Survivin Suppression Strengthens BZML-induced Mitotic Catastrophe to Overcome Multidrug Resistance by Removing Senescent A549/Taxol Cells

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Research

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Abstract

Background: Mitotic catastrophe (MC) of cancer cells induced by BZML, a novel colchicine-binding site inhibitor, exerts a significant advantage in overcoming multidrug resistance (MDR) in NSCLC. However, the long cellular death process resulting from MC is not beneficial for anticancer treatment. Here, we study the mechanisms underlying MC occurrence and development to promote the development of anticancer therapies based on drug-induced MC.

Methods: Cellular senescence was confirmed by morphological features, SA-β-Gal and C₁₂FDG staining. Cell cycle analysis and Hoechst 33342 staining were used to detect MC. Relevant signal transduction pathways and protein location were detected by qRT-PCR, western blot and immunofluorescence. The half-life of proteins was evaluated using the protein synthesis inhibitor cycloheximide. Flow cytometry, MTT assay, crystal violet staining, Hoechst 33342 staining and cell division detection were performed to determine the effects of BZML and/or YM155 on cell fate.

Results: We found that BZML induced p53-dependent cellular senescence in A549/Taxol cells, but not in A549, H1299 and MDA-MB-231 cells. Interestingly, BZML-induced senescence was a secondary effect of MC. In addition, the destruction of the protein-degradation system induced by BZML contributed not only to an increase in p53 protein but also to the accumulation of survivin in the nucleus of A549/Taxol cells. However, in A549 cells, the overexpression of survivin had no effect on apoptosis resistance against BZML and failed to promote BZML-induced MC. The inhibition of survivin did not prevent MC occurrence. Unexpectedly, targeting survivin with YM155 accelerated the death of the MC cells by eliminating senescent cells and strengthening the efficiency of BZML in overcoming the MDR of A549/Taxol cells.

Conclusions: Our data suggest that nuclear accumulation of survivin can delay cellular death during MC by promoting the survival of senescent BZML-treated A549/Taxol cells. Further, depending on the dose sequence, combination therapy with YM155 to inhibit survivin might be a new strategy for potentiating BZML-induced MC to overcome MDR during cancer treatment.

1. Background

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide \[1,2\]. Currently, chemotherapy is still an indispensable tool for millions of NSCLC patients worldwide \[3,4\]. Unfortunately, the occurrence of multidrug resistance (MDR) is becoming increasingly common in clinical treatment, leading to the failure of possible treatments \[5,6\]. Therefore, optimizing strategies to enhance therapeutic efficacy and reduce or eliminate MDR, as well as to achieve better prognosis and recovery, are urgently needed.

Currently, most anticancer drugs function by inducing the apoptosis of cells; however, the dysfunction of apoptosis during cancer cell evolution can cause MDR and result in therapy failure \[7\]. Importantly, a growing body of literature shows that apoptosis is not the sole anticancer mechanism and that the
activation of nonapoptotic cell death is a promising strategy for overcoming MDR \cite{8, 9}. Mitotic catastrophe (MC) is a newly identified type of anticancer mechanism in cancer treatment and MDR prevention and has received more attention in recent years \cite{10, 11}. Additionally, a number of studies have shown that MC and senescence are closely related and that cancer cell death by MC is often accompanied by a senescence-like phenotype \cite{12–14}. In general, cellular senescence is considered a tumor-suppressive mechanism that complements apoptosis \cite{14}. However, senescent cancer cells may not be efficiently eliminated by immune cells due to the impaired anticancer immune response resulting from the cancer microenvironment or cancer therapy \cite{15, 16}. This ineffective immune response makes therapy-induced senescence a double-edged sword, and senescent cancer cells that survive for a long time may cause inflammation and promote cancer recurrence, metastasis and MDR through the senescence-associated secretory phenotype of cancer cells \cite{15, 17}. Therefore, it is essential to intensively study the molecular mechanisms underlying the occurrence and development of MC and to find an effective way to remove these senescent cells immediately upon anticancer treatment to improve the outcome.

Survivin, encoded by the baculoviral IAP repeat-containing 5 (BIRC5) gene, is a multifunctional protein that typically exists in the cytoplasm and nucleus \cite{18}. Cytoplasmic survivin predominantly mediates anti-apoptotic functions, whereas nuclear survivin mediates mitotic function \cite{19, 20}. In addition, given its preferential expression in cancer cells and correlation with poor patient prognosis, survivin has been proposed as a biomedical target for cancer therapy \cite{20}. Interestingly, under certain conditions, survivin can be upregulated by various anticancer drugs and exogenous stress conditions, such as UV rays, adriamycin, docetaxel and cisplatin \cite{21–23}. Additionally, in addition to its association with apoptosis, the relationship between survivin and other anticancer mechanisms is still unclear. Therefore, it is necessary to clarify the significance of increased survivin in cancer treatment, which may provide new ideas and strategies for further cancer treatment.

5-(3, 4, 5-trimethoxybenzoyl)-4-methyl-2-(p-tolyl) imidazol (BZML) is a novel colchicine-binding site inhibitor (CBSI). Our recent studies have demonstrated that nanomolar concentrations of BZML exhibited anticancer activity against various chemosensitive and resistant cancer cells by targeting the colchicine-binding site to inhibit microtubule polymerization \textit{in vitro} and \textit{in vivo} \cite{24–26}. Interestingly, BZML can force A549 cells to die through apoptosis, whereas it mainly drives A549/Taxol cells to die by p53-independent MC, which overcomes MDR \cite{24}. However, the BZML induction of A549/Taxol cell death takes a long time because it does not cause instantaneous death while eliciting MC, and this long death process does not benefit anticancer treatment. Therefore, in this study, we aimed to focus on the molecular mechanisms underlying MC occurrence and development, which may provide a theoretical basis for optimizing therapeutic strategies to make BZML-induced MC a promising anticancer mechanism to overcome MDR in NSCLC.

