME and ATO on early and late apoptosis induction. 1 × 105 NB4 cells were seeded into six-well cell culture plates and treated with ZME (20 µg/mL), ATO (0.25 µM), and combination (20 µg/mL ZME and 0.25 µM ATO). After 24 h, the cells were harvested and washed with PBS. The Annexin-V Apoptosis Detection Kit (Mab Tag, AnxF100PI) was used for this technique, and the results were analyzed using FloMax software.

2.7. The prediction method of 4498 and 3156-5P microRNAs by bioinformatics method:

The Mirwalk, miRDB, Targetscan, and Diana databases were utilized to predict microRNAs that bind to the mRNA related to the PML gene, essential in developing acute promyelocytic leukemia. The miRDB database was used to rank microRNAs based on their ability to bind to the PML gene. The microRNAs with the highest score were then searched in the Excel output of other databases. Finally, 4498 and 3156-5P miRNAs were selected as they were present in all the databases.

2.8. Gene expression analyses

2.8.1. RNA extraction.

After treatment of the NB4 cells with ZME (20 µg/mL), ATO (0.25 µM), and a combination of both (20 µg/mL ZME and 0.25 µM ATO) for 24 hours, RNA extraction was performed using the YTzol Pure RNA kit (Yektatajhiz, Iran) following the manufacturer's protocol. Following purity, concentration, and quality testing, cDNA synthesis was done using the cDNA Synthesis kit (Yekta Tajhiz, Iran). Since miRNAs have short sequences of 22-25 nucleotides, making them difficult to track with standard primers, a stem-loop structure was utilized in the cDNA synthesis method to enable their tracking. The sequence of the stem-loop primers is detailed in Table 1.

2.8.2. Quantitative real-time PCR.

Real-time PCR was carried out using 6.5 µL of 2× Real-Time PCR qPCR Probe MasterMix (Yekta Tajhiz, Iran), 2 µL of cDNA, 1 µL each of forward and reverse primers (0.2 µM), 1.5 µL of nuclease-free water, and 0.5 µL of Taqman probe. The real-time PCR was conducted using the Rotor-Gene 6000 Real-time PCR System (QIAGEN, Germany). The expression of microRNAs 19a-3p, 23a-5p, 181b-5p, 3156-5p, and 4498 was analyzed using specific primers. The thermal cycles included an initial activation step at 95 °C for 5 minutes, followed by 40 cycles, each consisting of a denaturation step at 95 °C for 10 seconds and a combined annealing/elongation step at 60 °C for 30 seconds. Finally, the 2-ΔΔCT formula was used to calculate the fold changes and compare the results. The sequence of the primers is provided in Table 1.

2.8.3. Primer Design.

The specific primers for this research were designed by reviewing authentic articles and selecting primers from various sources. To ensure the selected primers were correct and did not anneal with other genes, their sequences were checked using http://www.ncbi.nlm.nih.gov/blast. The sequence of the desired primers was then ordered for synthesis from Pishgam Co. The primer sequences used are provided in Table 1.

2.9. Molecular docking

The 3D structure of the RAF1 protein (PDB ID: 8U1L) was sourced from the RCSB Protein Data Bank (https://www.rcsb.org). Ligands were removed using MVD software (Molegro Virtual Docker 7.0) to prepare the macromolecules for docking simulations. The structures of thymol (CID: 6989) and carvacrol (CID: 10364) were obtained from the PubChem compound database (https://pubchem.ncbi.nlm.nih.gov). For initial assessments of interactions, we performed blind docking with MVD and HDOCK software. Specific docking was carried out using amino acids from NCBI and RCSB PDB to target the active site. The grid box dimensions were optimized to allow free rotation of the ligands, with an estimated 100 docking runs. Poses were selected based on binding energy, and ligand interactions with the protein's amino acids were analyzed using LigPlot, illustrating 2D interactions. Finally, Chimera 1.12 software was used to visualize the 3D structures of the final interactions.

Results

3.1. Cellular studies

3.1.1. ZME boosts the impact of ATO on the survival and cellular metabolism activity of NB4 cells

The results indicated that There wasn't any significant difference in the survival and cellular metabolic activity of NB4-treated cells between the combined concentrations associated with ZM40 and the single concentration of ZM40. However, the combined concentrations associated with ZM20 µg/ml showed a notable reduction compared to the single dose. The most significant reduction was observed with the ZM20 µg/ml combined concentration + ATO0.25 µM within 24 hours (Fig. 1a and 1b). The data was analyzed using COMPUSYN software to assess synergism and antagonism, and the CI index was calculated for all compound doses. All CI values were found to be less than one, indicating a synergistic effect of the combined drug, with the best effect observed in the combination of ZM 20 µg/ml + ATO 0.25 µM within 24 hours (Fig. 1c). Furthermore, the results showed no toxicity for the considered concentrations on PBMCs after 24 hours of treatment (Fig. 1d).

