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Research paper

Chk1 inhibitors overcome imatinib resistance in chronic myeloid leukemia cells

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ABSTRACT

Drug resistance to tyrosine kinase inhibitors (TKIs) is currently a clinical problem of chronic myelogenous leukemia (CML). Bcr-Abl protein depletion is considered as a way to overcome drug resistance to TKIs. In our study, Chk1 inhibitors, AZD7762 and MK-8776, had strong antitumor effects on CML cell line KBM5 and imatinib-resistant form KBM5^{T315I}. Moreover, Chk1 inhibitors showed a strong cytotoxic effect on leukemia cells from primary CML and imatinib-resistance CML patients, but low cytotoxic effect on normal human mononuclear cells. Then, we found that Chk1 inhibitors induced apoptosis and increased DNA damage in CML cell lines with the degradation of the Bcr-Abl protein. Using the proteasome inhibitor and an immunoprecipitation assay, we found that Chk1 inhibitors triggered the degradation of Bcr-Abl through ubiquitination, which is depending on E3 ubiquitin ligase CHIP. At last, MK-8776 showed a significant tumor-suppressive effect of KBM5^{T315I} cell in xenograft tumor models. Taking together, these findings suggest that targeting Chk1 may overcome TKIs resistance for the treatment of CML.

1. Introduction

Chronic myelogenous leukemia (CML) is characterized by the Philadelphia (Ph) chromosome, which results from the t(9;22)(q34;q11) balanced reciprocal translocation. The molecular consequence of this translocation is the generation of the *BCR-ABL1* oncogene that encodes the chimeric Bcr-Abl protein with constitutive kinase activity. Bcr-Abl is believed to be the driving force for CML development [1–3]. Bcr-Abl kinase is specifically expressed in leukemic cells, but not normal cells, so inhibitors that target this kinase have been developed [4,5]. Imatinib, the first-generation tyrosine kinase inhibitor, competitively binds to the Bcr-Abl ATP-binding site and blocks the signal pathway. It is, most often, the first-choice treatment for patients with CML in the chronic phase [6,7]. However, some patients show primary resistance or relapse after several years of treatment (acquired resistance) [8]. Bcr-Abl kinase domain mutation, *BCR-ABL1* amplification and over-expression and clonal evolution with activation of additional oncogenic pathways are the most common causes of resistance [9]. Although many other TKIs (nilotinib, dasatinib, ponatinib et al) are used as second-line therapy for patients with resistance or intolerance to imatinib, several kinase domain mutations confer high-level resistance to one or more of these therapies [10,11]. Furthermore, TKIs cannot eliminate leukemia stem cells, because of the survival of CML stem cells does not depend on Bcr-Abl kinase activity [12]. Therefore, exploring novel therapeutic strategies to improve the treatment of CML are essential.

Checkpoint signaling is initiated following genotoxic insult by the proximal kinases, ATR and ATM, two phosphatidylinositol 3-kinase family members. Activation of these kinases leads to activation of checkpoint kinases 1 and 2 (Chk1 and Chk2) [13]. Upon activation, Chk1 and Chk2 phosphorylate downstream effectors and propagate checkpoint signaling, leading to intra-S phase and G2/M phase arrest. Checkpoint kinases inhibition abrogates DNA damage-induced cell cycle arrest allowing cells to enter mitosis despite the presence of DNA damage, which can lead to cell death [14].

AZD7762, an ATP-competitive Chk1/2 inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies [13]. It has been demonstrated that AZD7762 strongly sensitizes urothelial carcinoma cells to gemcitabine [15]. The dual Chk1/Chk2 inhibitor AZD7762 appears to inhibit only Chk1 protein in the cell, rather than by inhibiting both proteins following DNA damage. [16,17] In addition to potentiating cytotoxic chemotherapeutics, AZD7762 is also a potent radiation sensitizer of p53-compromised cells both *in vitro* and *in vivo* [18]. However, the phase I development of AZD7762 was not going forward owing to unpredictable cardiac toxicity in combination with gemcitabine with advanced solid tumors in US patients [19]. MK-8776

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sensitizes human AML cells to HDAC inhibitors by targeting the intra-S checkpoint and DNA replication and repair [20]. MK-8776 also enhances sensitivity to gemcitabine in multiple solid tumors, and the phase I study shows MK-8776 is well tolerated as monotherapy and in combination with gemcitabine, and the phase II study is processing [21,22]. Other works have been shown that Chk1 inhibitors are being developed not only as chemopotentiators, but also as single-agent therapies [14,23].

Bcr-Abl expression increases DNA double-strand damage after etoposide and leads to a defect in an intra-S phase checkpoint, which represents the disruption of a cell cycle checkpoint by Bcr-Abl [24]. In our study, we found that AZD7762 and MK-8776 exhibit dramatic cytotoxicity toward CML cell line KBM5 and its derived imatinib-resistant KBM5^{T315I}. Chk1 is overexpressed in primary and imatinib-resistant leukemia cells from CML patients, and AZD7762 and MK-8776 show significant cytotoxic effects on leukemic cells from patients. The compounds strongly induce DNA damage and increases γ-H_2AX expression, eventually leading to apoptosis. Interestingly, MK-8776 triggered the degradation of Bcr-Abl through ubiquitination pathway, which is depending on E3 ubiquitin ligase CHIP. MK-8776 showed a significant tumor-suppressive effect of KBM5^{T315I} cell in xenograft tumor models. These results may offer a novel approach to overcome TKIs resistance in CML cells.

