Inhibition of telomerase using BIBR1532 enhances doxorubicin-induced apoptosis in pre-B acute lymphoblastic leukemia cells

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ABSTRACT

Objectives: Interest into targeting telomerase in cancer has increased by the recent disclosure that elevated telomerase activity is associated with disease recurrence and poor outcome in cancers. In addition, cellular acquisition of unlimited replicative potential, which is closely related to the maintenance of telomeres mostly via the reactivation of telomerase, has been shown to confer loss of sensitivity to a wide range of anti-neoplastic agents.

Methods: To evaluate whether telomerase inhibition using non-nucleosidic inhibitor of telomerase BIBR1532 could enhance cytotoxic effect of doxorubicin in acute lymphoblastic leukemia, Nalm-6 pre-B ALL cells were subjected to combination treatment and subsequent cell viability, growth kinetics, caspase-3 activity, and transcriptional alteration of p73, p21, FOXO3a, c-Myc, hTERT, and other apoptosis-related target genes were investigated.

Results: Combination of BIBR1532 with doxorubicin produced a synergistic anticancer effect probably through induction of p73. Transcription factor p73 not only suppressed the proliferative capacity of the cells through induction of p21-mediated G1 arrest, but also down-regulated the mRNA level of hTERT and c-Myc. Our results also report that BIBR1532 induced a caspase-dependent apoptosis, at least partially, through heightened ROS levels, and noteworthy enhanced the pro-oxidant property of doxorubicin. In harmony, transcriptional repression of survivin could be a probable underlying mechanism for the induction of apoptosis through shifting the ratio of death promoters to death repressors via alteration of Bax and Bcl2 expression.

Conclusions: Overall, it seems that combination of BIBR1532 and doxorubicin could be a novel therapeutic strategy for acute lymphoblastic leukemia that may be clinically accessible in the near future.

KEYWORDS

Pre-B ALL; BIBR1532; doxorubicin; Nalm-6 cells; telomerase

Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent form of malignant neoplasia affecting children, accounting for approximately 25% of malignancies diagnosed in ages 0–14 [1]. In spite of endorsed and exponential researches to improve treatment strategies, efforts have not yet converted into better prospect for patients and still disease relapse remain a significant problem in ALL [2]. Doxorubicin is one of the most efficacious anticancer drugs, which takes apart as a fundamental agent in first-line treatment in ALL [3]. Although most of the patients with childhood leukemia treated with this anthracyclin-antibiotic achieve long-term survival, many of them experience wide range of serious treatment-related side effects such as congestive heart failure, which in turn lead to increased risk of sudden death or death from cardiac causes [4]. Due to its toxicity, the clinical use of doxorubicin is dose-limited by its safety profile [5]; providing opportunities for innovative combined-modality strategies that would enhance the clinical activity of the drug while reducing its toxic side effects.

Notwithstanding the fact that cancers display a great heterogeneity in clinical behavior, most human tumors share a set of limited phenotypical characteristics [6]. The immortality of tumor cells in nearly 90% of all human malignancies is guaranteed by the reactivation of a unique reverse transcriptase [7], telomerase, which counteracts progressive shortening of telomere during cellular replication [8]. Accumulating number of clinical studies have shown that elevated telomerase activity is associated with disease recurrence and poor therapeutic outcome in human cancers [9,10]. Our previous study showed that shortened telomere length and high telomerase activity in patients with acute promyelocytic leukemia (APL) correlate with disease progression and relapse [11]. Moreover, in a study conducted by Roth et al, both telomere length and telomerase activity introduced as attractive follow-up parameters to evaluate the treatment response for patients with T-cell prolymphocytic leukemia [12]. Of particular interest, it has also been reported that some anti-neoplastic agents,
such as doxorubicin can reversely increase telomerase activity in human cancer cells, giving them a chemoresistance phenotype [13]. Taking advantage of the fact that the sensitivity extent of cancer cells to chemotherapeutic agents correlates, at least partially, with telomerase activity, substantial efforts have been made to investigate the potential therapeutic value of anti-telomerase synergism with these drugs.

