Anti-myeloma activity of a multi targeted kinase inhibitor, AT9283, via potent Aurora Kinase and STAT3 inhibition either alone or in combination with lenalidomide.

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Translational relevance

Aurora Kinase A (AURKA) and B (AURKB) expression is correlated with cellular proliferation in multiple myeloma (MM) making these potential therapeutic targets in MM. In addition JAK/STAT pathway plays a critical role in MM pathophysiology. Blocking these pathways critical for MM cell survival represents a potential therapeutic strategy for MM patients. Our data demonstrates that AT9283, a small molecule with potent activity against AURKA, AURKB and JAK/STAT, has potent anti myeloma effect by inhibiting these three targets in vitro and in vivo. Importantly, AT9283 and lenalidomide are synergistic resulting in decreased proliferation, increased apoptosis and induction of downregulation of pSTAT3 and pERK in MM cells alone and in co-culture with bone marrow stromal cells (BMSCs), 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 with lenalidomide in MM patients.

Abstract

Purpose: Aurora Kinases, whose expression is linked to genetic instability and cellular proliferation, are under investigation as novel therapeutic targets in multiple myeloma (MM). Here, we investigated the preclinical activity of a small molecule–multi-targeted kinase inhibitor, AT9283, with potent activity against Aurora kinase A (AURKA), Aurora kinase B (AURKB) and Janus Kinase 2/3.

Experimental design: We evaluated the in vitro anti myeloma 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 demonstrated AT9283 induced cell growth inhibition and apoptosis in MM. Studying the apoptosis mechanism of AT9283 in MM, we observed features consistent with both
AURKA and AURKB inhibition, e.g. increase of cells with polyploid DNA content, decrease in phospho-Histone H3, and decrease of phospho-Aurora A. Importantly, AT9283 also inhibited STAT3 tyrosine phosphorylation in MM cells. Genetic depletion of STAT3, AURKA or AURKB 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 demonstrated decreased MM cell growth and prolonged survival in AT9283-treated mice compared to controls. Importantly, combination studies of AT9283 with lenalidomide showed significant synergistic cytotoxicity in MM cells, even in the presence of bone marrow stromal cells (BMSCs). Enhanced cytotoxicity was associated with increased inhibition of pSTAT3 and pERK.

**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 in patients with MM.

**Introduction**

Aurora Kinases are a family of serine/threonine kinases that play a crucial role in multiple steps of mitosis. Aurora A localizes to spindle poles and is required for spindle assembly, whereas Aurora B is a chromosome passenger protein required for phosphorylation of Histone H3 on serine 10, chromosome alignment and segregation, spindle checkpoint function and cytokinesis (1) (2, 3) (4). Inhibition of Aurora A and B activity induces distinct cellular behavior. Studies using selective Aurora A inhibitors or RNAi have shown that Aurora A inhibition leads to G2/M arrest, increased levels of H3 phosphorylation at ser-10 and the formation of unipolar spindles which in turn can lead to apoptosis (5). Ablation of Aurora B induces abrogation of spindle assembly checkpoints, rapid inhibition of H3 phosphorylation at ser-10, and failure of cytokinesis, resulting in cells acquiring enlarged polyploid nuclei and apoptosis (6, 7). Aurora kinases are often over-expressed in human tumors, indicating their involvement in tumor progression. In multiple myeloma (MM),
genetic instability and centrosome amplification are associated with the over-expression of proteins involved in cell cycle, G2/M checkpoints and Aurora kinase; as a result, Aurora kinase inhibitors have recently been studied as potential novel therapeutic targets in MM (8-13). Interestingly Chng et al. showed that high Aurora A and Aurora B gene expression correlated with high centrosome index and poor prognosis in MM demonstrating that Aurora kinase inhibitors may represent a novel therapy in MM (11).

