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Small molecule inhibitor of c-Myc 10058-F4 inhibits proliferation and induces apoptosis in acute leukemia cells, irrespective of PTEN status

Davood Bashash[a,*], Mohamad Sayyadi[b], Ava Safaroghli-Azar[b], Negar Sheikh-Zeineddini[b], Niknam Riyahi[b], Majid Momeny[c]

[a] Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Student Research Committee, Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Cancer Cell Signaling, Turku Center for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland

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ABSTRACT

Based on the frequent over-expression of c-Myc in hematologic malignancies and its crucial role in the regulation of diverse oncogenic pathways involved in leukomogenesis, intense interest has recently focused on this factor as an appealing therapeutically target in leukemia. In the present study, we aimed to investigate the anti-leukemic property of small molecule inhibitor of c-Myc 10058-F4 in two distinct acute leukemia cell lines consist of acute promyelocytic leukemia (APL)-derived NB4 cells (with wild-type PTEN) and acute lymphoblastic leukemia (ALL)-derived Nalm-6 cells (with down-regulated PTEN). 10058-F4 effectively reduced survival of leukemic cells; however, we found a different cell sensitivity pattern in the tested cell lines. To the best of our knowledge, no study has addressed the underlying mechanisms responsible for leukemic cell resistance to 10058-F4, and we propose for the first time that the effectiveness of c-Myc inhibition might be attenuated through over-activated phosphoinositide 3-kinase (PI3K) in less sensitive Nalm-6 cells. Notably, we failed to find an obvious correlation between PTEN status and cell sensitivity to the inhibitor in a panel of hematologic malignant cells. Beyond 10058-F4 cytotoxicity as a single agent, synergistic experiments also delineated that pharmaceutical targeting of c-Myc could potentiate the anti-cancer effect of both vincristine and Arsenic trioxide in ALL and APL cells, respectively. In conclusion, this study sheds light on the potent anti-leukemic characteristics of 10058-F4 and provide an interesting evidence to the application of this agent in combination with PI3K inhibitors especially in acute leukemia with over-activated PI3K, irrespective of PTEN status.

1. Introduction

The intensive molecular and genetic investigations have recently disclosed a unique as well as a strong role for c-Myc oncogene in the regulation of cell growth and differentiation in the hematopoietic system (Wilson et al., 2004). As the knowledge about the intercellular function of c-Myc grows, more association between the aberrant expression of this oncogene and leukomogenesis are discovered (Hoffman et al., 2002). It has been demonstrated that the amplification of c-Myc and its chromosome translocation are frequently involved in the initiation and progression of both myeloid and lymphoid acute leukemia (Delgado and León, 2010). The importance of this oncoprotein in acute leukemia has also loaned from the recent large-scale expression data reflecting that c-Myc acts as a bridge between various signaling pathways, foremost p53, nuclear factor (NF)-κB, and PI3K (Schlee et al., 2007; Sachdeva et al., 2009; Tsai et al., 2012). Notably, the prominence of c-Myc in acute leukemia is not restricted only to the maintenance of leukemic cell survival and recently, tremendous attention to this molecule is due to its fundamental role in inducing chemo-resistant phenotype (Xia et al., 2015). Given these, it is not surprising that c-Myc is considered as an appealing druggable factor and exploitation of small molecule inhibitors of this protein turns to be intensively debatable in future therapeutic approaches.

Among different mechanisms to halt c-Myc on the path of cancer formation, 10058-F4 perturbs the ability of c-Myc to form productive DNA-binding heterodimers (Fletcher and Prochownik, 2015). The favorable anti-cancer property of this inhibitor has been described in several studies, either in the context of *in vitro* or *in vivo* investigations

* Corresponding author at: Department of Hematology and Blood Banking, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail address: d.bashash@sbmu.ac.ir (D. Bashash).

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(Huang et al., 2006; Guo et al., 2009; Carabet et al., 2018). Lin et al. showed that 10058-F4 induces an apoptotic cell death in a panel of hepatocellular carcinoma cell lines through activation of mitochondrial pathway (Lin et al., 2007). Moreover, in a study conducted by Wang et al. (2014), it was demonstrated that suppression of c-Myc could reduce the intracellular level of ROS and thereby induce a caspase-3-dependent apoptosis in both ovarian carcinoma cell lines and primary cultures (Wang et al., 2014). Recently, the results of another study in hypopharyngeal carcinoma cells delineated that 10058-F4 could exerts its anti-cancer effects through modulation of glycolytic and glutaminolytic enzymes; suggesting that c-Myc suppression may be a promising strategy in head and neck carcinomas (Kleszcz et al., 2018). There are also several levels of evidence showing that 10058-F4 may find potential application to be used in a combined-modality strategy (Lin et al., 2007; Zhang et al., 2015). Although the results from the laboratory experiments provided a significant evidence for the prominent anti-cancer effects of 10058-F4, both the efficacy and the precise molecular mechanisms involved in leukemic cell response to the inhibitor have not yet been fully clarified. Apart from genetic aberrations in c-Myc, any disturbance in the PI3K or PTEN (the most important negative regulator of PI3K) has been shown to provide a signal which can increase the expression level of c-Myc in neoplastic cells (Zhang et al., 2016). Given these, the present study aimed to evaluate the anti-leukemic activity of c-Myc inhibition in two distinct acute leukemia cell lines consist of acute promyelocytic leukemia (APL)-derived NB4 cells (with wild-type PTEN) and acute lymphoblastic leukemia (ALL)-derived Nalm-6 cells (with down-regulated PTEN).

2. Materials and methods

2.1. Cell lines and reagents

To investigate the impact of cMyc inhibition on acute leukemia cells, pre-B ALL-derived Nalm-6 and APL-derived NB4 cells were chosen. The cells were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Life Technologies, Carlsbad, CA) in a humidified atmosphere with 5% CO_2 at 37 °C. A stock solution of 10058-F4 (Selleckchem) was diluted in culture medium to attain the concentrations of 50–200 μM. For extensive experiments, cell lines were also treated with desired concentrations of a pan-PI3K inhibitor (BKM120), isoform-specific PI3K inhibitor (CAL-101) and chemotherapeutic drugs (Vincristine and ATO), as well.

2.2. Trypan blue exclusion assay

The inhibitory effect of 10058-F4 on the viability and cell count of acute leukemia cell lines was assessed by the uptake of trypan blue dye by viable cells. Briefly, the cells were plated at the density of 250×10^3 cells/well and were incubated in the presence of the designated concentrations of 10058-F4, either alone or in combined modality. After indicated treatment intervals, one part of 0.4% trypan blue (Invitrogen) and one part of cell suspension were mixed and then the mixture was allowed to incubate for 1–2 min at room temperature. The total number of viable (unstained) and nonviable (stained) cells were manually counted and determined. Finally, percentage of viable cells was calculated.

