BIOCELL
2020 44(2): 183-192

Tech Science Press

Anti-proliferative effects of a small molecule inhibitor of CDK AT7519 on chronic myeloid leukemia (CML) cells through halting the transition of cells from G2/M phase of the cell cycle

Masoumeh OGHABI[1,2]; Ava SAFAROGHLI-AZAR[1,2]; Atieh POURBAGHERI-SIGAROODI[1,2]; Mohammad SAYYADI[3]; Mohsen HAMIDPOUR[1]; Mohammad Hossein MOHAMMADI[1]; Davood BASHASH[1,*]

[1] Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Student Research Committee, Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3] School of Allied Medical Sciences, Arak University of Medical Sciences, Arak, Iran

Key words: Chronic myeloid leukemia (CML), Cyclin-dependent kinase (CDK), AT7519, Autophagy, PI3K, c-Myc

Abstract: Pathogenesis of chronic myeloid leukemia (CML) has mostly been studied with regard to the oncogenic role of BCR/ABL fusion; however, recent disclosures have declared that the challenges with the treatment of CML patients would not be resolved until the role of other aberrancies is ignored. Given the involvement of cyclin-dependent kinases (CDKs) in the pathogenesis of CML, the present study aimed to investigate the effects of a multi-CDK inhibitor AT7519 on BCR/ABL-harboring CML-derived K562 cells. Our results showed that AT7519 effectively reduced the survival of K562 and induced its anti-proliferative effect through the induction of G2/M arrest due to elevated p21 and p27. The resulting data also revealed that either direct or indirect suppression of c-Myc using specific c-Myc inhibitor 10058-F4 and selective PI3K inhibitor CAL-101 resulted in a superior cytotoxicity, suggesting that the activation of PI3K pathway could attenuate antileukemic effects of the inhibitor, at least partly, through a c-Myc-dependent mechanism. To the best of our knowledge, to date, no study has addressed the effect of autophagy on CML cell response to AT7519, and, herein, we proposed for the first time that the suppression of autophagy boosted AT7519 cytotoxicity against K562. Overall, we suggested that selective CDK inhibitor AT7519 exerted antileukemic effect against CML cells and propose a novel therapeutic application for the inhibitor either as a single agent or in combination with c-Myc and/or PI3K inhibitors.

Introduction

Certain types of cancers are recently winning in retreat in the face of medical advances, among them chronic myeloid leukemia (CML) has taken the most advantages of the newly designed anti-cancer agents, foremost, tyrosine kinase inhibitors (TKIs) (Saussele *et al.*, 2017). For many years, CML pathogenesis has been mostly studied with regard to the oncogenic role of BCR/ABL fusion protein rather than any other oncogenic pathways; however, recent disclosures have declared that the challenges with the treatment of CML patients would not be resolved until the role of other aberrancies is ignored (Flis and Chojnacki, 2019). Uncontrolled cell proliferation serves as the universal hallmark of different phases of CML, which is mainly regulated by the activation and/or inactivation of different mediators (Perrotti *et al.*, 2010). Cyclin-dependent kinases (CDKs), a large family of serine/threonine kinases, vigorously control the progression of the cell cycle in the presence of their cyclin partners (Nurse, 2000). Since a wide variety of genetic and epigenetic events lead to the over-activity of CDKs in CML (Iolascon *et al.*, 1998), it seems that the exploitation of small molecule inhibitors of these molecules may offer an appealing opportunity for this malignancy.

The results of fragment-based screening techniques and structure-based design approaches on CDKs have led to the identification of multiple efficient and synthetically tractable sites for drug design, which has ultimately led to the development of the potent pan-CDK inhibitor AT7519 that is currently in the early phase of clinical development (Wyatt *et al.*, 2008). The wide cytotoxic activity of the agent on diverse cancer cells ranging from solid tumors (Kang *et al.*, 2018; Xi *et al.*, 2019) to hematologic malignancies (Squires *et al.*, 2010; Wang *et al.*, 2018) coupled with its favorable pharmacokinetic profile positions AT7519 as one of the most promising CDK inhibitors. Notably, in a study conducted in mice with neuroblastoma xenografts, it has

*Address correspondence to: Davood Bashash, d.bashash@sbmu.ac.ir
Received: 21 October 2019; Accepted: 16 December 2019

been demonstrated that the drug was stable in the plasma of mice under different conditions (Dolman *et al.*, 2014). In another study, the antitumor activity was also investigated in a clinical trial in refractory solid tumors, outlining that AT7519 at doses below to its dose-limiting toxic (DLT) effects could effectively suppress the activation of CDKs (Mahadevan *et al.*, 2011). Although previous studies reported the noticeable anti-tumor effects of AT7519, the precise mechanisms of action of this multi-CDK inhibitor in cancer cells has not yet been fully clarified. To the best of our knowledge, we report for the first time that the efficiency of CDK inhibition is reinforced in CML cells through both PI3K and c-Myc suppression. Moreover, we found that the activation of autophagy may act in opposition to the antileukemic effect of AT7519 and, thereby, blockage of this system could bring advantages for CDK inhibition in CML cells. Thus, we propose a plausible resistance mechanism for the inhibitor in leukemia.

Materials and Methods

Cell culture

K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37°C. Stock solutions of AT7519, CAL-101, 10058-F4, Bortezomib (Selleckchem), Imatinib, and an autophagy inhibitor, chloroquine (CQ) (Sigma), were provided. CML-derived cells were incubated with desired concentrations of each agent either alone or in a combined-modality. For negative control, K562 cells were also treated with equal concentrations of the solvent.