Janus Kinases (JAKs) have long been recognized to be involved in MM pathogenesis. JAKs are cytoplasmatic protein tyrosine kinases that are constitutively associated with several cytokines; IL-6 induces gp130 (IL-6 receptor) dimerization, thereby resulting in the autophosphorylation of JAK and activation of various signaling transducers and activators of transcription (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 multi-targeted kinase inhibitor with potent activity against Aurora A and Aurora B, and additional activity against Janus kinases (JAKs), Abl (T315I) and Flt3 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). Here, we show that AT9283 significantly inhibits cell growth and induces apoptosis in MM. Moreover, we demonstrate that apoptosis induced by AT9283 is due to activity against both Aurora A and Aurora B. Specifically, we observed an increase of cells with polyploidy DNA content and a decrease in phosphorylation of H3 typical of Aurora B inhibition; as well as a decrease of phospho Aurora A at thr 288, consistent with Aurora A inhibition. In addition, based on the in vitro kinase assays, we also investigated the effect of AT9283 on JAK/STAT3 pathway in MM cells: AT9283 decreases phosphorylation of STAT3, independent of Aurora A and B inhibition. In vivo xenograft studies confirmed our in vitro observations decreasing human MM cell growth and prolonging survival in a murine xenograft.
model of human MM. Importantly we found that AT9283 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 (Dex) sensitive (MM.1S) and Dex resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI8226 and U266 human MM cells were obtained from American Type Culture Collection (Rockville, MD). 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 interleukin-6 (IL-6, 10ng/ml). Fresh peripheral blood mononuclear cells (PBMNC) were obtained from four healthy volunteers. BM 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 Auto Macs magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). Bone marrow stromal cells (BMSCs) were generated as previously described (20). BMSCs were incubated in 96-well culture plates (10 000 BMSCs/well) for 24 h, after washing off the medium, MM cell lines were added to the wells (2x10⁴ cells/well) and incubated with media or with increasing doses of AT9283 for the specified time at 37°C.

AT9283, 1-Cyclopropyl-3-[3-(5-morpholin-4-ylmethyl-1H-benzoimidazol-2yl)-1H-pyrazol-4-yl]urea, was obtained from Astex Therapeutics Ltd, Cambridge, UK (Fig. 1A). It was dissolved first in dimethyl sulfoxide (DMSO; Sigma Chemical) at a concentration of 10mM, and then in culture medium (0.125-4 μM) immediately before use.

Lenalidomide (CC-5013) was obtained from Selleck Chemicals LLC (TX, USA). It was dissolved first in DMSO at a concentration of 10mM and then in culture medium immediately before use.
Cell viability and proliferation assays:

AT9283’s effects on viability of MM cell lines, primary MM cells, and PBMNCs was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrasodium bromide (MTT; Chemicon International, Temecula, CA, USA) dye absorbance as previously described (20). Studies with combinations of lenalidomide (1-2 μM) were similarly performed in 48 hr cultures and cytotoxicity was determined by MTT. PBMNCs from healthy donors were isolated and stimulated with 2.5μg/ml PHA for 72 hours in the presence of increasing concentrations of AT9283. DNA synthesis was measured by tritiated thymidine uptake (3H-TdR; Perkin Elmer, Boston, MA). MM cells (2-3 x 10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) with media and different concentrations of AT9283 and/or recombinant IL-6 (10 ng/mL) or IGF-1 (50 ng/mL) for 24 or 48 h at 37°C, and 3H-TdR incorporation was measured as previously described (20).

Cell cycle analysis and detection of apoptosis:

MM cells (1x10^6) were cultured for 24 and 48h in media alone or with varying concentrations of AT9283. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), fixed with 70% ethanol for 20 minutes, and pretreated with 10 μg/mL RNase (Sigma) for 20 minutes as previously described (20). Apoptosis analysis was also confirmed by using Annexin V/PI staining after MM cells were cultured in media or 0.5 μM of AT9283 at 37°C for 12, 24, 48 and 72 hours, as previously described (20). Annexin V^+PI^ apoptotic cells were enumerated by using the Epics flow cytometer. The percentage of cells undergoing apoptosis was defined as the sum of early apoptosis (AnnexinV-positive) and late apoptosis (Annexin V-positive and PI-positive) cells.

