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AZD7762 induces CRBN dependent BAG3 degradation through ubiquitin-proteasome pathway

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Protein degraders are currently under rapid development as a promising modality for drug discovery. They are compounds that orchestrate interactions between a target protein and an E3 ubiquitin ligase, prompting intracellular protein degradation through proteasomal pathway. More protein degraders identification will greatly promote the development of this field. BAG3 is widely recognized as an excellent therapeutic target in cancer treatments. Exploring protein degraders that target BAG3 degradation has profound implications. Herein, molecular docking was applied to assess binding energy between 81 clinical phase I kinase inhibitors and BAG3. BAG3 protein and mRNA level were detected by western blot and quantitative real-time PCR. CCK8 assay and colony formation assay were applied to detect the cell viability and proliferation rate. Cell death was accessed using flow cytometry combined with PI and Annexin V double staining. AZD7762, a Chk1 kinase inhibitor, was identified to induce BAG3 degradation in a ubiquitin-proteasome pathway. AZD7762-induced BAG3 degradation was not dependent on Chk1 expression or activity. CRBN, an E3 ligase, was identified to bind to BAG3 and mediated BAG3 ubiquitination in the presence of AZD7762. By targeting Chk1 and BAG3, two ideal therapeutic targets in cancer treatment, AZD7762 would be a powerful chemotherapy agent in the future. ***Anti-Cancer Drugs*** **35: 46–54**

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Introduction

BAG3, a 74 kDa protein, belongs to a family of co-chaperones, that was originally identified to be an HSP70 partner by yeast two-hybrid screening [1]. BAG3 is a conserved protein, which is highly expressed in the heart, skeletal muscles, and many cancers [2]. BAG3 contains a BAG domain, a WW domain, a proline-rich repeat (PXXP), and two conserved IPV (Ile-Pro-Val) motifs [3]. By interacting with different partners through different protein motifs, BAG3 participates in diverse cellular functions, including apoptosis, development, cytoskeleton organization, cell motility, and autophagy [4,5]. BAG3 protein is overexpressed in several tumor types, and promotes cell survival and regulates autophagy and motility in cancer cells. It is widely recognized as an excellent therapeutic target in cancer treatments [4–7]. Although 2,4-thiazolidinedione derivative was reported to modulate BAG3 activity through inhibit the binding to HSP70 [8], no drug can modulate BAG3 protein degradation.

In cardiomyocyte cells, BAG3 is critical to maintaining the Z-disk integrity and muscle contractility by several different mechanisms [4,6,9]. Mutations in BAG3 can present with a variety of cardiovascular phenotypes, including dilated cardiomyopathy, diffuse myocardial fibrosis, heart failure, hypertrophic cardiomyopathy with restrictive physiology, and a prolonged QT interval [10,11]. And polymorphisms of BAG3 may also play a role in the pathogenesis of tako-tsubo cardiomyopathy [12]. BAG3-deficient mice died before 4 weeks of age, with evidence of severe skeletal and cardiac muscle degeneration [13]. In addition, overexpression of BAG3 could improve left ventricular function in murine hearts with left ventricular dysfunction [2]. In 2015, Knezevic *et al.* put BAG3 on the map as a 'new player in the heart failure paradigm' [11]. These reports suggested that BAG3 is critical for myocardial function, and interrupting the expression of BAG3 protein might lead to myocardial toxicity.

Kinase inhibitors are a large group of drug targets, now account for a quarter of all current small molecule targeted drugs discovery research [14]. However, it has

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become more apparent that these drugs have numerous adverse effects, including cardiotoxicities associated with hypertension, cardiac dysfunction, and QT prolongation [15]. Thus, we hypothesize whether BAG3 is responsible for the cardiotoxicity induced by small Kinase inhibitors. Protein-ligand docking, one of the most frequently used structure-based drug design methods [16], was applied in this study to evaluate the affinity between different clinically Phase I evaluated Kinase inhibitors and BAG3 protein. By further validated with western blot, AZD7762 was found to selectively promote BAG3 degradation by Ubiquitin-Proteasome Pathway.

Materials and methods

Molecular docking

Download the 3D structure file according to drugs name in PubChem Database (https://pubchem.ncbi.nlm.nih.gov/), and the download *sdf* format file. Convert *sdf* format file into a *mol2* file using openBabel software (version 3.1.1) (https://openbabel.org/wiki/Main_Page). Batch converts *mol2* format ligand files into *pdbqt* files using Python language. In the RCSB-PDB database (https://www.rcsb.org/search), enter BAG3, and download its structure. Save BAG3 protein structure in *pdb* format. Open ligands and receptors by AutoDock2 software (https://autodock.scripps.edu/), adjust parameters and obtain docking pocket information. Finally, batch molecular docking was finished by AutoDock Vina (https://vina.scripps.edu/) using Python command.

To visualize the 3D structure of the complex in this paper, BAG3 protein structure in *pdb* format was imported into the PyMOL software to remove the ligands and water molecules. PyMOL software (version 2.6.0) (https://www.pymol.org/2/) was applied to visualize 3D structural images. The docking parameter settings are as follows. In the genetic algorithm parameters for finding the optimal solution through docking settings, modify the Number of GA Runs to 50. Select Lamarckian GA for docking output files, and set all other parameters and running options to default values.

