Antimyeloma Activity of a Multitargeted Kinase Inhibitor, AT9283, via Potent Aurora Kinase and STAT3 Inhibition Either Alone or in Combination with Lenalidomide

Loredana Santo1,2, Teru Hideshima2, Diana Cirstea2, Madhavi Bandi2, Erik A. Nelson2, Gullu Gorgun2, Scott Rodig1, Sonia Vallet1,2, Samantha Pozzi1,2, Kishan Patel2, Christine Unitt3, Matt Squires4, Yiguo Hu2, Dharminder Chauhan2, Anuj Mahindra1, Nikhil C. Munshi2, Kenneth C. Anderson2, and Noopur Raje1,2

Abstract

Purpose: Aurora kinases, whose expression is linked to genetic instability and cellular proliferation, are being investigated as novel therapeutic targets in multiple myeloma (MM). In this study, we investigated the preclinical activity of a small-molecule multitargeted kinase inhibitor, AT9283, with potent activity against Aurora kinase A, Aurora kinase B, and Janus kinase 2/3.

Experimental Design: We evaluated the in vitro antimyeloma activity of AT9283 alone and in combination with lenalidomide and the in vivo efficacy by using a xenograft mouse model of human MM.

Results: Our data showed that AT9283 induced cell-growth inhibition and apoptosis in MM. Studying the apoptosis mechanism of AT9283 in MM, we observed features consistent with both Aurora kinase A and Aurora kinase B inhibition, such as increase of cells with polyploid DNA content, decrease in phospho-histone H3, and decrease in phospho-Aurora A. Importantly, AT9283 also inhibited STAT3 tyrosine phosphorylation in MM cells. Genetic depletion of STAT3, Aurora kinase A, or Aurora kinase B showed growth inhibition of MM cells, suggesting a role of AT9283-induced inhibition of these molecules in the underlying mechanism of MM cell death. In vivo studies showed decreased MM cell growth and prolonged survival in AT9283-treated mice compared with controls. Importantly, combination studies of AT9283 with lenalidomide showed significant synergistic cytotoxicity in MM cells, even in the presence of bone marrow stromal cells. Enhanced cytotoxicity was associated with increased inhibition of phosphorylated STAT3 and phosphorylated extracellular signal–regulated kinase.

Conclusions: Demonstration of in vitro and in vivo anti-MM activity of AT9283 provides the rationale for the clinical evaluation of AT9283 as monotherapy and in combination therapy for treating patients with MM. Clin Cancer Res; 17(10); 1–13. ©2011 AACR.
Translational Relevance

Aurora kinase A and Aurora kinase B expression is correlated with cellular proliferation in multiple myeloma (MM), which makes these potential therapeutic targets in MM. In addition, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway plays a critical role in MM pathophysiology. Blocking these pathways that are critical for MM cell survival represents a potential therapeutic strategy for MM patients. Our data show that AT9283, a small molecule with potent activity against Aurora kinase A, Aurora kinase B, and JAK/STAT; has potent anti-myeloma effect by inhibiting these 3 targets in vitro and in vivo. Importantly, AT9283 and lenalidomide are synergistic, which results in decreased proliferation, increased apoptosis, and induction of downregulation of phosphorylated STAT3 (pSTAT3) and phosphorylated extracellular signal–regulated kinase (pERK) in MM cells alone and in coculture with bone marrow stromal cells, thus highlighting the role of this combination in overcoming the effect of BMSCs. These results provide the rationale for the clinical evaluation of AT9283 alone and in combination therapy with lenalidomide in MM patients.

Janus kinases (JAK) have long been recognized to be involved in MM pathogenesis. JAKs are cytoplasmic protein tyrosine kinases that are constitutively associated with several cytokines; interleukin 6 (IL-6) induces gp130 (IL-6 receptor) dimerization, thereby resulting in the autophosphorylation of JAK and activation of various STAT proteins (14, 15). JAK/STAT pathways are critically involved in the survival and proliferation of MM cells, and blocking this pathway may, therefore, also represent a novel therapeutic strategy for MM patients (16). AT9283 is a small-molecule multitargeted kinase inhibitor with potent activity against Aurora A and Aurora B, and additional activity against JAKs, AbI (T315I), and Flt3 as shown by in vitro kinase activity (17). AT9283 has already shown activity in various solid tumor cell lines and xenograft mouse models associated with Aurora B inhibition (18, 19). In this study, we show that AT9283 significantly inhibits cell growth and induces apoptosis in MM. Moreover, we show that apoptosis induced by AT9283 is due to activity against both Aurora A and Aurora B. Specifically, we observed an increase of cells with polyplody DNA content and a decrease in phosphorylation of HH3 typical of Aurora B inhibition; furthermore, we noted a decrease of phospho-Aurora A (p-Aurora A) at Thr 288, consistent with Aurora A inhibition. In addition, on the basis of in vitro kinase assays, we investigated the effect of AT9283 on JAK/STAT3 pathway in MM cells: AT9283 decreases phosphorylation of STAT3, independent of Aurora A and Aurora B inhibition. In vivo xenograft studies confirmed our in vitro observations of decreasing human MM cell growth and prolonging survival in a murine xenograft model of human MM. Importantly, we found that AT9283 when combined with lenalidomide triggered synergistic MM cytotoxicity, even in the context of the bone marrow microenvironment. Our studies provide the rationale for clinical evaluation of AT9283 both as monotherapy and in combination with lenalidomide in MM patients.