MTT assay

We applied MTT assay to evaluate the suppressive effects of AT7519 on the metabolic activity of K562 cells either alone or in combination with other agents. Cells were treated with relevant concentrations of drugs up to 48 h in 96-well plates. Next, the media were removed and MTT solution (5 mg/mL in PBS) was added to the cells. Using dimethyl sulfoxide (DMSO, Sigma), the resulting formazan was solubilized and the absorption was determined in ELISA reader at 570 nm.

Trypan blue staining assay

We applied trypan blue assay to evaluate suppressive effects of AT7519 on the growth kinetic and viability of K562 cells either alone or in combination with other agents. To this end, cell suspensions were centrifuged and cell pellets were re-suspended in serum-free complete medium. Next, we mixed cell pellets with trypan blue dye (Invitrogen). After 1 min, viable cell count was assessed using a Neubauer hemocytometer, and then the percentage of viability was determined.

DNA content analysis using PI staining

The inhibitory effect of CDK inhibition on cell cycle progression was investigated using flow cytometric analysis. After treatment for 48 h, cells were washed and fixed with 70% ethanol. In the next step, to stain DNA and degrade RNA, we used propidiumiodide (PI) and RNase, respectively. Finally, by using Windows FlowJo V10 software, DNA content was quantified and the data were interpreted.

Investigating DNA synthesis rate using BrdU assay

To assess the suppressive effects of AT7519 on cell growth and DNA synthesis, we performed BrdU assay using ELISA kit of 5-Bromo-2-deoxyuridine cell proliferation assay (Roche, Mannheim, Germany). After treatment of K562 cells with different concentrations of AT7519, cells were exposed to BrdU labeling solution, peroxidase-conjugated anti-BrdU antibody, and TMB. The reaction product was measured at 450 nm. The detailed procedure was reported previously by Bashash *et al.* (2013).

Calculation of combination index (CI) and dose reduction index (DRI)

To find whether AT7519 and Imatinib act in a synergistic or additive manner, we applied the classic isobologram equation [CI=(D)1/(Dx)1+(D)2/(Dx)2] to compute the combination index (CI). While (Dx)1 and (Dx)2 are the concentrations of single agents of Imatinib and AT7519 necessary to produce a given level of suppressive effect on viability, (D)1 and (D)2 indicate the concentrations of the agents required to inhibit viability at the same level in combination. CI values greater than, equal to, and less than 1 point out synergism, cumulative impact, and antagonism of the agent, respectively.

Apoptosis analysis using Annexin-V assay

The apoptotic effect of AT7519 on CML-derived cells was evaluated by Annexin-V staining assay. After treatment for 48 h, cells were washed with PBS and then suspended in the incubation buffer. Next, we added 2 µL/sample of Annexin-V-Flous and incubated for 20 min in the dark. Finally, the intensity of fluorescence was quantified using flow cytometry.

qRT-PCR analysis

High Pure RNA Isolation Kit (Roche) was used to extract total RNA after 24 h exposure to AT7519. Afterwards, cDNA Synthesis Kit (Takara Bio) was used to perform reverse transcription (RT) reaction. The expression of cell cycle- and autophagy-related genes were investigated by quantitative real-time PCR (qRT-PCR). To this end, we applied 40 cycles including an initial activation step (30 s at 95°C), a denaturation step (5 s at 95°C), and a combined annealing/extension step (20 s at 60°C). Fold change in the expression of each gene was calculated relative to GAPDH, as the internal control, according to $2^{-\Delta\Delta ct}$ formula. Melting curves were also analyzed to verify single PCR product of each primer.

Acridine orange staining assay

To investigate the contributory role of autophagy in AT7519 cytotoxicity on K562, cells were treated with the non-toxic concentration of autophagy inhibitor CQ (40 µM). After washing with PBS, we added 1 µg/mL acridine orange (Merck) to each well and incubated for 15 min in the dark. Finally, by using a fluorescence microscope (Labomed), we

provided images representing the differences in acidity of autophagic lysosomes and cytoplasm/nucleolus.

Statistical analysis

We present our data as the mean ± standard deviation (S.D.) of three separate experiments. Significant differences were determined using *t* student test, SPSS, and one-way variance analysis.

Results

CDK inhibition using AT7519 inhibited cell growth and induced G2 cell cycle arrest

Previous studies have demonstrated that G0 to G1 transition-related genes such as Myc, FOS, and Jun are overexpressed in BCR/ABL-positive cells, indicating that the cell cycle in CML cells is impaired (Jena *et al.*, 2002). Thus, it was reasonable that the inhibition of cyclin-dependent kinases (CDKs) and, consequently, their related cyclins in CML cells with BCR/ABL transformation would be an effective strategy to prevent the cell cycle progression. In light of the pan-CDK inhibitory effect of AT7519, we aimed to investigate the cell cycle inhibitory effect of this compound on CML-derived K562 cells using flow cytometric analysis. Consistent with the growth inhibitory effect of AT7519 on K562 cells, as evident by the decreased viable cell count and suppressed DNA synthesis (Fig. 1A), the percentage of cell population in S phase was decreased in inhibitor-treated K562 cells (Fig. 1B). As presented in Fig. 1, our results also show that exposure to CDK inhibitor AT7519 imposed cell accumulation in G2/M phase, which was in agreement with the induction of cyclin-dependent kinase inhibitors p21 and p27.