2. Materials And Methods

2.1. Chemical compounds and reagents
BZML was synthesized by our group (the purity ≥ 98%). Cellular senescence assay kit, carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), Hoechst 33342, Lyso-Tracker Red, Nuclear and cytoplasmic protein extraction kit, Crystal violet kit and 4’,6-diamidino-2-phenylindole (DAPI) were provided by Beyotime Biotechnology (Nanjing, P.R.C). Propidium iodide (PI), 5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside ($C_{12}$FDG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, USA). Paclitaxel, pifithrin-α and sepantronium bromide (YM155) were obtained from Selleck Chemicals (Houston, USA). pEX-3-BIRC5 + 3 × Flag plasmid, empty pEX-3 vector, p53 siRNA #1, p53 siRNA #2, survivin siRNA #1, survivin siRNA #2 and scrambled siRNA control were all purchased from GenePharma Corporation (Shanghai, P.R.C.). Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, USA). Annexin V-FITC/PI double staining kit was provided by KeyGen (Nanjing, P.R.C.). Primary antibodies against p53, p21, Rb, p-Rb, survivin, Lamin B1, DDDDK tag and β-actin, as well as HRP-conjugated, TRITC-conjugated and FITC-conjugated secondary antibodies were purchased from Proteintech Group (Chicago, USA). MDM2 and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). PMSA6 antibody was obtained from Bioworld Technology (Nanjing, P.R.C.). Proteasome 20S core subunits polyclonal antibody was obtained from Enzo Life Sciences (New York, USA).

2.2. Cell culture

Human cancer cell lines A549, H1299 and MDA-MB-231 were purchased from the Shanghai Institute of Cell Resource Center Life Science. A MDR cell line (A549/Taxol) was established from its isogenetic cell line A549 by stepwise selection, and 300 nM paclitaxel was used to maintain MDR in the culture medium \[24\]. These cells were tested by short tandem repeat analysis, validated to be free of mycoplasma, which were used within 6 months. In addition, the cells were cultured within 25 passages for all experiments. All cells were cultured in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO$_2$ and maintained in a logarithmic growth phase for all experiments.

2.3. Detection of senescence-associated β-galactosidase (SA-β-Gal)

Cells were exposed to BZML for indicated time and detected using the cellular senescence assay kit according to the manufacturer’s protocol. The percentages of SA-β-Gal positive cells were observed and counted in five random fields under an optical microscope (Olympus, Tokyo, Japan). Alternatively, SA-β-Gal activity was also measured by $C_{12}$FDG staining for 40 min at 37 °C in the dark, the levels of fluorescent product were measured immediately by flow cytometric analysis (FACS) (Accuri Cytometers, Inc., Ann Arbor, MI).

2.4. Cell morphology changes

A549/Taxol cells seeded in a 6-well plate were incubated with BZML for indicated time and then photographed by phase contrast microscopy (Olympus, Tokyo, Japan). Cellular area was evaluated by
Image J2 software.

Morphological changes of nucleus were detected by Hoechst 33342 staining for 30 min. Cells were photographed by a fluorescence microscope (Olympus, Tokyo, Japan) after washing off the unbound dye.

2.5. Cellular size and granularity

A549/Taxol cells were seeded into six well plates and then treated with BZML for indicated time. Forward scatter (FSC) and side scatter (SSC) values were measured by FASC (Becton Dickinson, NJ, USA).

2.6. Lysosomal staining

Changes of lysosomes in cells under the influence of BZML were detected by Lyso-Tracker Red staining. Briefly, A549/Taxol cells were seeded into six well plates. After BZML treatment, living cells were incubated with Lyso-Tracker Red at a concentration of 50 nM for 30 min at 37 °C. Fluorescence images of cells were observed using a fluorescence microscope.

2.7. MTT assay

Cells were treated with BZML for indicated time and then transferred to MTT solution for 4 h at 37 °C. Subsequently, the medium was discarded and DMSO was added to dissolve the formazan precipitate. The values of optical density (OD) at 492 nm were measured using a microplate reader (Thermo, Germany).

2.8. Crystal violet staining

Cells were seeded into six-well plates at density of 5000 cells/well and treated with BZML for indicated time. After fixing with 4% paraformaldehyde, cells were stained with crystal violet and photographed by a digital camera.

2.9. Cell division assay

CFDA-SE staining kit was used to track the division of senescent cells. A549/Taxol cells labeled with CFDA-SE for 24 h were stimulated by BZML for indicated time. Then, cells were collected or stained with DAPI. After washing with ice-cold PBS, the fluorescence intensity was analyzed and observed by FACS or fluorescence microscope.

2.10. Cell cycle analysis

Cells incubated with BZML at the indicated time periods were fixed with 70% ice-cold ethanol, washed with PBS and stained with PI (50 µg/ml) for 30 min at 4 °C in the dark. Next, the samples were analyzed by FACS.

2.11. Apoptosis analysis
Cells apoptosis was detected by Annexin V-FITC/PI double-staining. Briefly, cells were treated with BZML or paclitaxel for indicated time, then collected and stained using Annexin V-FITC/PI staining kit for 30 min at room temperature. Samples were analyzed by FACS.

2.12. siRNA and plasmid transfections

Cells (5 × 10^5 cells per well) were seeded into six well plates, and siRNAs (30 nM/well) or plasmids (3 µg/well) were transfected using lipofectamine 2000 according to the manufacturer's instructions. The sequences of siRNA were listed as following: p53 siRNA #1 (forward) 5'-UGU UCC GAG AGC UGA AUG ATT-3', (reverse) 5'-UCA UUC AGC UCU CGG AAC ATT-3'; p53 siRNA #2 (forward) 5'-GGA AAU UUG CGU GUG GAG UTT-3', (reverse) 5'-ACU CCA CAC GCA AAU UUC CUU-3'; survivin siRNA #1 (forward) 5'-UCC GGU UGC GCU UUC CUU UTT-3', (reverse) 5'-AAA GGA AAG CGC AAC CGG ACG-3'; survivin siRNA #2: (forward) 5'-CCG GUU GCG CUU UCC UUU CTT-3', (reverse) 5'-GAA AGG AAA GCG CAA CCG GAC-3'; Negative control (scrambled siRNA): (forward) 5'-UUC UCC GAA CGU GUC ACG UTT-3'; (reverse) 5'-ACG UGA CAC GUU CGG AGA ATT-3'. The efficiency of knockdown or overexpression of p53 and survivin in cells were identified by western blotting.