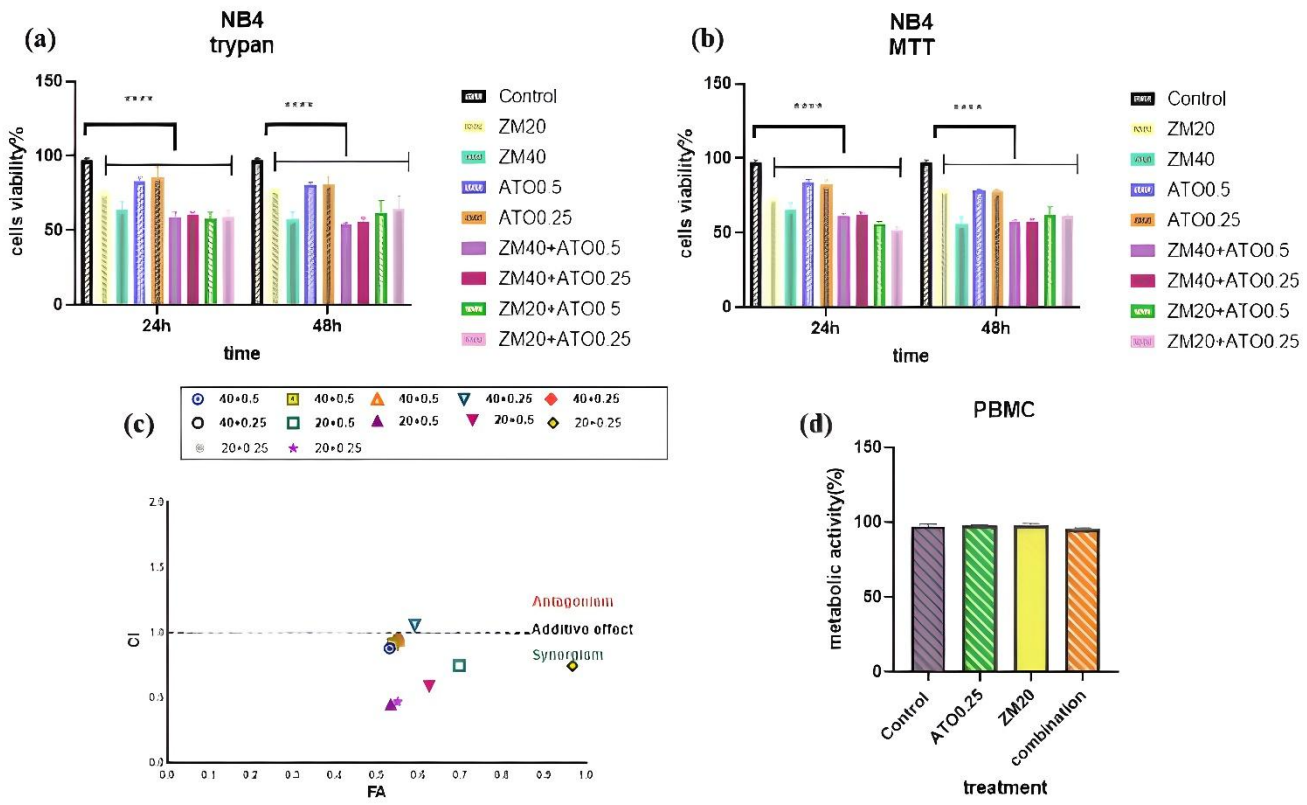

Figure 1. Effect of different doses of ATO (µM), ZM (µg/ml), and their combination on cell viability **(a)**, the effect of different doses of ATO (µM), ZM (µg/ml), and their combination on cell metabolic activity **(b)**, the synergistic effect of different combined concentrations **(c)**, the effect of considered doses of ATO (µM), ZM (µg/ml), and their combination on PBMCs **(d)**.