Concentration- and time-dependent inhibitory effects of AT7519 on K562 cell survival

Previous studies have reported that the activity of cyclin-CDK complexes is a prerequisite for viability and normal development of hematopoietic progenitors (Hofmann *et al.*, 2001). To explore whether the inhibition of CDKs using AT7519 is associated with the induction of anti-survival effect on K562 cells, both trypan blue and MTT assays were applied in the presence of increasing concentrations (0.5–1 μM) of the inhibitor at different time intervals. Our results reveal that the treatment of the cells with AT7519 decreased cell viability in a concentration-dependent manner. As represented in Fig. 2, 48-h treatment with the drug at the concentrations of 0.5, 0.75, and 1 μM reduced cell viability to 91%, 86%, and 71%, respectively. Time-dependent experiments also showed that K562 cell metabolic activity was hindered upon exposure to the inhibitor with maximal decrease observed in 1-μM-treated cells after 48 h (Fig. 2). Different experimental approaches strongly support the view that CDKs play a prominent role in apoptotic cell death that is distinct from their regulatory role in the cell division cycle. It has also been demonstrated that cyclins A, B, D, and E have a pro-apoptotic role (Borgne and Golsteyn, 2003). To investigate whether AT7519-induced cytotoxicity is mediated through the induction of apoptosis in K562, the appearance of phosphatidylserine (PS) residues on the surface of the cells was assessed by Annexin-V staining assay. In corroboration with the elevated cell population in sub-G1, we found that Annexin-V and Annexin-V/PI positivity increased after 48 h of treatment with 1 μM of the inhibitor (Fig. 2).

Stimulatory effect of autophagy inhibition on AT7519 antileukemic effect in K562 cells

It has been demonstrated that p21 and p27 cyclin-dependent kinase inhibitors may regulate autophagy, which proposes the existence of coordinating stress responses connecting autophagy and cell cycle arrest (Mathiassen *et al.*, 2017). Mouse hematopoietic precursor cells harboring BCR/ABL are also highly dependent on autophagy, although at a low basal level (Helgason *et al.*, 2011). The role of autophagy in CML cells together with the tight interaction between autophagy and progression of cell cycle tempted us to investigate the mRNA expression of autophagy target genes including ATG-7 and ATG-10 using qRT-PCR. Our results demonstrate that the incubation of K562 cells with AT7519 suppressed the mRNA expression levels of the aforementioned genes (Fig. 3), indicating that the inhibition of CDK in K562 cells was associated with the alteration of the autophagy system. Although different functions of autophagy have been reported in cell survival and cell death, several studies propose that autophagy mainly acts in favor of cell survival in the context of stress responses induced by anticancer agents (Borgne and Golsteyn, 2003). To investigate whether the inhibition of autophagy-related genes expression acts as a protection or execution mechanism of cell death, inhibitor-treated cells were exposed to the autophagy inhibitor chloroquine (CQ), and then the cell viability was assessed. The resulting data show that the autophagy inhibition, as revealed by the decreased excitation of acridin florescent, decreased the viability of inhibitor-treated K562 cells (Fig. 3), indicating that the inhibition of autophagy in AT7519-treated K562 cells serves as an executioner of cell death.

Inhibition of PI3K and c-Myc enhanced AT7519 cytotoxicity in K562 cells

Due to the over-activated PI3K in the BCR/ABL-positive K562 cells, it is likely that the cytotoxic effects of the CDK inhibitor were affected by this pathway. To investigate the PI3K interference on the cytotoxic effects of CDK inhibition, AT7519-treated cells were co-treated with PI3K inhibitor CAL-101 for 24 h. The resulting data show that the combination of CAL-101 and AT7519 had stronger cytotoxicity when compared to either drug alone (Fig. 4), indicating that the hyperactivated PI3K signaling pathway probably attenuated the cytotoxic effects of AT7519 on CML-derived K562 cells. Accordingly, our results reveal that the inhibition of c-Myc, as a significant target of PI3K, resulted in a superior cytotoxicity in AT7519-treated cells (Fig. 4), further strengthening our hypothesis pointing to the inhibitory effect of PI3K pathway on AT7519 cytotoxicity.

AT7519 induced cytotoxicity in CML-derived K562 cells in a proteasome-independent manner

According to previous studies, inhibition of transcriptional CDKs may induce cell death through the downregulation of