Immunofluorescence assay

MM.1S cells were cultured on tissue culture treated glass slides (BD Falcon) in the presence or absence of AT9283 0.5 μM. After 24 hours, cells were fixed in 4% paraformaldehyde/PBS for 15 min, and permeabilized in 0.05% Triton X-100/PBS for 5 min. After blocking with 5% bovine
serum albumin (BSA)/PBS and 0.01% Triton X-100/PBS for 1 hour, cell were stained with α-tubulin (Sigma-Aldrich St. Louis, MO, USA) 1:250 for 1 hour at room temperature. Cells were washed and incubated with Alexa-flour 488 goat anti mouse antibody (Invitrogen, CA, USA) for 1 hour. After subsequent washes, Hoechst 33342 (Invitrogen, CA, USA) was added for 10 min. The slides were mounted with Prolong Gold Antifade reagent (Invitrogen, CA, USA), and images were taken using Zeiss microscope equipped with Hamamatsu ORCA ER camera.

**Western blotting:**

MM cells were cultured with different concentrations of AT9283, harvested, washed, and lysed using lysis buffer as previously described (20). The protein concentration of lysate was measured, mixed with gel electrophoresis loading buffer, boiled for 5 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked in TBS plus 5% non fat milk powder and 0.1% TWEEN20 for 1 hour before incubating with the following antibodies overnight at 4°C: anti-Aurora A kinase (Santa Cruz Biotecnology, Santa Cruz, CA); Aurora B kinasse (Abcam); phospho Aurora A thr 288 (Cell Signaling, Beverly, MA); phospho Histone H3 (Millipore); c-Myc (BD Biosciences, San Diego, CA); as well as anti-cyclin B1, Caspase 3, 9 and 8, PARP, pSTAT3 and STAT3 (Cell Signaling, Beverly, MA). Antigen-antibody complexes were detected using secondary antibodies conjugated to HRP and visualized using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). Blots were stripped and reprobed with anti-α-tubulin, GAPDH or α-actin (Santa Cruz Biotecnology, Santa Cruz, CA) antibodies to ensure equal protein loading.

**siRNA transfection**

U266 cells were transiently transfected with indicated amounts of small interfering RNA (siRNA) SMART pool for Aurora A, Aurora B, STAT3 or nonspecific control duplexes (pool of four; Upstate Cell Signaling Solutions/Dharmacon RNA Technologies, Lafayette, CO) using the Cell
Line Nucleofector Kit V Solution (Amixa Biosystems, Cologne, Germany). Cells were collected 48 hours after transfection, and DNA synthesis was measured by tritiated thymidine uptake at indicated time points.

**MM xenograft mouse model:**

To evaluate the *in vivo* anti-MM activity of AT9283, male SCID mice were inoculated subcutaneously with $5 \times 10^6$ MM.1S cells in 100 μl serum-free RPMI 1640 medium. When tumors were measurable, mice were treated intraperitoneally (IP) with vehicle or AT9283 dissolved in saline 0.9%. The first group of 10 mice was treated with 15 mg/kg once a day for five days for 4 weeks, and the second group was treated with 45 mg/kg once a day twice a week for four consecutive weeks. The control group received the carrier alone at the same schedule. Tumor size was measured every alternate day in 2 dimensions using calipers, and tumor volume was calculated with the formula: $V = 0.5 \times a \times b^2$ (a= long diameter of the tumor, b= short diameter of the tumor). Animals were sacrificed when the tumor reached 2 cm$^3$ or when the tumor was 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 performed using 5-μm-thick formalin fixed paraffin embedded (FFPE) tissue sections. Slides were soaked in xylene, passed through graded alcohol, and put in distilled water. Slides were pretreated with 1mM ethylenediamine tetra-acetic acid 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 Biomedical, Concord, CA) at manufacturers recommended settings. All further steps are performed at room temperature in a hydrated chamber. The slides were blocked for endogenous peroxidase activity with peroxidase block (DAKO), washed 5 minutes in buffer, and followed by 20 minute incubation with serum free protein block (DAKO). The rabbit anti-Aurora B polyclonal antibody (Abcam, cat. Ab2254) was applied in
DAKO diluent at 1:200 dilution for 1 hour. The rabbit anti-phospho H3 (Ser10) polyclonal antibody (Millipore, cat. 06-570) was applied in DAKO diluent at 1:500 dilution for 1 hour. After washing, antibodies were detected using rabbit Envision kit (DAKO) and DAB and counterstained with Harris hematoxylin.