2.3. MTT assay

Microculture tetrazolium assay (MTT) was applied to explore the impeding effect of 10058-F4, either as a single agent or in combination with the indicated agents, on the ability of acute leukemic cells to metabolize thiazolyl blue tetrazolium bromide into formazan crystals. The cells were seeded at the density of 5000/well into 96-well plates and incubated for 24 h with the indicated concentrations of drugs. Afterwards, 100 μL of MTT solution (5 mg/mL in PBS) was added to each well and plates were incubated at 37 °C and 5% CO_2. The resulting formazan solubilized with DMSO and the absorption was measured at 570 nm in ELISA reader.

2.4. BrdU cell proliferation assay

Bromodeoxyuridine (BrdU)-based cell proliferation ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to assess the anti-proliferative effect of 10058-F4 on Nalm-6 and NB4 cells. Acute leukemia cells were grown in the presence of the different concentrations of c-Myc inhibitor in a 96-well plate and were incubated at 37 °C for 24 h. Afterwards, 100 μL/well of BrdU labeling solution was added, and the cells were re-incubated at 37 °C. The cells were then fixed and DNA was denatured using FixDenat solution. Following incubation with peroxidase-conjugated anti-BrdU antibody, wells were washed. Finally, the cells were exposed to 100 μL of substrate tetramethyl-benzidine (TMB) for 3 min at room temperature and the reaction product was quantified by measuring the absorbance at 450 nm in an ELISA reader.

2.5. Median-effect analysis of drug combinations

To evaluate the interaction between 10058-F4 and ATO or vincristine, the combination index (CI) was computed using the method developed by Chou and Talalay (Chou, 2010) and the computer software CalcuSyn according to the classic isobologram equation. CI = (D)1/(Dx)1 + (D)2/(Dx)2, where (Dx)1 and (Dx)2 indicate the individual dose of ATO/Vincristine and 10058-F4 required to inhibit a given level of viability index, and (D)1 and (D)2 are the doses of ATO/Vincristine and 10058-F4 necessary to produce the same effect in combination, respectively. The dose that may be reduced in a combination for a given level of effect, as compared with the concentration of the individual drug alone is defined as dose reduction index (DRI) and is calculated as follows: DRI_1 = (Dx)1/(D)1 and DRI_2 = (Dx)2/(D)2. We also calculated CI and DRI values to investigate the effects of PI3K inhibition on 10058-F4 cytotoxicity in Nalm-6 cells.

2.6. Assessment of apoptosis using flow cytometry

Annexin-V/PI staining was applied to investigate whether 10058-F4 as a single agent could induce apoptotic cell death in acute leukemia cell lines. After treatment with the designated concentrations of the inhibitor, the cells were harvested, washed with PBS and re-suspended in a total volume of 100 μL of the incubation buffer. Annexin-V-Flous (2 μL per sample) was added, and cell suspensions were incubated for 20 min in the dark. Fluorescence was then measured using flowcytometery. Annexin V-positive and PI-negative cells were considered to be in early apoptotic phase and cells having positive staining both for Annexin-V and PI were deemed to undergo late apoptosis or necrosis.

2.7. Caspase-3 activity assay

To determine whether 10058-F4-induced apoptosis is mediated through caspase-dependent cascade, we investigated the enzymatic activity of caspase-3 using a caspase-3 assay kit (Sigma). Briefly, the cells were treated with 150 μM and 200 μM of 10058-F4 and incubated at 37 °C for 24 h. Following centrifugation at 600 g for 5 min, the cell pellets were lysed and the lysates were centrifuged at 20,000 g for 10 min. In a total volume of 100 μL, 5 μg of the supernatant was incubated with 85 μl of assay buffer plus 10 μl of caspase-3 substrate in a 96-well plate at 37 °C. Cleavage of the peptide by caspase-3 released the chromophore pNA, which was quantified spectrophotometrically at a wave length of 405 nm.

2.8. Cell cycle distribution analysis

Flow cytometric analysis of DNA content and cell cycle distribution were ascertained by PI staining after 24 h incubation of Nalm-6 and NB4 cells with different concentrations of 10058-F4. In brief, 1×10^6 cells were harvested, washed twice with cold PBS, and then fixed in 70% ethanol overnight. After fixation, the cells were centrifuged to remove the ethanol, washed with ice-cold PBS, and re-suspended in staining solution containing 1 mg/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at 37 °C. After 30 min, cellular DNA content was quantified from the peak analysis of flow cytometric DNA histograms (Partec PASIII flow cytometry, Germany) and data were interpreted using the Windows TMFloMax® software.

2.9. Quantitative real-time PCR

To carry out qRT-PCR, the cells were treated with 10058-F4 even alone or in combination with BKM120, and then RNA was extracted by high pure RNA isolation kit (Roche) from the cultured cells and quantified by a Nanodrop instrument (Nanodrop ND-1000 Technologies). Thereafter, 1 μg of RNA from each sample was applied for reverse transcription using the revertAid First Strand cDNA synthesis kit (Takara BIO, Japan). The prepared cDNA was subjected to qRT-PCR using SYBR Premix Ex Taq technology (Takara BIO) on a light cycler instrument (Roche). Melting curves were analyzed to validate single PCR product of each primer, and the values for the relative quantification were calculated based on $2^{-\Delta\Delta ct}$ relative expression formula. Nucleotide sequences of the primers used for qRT-PCR were listed in Table 1.

2.10. Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. All tests were performed in triplicate. The significance of differences between experimental variables was determined by the use of two-tailed Student's *t* test and by one-way variance analysis. In order to compare between the control group and the drugs-treated cells, the Dunnett's multiple comparison test was used. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Suppressive effect of c-Myc inhibitor on cell survival and proliferative capacity of acute leukemia cells

The anti-survival and growth suppressive properties of c-Myc inhibition, as revealed by the down-regulation of c-Myc expression level (Fig. 1), using 10058-F4 in pre-B ALL-derived Nalm-6 and APL-derived NB4 cells were investigated by trypan blue and MTT assays. Exposing the cells to the increasing concentrations of the inhibitor remarkably reduced cell viability, metabolic activity and proliferative capacity of both cell lines; however, the anti-leukemic effect of 10058-F4 on NB4 was more profound as compared to Nalm-6 cells (Fig. 1). Previous study has shown that c-Myc is located at the down-stream of the PI3K pathway and thereby any perturbation in this signaling axis could increase the oncogenic properties of c-Myc in cancer cells (Asano et al., 2004). In addition, it has been demonstrated that Nalm-6 cells display over-activated PI3K due to the down-regulation of PTEN (Shen et al., 2005; Li et al., 2008). Given these, it was of great interest to investigate whether the resistance to 10058-F4 in Nalm-6 cells is mediated through PI3K activation. Based on the results of our previous study which showed that the PI3K inhibition using pan-PI3K inhibitor BKM120 could effectively inhibit PI3K axis in Nalm-6 cells (Bashash et al., 2016), we designed experiments to investigate the effect of 10058-F4 in combination with BKM120. Notably, the results of time- and concentration-dependent experiments showed that the suppression of PI3K using BKM120 enhanced the cytotoxic effect of 10058-F4 and produced a synergistic effect in Nalm-6 cells (Fig. 2A and B). The values of combination index (CI) and dose reduction index (DRI) are summarized in Table 2. Intriguingly, the results of RQ-PCR demonstrated that the endorsing effect of the PI3K inhibition in less sensitive Nalm-6 cells was coupled with the down-regulation of c-Myc mRNA expression (Fig. 2C). On the other hand and based on the higher sensitivity of NB4 cells harboring wild-type PTEN, it was of great interest to find whether there is a correlation between PTEN status and leukemic cell response to 10058-F4. Based on the results of our supplemental investigations on a panel of several hematologic malignant cell lines with different PTEN status, we found that there is no obvious association between cell sensitivity to 10058-F4 and the mutation/inactivation of PTEN (Fig. 3).