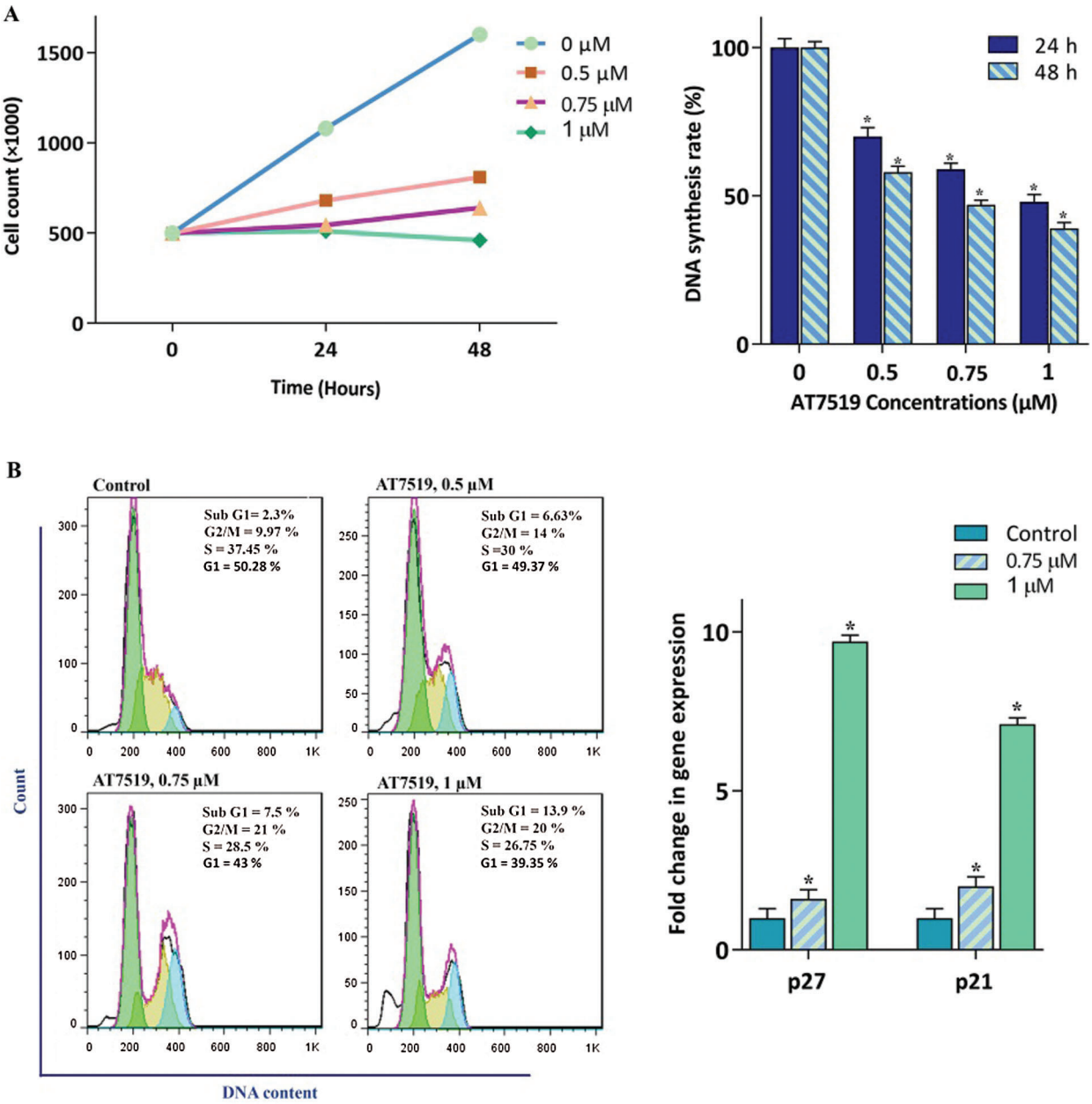

FIGURE 1. AT7519 reduced the proliferative effect of K562 cells via inducing G2/M arrest.
(A) Treatment of K562 cells with increasing concentrations of AT7519 reduced the number of K562 viable cells as well as the ability of cells to replicate DNA. (B) Evaluating the effect of the agent on the distribution of cells in different phases of the cell cycle revealed that incubation of the cells for 48 h with AT7519 halted the transition of cells from G2/M phase (blue-colored zone) of the cell cycle by elevating the expression levels of p21 and p27. Moreover, the distribution of cells in other phases of the cell cycle, including G1 (green-colored zone) and S (yellow-colored zone), declined significantly. Values are given as mean ± S.D. of three independent experiments. *$P \leq 0.05$ represents significant changes from untreated control.

several short-lived proteins, including the anti-apoptotic protein XIAP (Reed, 2006). Investigating the impact of the inhibitor on the mRNA expression level of this gene showed that the incubation of K562 cells with AT7519 suppressed the transcriptional level of XIAP (Fig. 5). It has been demonstrated that XIAP inhibition decreased the activity of proteasome in a caspase-dependent manner, which in turn stimulated the proteasome recovery pathway (Carter *et al.*, 2013). Given this and based on the important role of the XIAP/proteasome pathway in the cell cycle progression, we hypothesized that the inhibition of XIAP is likely associated with the increased proteasome activity. To test our hypothesis, we evaluated the effect of proteasome inhibition on AT7519 cytotoxicity by investigating K562 cell viability upon co-treatment with the CDK inhibitor and the well-known proteasome inhibitor bortezomib. As presented in Fig. 5, time-dependent experiments revealed that bortezomib induced a minimal effect on AT7519 cytotoxicity in K562, indicating that the cytotoxic effects of the inhibitor were probably not affected by the proteasome pathway.

