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Conferone, a coumarin from *Ferula flabelliloba*, induced toxic effects on ATL cells

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Abstract

Background: Adult T-cell leukemia/lymphoma (ATL) is a lymphoid malignancy caused by HTLV-1 infection, with distinct geographical distribution. Despite advances in cancer treatment, the average survival rate of ATL is low. Conferone is a natural coumarin extracted from *Ferula* species with a wide range of pharmaceutical effects. In search for a novel chemotherapeutic agent, we investigated the cytotoxicity of conferone on ATL cells.

Methods: To obtain conferone, the methanolic extract of the roots of *F. flabelliloba* was subjected to silica gel column chromatography, followed by ^{1}H- and ^{13}C-NMR to confirm its structure. For cytotoxicity assay, MT-2 cells were treated with different concentrations of conferone (2.5, 5, 10, 20, and 40 μM) for 24, 48, and 72 h, and viability was evaluated by a colorimetric assay using alamarBlue. Cell cycle was analyzed by PI staining and flow cytometry, and qPCR was used to study the expression of candidate genes.

Results and Conclusion: Obtained findings indicated that conferone induced considerable cytotoxic effects on MT-2 cells in a time- and dose-dependent manner. In addition, accumulation of cells in the sub-G_1 phase of the cell cycle was detected upon conferone administration. Moreover, conferone reduced the expression of *CDK6, c-MYC,* $CFLIP_L$, and *NF-κB (Rel-A)* in MT-2 cells. Accordingly, conferone could be considered as a potent agent against ATL, although complementary investigations are required to define more precisely its mechanism of action.

Keywords

conferone, adult T-cell leukemia/lymphoma, natural coumarin, cytotoxicity, in vitro

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a potentially aggressive neoplasm of mature T-lymphocytes associated with human T-lymphotropic virus type I (HTLV-I).[1] There are approximately 20 million HTLV-1-infected people across the world.[2] In tropical places such as South America, Central Africa, the Caribbean, South of Japan, and the Middle East, HTLV-I infection is endemic. ATL is common in individuals in their sixth and seventh decades.[3,4] There are four clinical subtypes for ATL, from indolent, slowly progressive disease (smoldering and chronic) to aggressive

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and life-threatening disease (lymphoma and acute). Despite improvements in therapeutic strategies, the prognosis of ATL remains poor and the survival rate of patients is still low.[5] Cyclophosphamide, Adriamycin, vincristine, and prednisolone have been used as the standard first-line treatments for ATL. Although many patients do achieve either partial or complete remission, clinical outcomes of current modalities remain unfortunate.[6] Among all chemotherapeutic regimes, high response rates (ranging from 58% to 92%) were seen for acute ATL after co-administration of interferon (IFN-α) and antiviral agent zidovudine and also IFN-α and arsenic trioxide (ATO), but these combinatorial treatments failed to achieve significant impacts on survival.[7] Several mechanisms including overexpression of efflux pumps, TP53 mutations, and deregulation of oncogenes in leukemic cells are involved in disease chemoresistance and relapse.[8]

Conferone ($C_{24}H_{28}O_4$, Figure 1) is a natural coumarin derived from the fruits and roots of self-growing *Ferula* species. As an anticancer agent, conferone has anti-angiogenic characteristics and suppresses p-glycoprotein-mediated drug efflux.[9] In addition, conferone possesses the potential to suppress cell proliferation, induce both apoptosis and necrosis, and generate free radicals in cancer cells.[10] To our knowledge, this is the first report studying the effects of conferone against ATL cells in vitro. In this regard, viability of MT-2 cells was assessed by alamarBlue assay, and changes induced on the cell cycle were detected by flow cytometry analysis upon propidium iodide (PI) staining. To ascertain the mechanism of conferone toxic action, quantitative polymerase chain reaction (qPCR) was used to investigate the expression of *CDK6*, *c-MYC*, *cFLIPL*, and *NF-κB (Rel A)*.