In the growing list of promising telomerase inhibitors, BIBR1532 is the most potent non-peptidic, non-nucleoside small molecule inhibitor of telomerase catalytic subunit (hTERT) discovered thus far. Its anticancer value has recently been evaluated in numerous preclinical studies, indicating strong ability of this inhibitor to suppress tumor cell growth in several types of cancers [14–16]. There is also evidence showing that BIBR1532 may find potential application as an adjunctive agent in cancer chemotherapy. Previous study suggested that pharmacologically inhibiting telomerase activity using BIBR1532 not only could sensitize drug-resistant cell lines to several DNA damaging agents, but also prevent the additional activation of telomerase in response to these drugs [17]. Meng et al. showed that targeted inhibition of telomerase activity with BIBR1532 in combination with carboplatin demonstrates synergistic effect in eliminating ovarian cancer spheroid-forming cells [18]. In another study, it was shown that treating drug-resistant leukemia and breast cancer cells with BIBR1532 led to increased sensitization to chemotherapeutic agents [19]. To address whether telomerase inhibition could enhance cytotoxic effect of the common anthracycline used in ALL treatment, we examined the effects of combination treatment of BIBR1532 and doxorubicin in human pre-B ALL-derived Nalm-6 cells.

**Materials and methods**

**Reagents and cell culture**

Nalm-6 (human pre-B ALL cell line) cells were cultured in RPMI 1640 medium complemented with 2 mM L-glutamine, 10% heat inactivated fetal bovine serum, 100 Unit/mL penicillin and 100 µg/mL streptomycin in a humidified 5% CO2 atmosphere at 37°C under standard cell culture conditions. A stock solution of BIBR1532 at a concentration of 1 mM was prepared by dissolving the compound in 0.1% sterile dimethyl sulfoxide (DMSO), divided into aliquots and stored at -20°C until use. For doxorubicin treatment, a relevant amount of stock solution of the drug was added to a culture medium to attain the concentrations of 70 and 100 nM.

**Telomeric repeat amplification protocol**

To investigate whether BIBR1532 treatment results in telomerase inhibition, the enzymatic activity of this unique reverse transcriptase was determined by Telo TAGGG telomerase kit according to the manufacturer’s protocol. For protein extraction, cells were lysed in lysis buffer, centrifuged and the protein extracts were subjected to telomeric repeat amplification protocol (TRAP assay). The PCR amplified telomerase products obtained in these assays were visualized on 8% PAGE and the ladder was detected by staining with silver nitrate (Sigma). The gel image was analyzed using Quantity One and Multi-analyist software (Bio-Rad Laboratories, Hercules, CA, U.S.A). Percentage of inhibition was calculated by comparing telomerase activity of inhibitor-treated cells with telomerase activity of untreated cells.

**Trypan blue exclusion test of cell count and viability**

To study the effects of doxorubicin-plus-BIBR1532 on cell viability index and logarithmic growth, Nalm-6 cells were seeded at 1.8 x 10^5 cells/mL and incubated in the presence of various concentrations of BIBR1532, doxorubicin and both agents as a combination therapy. In addition to the negative control (no inhibitor), cells were treated with the corresponding concentrations of DMSO as an alternative negative control. After 24 h, cell pellets were resuspended in phosphate buffered saline (PBS) and incubated with 0.4% trypan blue (Invitrogen) for 1–2 min at room temperature and eventually the number of viable cells were counted using Neubauer hemocytometer. Finally, cell viability was assessed as follows: viability (%) = viable cell count/total cell count x 100.