AT7519 showed no synergistic effect with Imatinib mesylate

It has been demonstrated that the synergistic interactions between CDK and signal transduction inhibitors in human

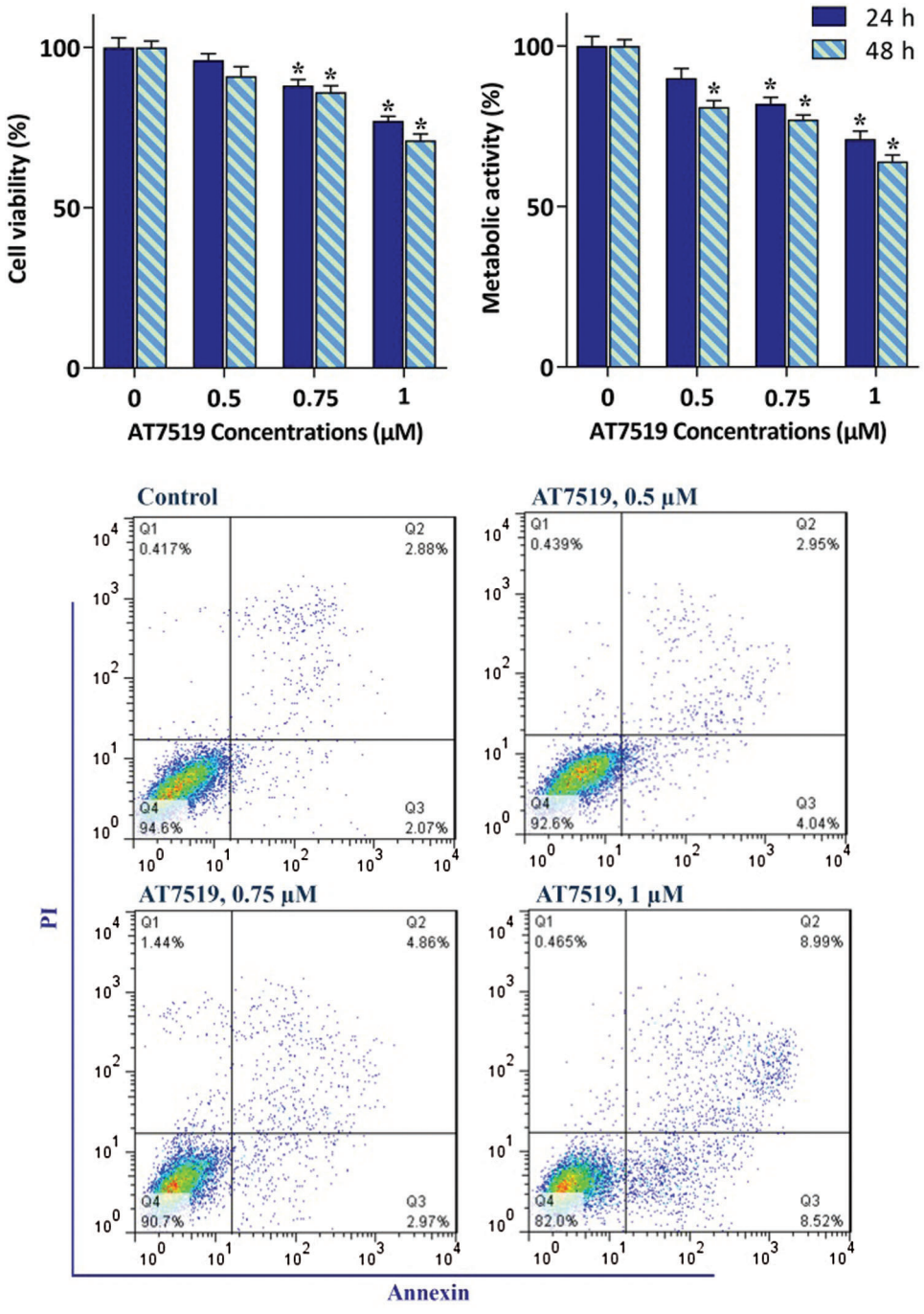

FIGURE 2. The suppression of CDK in K562 cells was coupled with induction of apoptotic cell death. AT7519 not only reduced the survival capacity of cells, but also elevated the percentage of Annexin/PI double positive cells in K562 cells. Values are given as mean ± S.D. of three independent experiments. *$P \leq 0.05$ represents significant changes from untreated control.

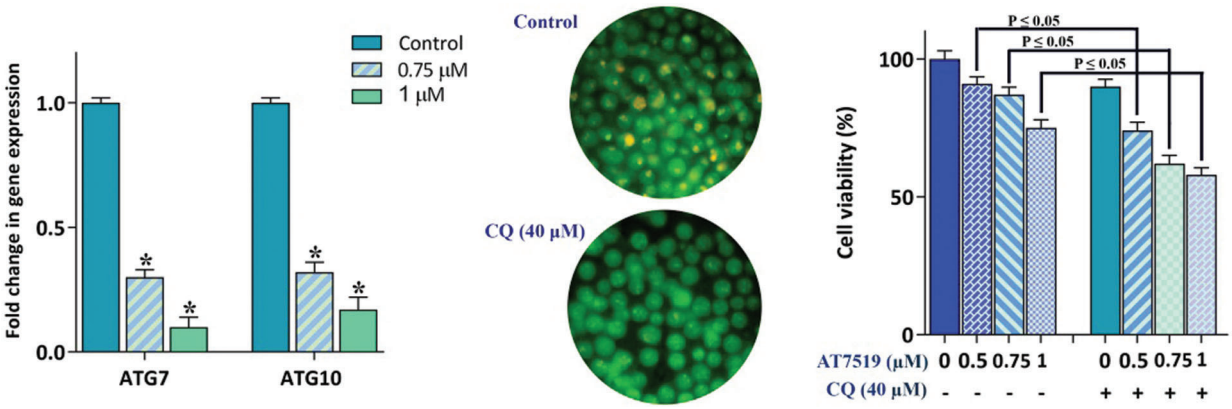

FIGURE 3. Enhanced cell death in combination of AT7519 and CQ. Not only did AT7519 reduce the mRNA of autophagy-related genes, but its effect was also enhanced upon autophagy inhibition using CQ, as evident by decreased red-to-green fluorescence. Values are given as mean ± S.D. of three independent experiments. *$P \leq 0.05$ represented significant changes from the control.