Materials and Methods

Cell lines and reagents

Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI-8226 and U-266 human MM cells were obtained from the American Type Culture Collection. Melphalan-resistant RPMI-LR5 (LR5) and doxorubicin-resistant RPMI-Dox40 (Dox40) cell lines were provided by Dr. William Dalton (H. Lee Moffitt Cancer Center, Tampa, FL). OPM1 cells were provided by Dr. P. Leif Bergsagel (Mayo Clinic, Tucson, AZ). All MM cell lines were cultured as previously described (20). INA-6 cells were grown in RPMI-1640 medium containing IL-6 (10 ng/mL). Fresh peripheral blood mononuclear cells (PBMCN) were obtained from 4 healthy volunteers. Bone marrow aspirates from MM patients were obtained following approval from the Institutional Review Board. After mononuclear cells were separated, MM cells were purified by positive selection using CD138 (Syndecan-1) Micro Beads and the autoMACS magnetic cell sorter (Miltenyi Biotec Inc.). Bone marrow stromal cells (BMSC) were generated as previously described (20). BMSCs were incubated in 96-well culture plates (10,000 BMSCs/well) for 24 hours, and after washing off the medium, MM cell lines were added to the wells (2 × 10⁴ cells/well) and incubated with media or with increasing doses of AT9283 for specified time periods at 37°C.

AT9283, 1-cyclopropyl-3-[3-(5-morpholin-4-ylmethyl)-1H-benzoimidazol-2-yl]-1H-pyrrozol-4-yl]-urea, was obtained from Astex Therapeutics Ltd. (Fig. 1A). It was initially dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical) at a concentration of 10 mmol/L and then in culture medium (0.125–4 μmol/L) immediately before use.

Lenalidomide (CC-5013) was obtained from Selleck Chemicals LLC. It was initially dissolved in DMSO at a concentration of 10 mmol/L and then in culture medium immediately before use.

Cell viability and proliferation assays

Effects of AT9283 on viability of MM cell lines, primary MM cells, and PBMCNs was assessed by measuring MTT (Chemicon International) dye absorbance as previously described (20). Studies with combinations of lenalidomide (1–2 μmol/L) were similarly conducted in 48-hour cultures and cytotoxicity was determined by MTT. PBMCNs from healthy donors were isolated and stimulated with 2.5 μg/ml phytohemagglutinin (PHA) for 72 hours in the presence of increasing concentrations of AT9283. DNA synthesis was measured by tritiated thymidine uptake (³H-FdR; Perkin Elmer). MM cells (2–3 × 10⁴ cells/well) were incubated in 96-well culture plates (Costar) with media and different concentrations of AT9283 and/or recombinant IL-6 (10 ng/mL) or insulin-like growth factor I (IGF-I, 10 ng/mL) immediately before use.
50 ng/mL) for 24 or 48 hours at 37°C, and \(^{3}\)H-TdR incorporation was measured as previously described (20).

**Cell-cycle analysis and detection of apoptosis**

MM cells (1 x 10^6) were cultured for 24 and 48 hours in media alone or with varying concentrations of AT9283. Cells were harvested, washed with ice-cold PBS, fixed with 70% ethanol for 20 minutes, and pretreated with 10 μg/mL RNase (Sigma) for 20 minutes as previously described (20). Furthermore, apoptosis analysis was confirmed by using Annexin V/propidium iodide (PI) staining after MM cells were cultured in media or 0.5 mmol/L of AT9283 for 72 hours. Viability was measured by MTT assays. Values represent the mean ± SD of triplicate cultures. D, MM.1S cells were treated with 0.4 μg/mL nocodazole for 12 hours washed and subsequently treated with 0.5 μmol/L AT9283 for 2 hours. Cell lysates were immunoblotted with indicated antibodies.