3.2. -F4 altered cell distribution in different phases of the cell cycle

To shed light on the mechanisms through which 10058-F4 induced its inhibitory effect on the logarithmic growth of acute leukemia cells, both Nalm-6 and NB4 cells were treated with designated concentrations of the inhibitor and the distribution of the cells in different phases of cell cycle was examined using PI staining. Although the sensitivity of the cells was different to the inhibitor, flowcytometric analysis revealed that 10058-F4 effectively altered the distribution of the cells in cell cycle of both cell lines. As depicted in Fig. 4A, following 24 h exposure to c-Myc inhibitor, the proportion of the cells in S phase decreased in inhibitor-treated NB4 and Nalm-6 cells. This finding was further confirmed by the results of BrdU incorporation assay, which revealed a significant dose-dependent decrease in the DNA synthesis rate of acute

Table 1
Nucleotide sequences of primers used for real-time RT-PCR.

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
p21	NM_000389	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT	130
p27	NM_004064	AACGTGCGAGTGTCTAACGG	CCCTCTAGGGGTTTGTGATTCT	139
P15	NM_004936	CGTTAAGTTTACGGCCAACG	GGTGAGAGTGGCAGGGTCT	302
Bax	NM_138761	CGAGAGGTCTTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG	242
Bad	NM_004322	CCCAGAGTTTGAGCCGAGTG	CCCATCCCTTCGTCGTCCT	249
FOXO3a	NM_001455	ACGGCTGACTGATATGGCAG	CGTGATGTTATCCAGCAGGTC	85
Bcl-2	NM_000633	CGGTGGGGTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC	90
Survivin	NM_001168	CCAGATGACGACCCCATAGAG	TTGTTGGTTTCCTTTGCAATTTT	152
MCL-1	NM_021960	AGAAAGCTGCATCGAACCAT	CCAGCTCCTACTCCAGCAAC	183
c-Myc	NM_002467	CCACAGCAAACCTCCTCACAG	GCAGGATAGTCCTTCCGAGTG	105
XIAP	NM_001167	ATAGTGCCACGCAGTCTACAA	AGATGGCCTGTCTAAGGCAAA	101
c-IAP1	NM_001166	AGCACGATCTTGTCAGATTGG	GGCGGGGAAAGTTGAATATGTA	102
c-IAP2	NM_001165	TCCTGGATAGTCTACTAACTGCC	GCTTCTTGCAGAGAGTTTCTGAA	160

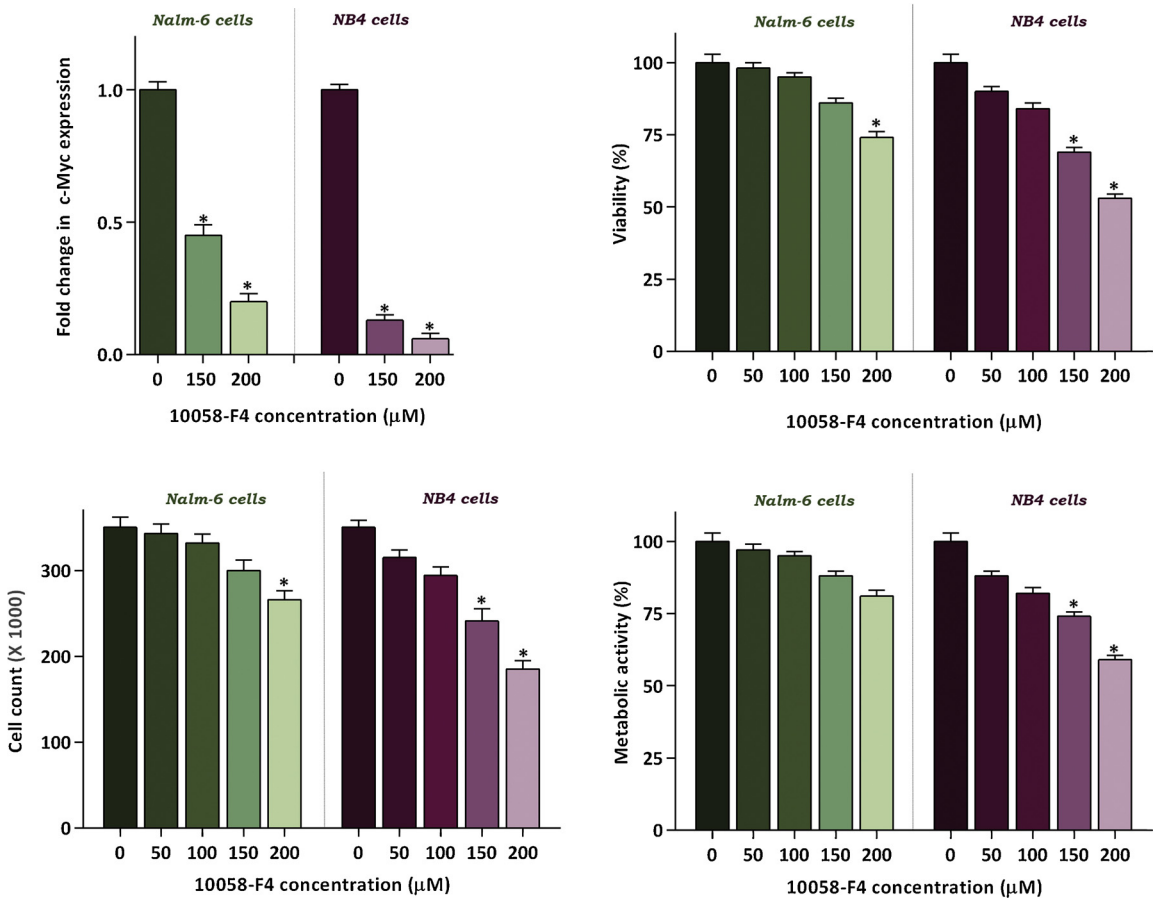

Fig. 1. Suppressive effect of c-Myc inhibition using 10058-F4 on the survival and growth potential of acute leukemia cell lines. Down-regulation of c-Myc reduced cell viability, cell count and the metabolic activity of both NB4 and Nalm-6 leukemic cells. Values are given as mean ± SD of three independent experiments. *, $P \leq 0.05$ represent significant changes from untreated control.

leukemia cells upon exposing to the inhibitor (Fig. 4A). Analysis of DNA content also showed that 10058-F4 halted the progression of cell cycle in Nalm-6, but not NB4 cells, through increasing the percentage of the cells in G1 phase. Consistently, evaluating the impact of the inhibitor on the mRNA expression level of the critical genes responsible for the transition of the cells from G1 to S phase also revealed that 10058-F4 increased the mRNA expression level of p21, p27, and p15 in wild-type p53 expressing Nalm-6 cells (Fig. 4B). Noteworthy, the expression levels of the aforementioned genes remained unchanged in NB4 cells harboring mutant p53 (Fig. 4B).