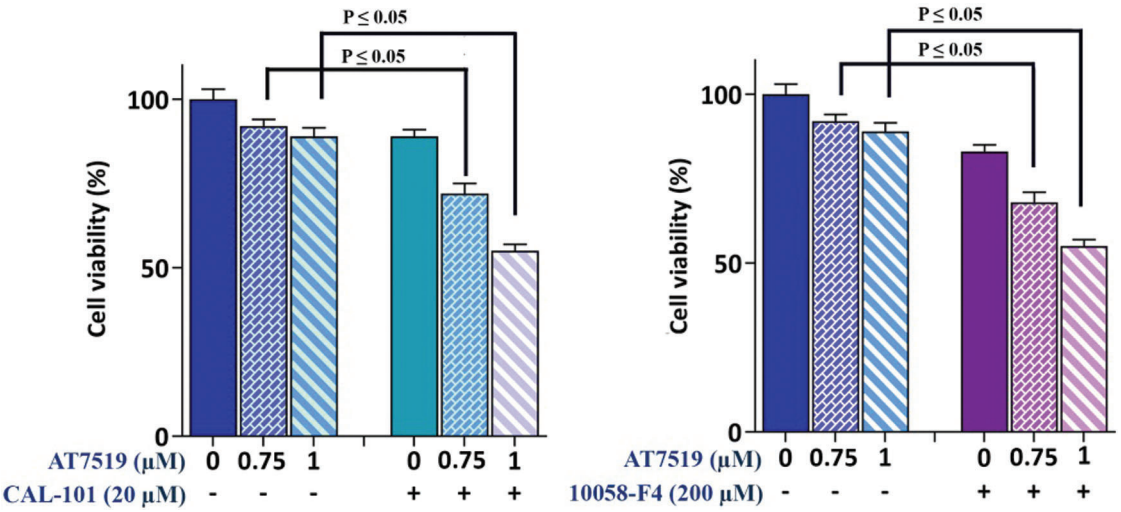

FIGURE 4. The effect of PI3K and c-Myc inhibitor on the antileukemic effect of AT7519.
The anti-survival effect of AT7519 on K562 cells was potentiated when the cells were co-treated with either PI3K inhibitor CAL-101 or c-Myc inhibitor 10058-F4. Values are given as mean ± S.D. of three independent experiments.

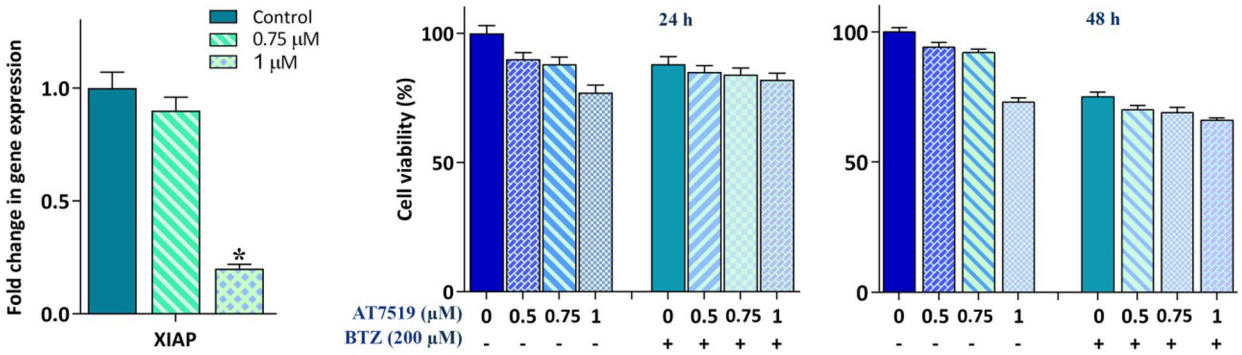

FIGURE 5. The effect of AT7519 on NF-κB anti-apoptotic target gene.
AT7519 could effectively reduce the expression level of XIAP, an important gene associated with NF-κB pathway. However, time-dependent experiments revealed that bortezomib could not potentiate AT7519 cytotoxicity in K562, indicating that the cytotoxic effects of the CDK inhibitor were probably not affected by the proteasome pathway.

cancer cells has led to the marked increase in mitochondrial damage, caspase activation, and apoptosis (Edamatsu *et al.*, 2000). To investigate whether co-treatment of K562 cells with AT7519 and Imatinib, as the first-line tyrosine kinase inhibitor used in CML, may result in an enhanced cytotoxicity, combinatorial experiments were conducted. AT7519-treated cells were subjected to Imatinib at a concentration of 500 nM, and then cell viability was assessed 24 and 48 h after treatment (Fig. 6). The results of this experiment show that there was no considerable difference in the viability of AT7519-treated cells after co-treatment with Imatinib. Our results were further confirmed by MTT assay, where we found that the combinatorial treatment of AT7519-treated K562 cells with Imatinib did not show any significant changes in cytotoxicity when compared with Imatinib alone. The values of CI and DRI achieved are presented in Tab. 1.