Table 1. Specifications of the primers used, the length of the bands of each gene

Gene	Forward primer	Tm (°C)
23a-5p	5'-GAAGTTCCTGGGGATGGGATTT-3'	54.8°C
19a-3p	5'-AGGGCGAATCTATGCAAAACTGA-3'	53.5°C
181b-5p	5'-AATTCATTGCTGTCGGTGGGT-3'	52.4°C
3156-5p	5'-AAGGATCTGGAAGTGGGAGACA-3'	54.8°C

4498	5'-TGGCAGGGCAAGTGCTG-3'	51.9°C
U6	5'-GCAAGGATGACACGCAAATTCG-3'	54.8°C
5S	5'-AATACCGGGTGCTGTAGGCT-3'	53.8°C
Gene	**RT stem-loop**	**Tm (°C)**
23a-5p	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACAAATCC-3'	76.7°C
19a-3p	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACTCAGTT-3'	76.7°C
181b-5p	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACACCCAC-3'	78.1°C
3156-5p	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACTGTCTC-3'	77.4°C
4498	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACCAGCAC-3'	78.1°C
U6	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACAAAAATATGG-3'	76 °C
5s	5'-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATCGAGCAGCATACAGCCTA-3'	77.4°C
	Revers universal	**Tm (°C)**
5'-GCTGCTACCTCGGACCCT-3'		54.9°C

3.1.2. ZME improves the ATO effect on NB4 cell-line apoptosis induction

The cytotoxic effects of ZM and ATO on NB4 cells were assessed by measuring apoptosis after 24 hours of treatment, both individually and in combination. For this purpose, a combined treatment of ZM 20 µg/ml + ATO 0.25 µM, which had the best synergistic effect, was selected. The findings demonstrated that both ZM and ATO individually induce apoptosis in NB4 cells, and the combined treatment with these drugs increased the percentage of annexin-positive and annexin/PI-positive cells, indicating early and late apoptosis, respectively, compared to the individual treatments. As depicted in Fig 2, the combined treatment of ZM20 µg/ml + ATO 0.25 µM induced 44% apoptosis in the studied cells, which is higher than the 9.32% induced by ATO at a dose of 0.25 µM and the 31.51% induced by a dose of 20 µg/ml of ZM alone. These results certified our primary investigations.

3.2. Molecular studies

3.2.1. ZME alters miRNA expression in NB4 cells

To assess changes in miRNA expression, NB4 cells were treated with a combined dose of ZM (20µM) + ATO (0.25µg/ml) and individual doses of ZM (20µg/ml) and ATO (0.25µM) for 24 hours. Following cDNA synthesis confirmation, mir-19a-3p, mir-23a-5p, mir-181b-5p, miRNA-3156-5p, and -4498 expressions were evaluated using quantitative real-time PCR. Results indicated high expression of these miRNAs in all control groups, showing their oncogenic effects in the studied cell line and significant differences between the control and other groups across most micro-RNAs (Fig. 3). Notably, mir-19a-3p exhibited the most significant decrease in expression when treated with ZM 20 µg/ml (Fig. 3a). Expression of mir-23a-5p was nearly absent in all groups(Fig. 3b). The combined concentration group displayed reduced mir-181b-5p expression, though the decrease was not statistically significant. Single doses showed expression patterns closer to the PBMC group compared to the combined concentration (Fig. 3c).

Regarding miRNA-3156-5p, the most substantial reduction was associated with the single ZM 20 µg/ml dose and the combination mode. In contrast, the expression in the single treatment of 0.25 µM ATO was closer to PBMC (Fig. 3d). As for miRNA-4498, the most significant reduction in expression was linked to the ZM 20 µg/ml treatment group, whereas the lowest significant reduction was associated with the ATO 0.25 µM treatment group. In the combined concentration group, the results indicated miRNA expression at the level of the PBMC group (Fig. 3e).

3.2.2. Molecular docking

The ligand binding residues of the RAF1 protein were obtained from NCBI and PDB databases. According to NCBI, active site of RAF1 protein was on 375-379, 383, 393, 395, 426, 441-444, 448, 488, 490, 492-493, 495, 506 sites and based on PDB, the active site was on 469 residue. Molecular docking was conducted using HDOCK and MVD software. Our results indicated that thymol and carvacrol, as ligands, can interact with the active sites of the RAF1 protein through several bonds with different energy levels (Table 2-7 and Fig. 4,5). These interactions suggest that these compounds may trigger the apoptosis pathway in NB4 cells treated with ZME, potentially confirming ZME's apoptotic effect on these cells.

Discussion

In the past two decades, the treatment of APL has involved combining ATRA with chemotherapy. For patients with lower risk, ATRA is combined with arsenic trioxide (ATO) while eliminating chemotherapy.[39] ATO and ATRA directly target the suppression of PML/RARα transcription and stability of the protein, leading to rapid differentiation in promyelocytes and clinical recovery of APL patients.[40] However, ATRA alone is insufficient for long-term remission, and despite continued treatment using ATRA and low-dose chemotherapy, most patients experience relapse in 6 months.[41] ATO has acted successfully in treating new cases and relapsed APL patients. It induces apoptosis in tumor cells by activating caspase-3, modulating intracellular glutathione reduction, causing oxidative damage, and inducing meiotic arrest by inhibiting spindle division and microtubulin formation.