Cell culture and reagents

Hela and 293T cells were maintained in DMEM (Gibco) basic medium and added with 10% fetal bovine serum (Gibco). A Cell incubator (Thermo) was used to culture the cells at 37 °C with 5% CO_2. Rebastinib, AEW-541, AZD7762, IMD-0354, MLN-8054, SGI-1776, PF-3758309, PF-562271, and SCH900776 were purchased from MedChemExpress.

Western blotting

293T cells were lysed on ice with RIPA lysate buffer (P0013B, Beyotime) containing PMSF (1 mM, Beyotime) for 20 min. After adding loading buffer, the lysates were boiled in a metal bath for 10 min, and then cooled on ice immediately for 5 min. The intensity of the protein fragments was quantified using QuantityOne software (Bio-Rad, Berkeley, California, USA). The antibodies against BAG3 (10599-1-AP), Actin (23660-1-AP), and Chk1 (25887-1-AP) were from Proteintech (China).

Co-IP and immunoprecipitation assay

Different plasmids were co-transfected into 293T. Thirty hours later, cells were harvested. The cell lysates were pre-precipitated with anti-Flag beads (Sigma, A2220) overnight at 4 °C. Then, beads were washed eight times with cold lysis buffer. The precipitated proteins were boiled in a metal bath for 10 min and submitted to Western blotting.

Quantitative real-time PCR

In order to obtain the cDNA, 2 μg of fresh RNA was utilized for the reverse transcription with M-MLV reverse transcriptase (Invitrogen). Then, the obtained cDNA was used as the template for quantitative real-time PCR with Power SYBR Green PCR Master Mix (Roche) and analyzed with the Applied Biosystems 7900 Real-Time PCR System. The corresponding mRNA was examined by the following primers: BAG3, forward, 5'-TGGGAGATCAAGATCGACCC-3', reverse, 5'-GGGCCATTGGCAGAGGATG-3'; actin, forward, 5'-CCAACCGCGAGAAGATGA-3', reverse, 5'-CCAGAGGCGTACAGGGATAG-3';

Cell count kit-8 assay

Cell viability was detected by CCK8 assay. Briefly, 3000 cells/well were seeded in 96-well plates and cultured for 24 h. Cells were transfected with different plasmids, and then added with the corresponding drugs for 24 h. After remove the culture medium, 90 μl fresh culture medium plus 10 μl of CCK8 reagent were added into wells (Beyotime, China). Place the 96-well plate in the incubator for 30 min, and use multifunctional microplate reader to detect the absorbance value. The absorption wavelength was set to 450 nm.