TUNEL staining was performed using ApopTag peroxidase in situ apoptosis kit (Millipore, cat. S7100) on formalin-fixed, paraffin embedded tissues according to the manufacturer’s directions.

**Statistical analysis:**

All *in vitro* experiments were performed in triplicate and repeated at least 3 times; a representative experiment was selected for figures. Statistical significances of differences were determined using Students t test, with minimal level of significance $P < 0.05$. Statistical significance of the *in vivo* growth inhibition observed in AT9283-treated mice compared with control group was determined using Students $t$ test. The minimal level of significance was $P < 0.05$. Overall survival was measured using the Kaplan-Meier method, and the results are presented as the median overall survival, with 95% confidence intervals. All statistical analyses were determined using GraphPad Prism software (GraphPad Software, Inc. San Diego, CA.)

**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 $^{3}$H Thymidine uptake using various cell lines, including LR-5, Dox 40, MM.1R, resistant to conventional therapy. Exposure of MM cells lines for 48 hours resulted in a dose-dependent inhibition of cell growth with $IC_{50}$s ranging from 0.25 to 1 μM. (Fig1B left). Cell growth inhibition was accompanied by a loss of viability. In fact, significant cytotoxic effect was demonstrated after 72 hours of AT9283 incubation
in MM cells lines by MTT assays. Cells were cultured in the presence of increasing doses of AT9283 (0-4 μM) for 72 h. AT9283 resulted in dose-dependent cytotoxicity with IC_{50}s ranging from 0.25 to 4 μM at 72 hours in MM cell lines with the most sensitive cell lines being MM.1S, U266, OPM1, INA-6 (0.25-0.5 μM) and the most resistant LR5 (4 μM) (Fig. 1B right). Significant cytotoxicity was noted in three out of four primary MM cells from patients after 72 hours of treatment with IC50 1-2 uM (Fig 1C left). AT9283 did not induce cytotoxicity in unstimulated PBMNC from healthy volunteers (data not shown) but it inhibited the viability of PHA-stimulated PBMNCs (Fig 1C right). This result is consistent with the fact that Aurora kinases are active during mitosis (21), therefore cells that proliferate rapidly are adversely affected by AT9283.

**AT9283 inhibits phospho Histone H3 and phospho Aurora A at Thr 288**

To ascertain the activity of AT9283 on Aurora A and B we evaluated the effect of AT9283 on phospho- H3 and on phospho Aurora A at Thr 288. It has been shown that selective Aurora A kinase inhibitors increase levels of H3 phosphorylation; conversely, Aurora B inhibition shows the opposite effect, decreasing H3 phosphorylation. Phosphorylation of H3 has been used as a marker for Aurora B inhibition (22-25). MM cells were treated with Nocodazole 0.4 μg/ml for 12 hrs to induce an M-phase block resulting in maximal phosphorylation of Histone H3; then cells were washed and subsequently treated with AT9283 for 2 hours. AT9283 decreased the phosphorylation of phospho Histone H3 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 treated with nocodazole and subsequently treated with AT9283 for 2 hours demonstrated a decrease in Aur 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.**
Since 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 μM) for 24 and 48 hrs. AT9283- treated MM.1S cells showed an increase of cells in G2/M, as well as 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 (13) (10, 21). Moreover, AT9283 induced morphological changes resulting in enlarged and multinucleated cells, as demonstrated by immunofluorescence (Fig 2A right). To investigate the molecular pathway activated by AT9283 we examined its effect on p53, p21, p27 and 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 hrs and 48 hrs respectively, when the cells undergo apoptosis; p21 upregulation and inhibition of Rb phosphorylation at ser 780 occurred after 12 hrs of treatment. This data is consistent with Aurora B inhibitory activity resulting in inhibition of Rb phosphorylation concomitant with polyploidy induction (Fig 2B).