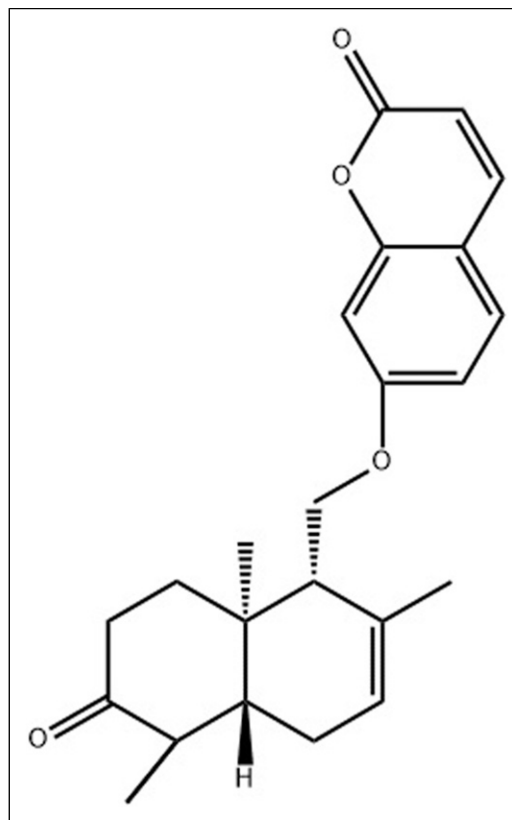

Figure 1. Chemical structure of conferone.

Materials and methods

Chemicals and reagents

alamarBlue and propidium iodide (PI) were purchased from Sigma-Aldrich (Germany). Dimethylsulfoxide (DMSO) and Triton X-100 were obtained from CinnaGen (Iran). RPMI-1640 was from Biosera (France), and fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine were from Gibco (Scotland). TriPure was produced by Roche (Germany), M-MuLV reverse transcriptase was from Thermo Scientific (USA), and SYBR green mix was from Takara (Japan).

Extraction of sesquiterpene coumarin conferone

Sesquiterpene coumarin conferone was extracted as previously described.[11] In summary, the roots of *F. flabelliloba* were collected, dried, powdered, and extracted with methanol by maceration method at ambient temperature. Then, the solvent was evaporated under vacuum pressure and a part of the extract was subjected to silica gel chromatography. Among obtained fractions, conferone (MW: 380.5 g/mol) was derived as white crystals form one fraction (18–21; PET: EtOAc [6:1]; 2.0 g; TLC [Hex: EtOAc 9 : 2.5]), and its structure was assigned by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra (Table 1). NMR experiments were run using Bruker DRX-500 and DRX-600 spectrometers at 300 K, and $CDCl_3$ was used as the solvent (Carlo Erba, Italy). To note, the spectra were calibrated using the solvent signal as the internal standard (^{1}H, d: 7.27 ppm; ^{13}C, d: 77.0 ppm).

Treatment of cells and viability assay

To assess the cytotoxic effects of natural coumarin conferone on human HTLV-1-infected T-cells, MT-2 cell line was purchased from Pasteur Institute (Tehran, Iran). For cell culture, RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 50 µl (W/V) penicillin/streptomycin, and 50 µl L-glutamine were used and cells were incubated at 37°C in 5% CO_2.

To prepare different concentrations of conferone, at first a stock solution was prepared and stored using DMSO as the solvent, and then, serial dilutions were made by complete culture medium right before use. Accordingly,

Table 1. ^{1}H-NMR data (600 MHz, δ ppm)* and ^{13}C-NMR data (125.7 MHz, δ ppm) obtained for conferone.

Position of proton		Position of carbon	
2	—	2	161.5
3	6.28 d (9.6)	3	113.4
4	7.66 d (9.6)	4	143.7
5	7.39 d (8.4)	5	129.2
6	6.86 dd (8.4, 2.3)	6	113.6
7	—	7	162.2
8	6.85 d (2.3)	8	101.7
9	—	9	156.3
10	—	10	113.0
1′	1.66 dd* β 2.30 m α	1′	38.8
2′	2.31 m β 2.75 ddd (15.3, 6.6) α	2′	34.8
3′	—	3′	216.4
4′	—	4′	47.9
5′	1.68 dd*	5′	51.5
6′	2.00 like brd β 2.18 like brt α	6′	24.3
7′	5.62 brs	7′	124.0
8′	—	8′	132.7
9′	2.28*	9′	53.4
10′	—	10′	36.2
11′	a 4.10 dd (9.80, 5.20) b 4.21 dd (9.80, 4.80)	11′	67.0
12′	1.75 s	12′	25.6
13′	1.17 s	13′	22.7
14′	1.13 s	14′	25.6
15′	1.18 s	15′	14.9

*Overlapped with other signals.