**Immunofluorescence assay**

MM.1S cells were cultured on tissue culture–treated glass slides (Falcon; Becton Dickinson) in the presence or absence of AT9283 0.5 μmol/L. After 24 hours, cells were fixed in 4% paraformaldehyde/PBS for 15 minutes and then permeabilized in 0.05% Triton X-100/PBS for 5 minutes. After blocking with 5% bovine serum albumin/PBS and 0.01% Triton X-100/PBS for 1 hour, cell were stained with \(\alpha\)-tubulin (Sigma–Aldrich) at a ratio of 1:250 for 1 hour at room temperature. Cells were washed and incubated with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) for 1 hour. After subsequent washes, Hoechst 33342 (Invitrogen) was added to the cells for 10 minutes. The slides were mounted with ProLong Gold Antifade Reagent (Invitrogen), and images were taken using a Zeiss microscope (Carl Zeiss) equipped with Hamamatsu ORCA-ER camera (Hamamatsu Photonics).

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**Figure 1.** AT9283 inhibits growth and induces dose-dependent cytotoxicity in MM cells. A, chemical structure of AT9283 and in vitro kinase assay table. B, dose-dependent inhibition in different MM cell lines shown by measuring \(^{3}\)H-TdR uptake during the last 8 hours of 48-hour cultures in the presence of increasing doses of AT9283 (left). AT9283 reduces MM cells viability in a dose-dependent manner. Cells were treated with increasing doses of AT9283 for 72 hours and cell viability was measured by MTT assay (right). C, CD138\(^{+}\) patient-derived MM cells were tested in cytotoxicity assays (MTT) at 72 hours (left). PBMCs from healthy donors were stimulated with PHA in the presence of increasing concentrations of AT9283 for 72 hours. Viability was measured by MTT assays. Values represent the mean ± SD of triplicate cultures. D, MM.1S cells were treated with 0.4 μg/mL nocodazole for 12 hours washed and subsequently treated with 0.5 μmol/L AT9283 for 2 hours. Cell lysates were immunoblotted with indicated antibodies.

**Table 1.** Kinase IC \(50\) (nmol/L)

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<thead>
<tr>
<th>Kinase</th>
<th>IC (50) (nmol/L)</th>
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<tr>
<td>Aurora A</td>
<td>52% @ 3 nmol/L</td>
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<tr>
<td>Aurora B</td>
<td>58% @ 3 nmol/L</td>
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<tr>
<td>JAK3</td>
<td>1.1</td>
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<td>JAK2</td>
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Western blotting

MM cells were cultured with different concentrations of AT9283, harvested, washed, and then lysed using lysis buffer as previously described (20). The protein concentration of the lysate was measured, and the lysate was mixed with gel electrophoresis loading buffer, boiled for 5 minutes, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked in TBS plus 5% non-fat milk powder and 0.1% Tween 20 (polyorbate 20) for 1 hour before incubating them overnight at 4°C with the following antibodies: anti-Aurora A kinase (Santa Cruz Biotechnology), Aurora B kinase (Abcam), p-Aurora A Thr 288 (Cell Signaling), phospho-HH3 (p-HH3; Millipore), c-Myc (Becton Dickinson Biosciences), as well as anti-cyclin B1, caspase-3, -9, and -8, PARP, phosphorylated STAT 3 (pSTAT3), and STAT3 (Cell Signaling). Anti-gen–antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence (GE Healthcare). Blots were stripped and reprobed with anti-α-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or α-actin (Santa Cruz Biotechnology) antibodies to ensure equal protein loading.

siRNA transfection

U266 cells were transiently transfected with specified amounts of SMARTpool siRNA for Aurora A, Aurora B, STAT3, or nonspecific control duplexes (pool of 4; Upstate Cell Signaling Solutions/Dharmacon RNA Technologies) using the Cell Line Nucleofector Kit V Solution (Amaxa Biosystems). Cells were collected 48 hours after transfection, and DNA synthesis was measured by 3H-TdR uptake at specified time points.

MM xenograft mouse model

To evaluate the in vivo anti-MM activity of AT9283, male severe combined immunodeficient (SCID) mice were inoculated subcutaneously with 5 × 10⁶ MM.1S cells in 100 μL serum-free RPMI-1640 medium. When tumors were measurable in size, mice were treated intraperitoneally with vehicle or AT9283 dissolved in saline 0.9%. After 5 days of treatment, once daily, 5 days in a week, for 4 weeks, and the second group was treated with 45 mg/kg once daily, twice a week for 4 consecutive weeks. The control group received the carrier alone according to the same schedule. Tumor size was measured every alternate day in 2 dimensions by using calipers, and tumor volume was calculated with the formula: V = 0.5a × b² (a = long diameter of the tumor; b = short diameter of the tumor). Animals were sacrificed when the tumors reached a size of 2 cm³ or became ulcerated. Survival and tumor growth were evaluated from the first day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemistry was done using 5-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections.