3.3. -F4 induced apoptosis and increased cell population in sub-G1

Consistent with the anti-proliferative effect of 10058-F4, the results of DNA content analysis also outlined that the blockage of c-Myc statistically increased the fraction of hypodiploid cells, an internucleosomal DNA fragmentation which is considered as an important hallmark of apoptosis, in sub-G1 phase (Fig. 5A). To assess whether 10058-F4-induced cytotoxic effect is likely due to the induction of apoptosis, inhibitor-treated cells were analyzed by Annexin-V staining assay. In corroboration with the elevated cell population in sub-G1, we found a significant elevation in the proportion of late apoptotic cells in both cell lines (Fig. 5B). As illustrated in Fig. 5B, 24 h treatment with the inhibitor resulted in the higher percentage of double positive Annexin-V/PI staining cells in NB4 (56.4%) as compared to Nalm-6 (27.8%), substantiating that NB4 cells are more sensitive to c-Myc inhibition than Nalm-6 cells. Induction of apoptosis was further investigated by caspase-3 activity assay, which disclosed that the cell death induced by 10058-F4 in both acute leukemia cell lines is primarily due to the induction of a caspase-3-mediated apoptosis (Fig. 5C).

3.4. Inhibition of c-Myc in acute leukemia cells is associated with the alteration in the expression level of apoptosis-related genes

To gain more insight into the molecular mechanisms responsible for the cell sensitivity pattern to c-Myc inhibition, we scrutinized the mRNA expression of the key target genes of apoptosis. As indicated in Fig. 6A, treatment of NB4 cells apparently altered the transcriptional activity of both anti- and pro-apoptotic target genes; however, analyzing the effect of the inhibitor on molecular feature of less sensitive cell line clearly showed that among wide-range of apoptosis-related genes, 10058-F4 mainly shifted the ratio of Bax and Bcl-2 expression (Fig. 6B). Interestingly, the results of RQ-PCR analysis showed that the combination of pan-PI3K inhibitor with 10058-F4 was able to alter the expression levels of all the indicated apoptosis-related genes (Fig. 6B); strengthening our finding which suggested that the effectiveness of the c-Myc inhibition could be overshadowed by the over-activated PI3K. It was reported that suppression of c-Myc in wild-type p53-expressing cell lines could provide a signal that up-regulates cyclin-dependent kinase inhibitors, which in turn activates the apoptotic pathway mainly through activation of FOXO3a (Chandramohan et al., 2004, 2008). As expected, under 10058-F4 treatment the mRNA expression of both p21 and FOXO3a raised steeply in a concentration-dependent manner in Nalm-6, but not in NB4 (Fig. 6C), which shed more light on the plausible p53-dependent pathway involved in induction of apoptosis in Nalm-6.

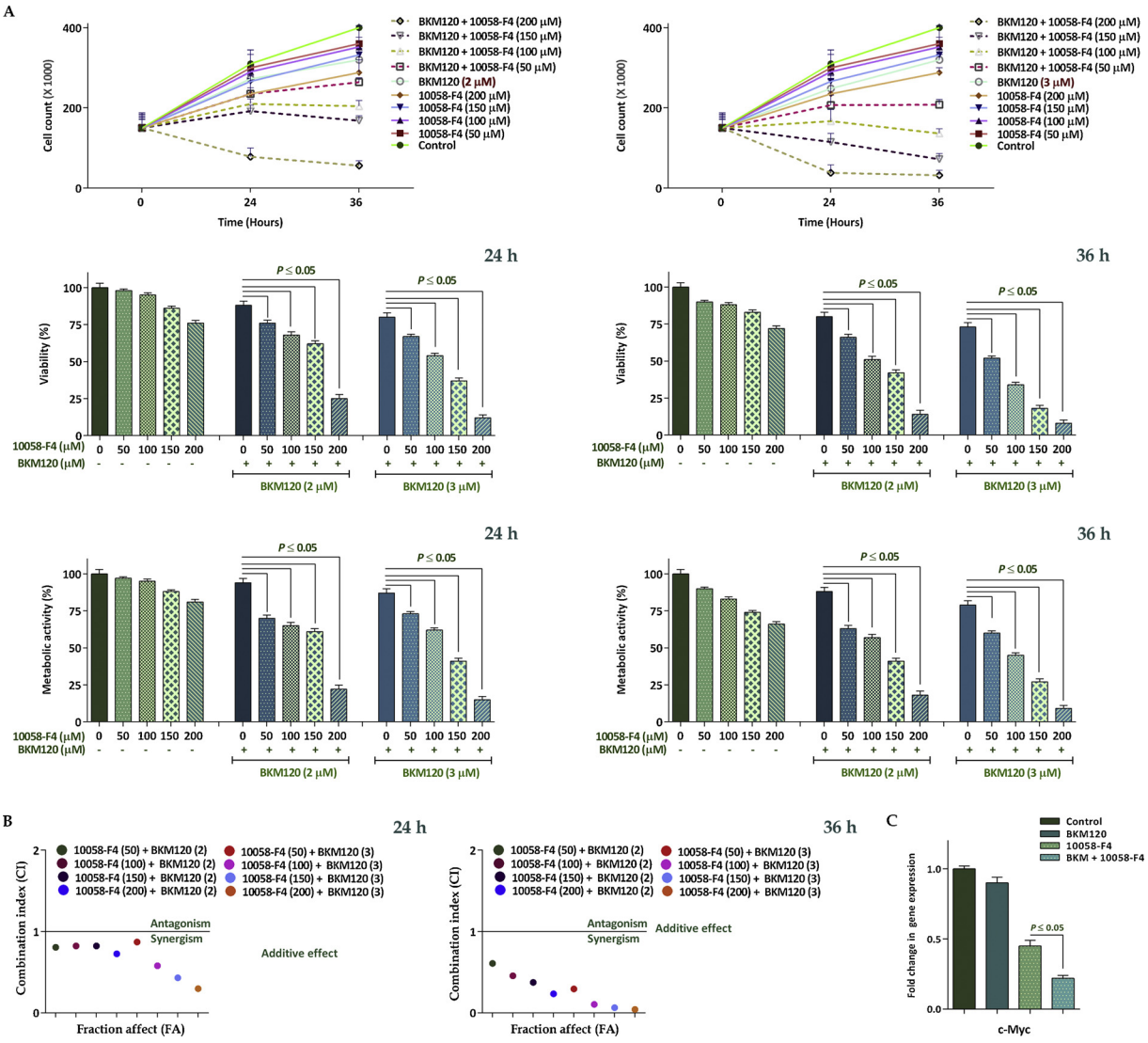

Fig. 2. Activation of the PI3K signaling pathway may be responsible for the less sensitivity of Nalm-6 cells to c-Myc inhibition. **A and B)** The results of time- and concentration-dependent experiments showed that the suppression of PI3K using BKM120 enhanced the cytotoxic effect of 10058-F4 and produced a synergistic effect in Nalm-6 cells. **C)** The results of RQ-PCR demonstrated that the endorsing effect of the PI3K inhibition was coupled with the down-regulation of c-Myc mRNA expression in Nalm-6 cells. Values are given as mean ± SD of three independent experiments.