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

**AT9283 inhibits STAT3 signaling pathway in MM cell lines**

Since AT9283 demonstrated a strong activity against JAKs by 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 STAT-dependent promoter (16). AT9283 inhibited STAT3-dependent luciferase activity with an EC50 of approximately 0.125 μM (Fig 3A). We further investigated the effect of AT9283 on STAT3 by evaluating the expression level of STAT3 tyrosine phosphorylation in MM.1S incubated with AT9283 (0.5 uM), phospho-STAT3 expression was downregulated starting at 30 minutes (Fig 3A). We confirmed this result by using U266 cells that have higher
constitutive STAT3 tyrosine phosphorylation than MM.1S cells; AT9283 (0.25 μM) induced a significant decrease in STAT3 tyrosine phosphorylation even in U266 cell lines. Downstream targets 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 bone marrow stromal cells and cytokines.**

The BM microenvironment enhances growth and survival of MM cells, mediated via direct MM cell-stromal cell contact as well as via cytokines (e.g. IL-6 and IGF-1) (15, 26, 27). To assess the effects of AT9283 on MM cells in the microenvironment, we analyzed the effect of MM.1S on phospho-STAT3, Aurora A and Aurora B in co-culture with BMSC. Consistent with the pro-survival effect of microenvironmental factors, co-culture 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 phospho-STAT3, 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 h in a dose-dependent manner, as well as inhibited the growth induced by IL6 and IGF-1 at 48h (Fig 3B). Therefore, AT9283 overcomes the proliferative advantage conferred by cytokines and the protective effect of BMSC.

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

We next sought to investigate whether the inhibition of STAT3, AURKA and AURKB was a direct effect of the compound, and if these activities were correlated. To this end, we suppressed the expression of STAT3, or Aurora A, or Aurora B using suitable siRNAs in U266 cells, and we verified the selective suppression by western blotting analysis (Fig. 4A). In cells with knocked-down STAT3, we did not observe any change in the expression level of AURKA and B; in cells with knocked-down AURKA and B, we observed a downregulation in the expression level of STAT3 likely due to the potential that STAT3 is downstream of Aurora Kinases. However, we
excluded physical interaction between AURKA and AURKB and STAT3 by performing immunoprecipitation (IP) (data not shown) (Fig 4A).

Fig. 4B shows the effect of AT9283 0.5 μM for 48 hrs on cell proliferation in the presence of genetic depletion of one of the three targets, STAT3, AURKA, and AURKB. Genetic depletion of each of the targets significantly reduced cell proliferation (knockdown grey bars vs control p<0.05). However, when each of the three targets are knocked down, AT9283 inhibited cell proliferation more but this was not statistically significant (grey vs white bars, p>0.05) indicating that STAT3, AURKA and AURKB are indeed targeted by AT9283. Alternately, genetic depletion of STAT3, AURKA and AURKB produce effects similar to AT9283 on MM cells making it difficult to delineate AT9283 induced inhibitory effect on the genetically depleted cells. Finally, comparing 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, AURKA and AURKB, 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 using a human MM xenograft mouse model. We used two different schedules of treatment: one group was treated with 15 mg/kg IP once a day for 5 days a week for 4 weeks; another group was treated with 45 mg kg IP once a day for 2 days a week for 4 weeks. These schedules were derived from the results of in vitro wash out experiments, which demonstrated that MM.1S cells are back 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 IP once a day for 5 days for 4 weeks did not show significant delay in tumor growth (data not shown).