2.13. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% (v/v) Triton X-100, and blocked with 5% BSA in PBS for 30 min. Primary antibodies were incubated overnight at 4 °C, followed by incubation with FITC- or/and TRITC-conjugated secondary antibodies for 2 h at room temperature. The nuclei were stained with DAPI and images were visualized with a confocal microscopy (Nikon C2, Japan).

2.14. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic proteins were extracted from BZML-treated A549/Taxol cells according to the manufacturer's protocol of the nuclear and cytoplasmic protein extraction kit.

2.15. Protein half-life assay

A549/Taxol cells were treated with BZML for 48 h and then followed by treatment with a protein synthesis inhibitor, CHX (100 µg/mL). The expression levels of p53 and survivin protein were detected by western blot at the indicated times after treatment with CHX.

2.16. Western blot
The protein samples were prepared in ice-cold RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor. Protein concentrations were measured using BCA protein assay. Cell lysate proteins were separated by SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% BSA or non-fat milk, and probed with primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. The signals were visualized by enhanced chemiluminescence and densitometry analysis was assessed using Image J2 software.

2.17. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed following a routine procedure [24]. The primers were listed as following: p53 (F) 5’- CCT CAG CAT CTT ATC CGA GTG G -3’ and (R) 5’- TGG ATG GTG GTA CAG TCA GAG C -3’; survivin (F) 5’- GCA TGG GTG CCC CGA CGT TG -3’ and (R) 5’- GCT CCG GCC AGA GGC CTC AA -3’; β-actin (F) 5’- CAT CAC CAT TGG CAA TGA GC -3’ and (R) 5’- TCG TCA TAC TCC TGC TTG C -3’. The 2^−ΔΔCt method was used for data analysis. β-actin expression level was used for comparison with the relative mRNA expression level of each sample.

2.18. Statistical analysis

Data are presented as mean ± SD and all experiments were done in triplicate. Comparison among groups was made by One-Way ANOVA Tukey Test of variance and Student t-test of unpaired data using SPSS 22.0 software. The p-value < 0.05 was defined as significant differences.

3. Results

3.1. BZML induced the senescence of the A549/Taxol cells

Cellular senescence is recognized as a potent anticancer mechanism, and it often occurs with MC [17, 27]. In our previous study, we found that BZML overcomes the MDR of A549/Taxol cells by inducing MC [24]. To determine whether the MC induced by BZML is accompanied by a senescence-like phenotype of A549/Taxol cells, we employed SA-β-gal staining to detect acidic β-gal activity at pH 6.0 because this activity is a known characteristic of senescent cells. As shown in Fig. 1a, BZML treatment increased the percentage of SA-β-gal-positive A549/Taxol cells in a time-dependent manner, as particularly evident at 72 and 96 h. In addition, an increase in SA-β-gal activity was also detected by FACS using its fluorogenic substrate, C12FDG. The percentage of fluorescent product-labeled cells, which indicates SA-β-gal activity, reached 70% in the A549/Taxol cells 72 h post-BZML treatment (Fig. 1b).

In addition, the typical morphological features of cellular senescence, such as an enlarged and flattened morphology with enhanced cytoplasmic granularity, were observed in the BZML-treated A549/Taxol cells (Fig. 1c and d). Furthermore, we also confirmed cell enlargement using a FASC dot plot analysis of
cellular granularity (SSC) and size (FSC) by observing the cell population. As expected, the cells treated with BZML for 48 h were much larger with more complex granularity than the cells treated with the vehicle control (Fig. 1e). In addition to morphological changes, the lysosomal mass was also significantly increased in the BZML-treated A549/Taxol cells (Fig. 1f).

3.2. BZML-induced senescence was a secondary effect of MC in the A549/Taxol cells

To further investigate the possible link between cells undergoing MC and senescence, we compared the proportion of polyploid cells and SA-β-gal-positive cells through costaining of the BZML-treated A549/Taxol cells. As shown in Fig. 2a, after BZML treatment for 24 and 48 h, the percentage of polyploid cells was higher than that of the SA-β-gal-positive cells. Importantly, all the SA-β-gal-positive cells (100%) showed polyploid features, but not all the polyploid cells showed staining indicative of positive SA-β-gal activity. In addition, this finding was consistent with our previous studies [24] showing that the proportion of polyploid cells exhibited a gradual increase for 12 h after BZML treatment and reached a peak at 36 and 48 h (Fig. 2b). Interestingly, as shown by the data presented in Fig. 1a and b, the A549/Taxol cells did not undergo senescence until 48 h post-BZML treatment. A time course analysis demonstrated that the MC occurred relatively early in this process and approximately parallel with cellular senescence in later development.

Additionally, BZML did not cause MC in A549 cells (p53-wild) [24], H1299 cells (p53-null) (Supplementary Fig. S1) or MDA-MB-231 cells (p53-mutant) (Supplementary Fig. S2), but induced classical apoptosis in these cells (Supplementary Fig. S3). Interestingly, BZML did not induce cellular senescence in A549, H1299 or MDA-MB-231 cells (Fig. 3c-e). These results indicate that cellular senescence caused by BZML may be a secondary effect of MC that counteracts the MDR of A549/Taxol cells.