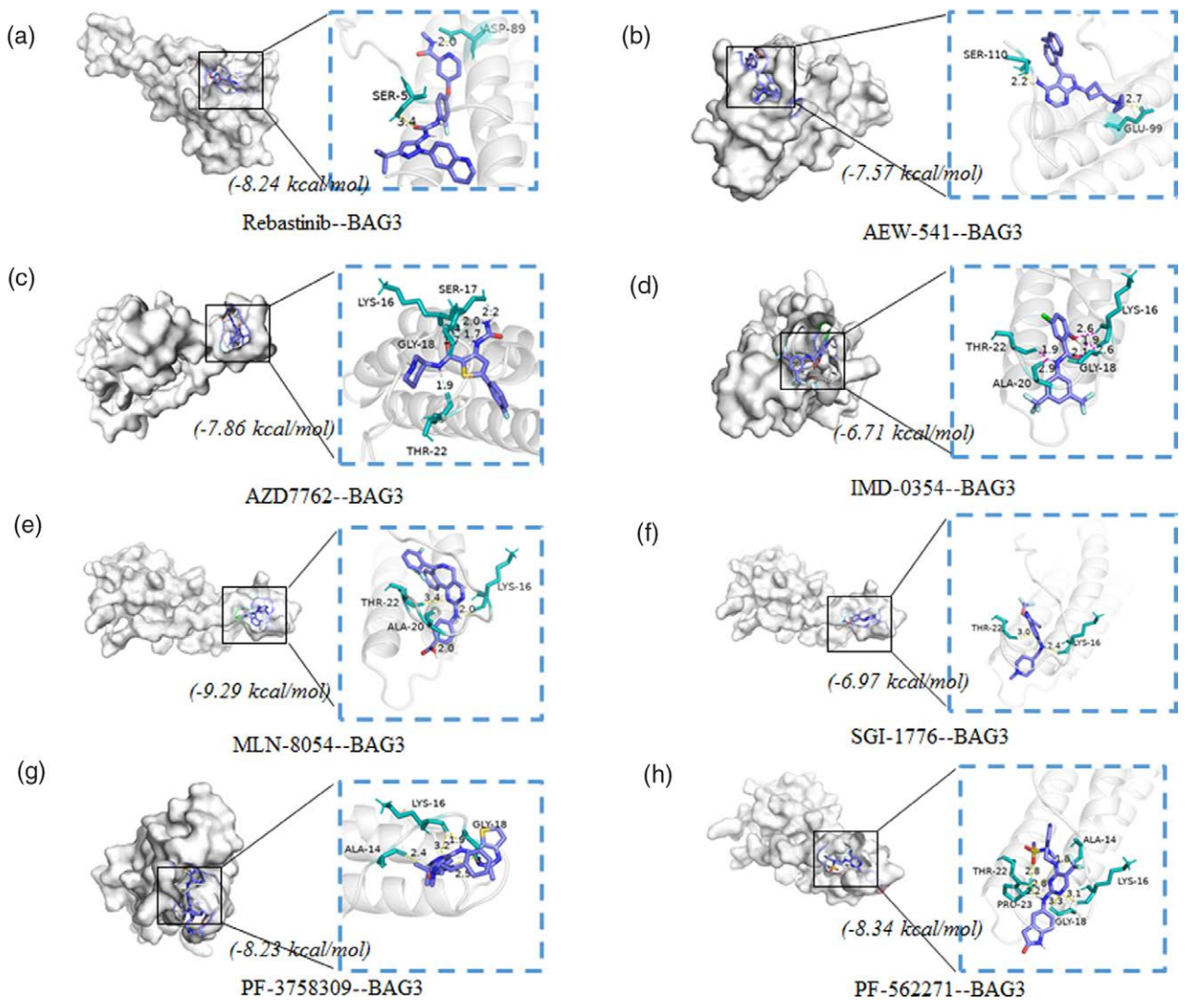
Clone formation assay

Cells were transfected with different plasmids. After 24 h, cells were digested and re-seeded into wells. The cells were treated with AZD7762 or AZD7762 plus Mln4924 for 24 h, and then cultured for 10 days. The culture media was replaced every third day. Cells were fixed with 4% paraformaldehyde, then stained with crystal violet. These experiments were performed in triplicate. The number of colonies that contain at least 50 cells each under inverted optical microscope were counted using Image J software.

Flow cytometry assay

Cells were transfected with different plasmids. After 24 h, cells were treated with DMSO, AZD7762, or AZD7762 plus Mln4924 for 24 h. Cells were digested by Trpsin without EDTA. Subsequently, clean the cells twice with pre-cooled PBS. 3 ml of cell suspension was

Fig. 1



Molecular docking between BAG3 and drugs. (a–h) 3D structures and affinity energy of BAG3-drugs complexes.

collected from each sample into a 5 mL centrifuge tube. Next, centrifugation was performed at 2000 rpm for 5 min, and the input medium was discarded, followed by centrifugation at 2000 rpm for 5 min, and the supernatant was removed. The cells were then re-suspended in 100 µl of binding buffer, and were successively added with FITC and propidine iodide, gently vortexed and incubated at room temperature without light for 15 min. Fluorescence intensity of dyes was detected by a flow cytometry to analyze the cell apoptosis rate.

Statistical analysis

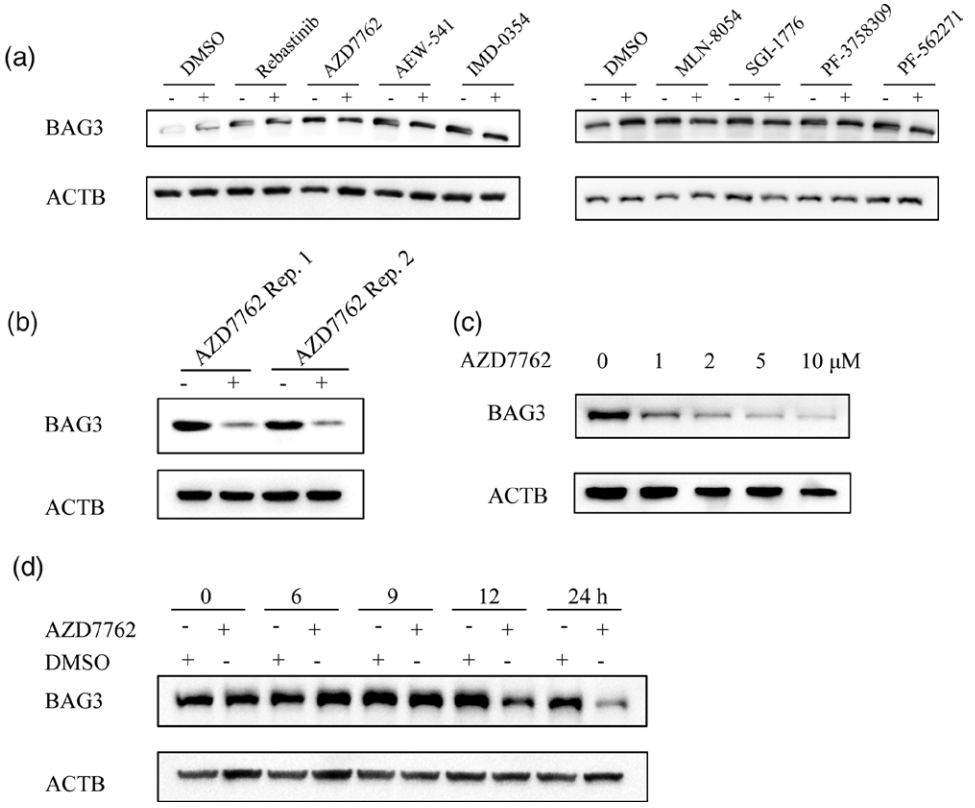
All experiments were performed three times, and the data were analyzed with GraphPad Prism 5 (La Jolla, California, USA). Results are presented as mean ± SD. A *P*-value of <0.05 was considered statistically significant.