As shown in Fig 5B, in the group treated with 45 mg/kg IP once a day for 2 days a week for 4 weeks, tumor growth was inhibited compared to controls (p =0.018). TUNEL assays on tumor
sections from treated versus control mice showed significantly increased apoptosis. Moreover IHC analysis of tumor taken from mice following administration of 2 cycles of AT9283 45 mg/kg 14 hours after drug administration confirmed decreased expression of phospho-Histone H3 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 Kaplan-Meier and log-rank analysis, the median overall survival (OS) of animals treated with 45 mg/kg IP once a day for 2 days a week for 4 weeks was significantly prolonged (32 days versus 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**

We next 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 concentrations of both agents at lower than their maximal cytotoxic concentrations. Increasing doses of AT9283 (0, 0.125 and 0.25 \( \mu \text{M} \)) were added to lenalidomide (0, 1 and 2 \( \mu \text{M} \)) and MM cytotoxicity was assayed by MTT at 48 hrs. The effects of combined therapy on DNA synthesis of MM.1S and INA-6 cells were determined by \(^{3}H\)-TdR uptake at 48 hrs. Significant decrease in viability and cell growth was observed with combined therapy compared to agents alone. Representative results for maximal synergistic effect are shown in Fig 6A. Synergism was confirmed by applying 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 lenalidomide by AnnexinV/PI staining. The analysis showed an increase (55.7\%) of cells in early and late apoptosis after 72 hours of exposure to combined therapy. Since 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 \( \mu \text{M} \) or lenalidomide (2 \( \mu \text{M} \)) or combination for 18 and 36 hours; cell lysates were...
subjected to immunoblotting using indicated antibodies. Combined 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 downregulation of pSTAT3 and pERK following 4 hours of treatment (Fig. 6C). Considering the role that the BM 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 BM 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 this data, we observed that AT9283 plus lenalidomide downregulated the expression of the p-STAT3 and p-ERK 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 Histone H3 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). JAK/STAT pathways have also attracted a lot of interest as a therapeutic target in MM (16) (33) because they are critically linked to survival and proliferation of MM cells (34, 35). We have studied AT9283, a multi kinase inhibitor with activity against Aurora A, Aurora B and STAT 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, i.e. enlarged polyploid nuclei due to failure of cytokinesis. Moreover, treatment for two hours with AT9283 after nocodazole treatment suppressed phosphorylation of Histone H3, another marker of Aurora B inhibition. The inhibition of phosphorylation of Histone H3 was also observed in our in vivo study, as demonstrated in histological sections of tumors from mice treated with AT9283. Although AT9283 seems to act predominantly as an Aurora B inhibitor also in MM cells, its molecular mechanism in this setting seems to be more complex, as 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 phospho Aurora A thr 288 was observed, suggesting activity of AT9283 against Aurora A. Additionally, we observed that AT9283 also decreases STAT3 tyrosine phosphorylation, which in turn led to c-myc downregulation (a STAT3 target), as observed by western blot analysis. The inhibition of these three pathways seems to be a direct effect of the compound. This effect was demonstrated by comparing AT9283 treatment of U266 cells with individually knocked down AURKA, AURKB, or STAT3 with respect to wild type U266 cells. Genetic depletion of each of these three targets independently did not change AT9283’s effect on cell proliferation. Importantly, when MM cells were adherent to BMSCs our data demonstrated an increase of STAT3, Aurora A and Aurora B expression which 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 retinoblastoma (Rb) at ser 780, consistent with previous studies showing that the induction of polyploidy or pseudo G1 arrest by small molecule inhibitors of Aurora Kinases is dependent on these pathways (2, 22). The retinoblastoma 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
post-mitotic checkpoints influences the effect of Aurora Kinase inhibitors on mitosis and polyploidy induction (19) (36). Treating cells with Aurora inhibitors when p53 function is intact leads to a post mitotic 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). Here we used a wild type p53 cell line MM.1S, and we observed substantial endoreduplication despite increased p53 and p21 protein levels. This data suggests that the response to Aurora inhibition is not solely determined by p53 status, although 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 polyploidy induction under Aurora B inhibition (22). Therefore p53-p21 could play a role in the endoreduplication and subsequential 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 non overlapping 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 (IMiDs) 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. Additionally 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 μM of AT9283 and 2 μM 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 p-ERK and p-STAT3; in particular, p-ERK is affected only by lenalidomide treatment and p-STAT3 is affected predominantly by AT9283. Finally, BMSCs support MM cell survival and proliferation both by adhesion and cytokine secretion. Here we confirmed a downregulation of phospho–STAT3 and phospho-ERK in the combined therapy when MM cells were co-cultured 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.