3.3. BZML-induced senescence was an irreversible anticancer mechanism of A549/Taxol cells

To determine whether cellular senescence secondary to BZML-induced MC is an adaptive survival response or contributes to anticancer effects, we investigated the final fate of BZML-treated A549/Taxol cells after treatment withdrawal (Fig. 3a). As shown in Fig. 3b and c, among the A549/Taxol cells pretreated with BZML for 48 h and then transferred to drug-free medium for another 24 or 48 h of incubation, the percentage of SA-β-gal-positive cells continued to increase in a time-dependent manner. Importantly, crystal violet staining showed that treatment with BZML for 24, 48 and 72 h did not further increase the number of A549/Taxol cells compared with the number of untreated cells when the culture time was extended (Fig. 3d). Furthermore, even when the A549/Taxol cells were washed to remove BZML after 48 h of coincubation, the cells did not continue to proliferate in the drug-free medium for the following 24 and 48 h of cultivation; in contrast, the number of cells decreased gradually (Fig. 3e). Importantly, the MTT assay indicated found that the OD value of the BZML-treated A549/Taxol cells
failed to increase after treatment withdrawal and that the decrease continued for the indicated times that the cells were cultured in drug-free medium (Fig. 3f).

Additionally, CFDA-SE staining was performed to track the division of the BZML-treated senescent A549/Taxol cells because the fluorescence intensity of the CFSE converted from CFDA-SE by an esterase is reduced by one-half with each division of tested cells [28]. As shown in Fig. 3g, BZML significantly inhibited the division of the senescent A549/Taxol cells, as indicated by the fluorescence failing to shift further leftward in the FASC plot. Similarly, the fluorescence intensity of CFSE in the BZML-treated A549/Taxol cells was much higher than that of the untreated cell groups both at 48 h and 72 h, as indicated by the fluorescence microscopy analysis (Fig. 3h). This suggests that BZML-induced senescence exerts an irreversible anticancer effect that prevents the MDR of A549/Taxol cells, even after it is withdrawn from the medium during transient incubation.

3.4. BZML-induced senescence of the A549/Taxol cells was dependent on p53

The p53-p21 and p16\(^{INK4a}\)-Rb pathways are believed to have important contributions to the regulation of cellular senescence [29, 30]. As shown in Fig. 4a, BZML induced an evident increase in p53 expression within 24 h of coincubation, and the expression of p53 in the A549/Taxol cells reached a peak after 48 h of treatment and was sustained for at least 72 h, findings in line with previous findings [24]. In addition, the expression of p21 and Rb was increased, but p-Rb expression was decreased in the BZML-treated A549/Taxol cells.

To further investigate the functional involvement of p53 in BZML-induced senescence, the p53 inhibitor pifithrin-\(\alpha\) and p53-siRNAs were used to abrogate the expression of p53 in BZML-treated A549/Taxol cells (Supplementary Fig. S4a and b). As shown in Fig. 1a, 4b and S5a, both pifithrin-\(\alpha\) and p53-siRNAs significantly decreased the percentage of SA-\(\beta\)-gal-positive A549/Taxol cells at different time points after BZML treatment. Similarly, the C\(_{12}\)FDG staining results showed that the increase in SA-\(\beta\)-gal-positive cells caused by BZML was also significantly downregulated by pifithrin-\(\alpha\) and p53-siRNAs pretreatment of the A549/Taxol cells (Fig. 4c and d). These data suggest that BZML-induced cellular senescence is dependent on p53 in A549/Taxol cells.

3.5. BZML activated p53 through multiple mechanisms in the A549/Taxol cells

Recent studies have proven that p53 function is tightly regulated by its cellular localization, and p53 is synthesized in the cytoplasm while exerting its transcriptional effect on downstream targets responding to cellular stress only after being transported to the nucleus [31]. The results from western blot analysis and immunofluorescence staining showed that in the BZML-treated A549/Taxol cells, the expression of p53 increased only slightly in the cytoplasm but was significantly upregulated in a time-dependent manner in the nucleus (Fig. 5a and Supplementary Fig. S5b).
In addition, BZML downregulated the expression of MDM2, which is a negative regulatory protein of p53 (Fig. 5b). Further, the expression of proteasome 20S core subunits and PMSA6, which are important components of the ubiquitin-proteasome system, was also significantly decreased in the BZML-treated A549/Taxol cells (Fig. 5b). The data above indicated that the protein degradation system might be destroyed by BZML in the A549/Taxol cells. Next, to further clarify how BZML affects p53 protein stability, we measured the half-life of the p53 protein using the protein synthesis inhibitor CHX. Notably, the half-life of p53 in the untreated cells was found to be less than 30 min, while it was extended approximately 2-fold by BZML in the A549/Taxol cells (Fig. 5c). Moreover, the expression of p53 at the mRNA level was also increased after BZML treatment (Fig. 5d).

### 3.6. Survivin mainly accumulated in the nucleus of the BZML-treated A549/Taxol cells

It is generally known that survivin is also a short-lived protein \[^{32}\]. Importantly, survivin may mediate MDR and modulate cellular mitosis; therefore, we further investigated the expression of survivin in apoptotic A549 cells and in MC A549/Taxol cells treated with BZML. As shown in Fig. 6a and b, the intrinsic expression of survivin was very low, and there was no significant difference between the untreated A549 and A549/Taxol cells at either the protein or mRNA level. Interestingly, BZML significantly decreased survivin expression in the A549 cells from 0 to 96 h of treatment. However, in contrast, the expression of survivin in the BZML-treated A549/Taxol cells was significantly increased from 0 to 48 h and then maintained at a high level for 72 h after the transient increase; subsequently, at 96 h, it rapidly decreased; see Fig. 6c. Furthermore, the immunofluorescence staining also showed that BZML caused a significant increase in survivin expression in the A549/Taxol cells but not in the A549 cells (Fig. 6d). Notably the increased survivin expression was mainly found in the nucleus in the BZML-treated A549/Taxol cells (Fig. 6d and 5b). Interestingly, the qRT-PCR results showed that the expression of survivin was only slightly increased at the mRNA level (Fig. 6e). Furthermore, to confirm whether the increase in survivin induced by BZML was attributable to the increase in protein stability in the A549/Taxol cells, CHX was used again in this study. As expected, the half-life of survivin was also significantly prolonged by BZML, suggesting that BZML improves the stability of the survivin protein in A549/Taxol cells (Fig. 5d).