Slides were soaked in xylene, passed through graded alcohol, and placed in distilled water. Slides were pretreated with 1 mmol/L EDTA buffer, pH 8.0 (for Aurora B staining), or with citrate buffer (for p-HH3 staining) in a steam pressure cooker (Biocare Decloaking Chamber CD2008US; Biocare Biomedica) at manufacturer-recommended settings. All further steps were done at room temperature in a hydrated chamber. The slides were blocked for endogenous peroxidase activity with Peroxidase Block (DAKO), washed for 5 minutes in buffer solution, and this was followed by a 20-minute incubation with Protein Block Serum Free (DAKO). The rabbit anti-Aurora B Polyclonal Antibody (Abcam; catalogue no. Ab2254) was applied in DAKO Antibody Diluent at 1:200 dilution for 1 hour. The rabbit anti-p-HH3 (Ser 10) polyclonal antibody (Millipore; catalogue no. 06-570) was applied in the DAKO Antibody Diluent at 1:500 dilution for 1 hour. After washing, antibodies were detected using rabbit EnVision Kit (DAKO) and 3,3’-diaminobenzidine (DAB) and counterstained with Harris Hematoxylin.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done using ApopTag Peroxidase In Situ Apoptosis Kit (Millipore; catalogue no. S7100) for FFPE tissues according to the manufacturer’s instructions.

Statistical analysis

All in vitro experiments were done in triplicate and repeated at least 3 times; a representative experiment was selected for figures. Statistical significances of differences were determined using Student’s t test, with minimal level of significance specified as P < 0.05. Overall survival (OS) was measured using the Kaplan–Meier method, and the results are presented as the median OS, with 95% CIs. All statistical analyses were determined using GraphPad Prism software (GraphPad Software, Inc.).

Results

AT9283 inhibits growth and induces dose-dependent cytotoxicity in MM cells

The effect of AT9283 (Fig. 1A) on MM cell proliferation was determined by 3H-TdR uptake, using various cell lines including LR-5, Dox 40, and MM.1R, resistant to conventional therapy. Exposure of MM cells lines for 48 hours resulted in a dose-dependent inhibition of cell growth with IC₅₀ values ranging from 0.25 to 1 μmol/L (Fig. 1B, left). Cell-growth inhibition was accompanied by a loss of viability. In fact, significant cytotoxic effect was shown after 72 hours of AT9283 incubation in MM cell lines by MTT assays. Cells were cultured in the presence of increasing doses of AT9283 (0–4 μmol/L) for 72 hours. AT9283 resulted in dose-dependent cytotoxicity with IC₅₀ values ranging from 0.25 to 4 μmol/L at 72 hours in MM cell lines, with the most sensitive cell lines being MM.1S, U266,
AT9283 inhibits p-HH3 and p-Aurora A at Thr 288

To ascertain the activity of AT9283 on Aurora A and Aurora B, we evaluated the effect of AT9283 on p-HH3 and on p-Aurora A at Thr 288. It has been shown that selective Aurora kinase A inhibitors increase levels of HH3 phosphorylation; conversely, Aurora B inhibition shows the opposite effect, that is, decreasing HH3 phosphorylation. Phosphorylation of HH3 has been used as a marker for Aurora B inhibition (22–25). MM cells were treated with nocodazole 0.4 μg/mL for 12 hours to induce an M-phase block resulting in maximal phosphorylation of HH3; thereafter, cells were washed and subsequently treated with AT9283 for 2 hours. AT9283 decreased the phosphorylation of p-HH3 in a dose-dependent manner, confirming the role of AT9283 on Aurora kinase B inhibition. Aurora A is autophosphorylated in its activation loop at Thr 288 (5). MM.1S cells treated with nocodazole and subsequently treated with AT9283 for 2 hours showed a decrease in Aurora kinase A phosphorylation at Thr 288, consistent with the effect of AT9283 on Aurora A inhibition (Fig. 1D). These findings suggest the dual activity of AT9283 against Aurora A and Aurora B.