Table 2
Combination index (CI) and dose reduction index (DRI) for drug combinations by 10058-F4 and BKM120.

10058-F4			BKM120			CI	
Concentration	DRI		Concentration	DRI			
	24 h	36 h		24 h	36 h	24 h	36 h
50 μM	4.252	7.466	**2 μM**	1.754	2.114	0.805	0.607
50 μM	5.316	14.997	**3 μM**	1.574	2.577	0.823	0.455
100 μM	2.598	7.866	**2 μM**	2.290	4.030	0.822	0.375
100 μM	3.501	18.241	**3 μM**	2.271	5.564	0.726	0.235
150 μM	1.978	8.093	**2 μM**	2.733	5.866	0.871	0.294
150 μM	3.306	33.731	**3 μM**	3.609	13.456	0.580	0.104
200 μM	3.297	36.170	**2 μM**	7.907	27.504	0.430	0.064
200 μM	5.167	76.565	**3 μM**	9.584	35.093	0.298	0.042

3.5. -F4 synergized with chemotherapeutic drugs to enhance cytotoxicity in acute leukemia cells

Intrigued by the remarkable anti-leukemic effect of 10058-F4, it was of particular interest to determine whether c-Myc inhibition could enhance the cytotoxic effect of the common chemotherapeutic drugs used in acute leukemia treatment. Nalm-6 and NB4 cells were treated with the 10058-F4 either separately or together with vincristine (VCR) and arsenic trioxide (ATO), respectively. As shown in Fig. 7, the combination of 10058-F4 with both ATO (1 μmol/l) and VCR (1 nmol/l) was more effective in inhibiting cell growth and survival as compared with either drug alone. To test whether the interactions between 10058-F4 and chemotherapeutic drugs were synergistic or caused by additive effect, the combination index (CI) and dose reduction index (DRI) were calculated. Notably, our results demonstrated that the combination of VCR and 10058-F4 had a lower impact on promoting cytotoxicity in Nalm-6, in comparison with the synergistic effect between the inhibitor and ATO in NB4, which may be due to the less sensitivity of Nalm-6 cells to the lower concentrations of the inhibitor. Values of CI and DRI are summarized in Table 3.

4. Discussion

The overriding posture of c-Myc in the pathogenesis of myriad type of cancers coupled with its undeniable role in disturbing the favorable

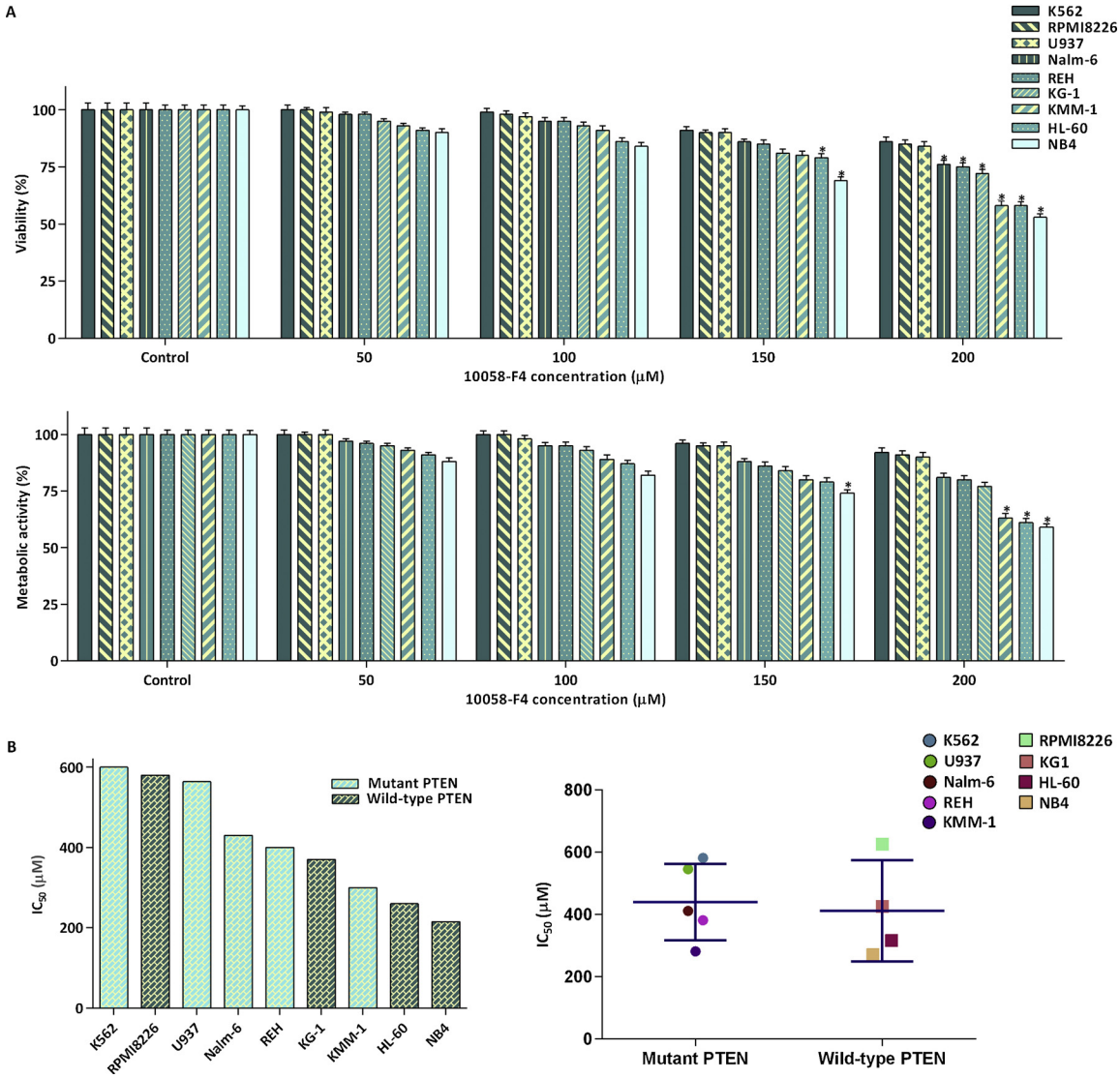

Fig. 3. Inhibitory effect of 10058-F4 on the survival and proliferative rate of hematologic malignant cell lines. **A)** After incubating the cells with increasing concentrations of the inhibitor for 24 h, the cell survival and metabolic activity were evaluated. Values are given as mean ± SD of three independent experiments. *, $P \leq 0.05$ represent significant changes from untreated control. **B)** A list of IC_{50} values of different leukemic cell lines to 10058-F4 based on their PTEN status was made. Dot blot showing correlation between PTEN status and in vitro drug sensitivity based on the IC_{50} of individual cell line. We failed to identify an obvious association between PTEN status and leukemic cell sensitivity to 10058-F4.