### 3.7. Overexpression of survivin failed to promote BZML to induce MC in A549 cells

Since survivin is an important anti-apoptotic protein and its specifically increased expression was detected in cells showing BZML-induced MC, we further evaluated the function of survivin on cells with BZML-induced MC. Here, A549 cells overexpressing survivin protein were used (Fig. 7a). Notably, transfected exogenous survivin was expressed in both the cytoplasm and nucleus (Supplementary Fig. S5c). In addition, the MTT assay showed that the A549 cells overexpressing survivin exhibited drug resistance against paclitaxel but not against BZML (Fig. 7b). Furthermore, at the normal anticancer dose (15 nM), pacitaxel failed to induce apoptosis in the A549 cells overexpressing survivin (Fig. 7c). In contrast, the rate of apoptosis induced by BZML was not changed in the A549 cells overexpressing
survivin (Fig. 7d). Importantly, the overexpression of survivin significantly decreased the percentage of cells in the sub-G1 phase, which had been increased by paclitaxel, but failed to promote BZML induction of MC in the A549 cells (Fig. 7e). Interestingly, with increases in dose and time, paclitaxel at a high dose also induced MC in the A549/Taxol cells (Supplementary Fig. S6). Therefore, we conclude that the nuclear accumulation of survivin is the result of MC rather than the result of BZML-induced MC.

3.8. YM155 treatment potentiated the effect of BZML in overcoming the MDR of the A549/Taxol cells

To further define the relationship between ultimate cell fate and BZML-induced survivin expression, we used a specific inhibitor (YM155) or survivin-siRNAs to inhibit BZML-induced survivin in A549/Taxol cells (Fig. 8a and Supplementary Fig. S7). Unexpectedly, pretreatment with YM155 did not affect the proportion of polyploid and sub-G1 A549/Taxol cells treated with BZML for 48 h (Fig. 8b). Similarly, survivin-siRNA transfection did not affect the cell cycle distribution of the A549/Taxol cells treated with BZML (Fig. 8c). Furthermore, Hoechst 33342 staining showed that pretreatment with YM155 or survivin-siRNAs had no effect on the number of multinucleated A549/Taxol cells treated with BZML for 48 h (Fig. 8d).

However, the proportion of polyploid cells was significantly decreased, a finding associated with an increase in sub-G1 A549/Taxol cells cotreated with BZML and YM155 for 72 h (Fig. 8e). In addition, after 48 h of incubation with BZML, the A549/Taxol cells were washed to remove BZML and then were added to YM155-containing or drug-free medium for another 24 h of incubation. Compared to that of the cells with BZML treatment alone for 48 h, the proportion of polyploid cells was significantly decreased, and the proportion of sub-G1 cells in the drug-free medium was increased; however, the percentage of sub-G1 cells was further increased after 24 h of culture in the presence of YM155 (Fig. 8f). Similarly, the MTT assay demonstrated that YM155, as a cotreatment or sequential treatment, enhanced the inhibitory effect of the 72-h BZML exposure on the proliferation of A549/Taxol cells (Fig. 8g and h). Furthermore, the percentage of SA-β-gal-positive cells was significantly decreased by sequence-dependent combination therapy with BZML and YM155 (Fig. 8i and g). Therefore, these results indicate that the inhibition of survivin cannot prevent MC but accelerates the death of cells undergoing MC by removing senescent cells in time.

4. Discussion

Despite considerable research efforts aimed at developing novel therapeutic approaches against NSCLC, including molecularly targeted therapy and immunotherapy, the frequency of MDR severely restricts the effectiveness of various anticancer drugs \[^3, 4\]. Currently, apoptosis tolerance-mediated MDR has been considered the most common and complex drug-resistant mechanism \[^5, 33\]. However, in addition to apoptosis, anticancer drugs can also trigger other anticancer mechanisms, such as autophagy, MC, ferroptosis, pyroptosis, necrosis and senescence, to inhibit the proliferation of cancer cells and kill them
by avoiding the apoptosis-resistant pathway \cite{11, 17, 34, 35}. In our previous studies, we confirmed that BZML has potent anticancer activity and overcomes MDR by inducing MC in A549/Taxol cells, an MDR cell line \cite{24}. However, a detailed and clear explanation of the mechanisms underlying MC occurrence and development in BZML-treated A549/Taxol cells was lacking.

Cellular senescence is a permanent state of cell cycle arrest and has been considered a novel anticancer mechanism \cite{14, 17}. In our study, BZML treatment induced the cellular senescence in A549/Taxol cells, showing a significantly increased proportion of SA-β-gal-positive cells and some other typical features of cellular senescence, such as flattened morphology, increased size and granularity and an elevated lysosomal mass in the cells. To further investigate whether these senescent cancer cells were dormant cells that can contribute to cancer redevelopment with the decrease in the concentrations of therapeutic drugs, the BZML-containing medium was replaced with drug-free medium at 48 h post-BZML treatment, and the results from the SA-β-gal staining and microscopy assays of the size and shape of the cells demonstrated that the A549/Taxol cells underwent senescence even after BZML withdrawal. Importantly, the senescent cancer cells neither reentered the normal cell cycle nor divided; thus, they lost normal proliferative potential, and the increase in cell death was significant after the withdrawal of the BZML treatment. Therefore, given the important role of senescence in cancer treatment, our findings support the possibility that BZML-induced senescence might act as an anticancer mechanism against MDR in NSCLC.