Discussion

The development of different generations of tyrosine kinase inhibitors (TKIs) during the past decades has intensely converted the prospect of treatment strategies for patients with chronic myeloid leukemia (CML) (Larson, 2015); however, they could not thoroughly overcome the major problem of drug resistance (Troadec *et al.*, 2015). Previous studies suggested that the reciprocal translocation between chromosomes 9 and 22 could activate multiple downstream signaling pathways, resulting in the survival and proliferation of CML cells, which in turn lead to the accelerated phase of CML (Ren, 2005). Growing studies on the molecular mechanisms involved in the proliferation of CML cells have recently highlighted the role of cyclin-dependent kinases (CDK) in the pathogenesis of this leukemia and culminated in the rigorous pursuit for small molecule inhibitors of CDK for therapeutic uses (Gesbert *et al.*, 2000; Moreno-Lorenzana *et al.*, 2016). The results of the present study show that selective CDK inhibitor AT7519 not only could effectively reduce the survival and proliferative property of BCR/ABL-expressing K562 cells, but also its antileukemic effect was coupled with the induction of G2/M cell cycle arrest as a result of the elevation in the expression levels of p21 and p27 cyclin-dependent kinase inhibitors. From the first description, the understanding of the

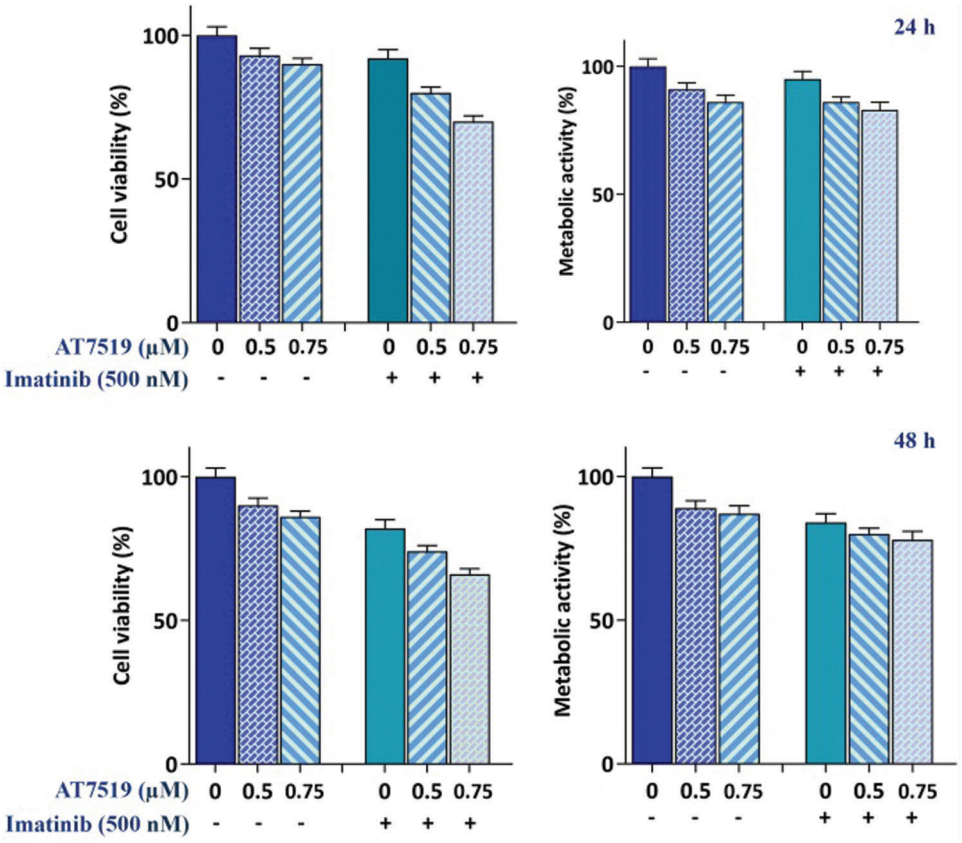

FIGURE 6. Evaluating the synergistic effect between AT7519 and Imatinib. The results of the experimental study reveal that there was no considerable difference in the viability of AT7519-treated cells after co-treatment with Imatinib.

TABLE 1

Combination index (CI) and dose reduction index (DRI) for drug combination by AT7519 and Imatinib

AT7519	Imatinib		CI
Concentration (μM)	Concentration (nM)	DRI	
24 h			
0.5	500	1.112	1.11
1	500	1.137	1.08
48 h			
0.5	500	1.07	1.006
1	500	1.11	0.99

regulatory potential of CDKs has altered dramatically and roles deviating from their canonical activity have been reported (Krystof and Uldrijan, 2010; Sohn *et al.*, 2006). Our data indicate that the inhibition of CDK in K562 was coupled with the induction of apoptotic cell death, as revealed by the elevation in the proportion of Annexin-V/PI stained cells and sub-G1 cell population. This finding is in agreement with the results of a recent study, which showed that AT7519 exerted a promising therapeutic effect on multiple myeloma through induction of apoptosis (Santo *et al.*, 2010).