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AUTORSHIP CONTRIBUTION

L.S. designed research, performed research, collected data, analyzed and interpreted data, performed statistical analysis, and/or wrote the manuscript; T.H., S.P. and S.V. analyzed and interpreted data; D.C., K.P., E.N., M.B., S.R., G.G., Y.H. and C.U. performed research, collected data; M.S. provided AT9283, A.M., N.C.M., D.C. and K.C.A. contributed vital new reagents or analytical tools, N.R. designed research, provided the environment and support and wrote the manuscript.

Conflict-of-interest disclosure: K.C.A., N.C.M. and N.R. are members of the Board of Directors or advisory committees for Millennium, Celgene, and Novartis. N.R. is a recipient of research grants...
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The remaining authors declare no competing financial interests.

REFERENCES:


Figure legend

Fig.1 AT9283 inhibits growth and induces dose dependent cytotoxicity in MM cells. A) Chemical structure of AT9283 1-Cyclopropyl-3-[3-(5-morpholin-4-ylmethyl-1H-benzoimidazol-2yl)-1H-pyrazol-4-yl]-urea and in vitro kinase assay table. B) Dose-dependent inhibition in different MM cell lines demonstrated by measuring thymidine [3H-TdR] uptake during the last 8 hours of 48-hour cultures in 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). PBMNCs 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 ± s.d. of triplicate cultures. D) MM.1S cells were treated with Nocodazole 0.4 μg/ml for 12 hrs washed and subsequently treated with AT9283 0.5 μM for 2 hours. Cell lysates were immunoblotted with indicated antibodies.

Fig 2 AT9283 increases G2/M phase and induces apoptosis of MM cells in a time-dependent manner. A) MM.1S cells were cultured with media alone and AT9283 (0.5 μM) for 24 and 48 h.
Cell cycle analysis show an increase of cells in G2/M as well as an increase in cells with polyploid DNA content (left). Immunofluorescence assays on MM.1S treated with AT9283 0.5 uM for 24 hr, fixed and stained for microtubules (green – alpha tubulin). DNA was stained with Hoechst. Representative examples of multinucleated cells are shown (right) B) MM.1S were treated with 0.5 uM of AT9283. At indicated time points, cells were harvested and cell lysates were immunoblotted. AT9283 induced downregulation of pRB and upregulation of p53, p21 and p27. Densitometry is shown for p53. C) Increase of apoptotic/necrotic cells after AT9283 exposure for 72 hours (left) associated with PARP, Caspase-8 and -9 cleavage (right).

Fig 3 AT9283 inhibits STAT3 signaling pathway in MM cell lines. A) STAT3 reporter cells were pretreated with AT9283 at the indicated concentration for 1 hour, after which cells were stimulated with IL6 for 6 hours. Luciferase activity was quantitated by luminometry. MM1S and U266 cells were incubated with 0.5 uM and 0.25 uM of AT9283; at indicated time points, cells were harvested and whole lysates were incubated with indicated antibodies. B) MM.1S cells were cultured in the presence or absence of IL-6 10 ng/ml and IGF-1 50 ng/ml (upper graph) and in the presence or absence of BMSCs (lower graph) for 48 hours with indicated concentration of AT9283. ^H thymidine incorporation was measured during the last 8 hours of incubation to measure DNA synthesis. MM.1S were cultured in presence or absence of BMSC and treated with AT9283 at indicated time points; cells were harvested and whole lysates were immunoblotted with indicated antibodies.

