Preclinical Development

The Checkpoint Kinase Inhibitor AZD7762 Potentiates Chemotherapy-Induced Apoptosis of p53-Mutated Multiple Myeloma Cells

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Abstract

DNA cross-linking agents are frequently used in the treatment of multiple myeloma–generating lesions, which activate checkpoint kinase 1 (Chk1), a critical transducer of the DNA damage response. Chk1 activation promotes cell survival by regulating cell-cycle arrest and DNA repair following genotoxic stress. The ability of AZD7762, an ATP-competitive Chk1/2 inhibitor to increase the efficacy of the DNA-damaging agents bendamustine, melphalan, and doxorubicin was examined using four human myeloma cell lines, KMS-12-BM, KMS-12-PE, RPMI-8226, and U266B1. The in vitro activity of AZD7762 as monotherapy and combined with alkylating agents and the novel drug bortezomib was evaluated by studying its effects on cytotoxicity, signaling, and apoptotic pathways. The Chk1/2 inhibitor AZD7762 potentiated the antiproliferative effects of bendamustine, melphalan, and doxorubicin but not bortezomib in multiple myeloma cell lines that were p53-deficient. Increased γH2AX staining in cells treated with bendamustine or melphalan plus AZD7762 indicates a greater degree of DNA damage with combined therapy. Abrogation of the G2–M checkpoint by AZD7762 resulted in mitotic catastrophe with ensuing apoptosis evidenced by PARP and caspase-3 cleavage. In summary, the cytotoxic effects of bendamustine, melphalan and doxorubicin on p53-deficient multiple myeloma cell lines were enhanced by the coadministration of AZD7762. These data provide a rationale for testing these combinations in patients with relapsed and/or refractory multiple myeloma. Mol Cancer Ther; 11(8); 1781–8. ©2012 AACR.

Introduction

Multiple myeloma is an incurable hematologic malignancy that arises from the dysregulated proliferation of plasma cells. While traditional chemotherapy regimens including alkylating agents and anthracyclines have modest benefits in this disease, high-dose melphalan supported by autologous stem cell transplantation has been shown to increase the incidence of complete and near complete remissions in approximately 50% of patients (1). However, almost all patients relapse and respond to subsequent therapy only temporarily. Dysregulation of FGFR3 and loss of expression or mutation in TP53 play a key role in determining tumor progression and drug resistance in multiple myeloma (2). The introduction of novel drugs such as thalidomide, bortezomib, and lenalidomide has dramatically improved patient outcomes both during the initial therapy and at relapse. In some studies these drugs overcome the poor prognosis associated with specific chromosomal lesions (3). However, there are no reliable data showing improved survival when patients with p53 abnormalities are treated with these agents or even allogeneic stem cell transplant (4). Therefore, new therapeutic strategies are needed.

Chemotherapy, commonly used to treat multiple myeloma, includes several DNA-damaging agents such as the nitrogen mustards, bendamustine and melphalan, and doxorubicin that is an anthracycline antibiotic. All 3 agents cause DNA cross-links and strand breaks thereby eliciting the DNA damage response (DDR), which ensures genomic stability by activating cell-cycle checkpoints and promoting DNA repair. Checkpoint kinase 1 (Chk1) is a critical component of the DDR to replicative stress. Once activated by ATR, Chk1 promotes cell-cycle arrest via phosphorylation of Cdc25 phosphatases resulting in their destruction or sequestration, preventing the reversal of inhibitory phosphorylation of the cyclin-dependent kinases (5). Chk1 also inhibits firing of replication origins...
(6), stabilizes stalled replication forks (7), and regulates proteins involved with DNA repair (8, 9). A structurally dissimilar checkpoint kinase, Chk2 is activated by ataxia telangiectasia mutated (ATM) in response to double strand breaks; it promotes both Cdc25 inactivation and p53-dependent cell-cycle arrest.

Chk1 inhibitors have been shown to potentiate conventional DNA-targeted chemotherapeutics both in vitro and in vivo (10, 11). AZD7762, an ATP-competitive Chk1/2 inhibitor that has been studied clinically, increases the level of DNA damage and abrogates cell-cycle arrest in cells treated with gemcitabine or irinotecan (12, 13). Given the known clinical activity of alkylating agents in multiple myeloma, we examined whether combining AZD7762 with bendamustine, melphalan, and doxorubicin could potentiate the cytotoxic effects of these agents on myeloma cell lines. We also investigated AZD7762 in combination with the proteasome inhibitor bortezomib, an active agent in multiple myeloma that is not known to elicit the DDR.