AT9283 increases G2/M phase and induces apoptosis of MM cells in a time-dependent manner

Because Aurora kinases play an important role in mitosis and proliferation, we next evaluated the effect of AT9283 on the cell-cycle profile of MM cell lines by flow cytometry. MM.1S cells were cultured with media alone and AT9283 (0.5 μmol/L) for 24 and 48 hours. AT9283-treated MM.1S cells showed both an increase of cells in G2/M and an increase in cells with polyploidy DNA content (Fig. 2A, left). These findings are consistent with the effects of other pan-Aurora kinase inhibitors studied (10, 13, 21). Moreover, AT9283 induced morphologic changes resulting in enlarged and multinucleated cells, as shown by immunofluorescence (Fig. 2A, right). To investigate the molecular pathway activated by AT9283, we examined its effect on p53, p21, p27, and retinoblastoma (Rb) on MM.1S cells. These proteins not only mediate cell-cycle control but also trigger the induction of pro-apoptotic genes. Treatment with AT9283 upregulated p53 and p27 levels at 72 and 48 hours, respectively, when the cells undergo apoptosis; p21 upregulation and inhibition of Rb phosphorylation at Ser 780 occurred after 12 hours of treatment. These data are consistent with Aurora B inhibitory activity resulting in inhibition of Rb phosphorylation concomitant with polyploidy induction (Fig. 2B).

PI and Annexin V staining showed the induction of apoptosis by AT9283, with maximal effect at 72 hours in MM.1S (Fig. 2C, left) and 48 hours in U266 and INA-6 (data not shown). Apoptosis was confirmed by PARP as well as caspase-9 and -8 cleavage (Fig. 2C, right).

AT9283 inhibits STAT3 signaling pathway in MM cell lines

Because AT9283 showed a strong activity against JAKs on in vitro kinase assay, we investigated the effect of AT9283 on STAT3 by using U3A cells stably expressing a luciferase reporter gene under the control of a STAT1-dependent promoter (16). AT9283 inhibited STAT3-dependent luciferase activity with an EC50 of approximately 0.125 μmol/L (Fig. 3A). Furthermore, we investigated the effect of AT9283 on STAT3 by evaluating the expression level of STAT3 tyrosine phosphorylation in MM.1S cells incubated with AT9283 (0.5 μmol/L); pSTAT3 expression was down-regulated starting at 30 minutes (Fig. 3A). We confirmed this result by using U266 cells that had higher constitutive STAT3 tyrosine phosphorylation than MM.1S cells; AT9283 (0.25 μmol/L) induced a significant decrease in STAT3 tyrosine phosphorylation even in U266 cell lines. The downstream target of STAT, c-Myc, was downregulated after 24 hours of AT9283 treatment, further confirming inhibition of STAT3 activity (Fig. 3A).

AT9283 overcomes the protective effect of BMSCs and cytokines

The bone marrow microenvironment enhances growth and survival of MM cells, mediated through direct MM cell–stromal cell contact as well as via cytokines (e.g., IL-6 and IGF-I; refs. 15, 26, 27). To assess the effects of AT9283 on MM cells in the microenvironment, we analyzed the effect of MM.1S cells on pSTAT3, Aurora A, and Aurora B in coculture with BMSCs. Consistent with the prosurvival effect of microenvironmental factors, coculture of MM cells with BMSCs leads to increased phosphorylation of STAT3 and increased expression of Aurora A and Aurora B. However, AT9283 treatment for 4 and 24 hours decreased expression of pSTAT3, Aurora A, and Aurora B, even in the presence of BMSCs. Moreover, AT9283 resulted in inhibition of DNA synthesis of MM cells adherent to BMSCs at 48 hours in a dose-dependent manner and inhibited the growth induced by IL-6 and IGF-1 at 48 hours (Fig. 3B). Therefore, AT9283 overcomes the proliferative advantage conferred by cytokines and the protective effect of BMSCs.

AT9283 directly affects STAT3 pathway, Aurora A, and Aurora B

Next, we sought to investigate whether the inhibition of STAT3, Aurora kinase A, and Aurora kinase B was a direct effect of the compound and whether these activities were correlated. To this end, we suppressed the expression of STAT3, Aurora A, or Aurora B by using suitable siRNAs in
U266 cells, and we verified the selective suppression by Western blot analysis (Fig. 4A). In cells with knocked down STAT3, we did not observe any change in the expression level of Aurora kinase A and Aurora kinase B; in cells with knocked down Aurora kinase A and Aurora kinase B, we observed a downregulation in the expression level of STAT3, which was likely due to the fact that STAT3 is downstream of Aurora kinases. However, we excluded physical interaction between Aurora kinase A and Aurora kinase B and STAT3 by conducting immunoprecipitation (data not shown; Fig. 4A).