Additionally, there is evidence that ATO may halt the growth and proliferation of tumor cells by inhibiting telomerase through suppressing hTERT.[41] Studies also suggest that ATO can influence the level of different miRNAs, such as miRNA-204.[42] MicroRNAs (miRNAs) are small RNA molecules that regulate the silencing of target genes after transcription and play a vital role in various physiological and developmental procedures.[29] They are important for the cellular response to environmental stress and are associated with human diseases such as cancer.[43] Despite their therapeutic properties, chemotherapy drugs have numerous side effects. For instance, ATO can lead to bone marrow suppression, liver dysfunction, digestive disorders, hyperleukocytosis, and differentiation syndrome.[44-46] Prolongation of the QT interval in the ECG, causing cardiac arrhythmia and cardiotoxicity, is a particularly significant side effect that requires careful monitoring.[47]

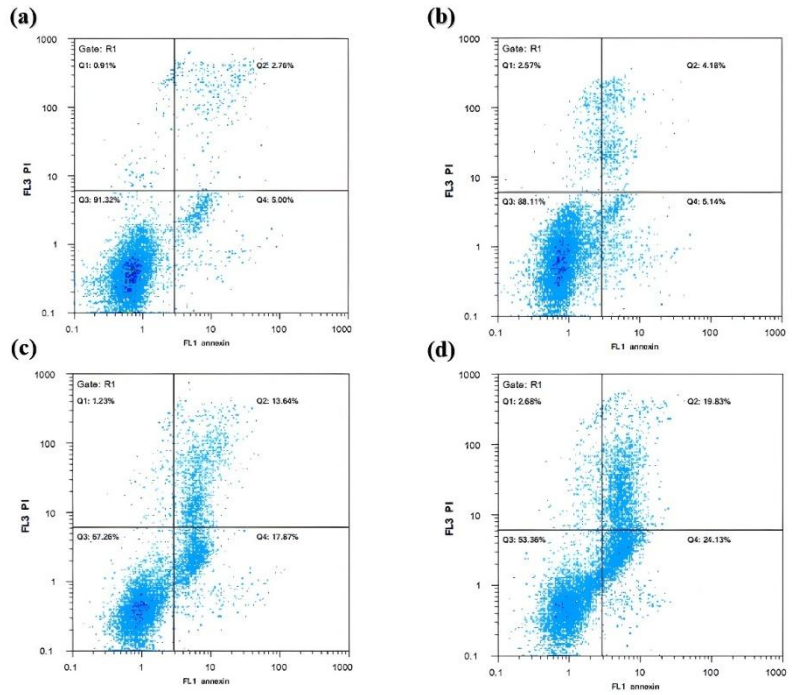

Figure 2. Cell apoptosis graphs, control **(a)**, ATO 0.25 μM **(b)**, ZM 20 μg/ml **(c)**, and ZM 20 μg/ml +ATO 0.25. μM **(d)**.