MC is a newly discovered form of tumor suppression that differs from other cell death modes and is characterized by unique nuclear alterations, such as multi and/or micronucleation \cite{24, 36, 37}. Interestingly, a number of studies reported that senescence is closely related to MC and that cells undergoing senescence may undergo polyploidization and/or become multinucleated \cite{37, 38}. In contrast, some studies showed that C85 cells senescence induced by methotrexate did not undergo polyploidization or multinucleation \cite{39}. Therefore, to elaborate the relationship between BZML-induced MC and the senescence-like phenotype of A549/Taxol cells, we first performed a time kinetic study to assess the dynamic changes of key parameters during MC and senescence. Notably, BZML-induced MC, indicated by the appearance of polyploid and/or multinucleated cells, occurred as early as 12 h, while an increase in SA-β-gal-positive cells was detected 48 h after BZML treatment. Analysis of these 2 events over the experimental time course indicated that BZML-induced MC occurred relatively early in the A549/Taxol cells. Moreover, SA-β-gal and Hoechst 33342 double staining revealed that almost all the SA-β-gal-positive cells were multi- and/or micronucleated, but not all the polyploid cells were stained positive for SA-β-gal activity. Interestingly, our previous study proved that BZML-induced MC was independent of p53 \cite{24}. As expected, regardless of p53 status, BZML did not induce MC in A549, H1299 or MDA-MB-231 cells, which are p53-wild-type, p53-null and p53-mutant cells, respectively. Importantly, these BZML-treated cells did not undergo senescence. In addition, in contrast to traditional cellular senescence, which is often accompanied by G0/G1 phase arrest \cite{37}, in our study, a substantial level of polyploidy was detected in the senescent A549/Taxol cells after BZML treatment. Together, these data strongly support the
supposition that BZML-induced senescence is downstream of MC and acts as an important phenotype associated with MC occurrence and development in A549/Taxol cells.

Interestingly, elevated ROS levels have been widely accepted as a major trigger of cellular senescence\cite{37,40}, and BZML was confirmed in our previous study to cause ROS generation in a time-dependent manner, but ROS are not inducers of MC occurrence or development in A549/Taxol cells\cite{25}. In addition, in this study, we also found that NAC did not reverse the BZML-induced senescence-like phenotype of the A549/Taxol cells (Supplementary Fig. S8a and b), suggesting that the senescence-like phenotype is secondary to the BZML-induced MC in A549/Taxol cells and may be attributed to mechanisms other than ROS. BZML-induced MC is independent of p53, but the expression of p53 was increased in BZML-treated A549/Taxol cells in a time-dependent manner. Given these considerations, p53-p21 pathway activation represents the trigger of senescence, and the p16\textsuperscript{INK4a}-Rb pathway is involved in maintaining senescence\cite{37,41}. As expected, the expression of p21 was gradually increased, followed by an increase in Rb expression and a decrease in Rb phosphorylation in the BZML-treated A549/Taxol cells. Moreover, our study also demonstrated that pifithrin-\alpha and p53-siRNAs can reverse the BZML-induced senescence-like phenotype, indicating that p53 might play a decisive role at the beginning of senescence in BZML-treated A549/Taxol cells.

Under normal conditions, p53 is a short-lived protein that shuttles between the nucleus and the cytoplasm in a cell cycle-specific manner\cite{31}. Relocation of p53 to the nucleus in response to cellular stress is a contributor to the inhibition of the growth of cancer cells\cite{42}. In this study, the expression of p53 was significantly increased in the BZML-treated A549/Taxol cells at both the mRNA and protein levels. Importantly, the results from the western blot analysis and fluorescence microscopy assay all indicated that BZML treatment increased p53 levels, mainly in the nucleus. In addition, increasing attention has been focused on MDM2 because it acts as an oncogene that negatively regulates the functions of the tumor suppressor p53 by inhibiting transcriptional activity and accelerating the degradation of p53\cite{43}. Interestingly, several reports have demonstrated that some CBSIs, such as combretastatin A-4 and SQ, exhibit a potential MDM2 inhibitory effect in breast cancer cells\cite{44}. Furthermore, in this study, BZML also significantly decreased the expression of MDM2 in the A549/Taxol cells. Additionally, it cannot be ignored that the ubiquitin-proteasome system (UPS) functions as the primary route of degradation for thousands of short-lived proteins\cite{45,46}. Here, its important components, proteasome 20S core subunits and PMSA6, were significantly downregulated in the BZML-treated A549/Taxol cells, indicating that BZML might cause the destruction of the UPS. In fact, the half-life of p53 was significantly prolonged by BZML in the A549/Taxol cells. Therefore, these results suggest that BZML can activate p53 via multiple mechanisms in the A549/Taxol cells and that the destruction of the UPS also contributes to the increase in p53 protein, at least in part.

In cancer cells, survivin located in the cytoplasm plays indispensable roles in cell proliferation and apoptosis inhibition\cite{32}. Interestingly, in this study, BZML treatment increased survivin mainly in the nucleus of the A549/Taxol cells. Given these considerations, survivin is also a short-lived protein and can
be polyubiquitylated and undergo proteasomal destruction [47]. Furthermore, nuclear survivin is significantly more unstable [20]. Interestingly, survivin was expressed at low levels under unstressed conditions; however, in response to BZML-induced MC, its stability in the nucleus was quickly increased, and its half-life was significantly prolonged at the same time. This outcome suggests that the increase in survivin in the nucleus may also be attributed to the destruction of the UPS in BZML-treated A549/Taxol cells. Notably, chemotherapy and radiation therapy can increase survivin expression, which is attributed to mitotic arrest at the G2/M phase and to the augmented stability of the survivin protein through its phosphorylation at Thr34 by a cdc2/cyclin B1 complex [21, 22, 48]. In the present study, the expression of survivin was also slightly increased at the mRNA level. In addition, our previous study showed that BZML treatment caused a transient decrease in cyclin B1 within 24 h, followed by a significant and stable increase in cyclin B1 expression during BZML-induced MC [24]. Remarkably, in this study, the expression of survivin and cyclin B1 changed in parallel during this process. Importantly, the overexpression of survivin neither mediated the apoptosis resistance against BZML nor promoted BZML to induce MC in the A549 cells, suggesting that survivin is not an inducer to the BZML induction of MC in A549/Taxol cells, at least in some settings. Therefore, we speculated that the increase in survivin in the nucleus may result from the increase in cyclin B1 and the destruction of the UPS, and the nuclear expression of survivin is an important biological phenotype associated with MC occurrence and development.