2. Materials and methods

2.1. Cell lines

KBM5 and KBM5^{T315I} cells were kindly provided by professor Jingxuan Pan. KBM5 and KBM5^{T315I} cells were cultured in IMDM supplemented with 10% heat-inactivated fetal bovine serum (Gemini, 900-108), penicillin (50 U/ml)/streptomycin (50 μg/ml) (Sangon Biotech, E607011) in 5% CO_2/95% air-humidified atmosphere at 37 °C. KBM5 expressing the 210 kD BCR-ABL protein and lacking normal c-ABL was derived from a female myeloid CML patient in blast crisis. KBM5^{T315I} line was derived from KBM5 by exposing to increasing concentrations of Gleevec, leading to the selection of survival clone harboring T315I mutation as previously described [25,26]. KBM5-T315I cells were routinely maintained in culture medium containing 1 μm Gleevec.

2.2. Reagents

Imatinib (STI571, S2475), AZD7762 (S1532), MK-8776 (S2735) and MG132 (S2619) were purchased from Selleck Chemicals. Annexin-V apoptosis detection kit (88-8007-74) was obtained from eBioscience. The mouse monoclonal antibody against β-actin (60008-1-Ig) was obtained from Proteintech. Antibodies against Chk-1(SC-8408), c-Abl (SC-887), Ub (SC-8017) and Mcl-1 (SC-819) were purchased from Santa Cruz Biotechnology. Cleaved caspase-3 (9661), caspase-9 (9502), γ-H2AX (9718) and CHIP (2080) antibody were purchased from Cell Signaling Technology.

2.3. Cell viability assay

CML cells were incubated with different concentrations of AZD7762, MK-8776 or imatinib for 24 and 48 h. Cell proliferation was assayed using a Cell Counting Kit 8 (CCK-8) (DOJINDO, CK04) according to the manufacturer's instructions. CCK-8 allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays. Peripheral blood from 3 cases of normal humans and bone marrow samples from 4 imatinib-resistances CML patients with T315I mutation were collected, and mononuclear cells were isolated through Ficoll-Hypaque (GE, B17544652) centrifugation and cultured with small molecule compounds for 48 h to assess cell proliferation.

2.4. Quantification of apoptosis

Apoptosis was measured using Annexin V Apoptosis Detection Kit (eBioscience, 88-8007-74) according to the manufacturer's instructions. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase.

2.5. Western blot analysis

Cells were lysed by using RIPA buffer (pH 7.4) containing protease inhibitor cocktail (Roche, 04693116001). Protein concentrations of the extracts were measured by BCA™ Protein Assay Kit (Pierce, 23225) and equal amount of total protein from each sample was separated with SDS-PAGE and then transferred to nitrocellulose membrane followed by probing with the indicated antibodies. The signals were detected using the chemiluminescence phototope-HRP kit (Cell Signaling) according to the manufacturer's instructions.

2.6. Real-time quantitative PCR

Total RNA was isolated from cells by using RNAiso Plus (Takara, 9108), 500 ng total RNA was used for reverse transcription by PrimeScript RT reagent Kit (Takara, DRR037A). Real-time quantitative PCRs were performed with SYBR Green PCR Master Mixture Reagents (Roche, 04913850001) with the ABI Prism 7300 system. The specific primers were shown as followed: *BCR-ABL1* forward 5′-CTGGCCCAAC GATGGCGA-3′ reverse 5′-CACTCAGACCCTGAGGCTCAA-3′ GAPDH forward 5′-CTTAGCACCCCTGGCCAAG-3′ reverse 5′- TGGTCATGAG TCCTTCCACG-3′. The comparative ΔΔ CT method was used to determine the quantification of gene expression. The primers were synthesized by Sangon Biotech (Shanghai, China).

2.7. Retroviral transduction and generation of stable cell lines

For gene silencing, CHIP specific shRNA and control shRNA were cloned into the pSIREN-RetroQ vector as described previously. [27] ShRNA oligonucleotides targeting CHIP was designed and synthesized. The targeting sequence was as followed: 5′-GGAGCAGGGCAATCGT CTG-3′.

2.8. Immunoprecipitation (IP)

Whole-cell extracts were prepared by using lysis buffer (Beyotime, China), then they were incubated with the appropriate antibodies overnight at 4 °C. Protein A&G beads (Abmart, USA) were added and the incubation was continued for 4 h at 4 °C. Beads were washed three times with PBS buffer, then separated by SDS-PAGE. For detection of c-ABL specific ubiquitination, KBM5^{T315I} cells were pretreated with 10 μM MG-132 for 1 h and then treated with DMSO or 10 μM MK-8776 for 2 h.

2.9. Tumor growth in xenografts

4×10^7 of KBM5^{T315I} cells were implanted subcutaneously into the right flank of female Balb/c (nu/nu) mice (Slac Laboratory Animal Co., Ltd., China). Tumor sizes were measured every three days using calipers and volumes were calculated using a standard formula (width^2 × length/2). In order to detect tumor cell proliferation and apoptosis, hematoxylin and eosin (H&E) staining and IHC were used. TUNEL assays and PCNA immunohistochemical (IHC) staining were detected. IHC section analysis was done using microscopy (Leica). 7 days after subcutaneous inoculation, when tumors were palpable (100–150 mm^3), mice were randomized to receive treatment with vehicle (10% DMSO, 10% Kolliphor® ELP and 10% propylene glycol in 0.9% saline water), MK8776 (25 mg/kg, injected intraperitoneally every day for 10 workdays) and imatinib (25 mg/kg, treated