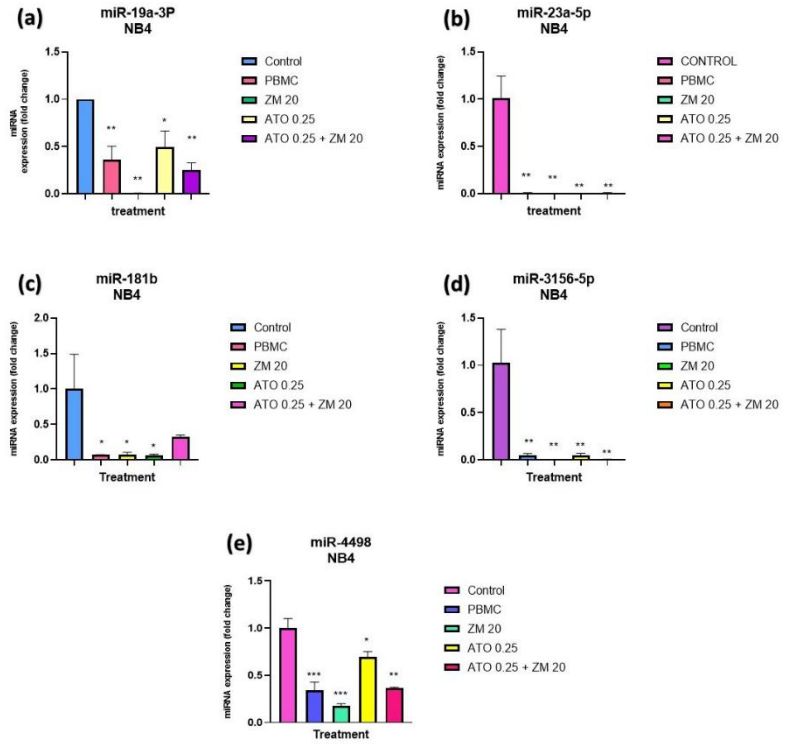

Figure 3. The effect of ATO 0.25 µM, ZM 20 µg/ml and their combination on mir-19a-3p **(a)**, mir-23a-5p **(b)**, mir-181b-5p **(c)**, miRNA-3156-5p **(d)** and -4498 **(e)** expression.

Table 2. Blind docking of RAF1 and thymol

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score
-79.82	0.19	169.38	-62.88	-2.5	-46.8
-78.42	0.19	168.69	-61.57	-2.4	-54.66
-77.65	0.19	177.08	-58.72	0	-51.24

Table 3. Blind docking of RAF1 and carvacrol

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score
-74.26	0.18	212.86	-68.01	-1.9	-47.64
-67.98	0.16	168.37	-65.97	-2.5	-47.24
-62.23	0.14	171.68	-64.98	-2.3	-40.49

Table 4. Specific docking of RAF1 and thymol (NCBI based active site)

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score
-58.15	0.13	181.33	-72.82	-2.1	-61.2
-68.01	0.16	185.87	-69.9	-2.4	-60
-66.82	0.15	192.5	-68.4	-3.3	-57.93

Table 5. Specific docking of RAF1 and carvacrol (NCBI based active site)

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score
-56.10	0.13	180.81	-77.83	-2.5	-54.3
-36.57	0.09	192.18	-77.05	0	-53.77
-69.01	0.16	169.79	-74.86	-2.5	-54.46

Table 6. Specific docking of RAF1 and thymol (PDB based active site)

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score
-33	0.08	167.4	-52.81	-2.3	-14.24
-24.43	0.07	174.15	-50.87	0	-44.85
-40.88	0.10	172.7	-49.30	-1.9	-35.97

Table 7. Specific docking of RAF1 and carvacrol (PDB based active site)

Hdock server			Molegro virtual docker		
Docking score	Confidence score	Ligand RMSD	Moldock score	H bond (kCal/mol)	Re-rank Score

-30.95	0.08	173.41	-63.9	-1.7	-44.79
-27.11	0.07	174.52	-59.79	0	-41.81
-24.09	0.07	173.79	-57.35	0	-39.97