0.4% DMSO was considered as the solvent control in all experiments. To determine viability, MT2 cells (50,000 cells per well in 96-well plate) were treated with increasing concentrations of conferone (5, 10, 20, 40, and 80 µM) for 24, 48, and 72 h. In addition, cells were treated with 4, 8, and 16 µM ATO (Sigma) as a standard chemical agent for ATL treatment. Then, alamarBlue (20 µl per well in the 96-well plate) was added at the end of each time point, and cells were incubated at 37°C. By using a microplate reader (Epoch), absorbance was measured at 600 nm, and cell viability (%) was determined according to the following equation: 100-((AT-AC)/(AB-AC) ×100), in which AT and AC were the absorbance of treated and untreated control cells, respectively, and AB was the absorbance of the blank control.

Detection of apoptosis

To further evaluate the effects of conferone on the cell cycle, PI staining followed by flow cytometry was applied. Briefly, MT-2 cells (50,000 cells per well in the 96-well plate) were incubated with conferone (40 µM) for 72 h. It should be noted that cells treated with 0.4% DMSO and untreated cells were considered as well. Briefly, MT-2 cells in each treatment were collected and centrifuged at 200 g for 5 min. Upon washing with cold phosphate-buffered saline (PBS) containing 5% FBS, cell pellets were resuspended in staining buffer containing 100 µg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate and incubated at 37°C in the dark for 30 min. Finally, flow cytometry (BD FACSCalibur) was carried out by FL2-H filter.

Gene expression analysis

To ascertain the molecular mechanism behind conferone cytotoxic action, the expression pattern of *NF-κB* (*REL-A*), *CDK6, c-MYC*, and $cFLIP_L$ was studied by qPCR. Briefly, the total cellular RNA was extracted from cells treated with 80 µM conferone and relevant controls using Triazole (Roche). Then, cDNAs were synthesized using random hexamer, dNTPs, and Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's protocol. qPCR was performed in Rotor-Gene 6000 detection system (Qiagen) with the SYBR green mix; primers are given in Table 2 for *c-MYC*, $cFLIP_L$, and *CDK6* genes. TaqMan probe and specific primers were employed for *NF-κB* (*REL-A*)**.** PCR cycling conditions were 94°C for 2 min, 94°C for 15 sec, 58°C for 30 sec, and 72°C for 45 sec. In all analyses, beta-2 microglobulin (B_2M) transcripts were used as the internal control, and data were analyzed by standard curve relative method using the following formula: efficiency of the target gene$^{(\Delta Ct\ target)}$/efficiency of the reference gene$^{(\Delta Ct\ reference)}$.

Statistical analysis

The statistical significance for viability assay was analyzed by one-way ANOVA, Dunnet, and Sidak multiple comparison tests using GraphPad Prism. In addition, results of flow cytometry were analyzed by WinMDI software. qPCR data were analyzed using GraphPad Prism and Kolmogorov–Smirnov statistical test [12]. All data were reported as mean ± SD, and *p*-values less than 0.05, 0.01, 0.001, and 0.0001 were considered significant for all comparisons.

Results

Viability of MT-2 cells was reduced after conferone treatment

Conferone decreased the viability of MT-2 cells in a time- and concentration-dependent matter. As presented in

Table 2. List of primers and probes used for qPCR analysis in the current study.

Name of gene	Length (bp)	5'→3'
B_2MG	127	**Forward:** AATTGAAAAAGTGGAGCATTCAGA **Reverse:** GGCTGTGACAAAGTCACATGGTT
c-MYC	159	**Forward:** ACTCTGAGGAGGAGGAACAAGAA **Reverse:** TGGAGACGTGGCACCTCTT
$cFLIP_L$	126	**Forward:** ATTGGCAATGAGACAGAGCTTC **Reverse:** CTCGGGCATACAGGCAAA
CDK6		**Forward:** GTTTCCAGATGGCTCTAACCTCAG **Reverse:** AAATATGCAGCCAACACTCCAGAG
Rel-A	145	**Forward:** ACCCCTTCCAAGTTCCTATAGAAGAG **Reverse:** CGATTGTCAAAGATGGGATGAGAAAG **Probe:** ACTACGACCTGAATGCTGTGCGGCTCT
B2MG	127	**Forward**: TTGTCTTTCAGCAAGGACTGG **Reverse:** CCACTTAACTATCTTGGGCTGTG **Probe:** TCACATGGTTCACACGGCAGGCAT

Figure 2, upon 24, 48, and 72 h treatment with 20 µM conferone, viability was calculated as 85%, 75%, and 60%, respectively. Likewise, after treatment with 40 µM conferone during the same consecutive time periods, cell viability significantly ($p < 0.0001$) reduced and was determined as 77%, 60%, and 45%, respectively. To note, the assessment of ATO effects indicated that its highest concentration (16 µM) significantly ($p < 0.0001$) decreased cell viability down to 79% and 51% after 48 and 72 h, respectively.