The sensitivity of tumor cells to apoptosis depends on the extent of the anti-apoptotic signals mostly transmitted through oncogenic pathways (Martini *et al.*, 2014). A mounting body of evidence indicates that the PI3K axis is activated in CML cells as a result of BCR/ABL fusion protein, providing a platform for malignant cells to survive and proliferate more robustly (Ding *et al.*, 2013). Of note, we found that, upon the inhibition of PI3K, AT7519 was able to more vigorously diminish the survival and proliferative rate of K562, which may indicate the involvement of the PI3K axis in the mechanism of action of the inhibitor. In accordance with our finding, other studies have also announced the ability of PI3K inhibitors to potentiate the cytotoxic effect of several small molecule inhibitors in human leukemia (Bashash *et al.*, 2019; Riyahi *et al.*, 2019), indicative of the attenuating role of the PI3K pathway on the death signals induced by different anticancer drugs. Dolman *et al.* (2015) indicated that AT7519 could exert a prominent anti-cancer effect in both MYCN-amplified neuroblastoma cell lines and AMC711T xenografts through reductions in the levels of

phosphorylated retinoblastoma. Another study has also declared that the suppression of c-Myc is the main mechanism through which AT7519 induces apoptotic cell death in multiple myeloma cells (Santo *et al.*, 2010). Due to its placement at the downstream of the PI3K network and given it is tightly knit with CDKs (Zhu *et al.*, 2008), we assumed that c-Myc is probably involved in the mechanism of action of AT7519. In accordance, our data reveal that the inhibition of c-Myc resulted in a superior cytotoxicity in AT7519-treated cells, further strengthening our hypothesis pointing to the inhibitory effect of PI3K signaling pathway on AT7519 cytotoxicity (Fig. 7).

Upon various transcription factors, foremost c-Myc, autophagy is activated and subsequently protects neoplastic cells from the death stimuli (Cianfanelli *et al.*, 2015). In agreement with the effect of c-Myc inhibition on the antileukemic property of AT7519, we found that CDK inhibition decreased expression levels of autophagy-relate genes ATG-7 and ATG-10. Although the oncogenic role of the autophagy system has been well-determined in different cancer cell types, in many cases, there are conflicting results. While the results of previous studies demonstrate that the suppression of autophagy could potentiate the antileukemic effect of several small molecule inhibitors in human leukemia (Livesey *et al.*, 2009; Sheikh-Zeineddini *et al.*, 2019), other studies have failed to find a correlation between this system and the acquisition of chemo-resistance phenotype (White and DiPaola, 2009). Thus, we aimed to investigate whether the inhibition of autophagy acts as a protection or execution mechanism of cell death. To the best of our knowledge, to date, no study has addressed the effect of autophagy on CML cell response to AT7519, and, herein, we propose for the first time that the suppression of autophagy boosted AT7519 cytotoxicity on K562, indicating that the inhibition of autophagy may act in parallel to the cytotoxic effect of AT7519. In conclusion, our preclinical results show that selective CDK inhibitor AT7519 had anti-tumor activity against CML cells and suggest that the efficacy of this agent may be potentiated in CML cells in the presence of either PI3K or c-Myc inhibitors. Since AT7519 is an ongoing drug in the therapeutic approaches of human cancers, the results of this study shed new light on the mechanism of action of this inhibitor in leukemic cells.

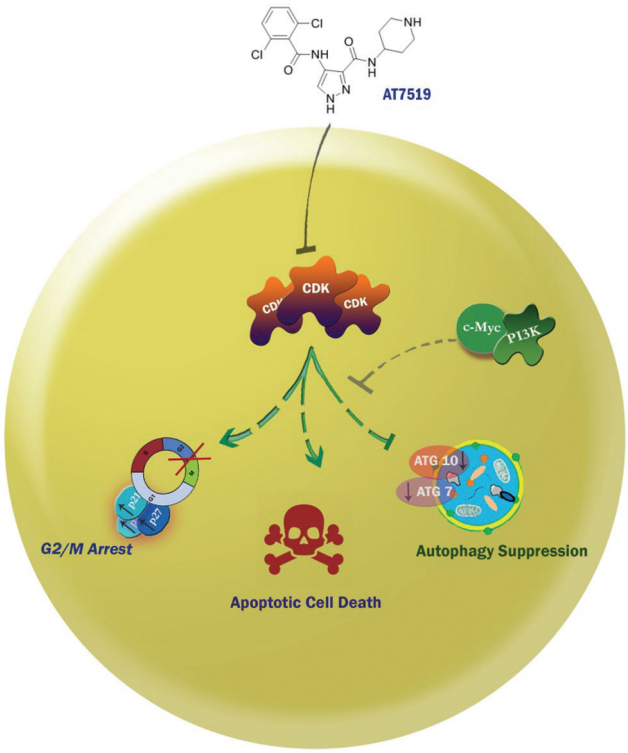

FIGURE 7. Schematic representation proposed for the plausible mechanism by which AT7519 induced apoptosis in CML cells.
By suppression of CDKs and elevating the expressions of p21 and p27, AT7519 induced its anti-proliferative effect in K562 cells by hampering the transition of K562 cells from G2/M phase of the cell cycle. Notably, scrutinizing the underlying molecular mechanisms of action of the inhibitor highlighted the attenuating role of c-Myc and/or PI3K pathway(s) on the efficacy of AT7519 in either suppression of autophagy or induction of apoptosis in K562 cells.

Acknowledgement: Authors would like to express their gratitude to Shahid Beheshti University of Medical Sciences (Tehran, Iran) for supporting this study.

Funding Statement: This study was funded by the grant number: 15425 from Shahid Beheshti University of Medical Sciences (Tehran, Iran) (http://en.sbmu.ac.ir/) achieved by D. B.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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