**Materials and Methods**

**Cells**

The KMS-12-BM and KMS-12-PE cell lines were purchased from the German Collection of Microorganisms and Cell Cultures and were characterized as per published guidelines (14). RPMI-8226, U266B1, and HCT116 colorectal cancer cell lines were purchased from the American Type Culture Collection (ATCC) and were verified according to established recommendations from the ATCC Technical Bulletin no. 8; Manassas: ATCC; 2007. The cells lines were not authenticated at our institution. All cells were maintained in RPMI with 10% FBS (HyClone) and 1% penicillin/streptomycin. Cells were grown at 37°C, 90% humidity, and 5% CO2. For all experiments, multiple myeloma cells were seeded at 4 x 10^5 to 6 x 10^5 cells/mL in fresh media.

**Drugs**

AZD7762 (12) was provided by AstraZeneca. Bendamustine, melphalan, and doxorubicin were purchased from Sigma. Bortezomib was commercially obtained from Millenium Pharmaceuticals, Inc. The concentrations of melphalan, bendamustine, doxorubicin, and bortezomib used in these studies were selected on the basis of in vitro combination studies in multiple myeloma (15, 16), and the concentrations and conditions selected for AZD7762 have been previously reported (12).

**Proliferation assays**

Cells were seeded at 4 x 10^5 to 6 x 10^5 cells/mL in fresh media in 96-well plates. After 24 hours, cells were treated with drug combinations concurrently for 72 hours and assayed with WST-8 (Dojindo Molecular Technologies, Inc.) as per manufacturer’s directions.

**Immunoblotting**

Immunoblotting was conducted as previously described (17). Mouse monoclonal antibodies were
Cdc25A (F-6), Chk1 (G-4), Chk2 (A-11), cyclin A (H-432), p53 (DO-1; Santa Cruz Biotechnology, Inc.); p21 (Ab-1; Calbiochem/Merck KGaA); PARP (19F4; Cell Signaling); and g-H2AX (JBW301; Millipore). Rabbit polyclonal antibodies were phospho-Ser 317 Chk1, phospho-Ser 345 Chk1 (133D3), phospho-Thr 68 Chk2, phospho-Ser 15 p53, a-tubulin (11H10), phospho-Ser 10 histone H3, cleaved caspase-3 (Asp175), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 14C10; Cell Signaling).

Flow cytometry
Cell-cycle distribution was analyzed using flow cytometry (fluorescence-activated cell-sorting analysis; FACS) for DNA content and mitotic marker MPM-2 (18).

Measurement of apoptosis and micronucleation
Cells were harvested, fixed in 3% paraformaldehyde, stained with 4',6-diamidino-2-phenylindole, and examined with fluorescence microscopy. Cells were scored as apoptotic on the basis of the presence of condensed fragmented chromatin. Micronucleated cells were defined as cells containing multiple (>3) small decondensed interphase nuclei. At least 500 cells were counted per sample. The percentage of apoptotic and micronucleated cells was measured using quantitative fluorescent microscopy (QFM; ref. 17).

Statistical considerations
All studies were done in at least triplicate unless otherwise indicated. Mean and SD were calculated and statistical significance was calculated by the Student t test.

Results
In vitro cytotoxicity of AZD7762 combinations on multiple myeloma cell lines

![Figure 2. Bendamustine, melphalan, and doxorubicin are potentiated by Chk1/2 inhibitor AZD7762. KMS-12-BM, KMS-12-PE, RPMI-8226, and U266B1 cells were treated with the indicated concentrations of bendamustine (A), melphalan (B), doxorubicin (C), or bortezomib (D) along with vehicle or 100 nmol/L AZD7762 for 72 hours before assay with WST-8 reagent.](attachment:image)
KMS-12-PE, RPMI-8226, and U266B1 cells. As a single agent, exposure to AZD7762 did not result in growth inhibition. However, as shown in Fig. 2, a fixed concentration of AZD7762 (100 nmol/L) potentiated growth inhibition induced by increasing concentrations of bendamustine (25–100 μmol/L; Fig. 2A), melphalan (5–25 μmol/L; Fig. 2B), or doxorubicin (0.5–1.5 μmol/L; Fig. 2C) on KMS-12-BM, KMS-12-PE, and RPMI-8226 cells with modest effects on U266B1 cells. Enhanced growth inhibition was not observed following treatment with the combination of AZD7762 (100 nmol/L) and bortezomib (at concentrations from 2.5 to 10 nmol/L; Fig. 2D).