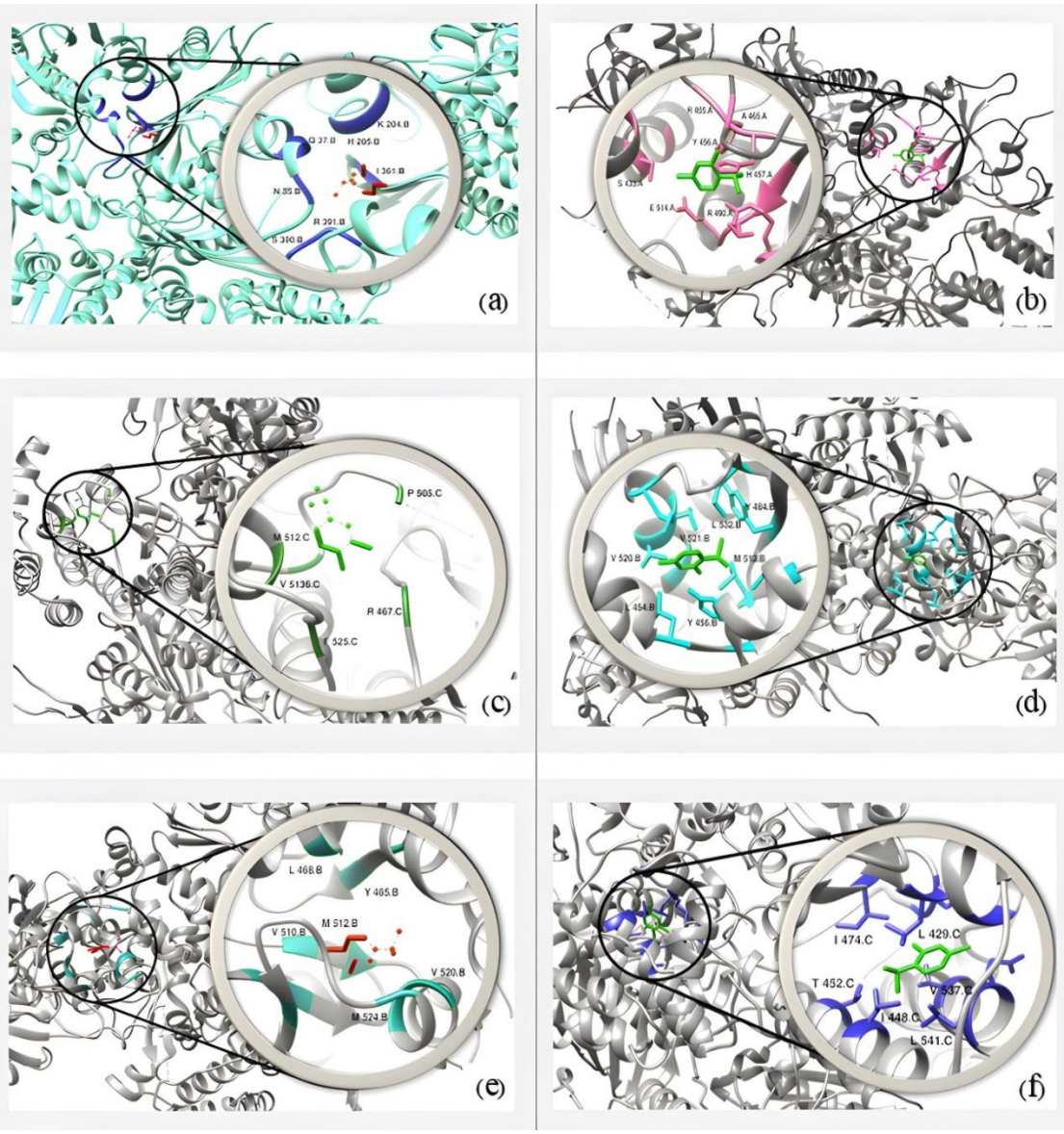

Figure 4. The 3D interactions of RAF1/carvacrol blind docking (**a**), RAF1/thymol blind docking (**b**), RAF1/carvacrol specific docking (NCBI active site based) (**c**) and RAF1/thymol specific docking (NCBI active site based) (**d**), RAF1/carvacrol specific docking (PDB active site based) (**e**), and RAF1/thymol specific docking (PDB active site based) (**f**).