Results

AZD7762 induce BAG3 protein level

In 2017, Klaeger *et al.* analyzed the cellular targets of 243 clinically studied kinase drug candidates using chemical proteomics. A good many kinase off-targets have been identified that may lead to side effects in the clinic [17]. To observe whether there were kinase inhibitors that could bind to BAG3, the affinity energy of 81 clinically Phase I evaluated small molecule kinase inhibitors [17] was calculated by molecular docking. Strikingly, almost half of the docking scores were lower than −7.0 kcal/mol, which means protein kinase inhibitors of these clinically Phase I drugs might be highly inclined to bind with BAG3. All the hit compounds with docking scores below −5.0 kcal/mol were listed in Supplementary Table S1, Supplemental digital content 1, http://links.lww.com/ACD/A507. Eight drugs, according to affinity values and

Fig. 2



AZD7762 decreases BAG3 protein level. (a) The effect of different drugs on the endogenous level of BAG3. 293T cells were treated with different drugs for 24 h. (b) 293T cells were treated with 10 μM AZD7762 for 24 h. BAG3 protein detected by western blot. (c) The effect of different concentrations of AZD7762 on the level of BAG3 protein. (d) The effect of different times of 10 μM AZD7762 on the level of BAG3 protein.

the frequency of PubMed literature reports were selected for further validation. The 3D structures of BAG3-Drugs complexes were presented in Fig. 1.

In the docking results, the Rebastinib-BAG3 complex has got highest affinity values. However, it just means that those drugs might bind with BAG3. Whether these drugs could target BAG3 degradation needs further verification. Western blot was applied to verify BAG3 protein level in the presence of the drugs individually. Surprisingly, AZD7762 showed to reduce BAG3 protein level significantly (Fig. 2a and b). What is more, AZD7762 treatment caused a dose- and time-dependent decrease of BAG3 protein (Fig. 2c and d). We found that short-time treatment of AZD7762 (no more than 12 h), did not reduce BAG3 protein. However, prolonged treatment of AZD7762 (longer than 12 h) could significantly decrease BAG3 protein level (Fig. 2d).

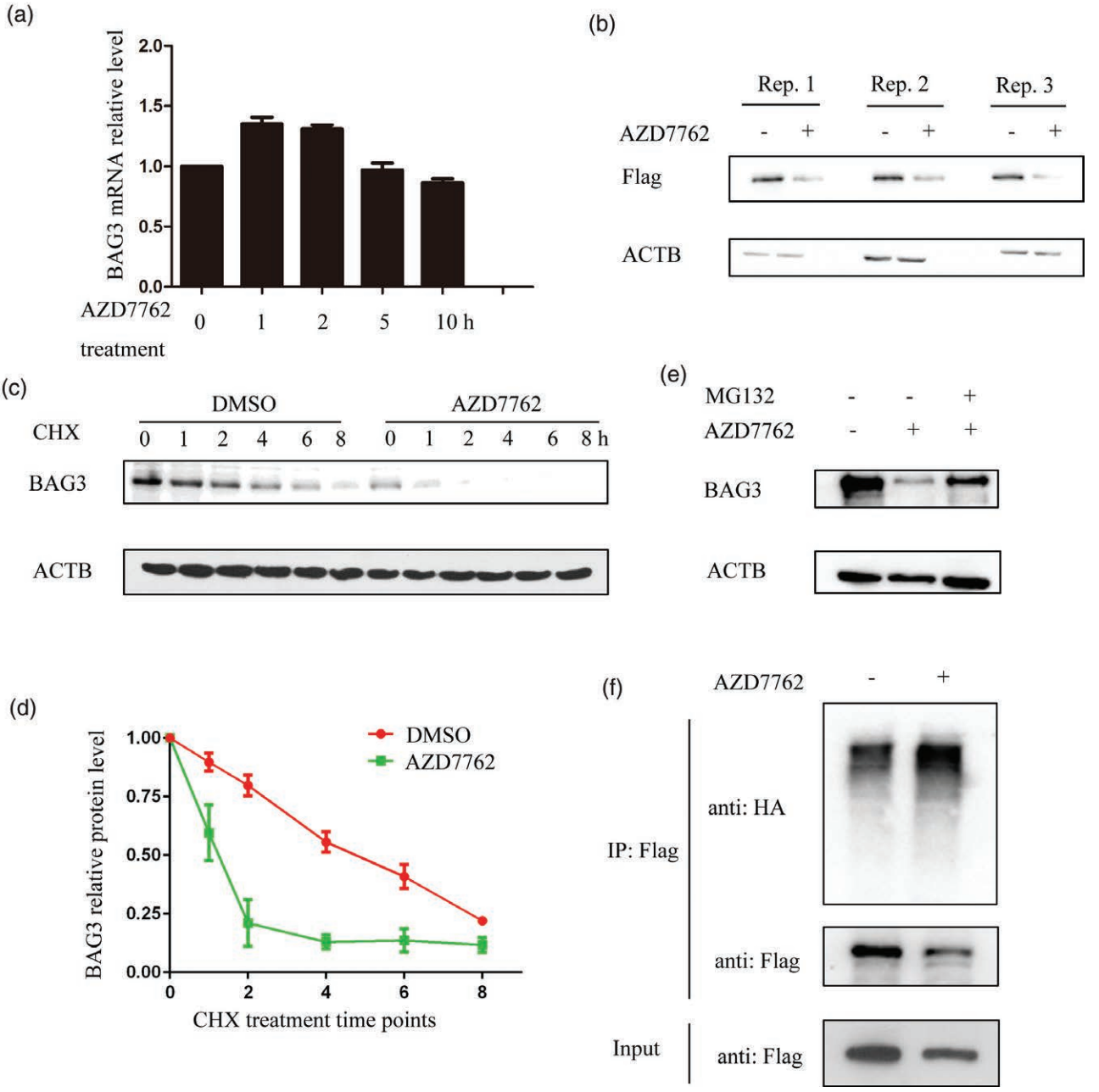
AZD7762 promotes BAG3 protein ubiquitin-dependent proteasome degradation