**Induction of apoptosis and micronucleation**

The induction of apoptosis and micronucleation was evaluated in KMS-12-PE cells treated with bendamustine (50 or 100 μmol/L) or melphalan (10 or 25 μmol/L) together with AZD7762 (100 nmol/L) for 48 hours (Fig. 3). This caused a dose-dependent increase in apoptosis from 3% (with 50 μmol/L bendamustine alone) to 25% with the combination, and from 5% (with 100 μmol/L bendamustine alone) to 35% with the combination (Fig. 3A). An increase in micronucleation was also seen at both concentrations of bendamustine with the greatest increase occurring when 50 μmol/L bendamustine was used (1%–50%; Fig. 3A). AZD7762 also increased apoptosis from 5% with 10 μmol/L melphalan alone to 25% with the combination and from 15% with 25 μmol/L melphalan alone to 45% with the combination (Fig. 3B). Micronucleation also increased with combination therapy, especially when 10 μmol/L melphalan was used (going from 1% to 35%). These results suggest a mitotic catastrophe with ensuing apoptosis (19, 20). Representative apoptotic and micronucleated cells are shown in Fig. 3C.

**Signaling and cell-cycle analyses**

Immunoblot analysis of KMS-12-PE cells treated with bendamustine for 24 and 48 hours showed that histone H2A.X was phosphorylated at Ser139 forming γH2AX, a marker of DNA damage (Fig. 4A). Consistent with activation by DNA damage, Chk1 and Chk2 underwent phosphorylation at Ser317/Ser345 and Thr68, respectively. Chk1 activation was accompanied by a diminution of both Cdc25A levels. In addition, bendamustine caused an accumulation of cyclin A and a decrease in the mitotic marker phosphorylated histone H3 indicative of S-phase cell-cycle arrest. Analysis via flow cytometry confirmed that cells were arresting in S-phase at 24 hours, with DNA content between 2N and 4N (Fig. 4B), accompanied by a loss of staining for mitotic marker MPM2. By 48 hours, cells accumulated in G2 consistent with S-phase arrest was more transient than arrest in either G1 or G2 (21).

Addition of 100 nmol/L AZD7762 to bendamustine treatment caused a significant induction of γH2AX at 24 hours and increased Chk2 Thr68 phosphorylation, suggesting Chk1 inhibition resulted in double strand breaks, which increased ATM activity. AZD7762 also abrogated cell-cycle arrest as indicated by stabilization of Cdc25A and decreased cyclin A. Flow cytometry confirmed S-phase checkpoint abrogation with the majority of cells having 4N DNA content. AZD7762 increased the percentage of MPM2-positive cells from 0.2% to 5.75%. There was also an increase in p-histone H3 at serine 10, indicative of cells entering mitosis and undergoing mitotic catastrophe.
consistent with the increase in micronucleated cells observed with the combination therapy (Fig. 3). Despite an increase in mitotic cells at 24 hours, a commensurate increase in G1 cells was not observed. Rather, at 48 hours, cells accumulated with sub-G1 DNA content. PARP and caspase-3 cleavage were also observed consistent with the increase in apoptosis noted with the QFM. The disappearance of Chk1 at 48 hours in the cells with combined treatment is probably a result of cleavage by active caspases, as the cells become highly apoptotic. This is consistent with the studies of Matsuura and colleagues, who showed that Chk1 is cleaved by caspase during apoptosis induced by genotoxic stress (22).

The benzimidazole ring system of bendamustine (Fig. 1C) endows it with antimetabolite properties and distinguishes it from most conventional alkylating agents (23). However, FACS and immunoblot analysis of KMS-12-PE cells treated with melphalan and AZD7762 showed analogous effects (Fig. 4C and D). Similar results were observed when RPMI-8226 cells were treated with bendamustine or melphalan in combination with AZD7762 (Supplementary Data S1). In contrast, when KMS-12-PE cells were treated with bortezomib and AZD7762, no increase in phospho-γH2AX was seen (Supplementary Data S2). Although PARP and caspase-3 cleavage can be induced with increased concentrations of bortezomib in the KMS-12-PE cells, this is not appreciably enhanced with the addition of AZD7762, especially with prolonged exposure of the drug (48 hours).

**Induction of p53**

The induction of p21 in multiple myeloma cell lines was studied following exposure to 10 Gy irradiation as a marker of p53 functionality. Relative to the induction of p21 in p53-proficient HCT116 colorectal cancer cells, U266B1 cells were the only multiple myeloma cells with...
relapsed disease have increased, the remission duration is limited, and its complications (27). Although options for treating patients eventually relapse and die from their disease or complications, improvements in supportive care (26). However, most studies have focused on the optimal use of stem cell transplantation and newer agents (thalidomide, bortezomib, and lenalidomide), as well as the use of checkpoint inhibitors to enhance antitumor activity of DNA-damaging agents, especially in tumors that are p53-deficient (17).