Figure 4B shows the effect of 0.5 µmol/L AT9283 for 48 hours on cell proliferation in the presence of genetic depletion of 1 of the 3 targets—STAT3, Aurora kinase A, and Aurora kinase B. Genetic depletion of each of the targets significantly reduced cell proliferation (knockdown gray bars vs. control; P < 0.05). However, when each of the 3 targets is knocked down, AT9283 inhibited cell proliferation more but this was not statistically significant (gray vs. white bars; P > 0.05), indicating that STAT3, Aurora kinase A, and Aurora kinase B are indeed targeted by AT9283. Alternately, genetic depletion of STAT3, Aurora kinase A, and Aurora kinase B produces effects similar to AT9283 on MM cells, making it difficult to delineate AT9283-induced inhibitory effect on the genetically depleted cells. Finally, comparison of the effect on cell proliferation (white bars) in the different genetic depletion conditions suggest that the various
conditions result in inhibition of proliferation but are not statistically different ($P > 0.05$).

These findings are consistent with a direct effect of AT9283 on STAT3, Aurora kinase A, and Aurora kinase B and suggest that STAT3 inhibition plays an independent role in the activity of this agent.

**AT9283 inhibits human MM cell growth in vivo**

We next examined the in vivo efficacy of AT9283 by using a human MM xenograft mouse model. We used 2 different schedules of treatment: one group was treated with 15 mg/kg i.p. once daily, 5 days in a week, for 4 weeks; another group was treated with 45 mg/kg i.p. once daily, 2 days a week, for 4 weeks. These schedules were derived from the results of in vitro washout experiments, which showed that MM.1S cells return to normal cell cycle with significant increase of sub-G1 population 72 hours after replacing medium (Fig. 5A). The group treated with 15 mg/kg i.p. once daily, 5 days in a week, for 4 weeks, did not show significant delay in tumor growth (data not shown). As shown in Figure 5B, in the group treated with 45 mg/kg i.p. once daily, 2 days a week, for 4 weeks, tumor growth was inhibited compared with controls ($P = 0.018$). TUNEL assays on tumor sections from treated versus control mice
showed significantly increased apoptosis. Moreover immuno-
histochemical (IHC) analysis of tumor taken from mice 
following administration of 2 cycles of 45 mg/kg AT9283 
14 hours after drug administration confirmed decreased 
expression of p-HH3 and Aurora B in treated animals 
(Fig. 5E). No significant difference was noted in pSTAT3 
and Aurora A expression (data not shown). Western blot 
analysis on tumor tissue showed decreased levels of 
pSTAT3, Aurora A, and Aurora B (Fig. 5F). Using the 
Kaplan–Meier method and log-rank analysis, the median 
OS of animals treated with 45 mg/kg i.p. once daily, 2 days 
in a week, for 4 weeks, was significantly prolonged (32 days vs. 
18 days, respectively; P < 0.0001; Fig. 5C). Importantly, 
treatment with AT9283 did not affect the body weight of 
the animals (Fig. 5D).

**AT9283 in combination with lenalidomide induces 
synergistic anti-MM activity**

Thereafter, we evaluated the activity of AT9283 in 
combination with lenalidomide. MM cell cytotoxicity 
was examined in the presence of AT9283 in combination 
with lenalidomide by using both agents at concentra-
tions lower than their maximal cytotoxic concentrations. 
Increasing doses of AT9283 (0, 0.125, and 0.25 μmol/L) 
were added to lenalidomide (0, 1, and 2 μmol/L) and 
MM cytotoxicity was assayed by MTT at 48 hours. The 
effects of combined therapy on DNA synthesis of MM.1S 
and INA-6 cells were determined by 3H-TdR uptake at 48 
hours. Significant decrease in viability and cell growth 
was observed with combined therapy compared with 
both agents used alone. Representative results for max-
imal synergistic effect are shown in Figure 6A. Synergism 
was confirmed by applying the Chou–Talalay method to 
calculate the combination index, as previously described 
(20). To further characterize the cytotoxic effect, we 
examined apoptosis induced by AT9283 plus lenalido-
mide therapy by AnnexinV/PI staining. The analysis 
showed an increase (55.7%) of cells in early and late 
apoptosis after 72 hours of exposure to combination 
therapy. Because it has previously been shown that 
lenalidomide-induced apoptosis is caspase-8 dependent 
(28), we next evaluated whether low concentrations of 
AT9283 and lenalidomide increased caspase-8 and PARP 
cleavage. MM.1S cells were incubated with AT9283
0.125 μmol/L or lenalidomide 2 μmol/L or a combination of both for 18 and 36 hours; cell lysates were subjected to immunoblotting using indicated antibodies. Combination therapy with AT9283 and lenalidomide increased cleavage of caspase-8 and PARP (Fig. 6B). Using Western blot analysis to delineate the molecular mechanism underlying this combination, we found that combination treatment resulted in down-regulation of pSTAT3 and phosphorylated extracellular signal-regulated kinase (pERK) following 4 hours of treatment (Fig. 6C). In view of the role that the bone marrow microenvironment plays in growth and survival of MM cells, we examined whether the combination of low-dose AT9283 plus lenalidomide induced MM cell death even in the presence of the bone marrow microenvironment. MM.1S cells were cultured with or without BMSCs in the presence or absence of AT9283, lenalidomide, or AT9283 plus lenalidomide. Combined therapy inhibited ³H-TdR uptake of MM.1S cells cultured in the presence of BMSCs. Interestingly, consistent with these data, we observed that AT9283 plus lenalidomide down-regulated the expression of the pSTAT3 and pERK in MM.1S cells cultured with BMSCs (Fig. 6D).