To confirm that AZD7762 represses BAG3 mRNA production or protein stability, RT-PCR was applied to detect the mRNA level after AZD7762 treatment. As shown in Fig. 3a, a low concentration of AZD7762 seems to induce BAG3 mRNA level. However, as the concentration increased, BAG3 mRNA was not decreased, which suggested that AZD7762-induced BAG3 protein decrease is not dependent on transcriptional inhibition. Indeed, exogenous expression of Flag-BAG3 protein was repressed under AZD7762 treatment in 293T cells (Fig. 3b). To confirm that AZD7762 was indeed promoted BAG3 proteolysis, we performed a cycloheximide (CHX) chase experiment. We found that in the presence of AZD7762, the half-life of BAG3 protein was shortened (Fig. 3c and d). Indeed, the proteasome inhibitor MG132 significantly restored the downregulation of BAG3 induced by AZD7762 (Fig. 3e). In addition, we found that adding AZD7762 could promote BAG3 ubiquitination significantly (Fig. 3f). Altogether, these results indicate that AZD7762 can promote proteasome degradation of BAG3 through ubiquitination modification.

AZD7762-induced BAG3 protein instability is not dependent on Chk1

As AZD7762 is a Chk1 kinase inhibitor, we hypothesize that Chk1 might involve in AZD7762-directed BAG3 degradation. To our surprise, SCH900776, another Chk1 inhibitor, was not able to induce BAG3 degradation

Fig. 3



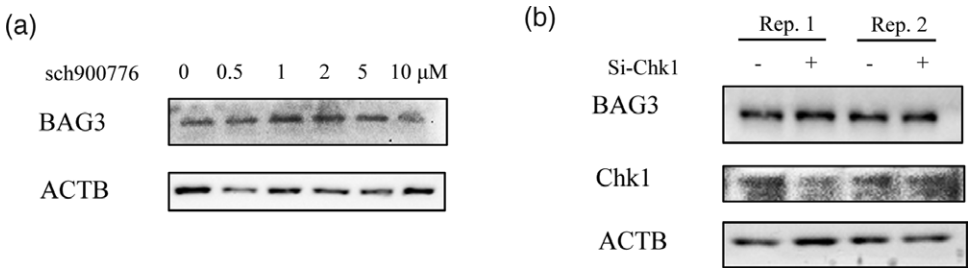
AZD7762 regulates the stability of BAG3 protein through ubiquitination. (a) The effect of AZD7762 on the mRNA level of BAG3. (b) AZD7762 (5 μM) regulates the level of exogenous BAG3 protein and promotes its degradation. (c) Cycloheximide (25 μg/ml) was added to inhibit the intracellular translation, and the effect of AZD7762 on the half-life of endogenous BAG3 protein was detected. (d) MG132 (10 μM) treatment can inhibit the degradation of BAG3 induced by AZD7762. (e) AZD7762 promotes ubiquitination of exogenous expressed BAG3.

(Fig. 4a), which suggested that Chk1 kinase activity is not essential for AZD7762-induced BAG3 protein degradation. Then, siRNA was applied to knock down Chk1 expression. As shown in Fig. 4b, Chk1 protein reduced significantly. However, the BAG3 protein level was not affected. These results supported that AZD7762-induced BAG3 protein instability is not dependent on Chk1.