Changes induced on the cell cycle were observed upon conferone treatment

To determine whether conferone induced changes on the cell cycle, the DNA content of MT-2 cells was analyzed by flow cytometry. As presented in Figure 3, in untreated MT-2 cells, 3.3%, 41.2%, 9.2%, and 46.3% of cells were detected in the sub-G_1, G_1, S, and G_2/M phases of the cell cycle, respectively. Upon treatment with DMSO solvent, 10.4%, 28.3%, 10.2%, and 51.1% of cells were detected in the sub-G_1, G_1, S, and G_2/M phases of the cell cycle, respectively. Surprisingly, conferone treatment altered the distribution of cells in the cell cycle, as 30.1% of cells were detected in the sub-G_1, 33% were detected in the G_1, 12.2% of cells were detected in the S, and 24.7% of cells were detected in the G_2/M phase of the cell cycle. This observation was in agreement with findings of the viability assay and confirmed cytotoxic effects of 40°µM conferone.

Conferone downregulated the expression of NF-κB (REL-A), CDK6, c-MYC, *and* $cFLIP_L$

To ascertain molecular mechanisms underlying the effects of conferone, the expression patterns of *NF-κB (REL-A), CDK6, c-MYC,* and *$cFLIP_l$*, all of which contributed to the proliferation and survival of ATL cells, were studied by qPCR. As shown in Figure 4, conferone decreased *NF-κB (REL-A)* expression and significantly ($p < 0.001$) reduced the expression of *CDK6* to lower levels in comparison with DMSO control. Significant ($p < 0.05$) downregulation in *c-MYC* expression was also observed when conferone treatment was compared with its relevant control. Last but not least, conferone downregulated the expression of *$cFLIP_L$* in comparison with other groups.

Discussion

ATL is known as a T-cell neoplasm associated with HTLV-1. Patients with the acute or lymphoma subtypes of ATL require therapeutic intervention, and although various chemotherapeutic regimens are currently available, clinical outcomes remain dismal.[12] To introduce a novel and more effective natural agent against ATL, the aim of present research was to investigate cytotoxic activity of conferone isolated from *F. flabelliloba* for the first time. Our findings revealed that conferone reduced the viability of MT-2 cells and attributed considerable accumulation of cells in the sub-G_1 phase of the cell cycle. These findings were confirmed by molecular analysis, since significant downregulation of genes involved in the survival and proliferation of ATL cells was detected after conferone administration.

The NF-B (REL-A) transcription factor family regulates the expression of genetic networks critical for cell survival, proliferation, inflammation, and T-cell transformation.[13–15] HTLV-1-Tax acts as a transcriptional activator via activation of nuclear factor-kB (NF-kB). Unlike normal T-cells, Tax-expressing T-cells constitutively express *NF-kB*.[16] Interestingly, our findings revealed that conferone was able to significantly reduce the expression of *NF-B (REL-A)* in MT-2 cells.