Discussion

Aurora kinases play a crucial role in multiple aspects of mitosis. Aurora A is required for spindle assembly, whereas Aurora B is required for HH3 phosphorylation, chromosome segregation, and cytokinesis (29–31). Aurora kinase inhibitors have received a lot of interest as potential therapeutic targets in MM because they trigger significant anti-MM activity in preclinical studies (10, 11, 13, 21, 32). Furthermore, JAK/STAT pathways have attracted a lot of interest as a therapeutic target in MM cells (16, 33) because they are critically linked to survival and proliferation of...
Figure 6. AT9283 in combination with lenalidomide enhances the toxicity of MM cells. A, AT9283 (0.125 µmol/L) was added with lenalidomide (2 µmol/L) to MM.1S and INA-6 cells for 48 hours. Cytotoxicity was assayed by MTT (left). Growth inhibition was assayed by 3H-TdR uptake. *, 95% CI < 0.9. To calculate the 95% CI, MM.1S and INA-6 were treated with AT9283 (0.125–0.25 µmol/L) and/or lenalidomide (1–2 µmol/L) for 48 hours. Isobologram analysis using CaluSyn software showed synergistic anti-myeloma effect of the combination AT9283 and lenalidomide. B, MM.1S were treated with AT9283 (0.125 µmol/L), lenalidomide (2 µmol/L), or combination therapy for 72 hours. Annexin/PI staining shows increased apoptosis associated with caspase-8 and PARP cleavage after 18 and 36 hours of exposure. C, MM.1S cells were treated with AT9283 (0.125 µmol/L), lenalidomide (2 µmol/L), or combination therapy for 4 hours. Whole lysates were immunoblotted with indicated antibodies. D, MM.1S cells were cultured for 48 hours in the presence or absence of BMSCs with control media, AT9283, lenalidomide, or AT9283 plus lenalidomide. Cell proliferation was assessed by 3H-TdR uptake (left). MM.1S cells were cultured in the absence or presence of BMSCs and treated for 4 hours with drugs alone or in combination. Whole lysates were immunoblotted with indicated antibodies.
MM cells (34, 35). We have studied AT9283, a multikinase inhibitor with activity against Aurora A, Aurora B, and STAT3 pathway, and showed synergistic anti-MM activity when combined with lenalidomide.

In solid tumors, AT9283 has already shown significant antitumor activity acting mainly as an Aurora B inhibitor (19). Consistent with these findings, we observed the typical phenotype of Aurora B inhibition in MM cells after AT9283 treatment, that is, enlarged polyplloid nuclei due to failure of cytokinesis. Moreover, treatment for 2 hours with AT9283 after nocodazole treatment suppressed phosphorylation of HH3, another marker of Aurora B inhibition. Furthermore, inhibition of phosphorylation of HH3 was observed in our in vivo study, as shown in histologic sections of tumors from mice treated with AT9283. Although AT9283 seems to act predominantly as an Aurora B inhibitor in MM cells, its molecular mechanism in this setting appears to be more complex because it also inhibits Aurora A and STAT3 pathways that independently lead to MM cell apoptosis. In fact, when cells were pretreated with nocodazole, a significant decrease in p-Aurora A Thr 288 was observed, suggesting activity of AT9283 against Aurora A. In addition, we observed that AT9283 decreases STAT3 tyrosine phosphorylation, which, in turn, led to c-Myc downregulation (a STAT3 target), as observed on Western blot analysis. The inhibition of these 3 pathways seems to be a direct effect of the compound. This effect was shown by comparing AT9283 treatment of U266 cells with individually knocked down Aurora kinase A, Aurora kinase B, or STAT3 with respect to wild-type (WT) U266 cells. Genetic depletion of each of these 3 targets independently did not change the effect of AT9283 on cell proliferation. Importantly, when MM cells were adherent to BMSCs, our data showed increased expression of STAT3, Aurora A, and Aurora B that was downregulated in a time-dependent manner by AT9283 treatment. In addition, we observed that AT9283 effectively overcame the protective effect of BMSCs and the proliferative advantage conferred by cytokines.