effects of anti-cancer agents have increased the demand for innovation and incorporation of small molecule inhibitors of c-Myc into an unbounded route of targeted therapies (Little et al., 1983; He et al., 1998; Dang et al., 2009). In this study, we aimed to investigate the anti-leukemic effect of a potent c-Myc inhibitor 10058-F4 on two distinct and well-characterized acute leukemia cell lines with different myeloid (NB4) and lymphoid (Nalm-6) origins. The results obtained in the present study showed that 10058-F4 effectively reduced the survival and proliferative capacity of both pre-B ALL-derived Nalm-6 and APL-derived NB4 cells; however, the anti-leukemic effect of the inhibitor in deficient PTEN-expressing Nalm-6 cells were achieved at the higher concentrations as compared to NB4 cells harboring wild-type PTEN. To the best of our knowledge, to date, no study has addressed the association between leukemic cell response to 10058-F4 and the molecular status of PTEN, and, herein, we proposed for the first time that there is no obvious correlation between sensitivity to the inhibitor and the mutation/inactivation of PTEN, a tumor suppressor phosphatase which negatively regulates PI3K (Song et al., 2005). Notwithstanding this fact, our investigations declared that the effectiveness of c-Myc inhibitor was overshadowed, at least partially, by the overactivated PI3K. We found that abrogating PI3K pathway using either pan-PI3K inhibitor or isoform-specific δ inhibitor significantly enhanced the anti-leukemic effect of 10058-F4 in less sensitive Nalm-6 cells (Fig. 8), which is in consistent with the recent study by tan et al. who showed that co-targeting PI3K and c-Myc not only produced a synergistic effect in Lck-Dlx5 mice but also remarkably boosted the cytotoxicity of chemotherapeutic drugs (Tan et al., 2015). Intiguingly, the results of our synergistic experiments also showed that inhibition of c-Myc in Nalm-6 and NB4 cells could vigorously enhance the cytotoxic effects of vincristine and ATO, respectively.

Beneath the complexity of every cancer, a number of common hallmarks including unlimited proliferative potential have propelled tumor cells into uncontrolled expansion and invasion (Cairns and Mak, 2016). The importance of c-Myc oncoprotien in tumorgenesis is borrowed not only from its key role in the regulation of cell cycle and proliferation but also from its influence on the expression of a wide variety of apoptosis-related genes (Dang, 2012). Our data showed that c-Myc inhibition resulted in a caspase-3-dependent apoptotic cell death

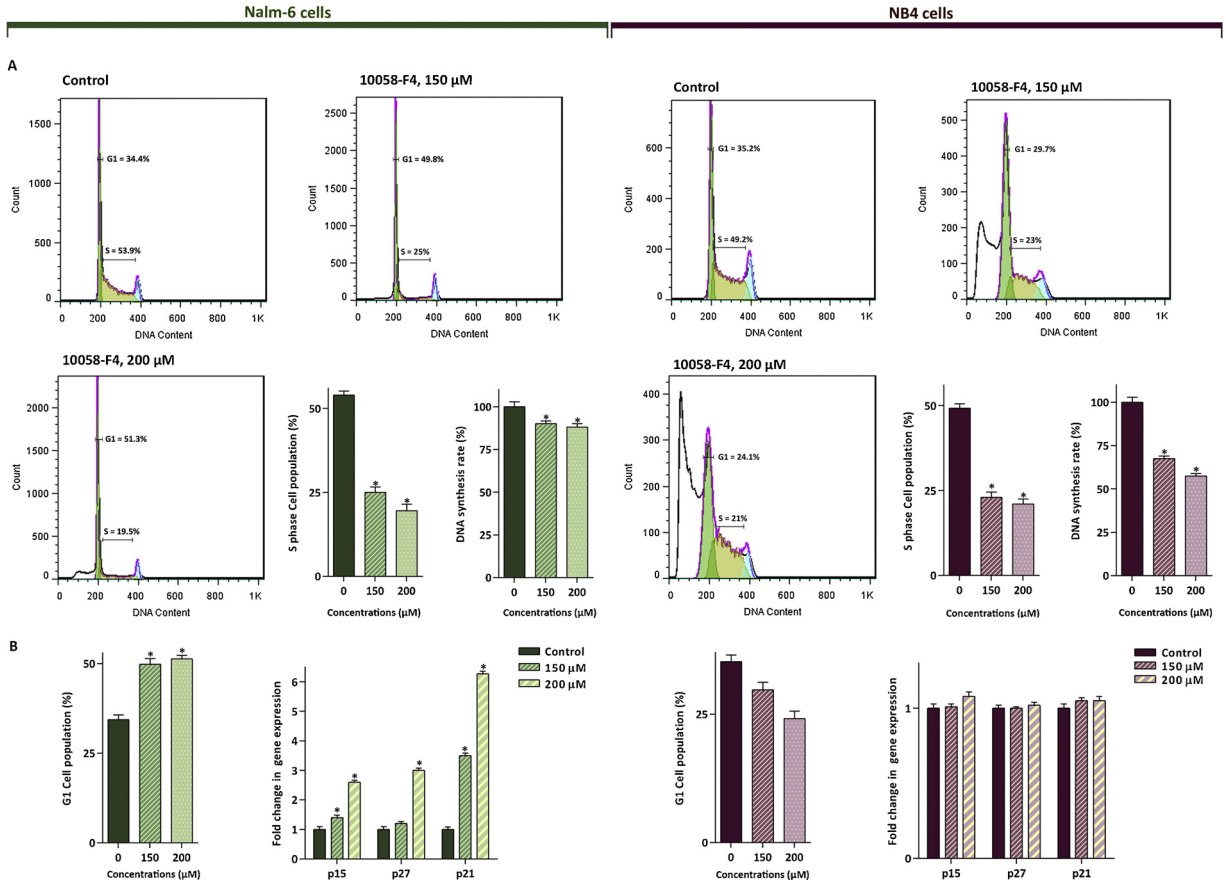

Fig. 4. A) Analysis of PI staining revealed that escalated doses of 10058-F4 reduced the number of acute leukemia cells in the S phase of cell cycle. The anti-proliferative capacity of 10058-F4 was further assessed by determining the extent of bromodeoxyuridine (BrdU) incorporation into the DNA of both Nalm-6 and NB4 cells using BrdU cell proliferation assay. As shown, the suppressive effect of the agent on cell cycle progression was coupled with decreased DNA synthesis rate of the cells in a dose-dependent manner. **B)** 10058-F4 not only increased the percentage of Nalm-6 cells in G1 phase but also elevated the mRNA expression level of p15, p27 and p21. However, this agent increased neither the proportion of NB4 in G1 phase nor the mRNA expression level of cell cycle-related genes. Values are given as mean ± SD of three independent experiments. *, $P \leq 0.05$ represent significant changes from untreated control.