**Determination of combination index and dose reduction index**

To evaluate the interaction between doxorubicin and BIBR1532, the combination index (CI) was calculated using CalcuSyn Software according to the classic isobologram equation: CI = (D1)/(Dx1) + (D2)/(Dx2), where (Dx1) and (Dx2) indicate the individual dose of doxorubicin and BIBR1532 required to inhibit a given level of viability index, and (D1) and (D2) are the doses of doxorubicin and BIBR1532 necessary to produce the same effect in combination, respectively [20]. Since different CI values can be observed at different levels of growth inhibition (fraction affected, FA), CI versus FA plots was applied to present the data using MS Excel. The dose which may be reduced in a combination for a given level of effect as compared to the concentration of individual drug alone defined as dose reduction index (DRI) and calculated as follow: (DRI)1 = (Dx1)/(D1) and (DRI)2 = (Dx2)/(D2).

**BrdU cell proliferation assay**

The suppressive effect of BIBR1532/doxorubicin on the growth and proliferation of pre-B ALL cells was
measured using BrdU cell proliferation kit (Roche). Briefly, cells (5000 cells/well) were treated with desired concentrations of BIBR1532 and doxorubicin in the up to 36 h and then incubated with 100 μL/well of BrdU labeling diluted solution. The cells were then fixed and DNA was denatured using Fix Denat solution. Following incubation with peroxidase-conjugated anti-BrdU antibody, wells were washed, and then, the cultures were exposed to substrate (TMB). The samples were read at 450 nm in an ELISA reader at several time points. The effect of combination treatment on the rate of DNA synthesis was measured via ODexp/ODcon × 100, where ODexp and ODcon are the optical densitometries of treated and untreated cells, respectively.

MTT assay

MTT assay was performed to evaluate the inhibitory effect of BIBR1532 and doxorubicin, both as a single agent or in combined modality on the ability of Nalm-6 cells to metabolize thiazolyl blue tetrazolium bromide into formazan crystals. Cells were seeded at the density of 5000/well into 96-well plates and incubated up to 36 h with 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% CO2. After 4 h, the formazan crystals were dissolved with 100 μL of DMSO and absorbance was measured at 570 nM with an enzyme-linked immunosorbent assay (ELISA) reader.

Flow cytometric analysis of DNA content

For detection of DNA content, propidium iodide staining of drugs-treated cells was used. In brief, cells at the concentration of 1 x 10⁶ were seeded into six-well plates and were treated with BIBR1532 and doxorubicin. After 24 h, cells were harvested, washed with PBS and fixed with 70% ethanol at −20°C overnight. Afterward, cells were treated with 0.5 μg/mL RNase in PBS and incubated at 37°C for 30 min before staining with 50 μg/mL PI for 30 min. The cellular DNA content were quantified from the peak analysis of flow cytometric DNA histograms (Partec PASIII flow cytometry, Germany). Data were interpreted using the Windows™ FloMax® software.

Phosphatidylserine externalization (annexin-V assay)

To explore the effect of BIBR1532-plus-doxorubicin on induction of programmed cell death, cells were subjected to apoptosis analysis. Briefly, Nalm-6 cells were seeded into 6-well cell culture plates and 24 h after treatment the cells were collected. The cell aliquots were washed with PBS and a total of 1 x 10⁶ cells per sample were resuspended 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 flow cytometry. 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.

Caspase-3 activity assay

To explore whether BIBR1532-plus-doxorubicin-induced apoptosis is mediated through enzymatic activity of caspase-3, cells were subjected to apoptosis analysis using caspase-3 assay kit (Sigma). Briefly, cells were treated with BIBR1532/doxorubicin and incubated for 24 h. Following centrifugation and washing the cells with ice-cold PBS, 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 acetyl-Asp-Glu-Val-Asp p-nitroanilide. Cleavage of the peptide by caspase released the chromophore pNA, which was quantified spectrophotometrically at a wave length of 405 nm.