Next, we conducted a detailed investigation of the molecular signaling pathways associated with AT9283-induced apoptosis. We found increased levels of p21 and p53 and decreased phosphorylation of Rb at Ser 780, consistent with previous studies showing that the induction of polyplploid or pseudo G1 arrest by small-molecule inhibitors of Aurora kinases is dependent on these pathways (2, 22). The Rb protein acts as a tumor suppressor by blocking S-phase entry and cell growth, thus disrupting the cell cycle (22). Through the induction of p21 by p53, endoreduplication is blocked and cell cycle is arrested. The status of p53-dependent postmitotic checkpoints influences the effect of Aurora kinase inhibitors on mitosis and polyplploid induction (19, 36). Treating cells with Aurora kinase inhibitors when p53 function is intact leads to a postmitotic cell-cycle arrest with 4N DNA content; on the other hand, tumor cells with limited p53 functionality fail to arrest this postmitotic checkpoint; as a result, they proceed to additional cell cycle characterized by aberrant mitosis and failed cytokinesis leading to cell death (23, 36–38). In this study, we used a WT p53 cell line (MM.1S), and we observed substantial endoreduplication despite increased p53 and p21 protein levels. These data suggest that the response to Aurora kinase inhibition is not solely determined by p53 status; however, further studies are needed to confirm this on different myeloma cell lines (22). Moreover, decreased phosphorylation of Rb is consistent with previous studies showing Rb promoted cell-cycle progression with polyplploid induction under Aurora A inhibition (22). Therefore, p53 and p21 could play a role in the endoreduplication and subsequent apoptosis induced by AT9283.

Despite recent advances with new drugs such as bortezomib, thalidomide, and lenalidomide, MM remains an incurable disease. Used as single agents, these compounds have shown marked antitumor activity, but the number of patients with relapsed and refractory disease remains high (8). The combination of different classes of drugs with nonoverlapping toxicities might lead to improved patient outcome. We, therefore, evaluated the effect of AT9283 in combination with various established MM drugs and found that the best synergistic effect was when AT9283 was combined with lenalidomide. Immunomodulatory derivatives (IMiD) have been shown to improve clinical responses in patients with relapsed and refractory disease (39). Despite the encouraging results in the clinical setting, lenalidomide treatment results in side effects, necessitating dose reduction. In addition, patients eventually relapse even after lenalidomide-based therapies. Combining low doses of lenalidomide with low doses of AT9283 might, therefore, represent an opportunity to improve patient outcome by overcoming drug resistance and improving drug-toxicity profile. In our in vitro experiments, maximum synergism was observed with a dose of 0.125 μmol/L of AT9283 and 2 μmol/L of lenalidomide, which are both pharmacologically easily achievable. Using the doses triggering maximum synergy, we delineated downstream signaling cascades targeted by lenalidomide and AT9283, alone and in combination. We observed that the combination markedly decreased pERK and pSTAT3; in particular, pERK is affected only by lenalidomide treatment and pSTAT3 is affected predominantly by AT9283. Finally, BMSCs support MM cell survival and proliferation both by adhesion and by cytokine secretion. In this study, we confirmed a downregulation of pSTAT3 and pERK in the combined therapy when MM cells were cocultured with BMSCs, highlighting the role of this drug combination in overcoming the protective effect of BMSCs. These results provide the rationale for the clinical evaluation of AT9283, as monotherapy and in combination with lenalidomide, to improve patient outcome in MM.

Authors’ Contributions

L. Santo designed research, carried out research, collected data, analyzed and interpreted data, conducted statistical analysis, and wrote the
manuscript; T. Hideshima, S. Pozzi, and S. Vallet analyzed and interpreted data; D. Cirstea, K. Patel, E. Nelson, M. Bandi, S. Rodig, G. Gorgun, Y. Hu, and C. Units carried out research and collected data; M. Squires provided AT9283; A. Mahindra, N. C. Munsu, D. Chauhan, and K. C. Anderson contributed vital new reagents or analytical tools; and N. Raje designed research, provided the environment and support, and wrote the manuscript.

Disclosure of Potential Conflicts of Interest

K. Anderson, N. Munshi, consultants, Millennium, Celgene, and Novartis; N. Raje, consultant, Celgene, recipient of research grants from AstraZeneca and Acelynol. M. Squires is employed by Astes Therapeutics, Ltd. The remaining authors declare no competing financial interests.

References


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Antimyeloma Activity of a Multitargeted Kinase Inhibitor, AT9283, via Potent Aurora Kinase and STAT3 Inhibition Either Alone or in Combination with Lenalidomide

Loredana Santo, Teru Hideshima, Diana Cirstea, et al.

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