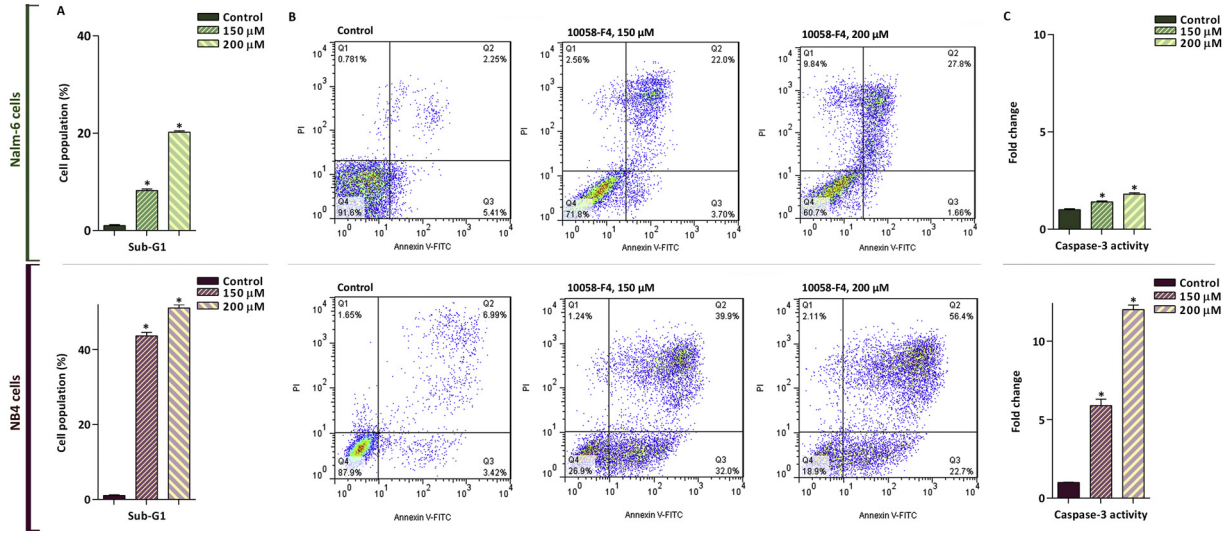

Fig. 5. A) Measuring the effect of 10058-F4 on the population of the cells in sub-G1 phase of cell cycle revealed that inhibition of c-Myc in both acute leukemia cell lines elevated the fraction of hypodiploid cells, with more prominent effect in NB4. **B)** The percentage of Annexin-V/PI double-positive inhibitor-treated cells was increased in response to drug treatment in both cell lines, as compared with the untreated group. C) To investigate the contribution of caspase-3 toward 10058-F4-induced apoptosis, inhibitor-treated Nalm-6 and NB4 cells were subjected to caspase-3 assay. As presented, 10058-F4 imposed a considerable elevation in caspase-3 activity with maximal escalation observed at 200 μM (NB4 > Nalm-6), suggestive of a caspase-3-dependent apoptotic effect of the compound on acute leukemia cell lines. Values are given as mean ± SD of three independent experiments. *, $P \leq 0.05$ represent significant changes from untreated control.

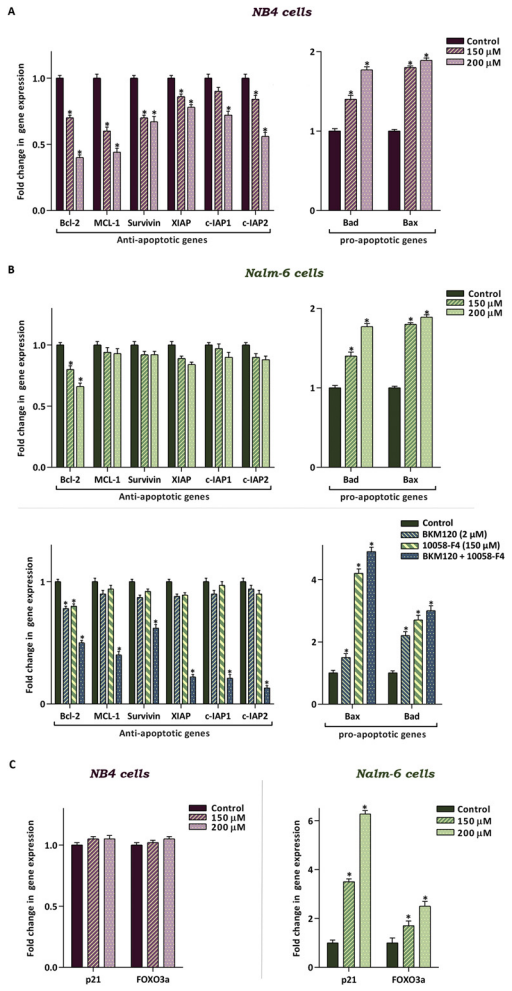

Fig. 6. Suppression of c-Myc in acute leukemia cells was associated with the alteration in the expression level of apoptosis-related genes. **A)** Treatment of NB4 cells with increasing concentrations of c-Myc inhibitor not only reduced the mRNA expression level of anti-apoptotic genes but also elevated the expression level of pro-apoptotic genes. **B)** In the less sensitive Nalm-6 cells, c-Myc inhibition only shifted the ratio of Bax and Bcl-2 expression. Noteworthy, co-treatment of the cells with PI3K inhibitor and 10058-F4 altered the expression levels of all the indicated apoptosis-related genes. **C)** Upon exposure to 10058-F4, the mRNA expression of p21 and FOXO3a raised in Nalm-6 but not in NB4 cells. Values are given as mean ± SD of three independent experiments. *, $P \leq 0.05$ represent significant changes from untreated control.

in both APL and pre-B ALL cells; however, the underlying mechanisms contributed in the anti-proliferative effect of 10058-F4 found to be different with respect to the molecular status of p53. According to the direct impact of functional p53 on the expression of cell cycle regulatory genes, and based on the inductive effect of the inhibitor on the expression of p21, p27, and p15 in wild-type p53-expressing Nalm-6 but not NB4 cells, we suggested that probably the growth suppressive effect of the inhibitor on leukemic cells harbouring wild-type p53 could be partially due to the induction of p21-mediated G1 arrest. Our finding is in accordance with the recent reports showing that abrogation of PI3K using pan-PI3K inhibitor BKM120 induced differential forms of cell death in either hematologic malignant (Bashash et al., 2016) or solid tumor cells (Ren et al., 2012) with respect to p53 status. As a central molecule involved in p53-dependent apoptosis, FOXO3a engages the mitochondria-mediated pathway of death signaling via conformational changes in Bcl-2 family (Birkenkamp and Coffer, 2003; Zhang et al., 2011). Noteworthy, the results of RQ-PCR showed that 10058-F4 not only elevated the mRNA expression level of FOXO3a in Nalm-6 but not NB4 cells, but also shifted the ratio of the death promoter to death repressor genes via alteration of Bax and Bcl-2 expression.