Additionally, some studies have demonstrated that MC and senescence play important and paradoxical roles in the process of cancer treatment [36, 37]. In some circumstances, senescent cells cannot be cleared at the time of treatment in time, which may result in the resumed division and apoptosis of drug-resistant cancer cells, and eventually potentiate cancer progression [49, 50]. Therefore, the development new strategies to remove cells undergoing MC and senescence in a timely manner after the initial anticancer response are urgent. Our data showed that survivin was not required for a cell to undergo MC, but once cellular senescence, secondary to MC, was induced, the upregulation of survivin may provide additional vulnerability to and critical opportunities for sequentially applied therapies. Interestingly, in this study, there was no synergistic effect of YM155 treatment in response to BZML-induced MC during short periods, but the inhibition of survivin by YM155 significantly enhanced the efficiency of BZML in overcoming the MDR of the A549/Taxol cells after 72 h of treatment. Importantly, after BZML-induced MC associated with senescence, dose-sequence-dependent combination therapy with YM155 exhibited a synergistic lethal effect. This suggests that the synergistic effectiveness of BZML and YM155 occurs in the context of the MC-associated senescence induced by BZML. Combinational treatment is typically employed to achieve a better response rate than monotherapy and is based on the use of drugs with different cytotoxicity-inducing mechanisms [22, 23]. Herein, sequential monotherapy not only retained the synergistic effect but also further reduced the toxicity of the anticancer drugs in clinical application. Due to the short half-life of YM155 in the human body [23], we proposed a "one-two punch" approach to cancer treatment based on our findings. That is, at the beginning of cancer treatment, MC-associated senescence is selectively induced in cancer cells; subsequently, in consecutive therapy, these senescent cells are killed by dose-sequence-dependent molecularly targeted drugs. Thus, MC-associated irreversible senescence induced by BZML may provide a treatment window for the opportunistic cell elimination.
using synergistic YM155. In addition, considering that the aberrant activation of an oncogene can cause cellular senescence, survivin, as an oncogene, may also promote the phosphorylation of Rb to maintain the senescence phenotype \[17,20,37\]. Subsequently, we tried to clarify whether the nuclear accumulation of survivin was critical for the senescence caused by BZML-induced MC. Notably, YM155 significantly decreased the percentage of SA-β-gal-positive cells among the BZML-treated A549/Taxol cells, suggesting that the nuclear accumulation of survivin may mediate self-protection by inducing a senescence-like phenotype during MC. Our data provide strong evidence for targeting survivin as a strategy for enhancing the efficiency of BZML because it induces MC to overcome the MDR of A549/Taxol cells.

**Conclusion**

BZML-induced senescence is a secondary effect of MC in A549/Taxol cells, and it also exhibits a potent and irreversible anticancer effect against MDR in NSCLC. Furthermore, cellular senescence is dependent on p53, and the activation of p53 is involved in the regulatory effects on its transcription, translation, and posttranslational modification in BZML-treated A549/Taxol cells. Interestingly, the destruction of the protein degradation system not only contributes to the increase in p53 protein but also promotes survivin accumulation in the nucleus of the BZML-treated A549/Taxol cells. However, the overexpression of survivin cannot lead to apoptosis resistance against BZML and fails to promote BZML to induce MC in the A549 cells. Importantly, the inhibition of survivin cannot prevent MC occurrence but accelerates cell death during MC by promptly removing the senescent cells, potentiates the effect of BZML in overcoming the MDR of A549/Taxol cells (Fig. 9). Therefore, our findings not only reveal the relationships between BZML-induced MC and senescence but also provide novel insights into the mechanisms of MC occurrence and development. Moreover, they will likely open a new area for drug discovery research through the induction of MC.

**Abbreviations**

BIRC5: baculoviral IAP repeat-containing 5; BZML: 5-(3, 4, 5-trimethoxybenzoyl)-4-methyl-2-(p-tolyl) imidazol; C12FDG: 5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside; CBSI: colchicine-binding site inhibitor; CFDA-SE: carboxyuorescein diacetate succinimidyl ester; CHX: cycloheximide; DAPI: 4',6-diamidino-2-phenylindole; FSC: Forward scatter; MC: Mitotic catastrophe; MDR: multidrug resistance; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OD: optical density; PI: Propidium iodide; PVDF: polyvinylidene fluoride; SA-β-Gal: senescence-associated β-galactosidase; qRT-PCR: Quantitative real-time PCR; SSC: side scatter; UPS: ubiquitin-proteasome system.

**Declarations**

**Acknowledgement**
Authors’ contributions

ZS.B., LM.M., and YR.Z. contributed to the manuscript and designed the experiments, and performed experiments, analyzed and interpreted data, prepared figures and wrote the manuscript. XY.Y. performed some experiments. Q.G. and WG.Z. performed synthesis of chemicals. ZS.B. and LM.M. analyzed data and revised the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki principles. It was approved by the Ethics Committee of the Jiangsu Cancer Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


**Figures**

**Figure 1**

BZML induced the senescence of the A549/Taxol cells. (a) SA-β-gal staining of cells pre-treated with pifithrin-α (10 μM) or DMSO for 2 h and continually incubated with BZML (60 nM) for another 48 - 96 h. Left: the representative micrograph, scale bar = 100 μm; right: SA-β-gal positive ratio (%). Black arrows: typical SA-β-gal positive cells. (b) C12FDG staining of cells treated with BZML (60 nM) for 0-72 h was detected by FACS. Right: the original pictures; left: the quantification of C12FDG positive cells. (c) Representative phase contrast microscopic pictures showing the effect of BZML (60 nM) on the morphology of A549/Taxol cells (scale bar = 10 μm). (d) Cellular area was calculated by Image J2 software. (e) Morphological change of BZML-treated cells was measured by FACS dot plots. FSC (x-axis) is an indicator of size and SSC (y-axis) is an indicator of granularity. (f) Changes of lysosomes in A549/Taxol cells in the presence of BZML (60 nM) incubation were stained with Lyso-Tracker Red and
observed under a fluorescence microscope. (scale bar = 20 μm). **p < 0.01 vs control; ##p < 0.01 vs BZML treatment alone group.