AZD7762 promotes BAG3 degradation through CRBN

Cullin-E3 ubiquitin ligase is a large family in charge of protein turnover in cells. We have previously reported that Cul1-FBXO22 could promote BAG3 degradation through the ubiquitin-proteasome pathway [18]. To test the possibility that Cullin-E3 ubiquitin ligase is responsible for the degradation of BAG3 in the presence of AZD7762, Mln4924 was added to block Cullin family E3

Fig. 4



AZD7762 promotes BAG3 degradation independent of Chk1. (a) 293T cells were treated with different concentrations of sch900776 and Western blot was applied to detect the level of BAG3 protein. (b) The expression of Chk1 was interfered with by small RNA interference, and the BAG3 protein level was observed by Western blot.

ubiquitin ligase activity. Indeed, Mln4924 could block AZD7762-mediated BAG3 degradation (Fig. 5a). To clarify whether FBXO22 is the key E3 ligase involved in AZD7762-mediated BAG3 protein degradation, FBXO22 knockout cell and control cells were treated with AZD7762. As shown in Fig. 5b, AZD7762 could reduce BAG3 protein in FBXO22-KO cells, which suggested that FBXO22 is not indispensable for AZD7762-directed BAG3-degradation.

Consistently, when the dominant-negative Cullin 4 (dn-Cul4) was overexpressed to inactivate the E3 complexes, BAG3 protein was significantly accumulated under AZD7762 treatment (Fig. 5c). These results suggested that cullin4a is important for the degradation. DCAFs are responsible for the substrate specificity of Cul4-E3 complex. DCAF7 was overexpressed when cells were exposed to AZD7762. However, DCAF7 could not affect the progress of AZD7762-induced BAG3 degradation (Fig. 5d). Thalidomide and its derivatives, as a reported protein degraders, could promote the degradation of diverse multiple neo-substrates through Cul4-CRBN complex [19].To clarify whether AZD7762 is a novel protein degrader by promoting Cul4-CRBN mediated BAG3 degradation, GFP-CRBN was overexpressed with or without AZD7762 treatment. As shown in Fig. 5e, CRBN could not induce BAG3 degradation alone. However, in the presence of AZD7762, CRBN promoted BAG3 degradation significantly. We next investigated the ubiquitination of BAG3 with or without CRBN. The results showed that CRBN promotes BAG3 ubiquitination in the presence of AZD7762 (Fig. 5f). By immunoprecipitation test, we revealed that AZD7762 could enhance the binding between CRBN and BAG3 (Fig. 5e). Altogether, these results suggested that AZD7762 promotes BAG3 degradation through CRBN-directed ubiquitination.

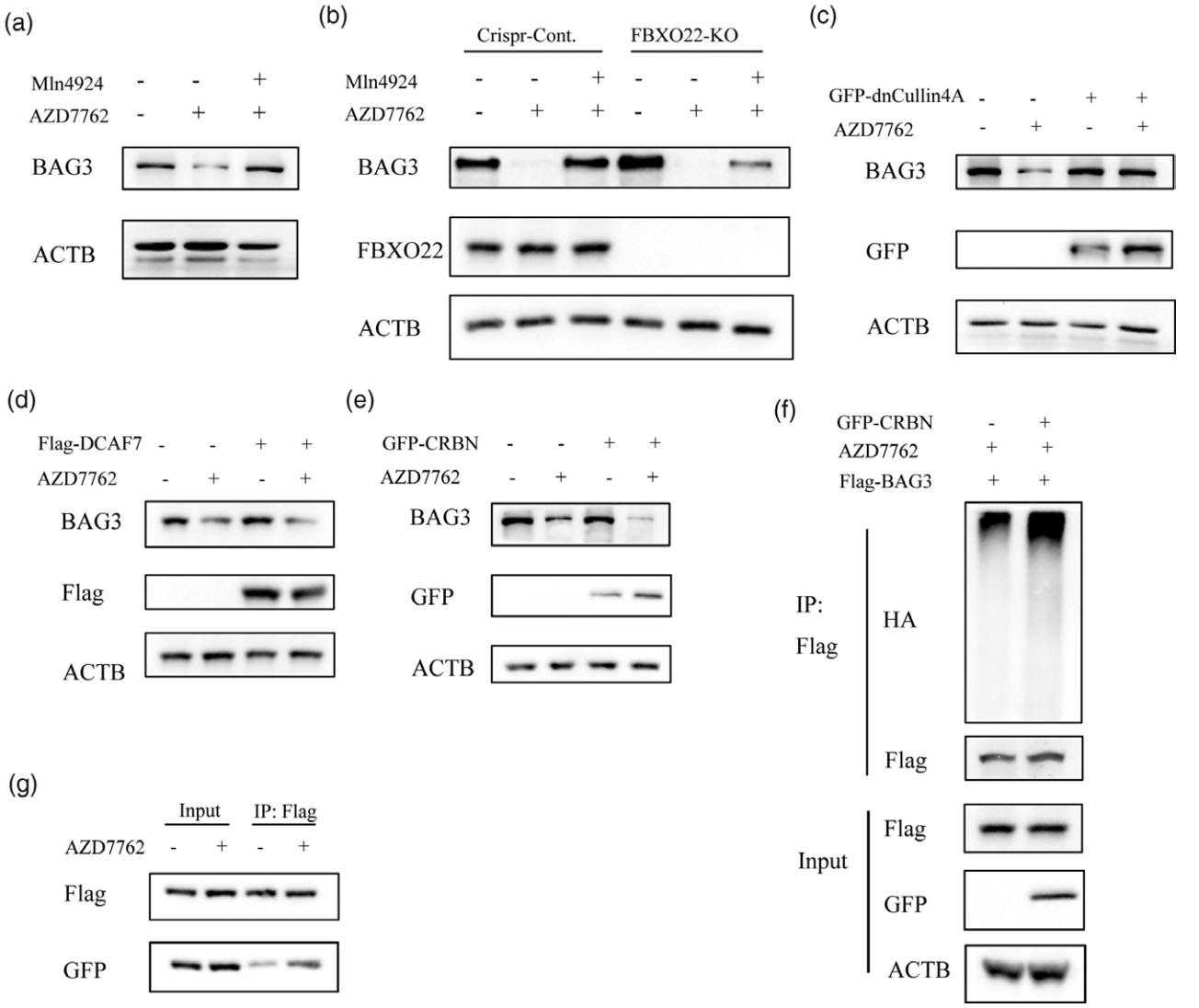
AZD7762 induces cancer cells death through BAG3