Perturbation of wide range of signaling pathways, which are crucial to many aspects of cell growth and survival, could determine the fate of cancer cells in response to the anti-cancer agents (Cox and Der, 2002; Altomare and Testa, 2005; Takahashi-Yanaga and Sasaguri, 2007). While functional p53 plays nemesis to most cancers, activation of nuclear factor-κB could potentiate the anti-survival capacity of malignant cells (Colman et al., 2000). Although it is early to hazard a conjecture on the mechanism by which 10058-F4 reduced the viability of mutant p53-expressing NB4 cells, a possible candidate would be NF-κB, a key cellular transcription factor that engages in a cross-talk with c-Myc (La Rosa et al., 1994). We found that 10058-F4 could apparently decrease transcription of the anti-apoptotic target genes of NF-κB in APL-derived NB4 cells; however, analyzing the effect of the inhibitor on molecular feature of less sensitive Nalm-6 cells unraveled the inaptitude of 10058-F4 on NF-κB modulation. Interestingly, the resulting data declared that the combination of pan-PI3K inhibitor with 10058-F4 was able to suppress NF-κB network; strengthening that the effectiveness of the c-Myc inhibition could be diminished, at least partially, by the over-activated PI3K. In conclusion and based on the broad pre-clinical effectiveness of 10058-F4, it is assumed that this inhibitor is a promising anti-cancer agent, either as a single agent or in a combined-modality strategy; however, *in vivo* investigations are warranted to determine the

Table 3
Combination index (CI) and dose reduction index (DRI) for drug combinations by 10058-F4 and VCR/ATO.

10058-F4		Chemotherapeutic drugs		CI
Concentration	DRI	Concentration	DRI	
150 μM	1.884	**VCR (2 nM)**	2.399	0.948
150 μM	2.041	**ATO (1 μM)**	3.357	0.788

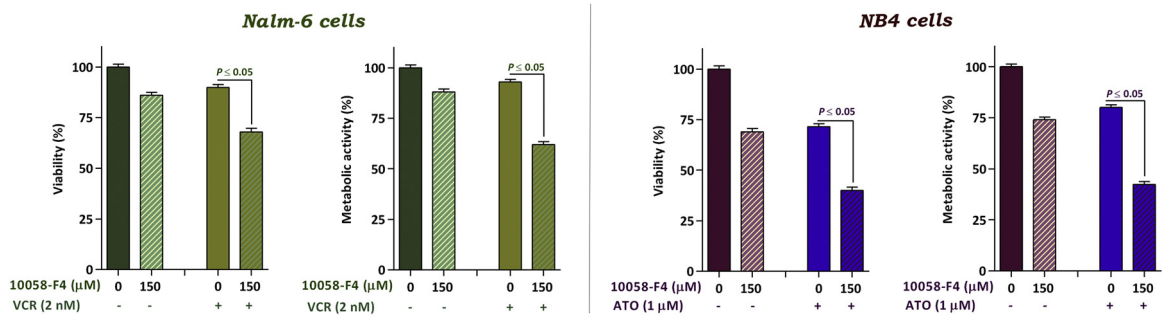

Fig. 7. Synergistic effect of 10058-F4 with the common chemotherapeutic drugs used in acute leukemia treatment. Nalm-6 and NB4 cells were treated with 10058-F4 in combination with vincristine (VCR) and ATO, respectively. The results of synergistic experiments showed that suppression of c-Myc could potentiate the cytotoxic effect of the chemotherapeutic drugs in both Nalm-6 and NB4 leukemic cells.

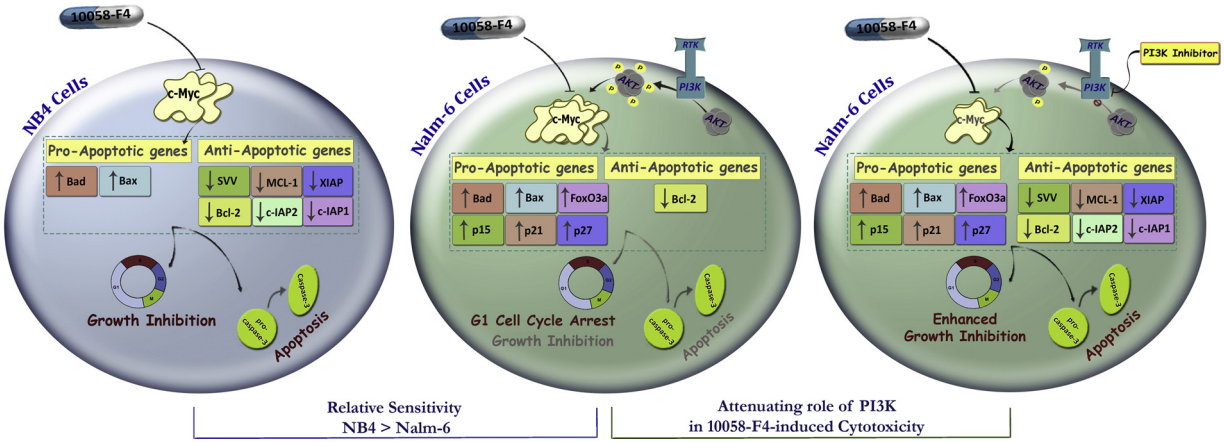

Fig. 8. Schematic representation proposed for the plausible mechanisms of action of 10058-F4 in acute leukemia cells. Abrogation of c-Myc in both NB4 and Nalm-6 cells reduced the survival rate; however, as presented, the anti-leukemic effect of 10058-F4 on NB4 was more profound as compared to Nalm-6 cells. While in wild-type p53-expressing Nalm-6 cells inhibition of c-Myc could induce G1 arrest and up-regulate the mRNA expression of cell cycle-related genes, no significant suppressive effect was found on the transition from G1 phase in NB4 cells harboring mutant p53. Our results also demonstrated that PI3K inhibition in less sensitive Nalm-6 cells potentiated the anti-leukemic effect of 10058-F4; suggesting that the effectiveness of the inhibitor could be overshadowed, at least partially, through activation of the PI3K pathway in pre-B ALL Nalm-6 cells.

usefulness of this inhibitor in cancer therapeutics, in particular for the treatment of acute leukemia.

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Conflict of interest

Davood Bashash has received research grants from Shahid Beheshti University of Medical Sciences. The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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