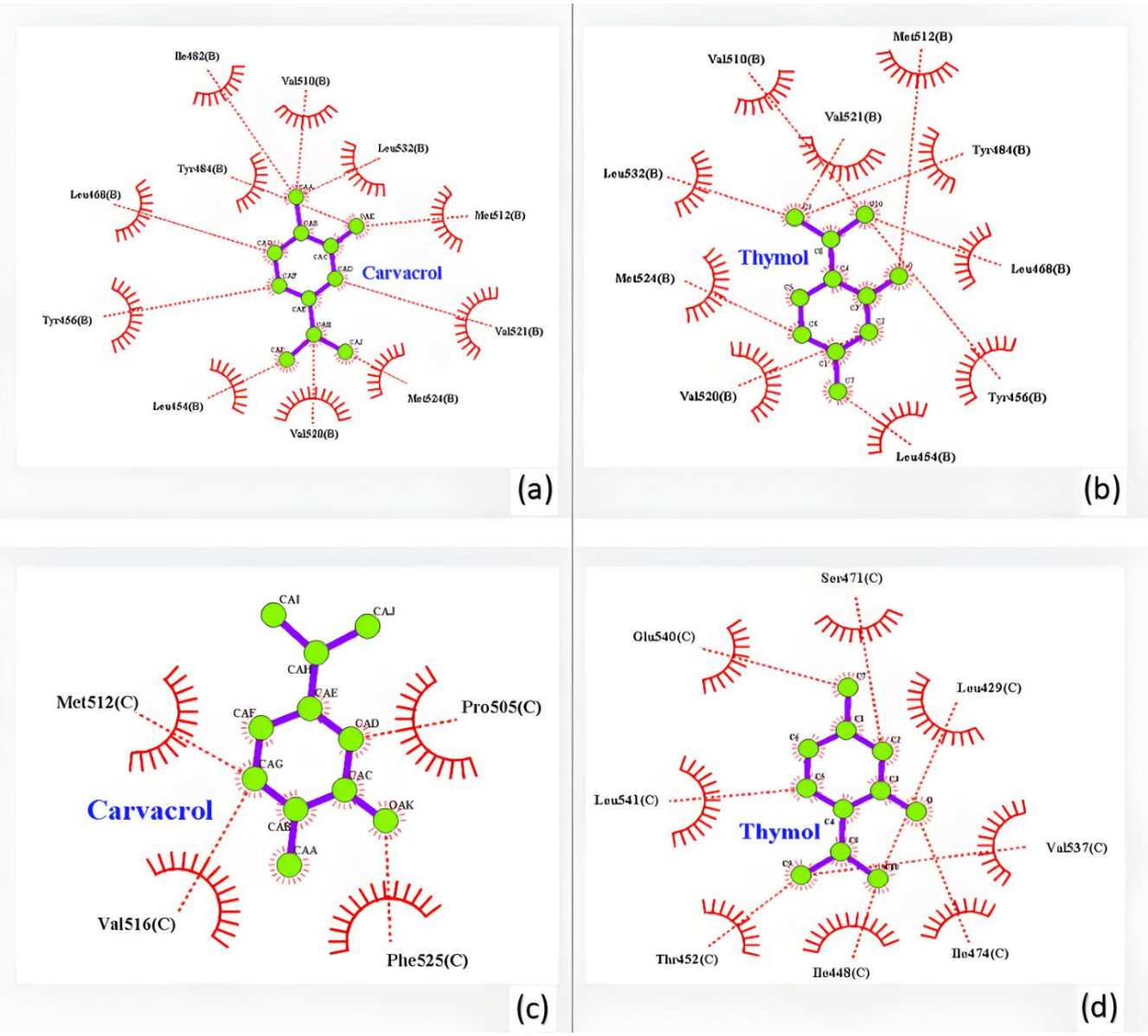

Figure 5. The 2D interactions of RAF1/carvacrol (NCBI active site based) (**a**), RAF1/thymol (NCBI active site based) (**b**), RAF1/carvacrol (PDB active site based) (**c**) and RAF1/thymol (PDB active site based) (**d**).