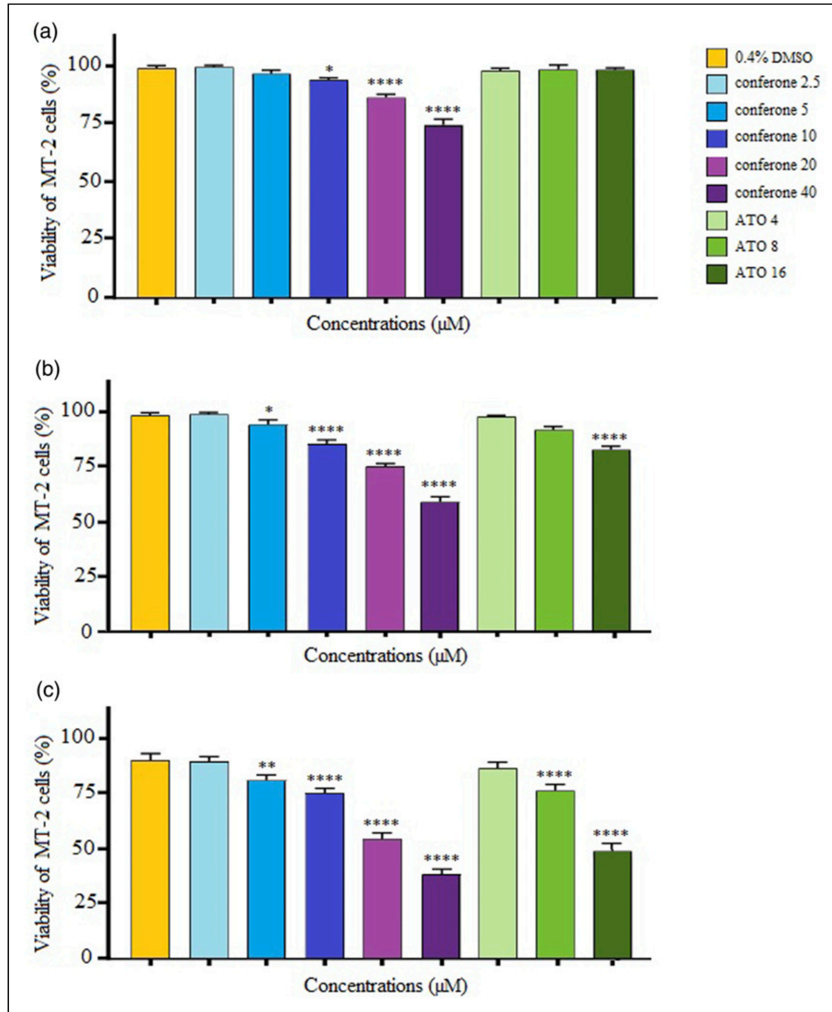

Figure 2. Viability assessment of MT-2 cells. Cells were treated with 2.5, 5, 10, 20, and 40 µM conferone and 4, 8 and 16 µM ATO and viability was assessed after 24 h (a), 48 h (b), and 72 h (c). alamarBlue assay was carried out for at least three times and results are presented as mean ± SD. (*$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$).

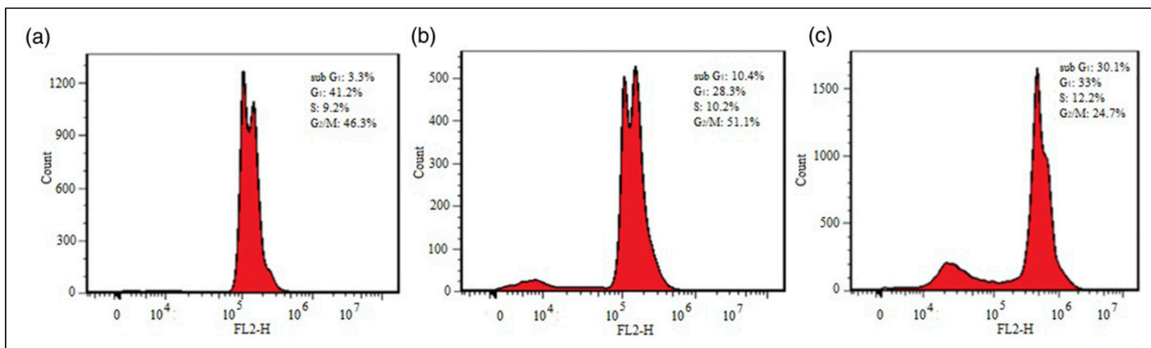

Figure 3. MT2 cell cycle analysis by PI staining. Untreated cells (a), cells treated with 0.4% DMSO (b), and 40 µM conferone (c). Sub-G_1 peak, as an indicative of dead cells, was specifically induced after conferone treatment.