Intracellular ROS detection

Cellular reactive oxygen species (ROS) detection assay uses a cell permeant and fluorogenic dye, DCFH-DA, for measuring hydroxyl, peroxyl, and other ROS activity within the cell. After diffusion into the cell, DCFDA is cleaved by nonspecific esterases to create a non-fluorescent compound, DCFH, which is later oxidized by ROS into the highly fluorescent compound, DCF, which can be detected by fluorescence spectroscopy. After incubation with BIBR1532 and/or doxorubicin for 24 h, cells were exposed to DCFH-DA and then incubated at 37°C for 30 min. Finally, fluorescence intensities of the samples detected by fluorescence spectrophotometer (Cary Eclipse, U.S.A) with excitation at 485 nm and emission at 530 nm.

RNA purification, reverse transcription, and real-time PCR amplification

Total RNA was isolated 24 h after treatment with BIBR1532 and doxorubicin using high pure RNA isolation kit (Roche). The reverse transcription reaction was performed using the revertAid First Strand cDNA synthesis kit (Takara BIO, Japan). The cDNA was subjected to qRT-PCR using SYBR Premix Ex Taq technology (Takara BIO) on a light cycler instrument (Roche Diagnostic). Thermal cycling conditions included an initial activation step for 30 s at 95°C followed by 40 cycles, including a denaturation step for 15 s at 95°C and a combined annealing/extension step for 60 s at
60°C. A melting curve analysis was applied to verify the specificity of the products, and the values for the relative quantification were calculated based on $2^{-\Delta\Delta Ct}$ relative expression formula. Each sample was analyzed in triplicate, and representative data sets are shown. Nucleotide sequences of the primers used for quantitative RT-PCR were listed in Table 1.

**Statistical analysis**

Experimental data are expressed by mean ± standard deviation of three independent assays. All tests were done in duplicate or triplicate. An independent t test was conducted for comparison between groups. Statistical significance was calculated using paired two-tailed Student’s t-tests. Statistically different values were defined significant at *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$.

**Results**

**BIBR1532 potently reduced telomerase activity in Nalm-6 pre-B ALL cells**

To investigate the inhibitory effect of BIBR1532 in pre-B acute lymphoblastic leukemia, Nalm-6 cells were treated with the inhibitor, and then, both the transcriptional activity of hTERT and the enzymatic activity of telomerase were determined using RQ-PCR and TRAP assay, respectively. As shown in Figure 1(a), exposure to BIBR1532 for 24 h resulted in a considerable decrease in the mRNA expression level of hTERT with maximal repression observed at 60 μM, suggestive of a dose-dependent suppressive effect of the inhibitor on telomerase catalytic subunit. In addition, concentration-dependent experiment showed that telomerase activity considerably hindered upon exposure of Nalm-6 cells to BIBR1532 after 24 h. As presented in Figure 1(b), despite lower concentrations which partially inhibit telomerase, we found that treating cells with higher doses of BIBR1532 resulted in marked telomerase inhibition.

**Investigation of the synergistic effect of BIBR1532 and doxorubicin on Nalm-6 cells**

To address whether telomerase inhibition could enhance cytotoxic effect of the common anthracycline used in ALL treatment, we examined the effects of both individual and combination treatments of BIBR1532 and doxorubicin on the viability and cell count of...
Nalm-6 cells. As shown in Figure 2(a), single agents of BIBR1532 and doxorubicin exerted dose-dependent growth suppressive and cytotoxic effects in this cell line. Our data also demonstrated that BIBR1532 markedly increased the sensitivity of cells to doxorubicin at 70 and 100 nM, as revealed by the decreased viability and number of viable cells in combination series (Figure 2(a)). Besides, to investigate to what extent BIBR1532 and doxorubicin act synergistically, we conducted the isobologram and CI analysis. As shown in Figure 2(b), isobologram analysis demonstrated that all of the points are below the line of additive effects, indicative of a potent synergistic effect between BIBR1532 and doxorubicin. Moreover, the fraction effect (FA) versus combination index (CI) curve. Since different CI values can be observed at different levels of growth inhibition (fraction affected, FA), CI versus FA plot for set of six BIBR1532/doxorubicin combinations was applied to present the data. CI < 1, = 1, and > 1 indicate synergism, additive effect (solid line), and antagonism, respectively.