Figure 2

BZML induced-senescence was a secondary effect of MC in A549/Taxol cells. (a) SA-β-gal and Hoechst 33342 double-staining of A549/Taxol cells treated with BZML (60 nM) for 24 and 48 h; quantification (right) (scale bar = 100 μm). Black arrows: typical SA-β-gal positive cells; white arrows: multiple micro-nuclei of different sizes. (b) FACS analysis for the cell cycle distribution of A549/Taxol cells after BZML (60 nM) treatment for 0-72 h. Left: the original pictures; right: the quantification of cells percentage in...
Figure 3

BZML-induced senescence was an irreversible anti-cancer mechanism in A549/Taxol cells. (a) Schematic overview for experiments described in (b)–(h). (b and c) SA-β-gal or C12FDG staining for cellular senescence study in A549/Taxol cells pre-incubated with BZML for 48 h and subsequently incubated with
drug-free medium for another 24 and 48 h. Left: the representative micrograph; right: the quantification of senescent cells. Black arrows: typical SA-β-gal positive cells (scale bar = 100 μm). (d-f) Crystal violet staining and MTT assay in A549/Taxol cells treated with BZML (60 nM) for different time. (g and h) CFDA-SE staining of A549/Taxol cells treated with BZML (60 nM) for the indicated time was performed by FACS and fluorescence microscope, respectively (scale bar = 50 μm). **p < 0.01 vs control.

Figure 4

BZML induced-senescence was dependent on p53 in A549/Taxol cells. (a) Western blot assay for the expression changes of senescence-related proteins in BZML-treated A549/Taxol cells; quantification (right). (b-d) SA-β-gal or C12FDG staining for cellular senescence in A549/Taxol cells with or without p53-si-RNA transfection followed by BZML (60 nM) exposure for 48 h. Left: the original pictures, scale bar = 100 μm; right: senescent cell ratio (%). **p < 0.01 vs control; ###p < 0.01 vs BZML treatment alone group.
Figure 5

BZML activated p53 through multiple mechanisms in A549/Taxol cells. (a) Western blot assay for the expression of p53 and survivin in the cytoplasmic and nucleus in BZML-treated A549/Taxol cells. (b) The expression of MDM2, proteasome 20S core subunits and PMSA6 were detected by western blot in A549/Taxol cells treated with BZML (60 nM) for 0-96 h; quantification (right). (c) After treatment with BZML or vehicle for 48 h, cells were continually incubated with the protein synthesis inhibitor
cycloheximide (CHX, 100 μg/mL) and the expression of p53 and survivin was detected by western blot. Down panel: the expression levels quantified by Image J software. (d) qRT-PCR for p53 mRNA expression after BZML treatment. **p < 0.01 vs control.

Figure 6

Survivin mainly accumulated in the nucleus of BZML-treated A549/Taxol cells. (a) The expression of survivin was detected by western blot in A549 and/or A549/Taxol cells; quantification (right). (b) qRT-PCR
for survivin mRNA expression in A549 and/or A549/Taxol cells. (c) Western blot assay for survivin expression in A549 and/or A549/Taxol cells treated with BZML (20 or 60 nM) for 0-72 h; quantification (right). (d) Confocal microscopy analysis for the expression and location of survivin in BZML-treated A549 and/or A549/Taxol cells. Green: microtubules visualized by anti-α-tubulin antibody; Red: survivin visualized by anti-survivin antibody; blue: nuclei visualized by DAPI; Scale bar = 50 μm. (e) qRT-PCR for survivin mRNA expression in A549/Taxol cells treated with BZML (60 nM) for different times. **p < 0.01 vs control; NS = No Significance.

Figure 7
Overexpression of survivin failed to promote BZML to induce MC in A549 cells. (a) Western blot analysis for survivin expression in A549 cells transfected with survivin-overexpressing plasmid; quantification (right). (b) The inhibitory effects of BZML (20 nM) or paclitaxel (15 nM) on the growth of survivin-overexpressing A549 cells were measured by MTT assay. (c and d) The survivin-overexpressing A549 cells were incubated with paclitaxel (15 nM) or BZML (20 nM) for 48 h to detect the apoptosis rates by FACS; quantification (right). (e) The survivin-overexpressing A549 cells were incubated with paclitaxel (15 nM) or BZML (20 nM) for 48 h. The percentage of cells in specific cell cycle phase was quantified (right) and original figures are shown (left). **p < 0.01 vs control; ##p < 0.01 vs paclitaxel treatment alone group; NS = No Significance.

Figure 8

YM155 treatment potentiated the role of BZML in overcoming MDR in A549/Taxol cells. (a) Schematic overview for experiments. (b) After pretreatment with YM155 (10 mM) for 3 h, A549/Taxol cells were
continually treated with BZML (60 nM) for 48 h. The cell cycle distribution was measured by FACS; quantification (right). (c) The survivin-siRNA-transfected A549/Taxol cells were incubated with BZML (60 nM) for 48 h, and the cell cycle distribution was then measured by FACS and quantified (right). (d) After the transfected or pre-treated cells were exposed to BZML (60 nM) for 48 h, nucleus morphology changes in the cells were visualized by Hoechst 33342 staining (scale bar = 100 μm). (e) FACS analysis showing the cell cycle distribution after treatment with BZML (60 nM) for 72 h in YM155-pretreated (10 mM) A549/Taxol cells; quantification (right). (f) After pre-incubation with BZML for 48 h and subsequent incubation with YM155 (10 mM) for another 24 h, the percentage of A549/Taxol cells in the specific cell cycle phase was quantified (right), and the original figures are shown (left). (g) MTT assay showing the cell viability of the A549/Taxol cells co-treated with BZML (60 nM) and YM155 (10 mM) for 72 h. (h) A549/Taxol cells were treated with BZML (60 nM) for 48 h and continually treated with YM155 (10 mM) for 24 h before MTT assay. (i and g) Cells were co-treated with BZML (60 nM) and YM155 (10 mM) for 72 h and then cellular senescence was detected by SA-β-gal or C12FDG staining. Black arrows: typical SA-β-gal positive cells. **p < 0.01 vs control; ##p < 0.01 vs BZML treatment alone group; NS = No Significance.

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