Since BAG3 is critical for the survival of different types of human cancer cells, we speculated that AZD7762-induced BAG3 protein instability might sensitize cancer cells to cell death. To this end, we first test the cell viability by CCK8 assay in Hela cells. Indeed, AZD7762 reduces cell viability in a dose-dependent manner. Consistent with our previous results, Mln4924 or forced expression of BAG3 could rescue cell viability very well (Fig. 6a). By cell clone formation experiment, the effect of AZD7762 on cancer cell proliferation was confirmed (Fig. 6b). These results implied that AZD7762 inhibits its cancer cell growth by inducing BAG3 degradation. Moreover, cell death was assessed by Annexin-V/PI double staining-based flow cytometry analysis. Hela cells were treated with AZD7762 or AZD7762 plus Mln4924. As depicted in Fig. 6c, AZD7762 could induce cell death in Hela cells. Overexpression of BAG3, or Mln4924 treatment could inhibit cell death induced by AZD7762. Together, these data showed that AZD7762 displayed anti-tumor activity through BAG3 protein modulation in vitro.

Discussion

AZD7762 was discovered as a potent and selective Chk1 kinase inhibitor, which could inhibit DNA damage response and induce tumor cell death [20]. However, the development of AZD7762 is not going forward owing to unpredictable cardiac toxicity in clinical trials [21]. Nevertheless, the cardiac defects induced by AZD7762 were not found in Chk1 knockout mice [22]. In addition, a cardiomyocyte impedance assay suggests that Chk1 did not correlate with the cardiovascular effects induced by AZD7762. Therefore, cardiac toxicity induced by AZD7762 might cause by off-target [23]. Nevertheless, the molecular mechanism induced by AZD7762 in myocardial toxicity is not clear. BAG3 works as an important cardiotoxicity biomarker, and was reported to be responsible for anthracycline-linked cardiotoxicity [24]. By using molecular docking, we identified that BAG3 is a potential target of AZD7762. Further validation revealed that AZD7762 could promote BAG3 degradation through the ubiquitin-proteasome pathway. This off-target identification may offer opportunities to explain undesired myocardial toxicity side effects.