**Table 2.** CI and DRI for drug combination by doxorubicin and BIBR1532.

| BIBR1532 Concentration (μM) | DRI | Doxorubicin Concentration (μM) | DRI | CI  |
|-----------------------------|-----|-------------------------------|-----|-----|
| 10                          | 6.733 | 70                           | 1.429 | 0.849 |
| 10                          | 7.623 | 100                          | 1.268 | 0.920 |
| 30                          | 3.511 | 70                           | 3.366 | 0.582 |
| 30                          | 4.386 | 100                          | 3.607 | 0.505 |
| 60                          | 4.554 | 70                           | 20.869 | 0.268 |
| 60                          | 7.364 | 100                          | 36.658 | 0.163 |

BIBR1532 enhanced doxorubicin-induced growth suppressive effect

To determine to what extent the synergistic effect between BIBR1532 and doxorubicin would affect the proliferative activity of pre-B ALL cells, DNA synthesis rate of drugs-treated cells was assessed using BrdU cell proliferation assay. As shown in Figure 3(a), using each agent alone only modestly decreased the proliferation capacity of the cells (5–13%), whereas combined exposure to the drugs led to significant reduction in the DNA synthesis rate by 35 and 56% after 24 and 36 h, respectively. Moreover, time and concentration-dependent experiments using MTT assay showed that the maximum cytotoxic effect obtained when doxorubicin used in combination
with BIBR1532 after 36 h (Figure 3(b)). Taken together, these findings suggest that BIBR1532 could potentiate doxorubicin-induced growth suppressive effect in Nalm-6 leukemic cells.

**BIBR1532 enhanced doxorubicin-induced apoptosis in Nalm-6 cells**

To determine whether cytotoxic effect induced by the drug combination is mediated through induction of apoptosis, drugs-treated cells were subjected to annexin-V-staining assay. We found that simultaneous treatment of cells with BIBR1532 and doxorubicin resulted in a significant increase in the percentage of Annexin-V and Annexin-V/PI double positive cells as compared to the doxorubicin-treated group, which indicated that BIBR1532 enhanced doxorubicin-induced apoptosis in Nalm-6 cells (Figure 4(a,b)). Afterwards, identical drug concentrations used for the annexin-V assay were used for the measurement of caspase-3 activity. As shown in Figure 4(c), our results demonstrated that the enzymatic activity of caspase-3, as an important executioner of the apoptotic pathway, was greatly increased upon exposing cells to the combination of the drugs.

**BIBR1532/doxorubicin-induced G1 arrest in Nalm-6 cells**

Measuring the effects of BIBR1532 and/or doxorubicin on cell cycle progression revealed that the combination of BIBR1532 and doxorubicin increased sub-G1 cell population (Figure 5(a)), which is indicative of the pro-apoptotic potential of drugs combination in Nalm-6 cells and is in agreement with the results obtained from annexin-V-staining assay. Moreover, analysis of the cell cycle also showed that treatment of the cells with BIBR1532 and doxorubicin significantly increased the percentage of cell populations in G1 phase coupled with decreased percentage of cells in S and G2/M phases, as well (Figure 5(a)). Recent studies have been demonstrated that induction of p73, a protein related to the p53 tumor protein, contributes in transition of cells from G1 to S phase of cell cycle mostly via up-regulation of p21 cycline-dependent kinase inhibitor and forkhead transcription factor FOXO3a [21]. Interestingly, 24 h of treatment with the combination of the drugs led to a significant increase in the mRNA expression level of p73, p21, and FOXO3a (Figure 5(b)).