Fig 4 AT9283 directly affects STAT3 pathway, Aurora A and Aurora B. A) Specific downregulation of STAT3, AURKB and AURKA by transient transfection of STAT3 siRNA, AURKB siRNA and AURKA siRNA, respectively, in U266 cells. B) U266 cells were transiently transfected with scramble siRNA, STAT3 siRNA, AURKA siRNA and AURKB siRNA and treated with AT9283 0.5 uM for 48 hours. ^H-TdR incorporation was measured during the last 8 hours of incubation to measure DNA synthesis.

Fig. 5 AT9283 inhibits human MM cell growth in vivo A) MM.1S cells were treated with 0.5 μM of AT9283 for 24 hrs, then replaced in drug-free medium, and collected for cell cycle analysis after 24, 48 and 72 hours. B) CB17 SCID mice were treated with either saline (n=8) or AT9283 (n=8) at 45 mg kg IP once a day for 2 days a week for 4 weeks. Tumor growth was inhibited in the treated group compared to controls (p =0.018). C) Using Kaplan-Meier and log-rank analysis, the median overall survival (OS) of animals treated with 45 mg kg IP once a day for 2 days a week for 4 weeks was significantly prolonged (32 days versus 18 days respectively; p < 0.0001) D) Treatment with AT9283 did not affect the body weight of the animals. E) TUNEL assays on tumor sections from
treated versus control mice showed significantly increased apoptosis. IHC analysis confirmed decreased expression of phospho-Histone H3 H3 (representative immunohistochemical positive expression for p-HH3 is indicated by the arrow) as well as Aurora B in treated animals. F) Western blot analysis showed decrease in pSTAT3, Aurora A and Aurora B.

Fig 6 AT9283 in combination with lenalidomide enhances the toxicity of MM cells A) AT9283 (0.125 μM) was added with lenalidomide (2 μM) to MM.1S, and INA-6 cells for 48 hrs. Cytotoxicity was assayed by MTT (left). Growth inhibition was assayed by ³H-TdR uptake.* CI<0.9. To calculate the CI, MM.1S and INA6 were treated with AT9283 (0.125-0.25 μM) and/or lenalidomide (1-2 μM) for 48 hours. Isobologram analysis using CaluSyn software showed synergistic anti-myeloma effect of the combination AT9283 and lenalidomide. B) MM1S were treated with AT9283 (0.125 μM), lenalidomide (2 μM) or combined therapy for 72 hours. Annexin/PI staining show 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 μM), lenalidomide (2 μM) or combined 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.
Kinase | IC50 (nM)
---|---
Aurora A | 52% @ 3nM
Aurora B | 58% @ 3nM
JAK3 | 1.1
JAK2 | 1.2
A

![Graph showing relative luciferase units for STAT3 at different AT9283 concentrations.](image)

**MM.1S**
- C
- 30' 2hr 4hr 6hr

- **phospho STAT3**
- **STAT3**
- **GAPDH**

**U266**
- C
- 30' 2hr 4hr 6hr

- **phospho STAT3**
- **STAT3**
- **GAPDH**

**MM.1S**
- C 2 4 6 12 24 48 hrs

- **c-myc**
- **GAPDH**

B

![Bar chart showing percent control of MM.1S, MM.1S + IL-6 10 ng/ml, and MM.1S + IGF-1 50 ng/ml at different AT9283 concentrations.](image)

**MM.1S**
- +

**BMSCs**
- -

- **AT9283**
- C 0 4 24 hrs

- **phospho STAT3**
- **STAT3**
- **Aurora A**
- **Aurora B**
- **GAPDH**

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Anti-myeloma activity of a multi targeted 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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