Fig. 5

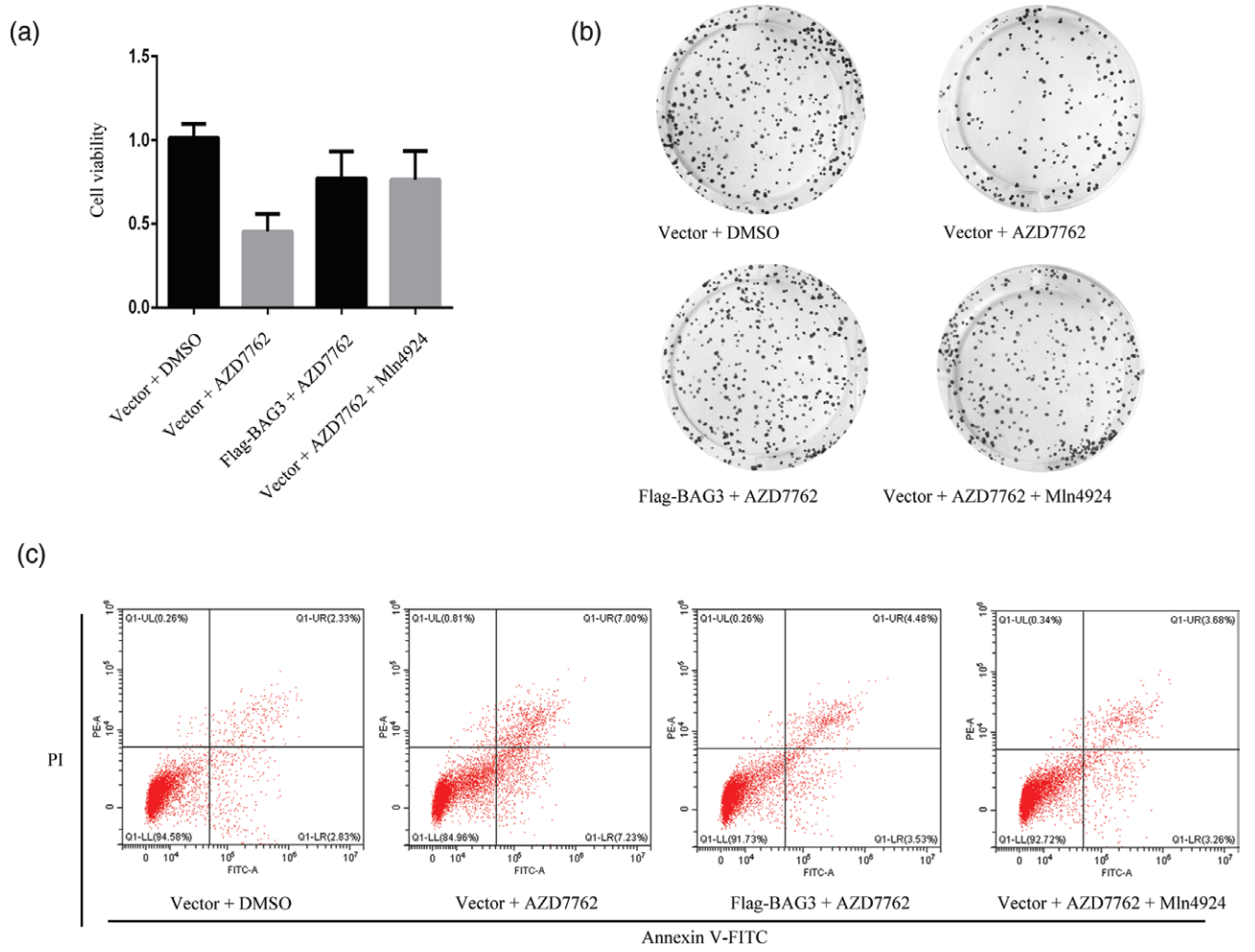


AZD7762 promotes BAG3 degradation through CRBN. (a) AZD7762-induced BAG3 degradation was abolished by Mln4924 (1 μM). (b) AZD7762 and MLN4924 were added to FBXO22-knockout cell lines and control cell lines respectively, and BAG3 expression was detected by Western blot. (c) The dn-Cul4a inhibits the degradation of BAG3 induced by AZD7762. (d) Overexpression of DCAF7 does not affect the regulation of BAG3 by AZD7762. (e) Overexpression of CRBN promotes the degradation of BAG3 by AZD7762. (f) Overexpression of CRBN promotes ubiquitination of exogenous BAG3 in the presence of AZD7762. GFP-CRBN, Flag-BAG3, and HA-Ub were transfected into 293T cells, and Flag-BAG3 was immune-enriched using anti-Flag beads. Cells were treated with AZD7762 for 24 h. Proteins were detected by Western blot. (g) GFP-CRBN and Flag-BAG3 were transfected into cells, and Flag-BAG3 was immune-enriched using anti-Flag beads. GFP-CRBN and Flag-BAG3 were detected by Western blot.

Protein degraders are currently under rapid development as a promising modality for drug discovery, which are compounds that selectively knock down target proteins via intracellular protein degradation pathways. The conventional small molecule compounds require a suitable binding pocket for the substrate, which makes over 85% of the proteome undruggable [25]. Protein degraders represent a novel class of therapeutic agents that employ a different mechanism of action than conventional small molecule compounds, and make it possible to target many proteins [19,26]. Thalidomide derivatives are the first, and currently the only, protein degraders with demonstrated clinical benefit [19]. The identification of more protein degraders would greatly promote the discovery of novel drugs used for cancer treatment.

BAG3 protein is constitutively expressed in multiple tumor types, which can promote cell survival, and regulate autophagy and motility in cancer cells [4]. By weakening HSP70 interaction with anti-apoptotic proteins, BAG3 improve Akt, IκB kinase, BRAF, Mcl-1, Bcl-2, and Bcl-XL protein level, resulting in tumor cell survival and growth. BAG3 also promotes cancer invasiveness by being

Fig. 6



AZD7762 induces cancer cell death through BAG3. Vectors and Flag-BAG3 were transfected into Hela cells. Then cells were treated with AZD7762 (10 μM) or Mln4924 (1 μM). Cell viability, proliferation rates, and cell death were detected by CCK8 (a), cell colony formation assay (b), and flow cytometry (c).

associated with FAK, Rac1, PDZGEF2, E/N-cadherins, and Vimentin. Accordingly, BAG3 is widely recognized as an excellent therapeutic target in cancer treatments [4–7]. Herein, we have identified that AZD7762 could prominently modulate BAG3 protein stability by ubiquitination. With modern drug delivery systems developed, which could release drugs in the right place and at the right time [27,28], AZD7762 or the derivative would be a promising drug for cancer treatment in the future.

Conclusion

Altogether, AZD7762 was found to be a protein degrader, which could promote BAG3 binds to CRBN, and promote BAG3 degradation through the Ubiquitin-Proteasome Pathway.

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Y.L. and C.Y. conceived the study; S.L. and X.Y. designed the experiments. Y.L., C.Y., M.H., W.X., D.L., R.W., and S.Z. performed experiments. Y.L., Y.Q., and W.W. analyzed the data. Y.L. and S.L. wrote the manuscript. S.L. supervised the study. All authors have read and approved the final version for publication.

Conflicts of interest

There are no conflicts of interest.

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