**BIBR1532/doxorubicin suppressed transcriptional activity hTERT and c-Myc**

Apart from cell cycle regulation, both p73 and p21 have been shown to inhibit telomerase activity via restriction of hTERT mRNA level [22,23]. Given this notion and on the basis of upregulated p73 and p21, it was tempting to evaluate the transcriptional activity of human telomerase reverse transcriptase (hTERT). Interestingly, our results showed that doxorubicin-plus-BIBR1532 not only suppressed the transcriptional activity of hTERT, but also decreased the mRNA expression level of c-Myc, as the most predominant positive regulator of hTERT gene transcription. As shown in Figure 6, although both doxorubicin and BIBR1532 individually decreased the mRNA level of c-Myc and hTERT, exposure to the drugs combination for 24 h resulted in a premiere decrease in transcriptional activity of these genes. Taken together, our results suggest that...
the antiproliferative effect of doxorubicin/BIBR1532 in Nalm-6 cells is probably mediated through induction of p73 and p21, which is coupled with G1 cell cycle arrest and transcriptional suppression of c-Myc and hTERT.

BIBR1532/doxorubicin-induced augmentation of ROS is coupled with alteration in apoptosis-related genes expression

Being a vital product of mitochondria, ROS participates in the regulation of physiological cell signaling; however, produced excessively, it might lead to cell death. Since doxorubicin or other DNA damaging agents may trigger the apoptotic pathway through the production of ROS, it was reasonable to hypothesize that telomerase inhibition using BIBR1532 may enhance doxorubicin-induced apoptosis through escalation of intracellular ROS. As represented in Figure 7(a), ROS was elevated after treating cells with either agent; however, the combination treatment resulted in a significant higher level of ROS as compared with the single agent treatments. It has been reported that increased intracellular ROS triggers the mitochondria-mediated pathway of death signaling via interacting with survivin, as the smallest member of the IAP family, which subsequently alter the mRNA expression levels of Bcl-2 family members [24]. As illustrated in Figure 7(b,c), we found that BIBR1532/doxorubicin not only repressed the transcriptional activity of survivin and Bcl-2, but also enhanced the mRNA expression level of pro-apoptotic members of Bcl-2 family, such as Bad and Bax.

Discussion

Doxorubicin, the most well-known anthracycline used in chemotherapeutic regimens for different types of cancers, is also considered as a cornerstone in the first-line treatment of acute lymphoblastic leukemia (ALL) [25]. Unlike the broad spectrum efficacies, a dose-related side effects, above all cardiotoxicity, limited the application of this agent in general therapeutic approaches [26]. Moreover, recent evidence suggests that resistance to doxorubicin is a common clinical problem encountered in the treatment of ALL [27]. Cellular acquisition of unlimited replicative potential, which is closely related to the maintenance of telomeres mostly via the reactivation of telomerase, confers loss of sensitivity to a wide range of anti-neoplastic agents, such as doxorubicin [28]. Therefore, several strategies, such as combining chemotherapeutic drugs with small
Figure 5. Evaluation of the combination effect of doxorubicin and BIBR1532 on cell cycle distribution of Nalm-6 cells. (a) Percentages of cell populations in different phases of cell cycle are plotted at different treatments. (b) Effect of BIBR1532 together with doxorubicin on the transcriptional activity of p73, FOXO3a, and p21. After the treatment of cells, the mRNA expression level of aforementioned genes was measured using real-time RT-PCR. Values are given as mean ± SD of three independent experiments. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 represent significant changes from untreated control.
molecules that target telomerase have been developed to enhance the clinical activity of these agents. Our previous study showed that inhibiting telomerase using BIBR1532, the lead compound of non-nucleosidic inhibitor of telomerase, causes rapid cell death in pre-B ALL cells [29]. In an effort to enhance the effectiveness of pre-B ALL treatment and to investigate the potential therapeutic value of anti-telomerase synergism with chemotherapy, we designed experiments to evaluate the effect of doxorubicin in combination with BIBR1532 for the treatment of Nalm-6 leukemic cells.