Efforts to reduce these adverse effects include replacing or reducing the dosage of such drugs, including the combined use of chemotherapy drugs with herbal medicines. Compared to chemotherapy drugs, Herbal medicines cause fewer side effects and less toxicity for non-tumor cells[48] In this study, the impact of the methanolic extract of Shiraz thyme (ZM) and its combination with the ATO drug on the expression of particular miRNAs and TS-miRNAs in the acute promyelocytic leukemia cell line (NB4) is examined. Recent research has highlighted the diverse effects of ZM, including analgesic, anti-inflammatory, antimicrobial, and antioxidant properties, as well as anti-tumor and anti-proliferative effects on conditions such as glioblastoma.[49] Our study demonstrated a notable reduction in NB4 cell metabolic activity when treated with 20 µg/ml Shirazi thyme, 0.25 µM ATO, and their combination. Time did not significantly impact survival or metabolic activity. Additionally, PBMCs were used to assess extract toxicity on normal cells, with results showing no significant toxicity at the tested doses. Like the same, a 2015 study by Janitrami and colleagues showed a reduction in AGS, HeLa, and MCF-7 cell lines metabolic activity after treatment with the Shirazi thyme extract, in a dose-dependent way, using the MTT method at various time points.[50-52] Similar to the 2022 study by Lashkari and colleagues,[35] our research confirms that chemotherapy may be more efficient in combination with ZME. In our study, Cell viability and metabolic activity in combined doses, particularly in the case of the combination of 0.25 µM ATO with 20 µg/ml ZM in 24 hours, experienced a notable decrease, as well as cell apoptosis in the same combination. Various studies, including a 2018 one by Chen et al., indicate that ATO causes apoptotic or autophagic death in cancer cells through oxidative stress- activated signaling pathways.[53] Our study also revealed that ATO can significantly reduce cell survival and metabolism in NB4 cancer cells over 24 and 48 hours and induce apoptosis in these cells. When combined with ATO at a concentration of 0.25 µM, thyme at 20 µg/ml showed significant synergy, decreasing the survival and metabolic activity of cancer cells while increasing apoptosis. So, ZME can be an eligible adjuvant along with ATO. In 2019, Wei and colleagues introduced melatonin as an applicable adjuvant substance, increasing the sensitivity of cancer cells to ATO.[54] Additionally, a 2019 study by Wang et al. showed that ATO increases apoptosis by altering the miRNA-204 expression levels;[42] Our study also investigated the expression of various miRNAs, suggesting that ATO may influence miRNA expression levels. Furthermore, a 2020 study by Farzadfard et al. explored the use of circulating miR19a-3p as an oncogenic miRNA in CLL diagnosis.[55] Lastly, a 2013 study by Lepore et al. on human acute myeloid leukemias demonstrated that Vorinostat HDACi reduces the mRNA levels of BARD1—an oncoprotein associated with various cancers—by up-expressing miR-19a and miR-19b.[56] In our study, in contrast to the previous one, the expression level of miRNA-19a-3p was notably higher in control cells (NB4) compared to healthy PBMC cells. This level returned to normal after treatment with a combination of ZM20 µg/ml + ATO0.25 µM. Chemotherapy resistance is a major challenge in treating acute myeloid leukemia (AML). A 2020 study by Hatzl et al. revealed that increased miR-23a expression can cause AraC chemoresistance and is associated with poorer outcomes in AML patients treated with AraC;[57] However, a 2009 study by Saumet et al. in APL patients showed that PML/RARα fusion suppresses miRNA-23a expression, which then increases again after treatment with ATO and ATRA.[58] In our study, unlike the recent one, a significant increase was observed in the expression level of miRNA-23a-5p in control cells (NB4) compared to healthy PBMC cells, which returned to normal after treatment with single doses and a combined concentration of ZM20 µg/ml + 0.25 µM ATO. Like a 2021 study by Yan and colleagues, which high expression of miRNA-181b-5p was confirmed in ALL cell lines,[59] our study, showed a notable rise in the expression level of miRNA-181b-5p in control cells (NB4) compared to healthy PBMC cells. This returned to normal after single-dose treatment, but in the combined concentration of ZM20 µg/ml + ATO0.25 µM, the reduction was not significant like the other treatment groups. In our study, unlike the study performed by Kooblall et al. on multiple endocrine neoplasia type 1 (MEN1) biomarkers in 2021,[60] the level of miRNA-3156-5p in control cells (NB4) showed a significant increase compared to healthy PBMC cells, which returned to normal after treatment with a single dose of 0.25 µM ATO. Also, a notable decrease in the expression level of this miRNA was seen in the single concentration of ZM20 and the combination of ZM20 µg/ml + ATO 0.25 µM compared to the normal state. In our study, like what is done in 2018 by Jiang and his colleagues to investigate the expression profile of different miRNAs in patients with multiple myeloma (MM),[61] the level of miRNA-4498 in control cells (NB4) showed a significant increase compared to healthy PBMC cells, which returned to normal after treatment with the combined concentration of ZM 20 µg/ml + ATO 0.25 µM. miR-19 is the main oncogenic element of the miR-17-92 cluster, indicating that its targets likely drive the cluster's oncogenic effects.[62] As said, RAF1 is a probable target for miRNA-19a confirmed by TargetScan analysis.[38] Our study showed that thymol and carvacrol, as ligands, can interact with the active sites of the RAF1 protein. These interactions suggest that these compounds may trigger the apoptosis pathway in NB4 cells treated with ZME, potentially confirming ZME's apoptotic effect on these cells.

Conclusion

Our study has shown that using single doses of Shirazi thyme and ATO can decrease the survival and metabolic activity of NB4 cells and increase the percentage of apoptosis. However, when these treatments are combined, the results are even better. Moreover, the treatment significantly decreased the expression of miRNAs 19a-3p, 23a-5p, 181b-5p, 4498, and 3156-5p. When ATO was combined with Shirazi thyme extract, the reduction in the miRNAs expression (except miR-181b) was even more significant than using ATO alone. This indicates that Shirazi thyme extract can be used as a helpful adjuvant in combination with ATO for treating acute promyelocytic leukemia. Our study also showed that thymol and carvacrol, as ligands, can interact with the active sites of the RAF1 protein. These interactions suggest that these compounds may trigger the apoptosis pathway in NB4 cells treated with ZME, potentially confirming ZME's apoptotic effect on these cells.

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Author Contributions

Roohollah Mirzaee Khalilabadi was responsible for the study design, supervising the data collection and study process, and revising the manuscript.

Mahsa Asghari was responsible for data collection, performing the study, doing all the study steps, and writing the manuscript.

Ali Afgar was responsible for data analysis, bioinformatic protocol design, and supervising the data analysis.

Hajar Mardani Valandani was responsible for the study design, supervising the data collection and study process
Amir Mohammad Zahedi was responsible for performing the bioinformatic part and preparing the bioinformatic graphs.

Alireza Farsinejad was responsible for supervising the data collection and study process, and revising the manuscript.
Mohsen Ehsan was responsible for supervising the study process, and revising the manuscript.

Competing Interests

Authors declare no competing interests.

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None

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