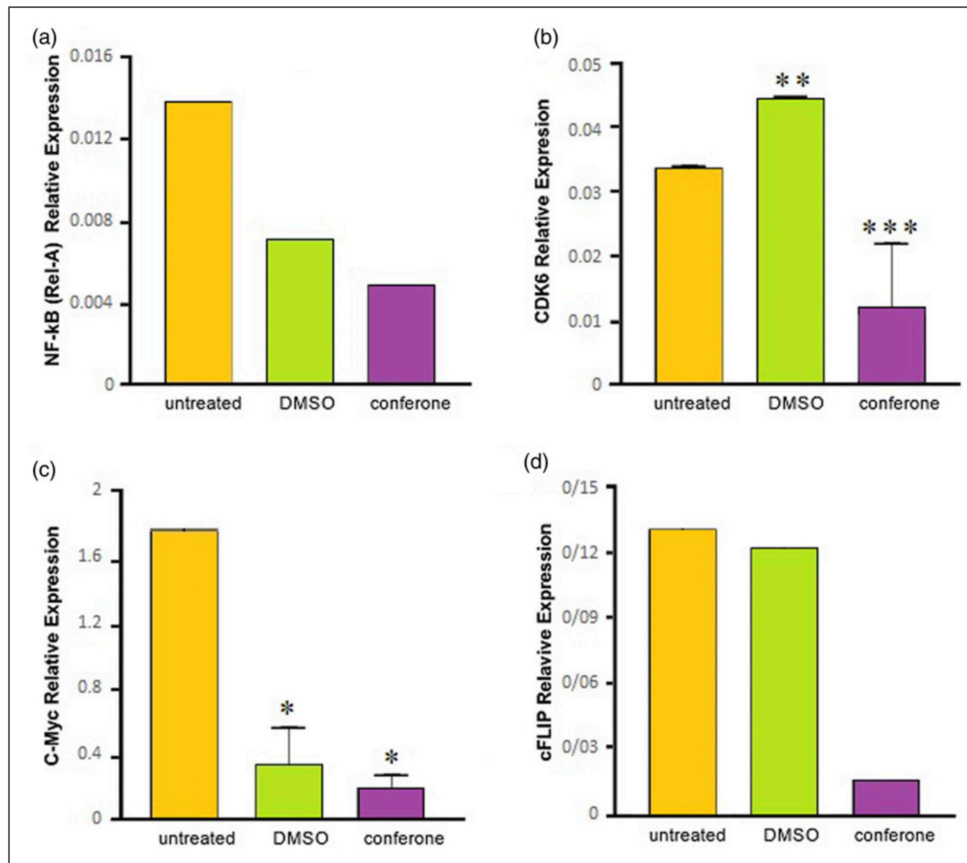

Figure 4. qPCR analysis of *NF-κB (REL-A)* (a), *CDK6* (b), *c-MYC* (c), and *cFLIP$_L$* (d) expression 72 h after the treatment of MT2 cells with conferone. To note, relative expression was compared with relevant control. (*p < .05, **p < .01, and ***p < .001).

CDK6 is a family of cell cycle kinases that form complexes with D-type cyclins to help cells proceed through the early G_1 phase of the cell cycle. Components of the CDK6-Cyclin D complexes are frequently altered in hematological malignancies.[17–21] *CDK6* inhibitors have various biological effects on cancer cells that can be exploited for therapeutic purposes, such as modulation of mitogenic kinase signaling, formation of a senescence-like phenotype, and increased immunogenicity of cancer cells.[22] In the present study, we demonstrated that conferone decreased the expression of *CDK6* significantly.

c-MYC is a key transcription factor that promotes cell proliferation in the G_1 phase by interacting with cyclin D and cyclin-dependent kinases-4/6.[23,24] High level of *c-MYC* expression is required for carcinogenesis and maintenance of ATL.[25–27] In addition, c-MYC protein and mRNA expression were substantially higher in lymphoma and acute types of ATL patients than in smoldering and chronic types.[28] Findings of the current study revealed significant reduction in *c-MYC* expression, implying that conferone has much potential as a negative regulator for this gene in ATL cells.

c-FLIP$_L$ is an anti-apoptotic protein with substantial expression in hematologic malignancies.[29,30] *c-FLIP$_L$* is highly expressed in bone marrow mononuclear cells obtained from ATL patients and is associated with prognosis of this malignancy. Moreover, it has been indicated that patients with peripheral T-cell lymphoma had elevated *c-FLIP$_L$* expression.[31] Present results revealed that conferone significantly reduced the expression of *c-FLIP$_L$*, that explains, to some extent, our observations regarding reduced viability and cell cycle changes.

The current research was carried out during COVID-19 pandemic, and due to global lockdown, laboratory work was profoundly restricted. Accordingly, there were limitations to develop this study on more ATL cell lines and/or carry out complementary analysis.

Conclusion

Findings of the present study indicated, for the first time, that conferone acts as a natural coumarin against ATL cells, and thus, could be considered as a potent pharmaceutical agent. Nevertheless, complementary studies on other cell lines are required to confirm observed effects of conferone in our study.

Ethical Statement

Ethical approval

This study was approved by the Research Council of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC. 970306).

Author Contributions

H. Rafatpanah supervised the project, M. Golizadeh and M. Mahdifar carried out the experiments, S. Mahdavi wrote the first draft, M. Iranshahi advised the project, and F.B. Rassouli designed and supervised the project and revised the manuscript.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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