On the basis of synergistic experiments, we found an enhanced reduction in the viability of Nalm-6 cells when doxorubicin was used in combination with BIBR1532. Our results also delineated that BIBR1532 augments doxorubicin-induced apoptosis and its antiproliferative effect, as evidenced by the increased externalization of phosphatidylserine (PS), elevated sub-G1 cell population, and decreased number of inhibitor-treated viable cells. Consistently, Shi et al. showed that combination of BIBR1532 and paclitaxel synergistically inhibits cell proliferation in breast cancer cell lines [30]. In another study, it was reported that simultaneously treatment of APL-derived NB4 cells with ATO-plus-BIBR1532 resulted in caspase-3 activation and eventually increased apoptotic cell death [31]. Herein, we found that mRNA expression level of p73 increased upon exposing Nalm-6 cells to doxorubicin when combined with BIBR1532. The transcription factor p73, which is a poten surrogate for p53, elicits anticaner effects through either activation of programmed cell death or induction of cell cycle arrest by regulating the expression level of a large cohort target genes [32]. It has been reported that p73 could provide a signal that up-regulates FOXO3a [33], which in turn induces G1 cell cycle arrest mainly through activation of p21, as a key regulator of cell cycle progression at G1 phase [34]. Consistent with this, our data showed that BIBR1532-plus-doxorubicin not only elevated the transcriptional activity of both FOXO3a and p21, but also induced a robust increase in cell population of G1 phase.

Apart from cell cycle regulation, both p73 and p21 have been shown to inhibit telomerase activity via restriction of hTERT mRNA level [22,23]. Moreover, Kartasheva et al. revealed that the mRNA level of c-Myc, as a strong regulator of hTERT transcription, was reduced by p73 [35]. In agreement with the inductive effect on p73, our results showed that combination of BIBR1532 and doxorubicin significantly suppressed both c-Myc and hTERT mRNA level. Hitherto, roles deviating from canonical activity of hTERT have been demonstrated in a fair number of studies [36,37]. Indran et al. indicated that the over-expression of hTERT reduces both basal and endogenous intracellular level of reactive oxygen species (ROS) in response to stimuli, which in turn endows cancer cells with a survival advantage by blunting apoptotic signals [38]. Interestingly, this study reports for the first time that BIBR1532-induced apoptosis is mediated through heightening ROS levels and noteworthy this agent enhances the pro-oxidant property of doxorubicin, as well. Although it is early to hazard a conjecture on the mechanism by which doxorubicin-plus-BIBR1532 decreased survival of Nalm-6 cells, a possible candidate would be survivin [24], a key cellular transcription factor that engages in a unique cross-talk with ROS. The transcriptional activity of survivin could be modulated by intracellular ROS level and has been shown to alter the mRNA expression level of several apoptotic target genes of Bcl-2 family. Of particular interest, we found that combination of doxorubicin and BIBR1532 not only reduced the mRNA expression level of survivin, but also shifted the ratio of the death promoter to death repressor genes via alteration of Bax and Bcl-2 expression levels.

Taken together, our study demonstrated that combination of doxorubicin with BIBR1532 may produce a synergistic anticaner effect in Nalm-6 cells through induction of ROS coupled with up-regulated Bax/Bcl-2 molecular ratio. Moreover, induced p21, subsequent G1 cell cycle arrest and transcriptional suppression of p73-mediated c-Myc and hTERT expression may contribute to the enhanced growth suppressive effect of the drugs combination. Overall, it seems that combination of BIBR1532 and doxorubicin could be a novel therapeutic strategy that may be clinically accessible in the near future; however, further investigation, including clinical trials, are warranted to determine the safety and the efficacy of this combination therapy for the treatment of pre-B ALL patients.
Disclosure statement
No potential conflict of interest was reported by the authors.

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