Recent data on the genetic and molecular pharmacology of a potent Chk1 inhibitor (AZD7762) administered in combination with commonly used chemotherapy to treat human myeloma cells that contain mutated p53.

AZD7762 (100 nmol/L) not only potentiated the growth inhibitory effects of bendamustine, melphalan, and doxorubicin on human multiple myeloma cell lines, but it also initiated apoptosis and led to micronucleation when combined with bendamustine and melphalan (17). Chk1 inhibitors are known to predominantly sensitize p53-deficient cancer cells to DNA damage (12, 29, 30). KMS-12-BM and KMS-12-PE cells harbor only one copy of p53, and RPMI-8226 and U266B1 cells each have distinct p53 mutations (data not shown). However, U266B1 cells can activate p21 and Gadd45 following genotoxic stress (31), and in our experiments, an amplified DNA damage response and intact p53 axis, characterized by induction of p21, was detected in U266B1 cells. This may correlate with the reduced sensitivity of U266B1 cells to the combination of AZD7762 and alkylating agent compared with the other cell lines tested. The staurosporine derivative UCN-01, a multityrosine kinase inhibitor, is also a potent Chk1 inhibitor. Interestingly, interfering with the NF-κB pathway with Bay 11-7082 enhanced the apoptotic effects of UCN-01 on multiple myeloma cell lines (32). Although the activity of bortezomib on multiple myeloma cells is, at least in part, mediated through NF-κB inhibition (33), we did not observe increased potency when we combined bortezomib and AZD7762. Thus, the previous reported effect of UCN-01 may not have been Chk1-dependent (32).

All data generated were with concurrent treatment. The importance of sequence has been shown in vitro, especially with the sequential treatment with the topoisomerase I inhibitor irinotecan, followed by a Chk1 inhibitor, such as CHIR-124 (17). Although, concomitant therapy shows a very strong proapoptotic effect, our data do not address whether sequential treatment would have an even greater effect.

In summary, the current study shows that pharmacologic disruption of checkpoint function sensitizes multiple myeloma cells with intrinsic checkpoint defects to genotoxic stress imparted by standard chemotherapy. When combined with bendamustine or melphalan, AZD7762 induced apoptosis and mitotic catastrophe. Drugs like the Chk1/2 inhibitor AZD7762 represent novel therapeutic agents for patients with multiple myeloma, perhaps even for those with p53 mutations, a population with limited and suboptimal treatment options. Studies to examine the clinical benefit of adding a checkpoint kinase inhibitor to standard DNA-damaging agents should be conducted in patients with relapsed and refractory multiple myeloma.

**Discussion**

Dysregulation of cell-cycle checkpoints is a salient feature of malignant transformation; however, this abnormality in checkpoint control may selectively sensitize tumor cells to genotoxic stress. Indeed, this is the rationale for evaluating therapeutic strategies that combine conventional cancer treatments with inhibitors of cell-cycle checkpoints. Chk1 is a serine/threonine kinase involved in the induction of cell-cycle checkpoints in response to DNA damage and replicative stress and is essential for both S and G2–M phase checkpoints. Chk1 is a potential target for combination approaches to enhance antitumor activity of DNA-damaging agents, especially in tumors that are p53-deficient (17).

Mutation or deletion of the TP53 gene is rarely detected at diagnosis in patients with multiple myeloma, but such abnormalities become more frequent in advanced disease (25). The survival of patients with multiple myeloma has improved over the last decade due to active new agents (thalidomide, bortezomib, and lenalidomide), as well as the optimal use of stem cell transplantation and improvements in supportive care (26). However, most patients eventually relapse and die from their disease or its complications (27). Although options for treating relapsed disease have increased, the remission duration decreases with each salvage regimen. p53 inactivation is associated with resistance to therapy in multiple myeloma, even with proteasome inhibitors, which are arguably the most active class of drugs for multiple myeloma (3, 28). In this report, we show in vitro data on the addition of a potent Chk1 inhibitor (AZD7762) administered in combination with commonly used chemotherapy to treat human myeloma cells that contain mutated p53.

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Disclosure of Potential Conflicts of Interest
R.L. Comenzo and H.J. Landau served on the advisory board for Millennium Pharmaceuticals. R.L. Comenzo is a consultant/advisory board member for Millennium Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): H.J. Landau, S.C. McNeely, T. Asai, H. Friedman, S.C. Jianwar, G.K. Schwartz

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