

AT9283, a Multi-Targeted Kinase Inhibitor has Potent Activity in AML Cell Lines and Patient Samples

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INTRODUCTION

- AT9283 is a potent inhibitor of JAK2, JAK3, mutant Abl kinase (T315I Abl) and Aurora kinases A and B all of which have an IC₅₀ <5nM. In addition to these activities, AT9283 also inhibits Flt-3 (IC₅₀ ~15nM) and c-kit (IC₅₀ ~250nM) (Table 1).
- Recent reports have described the activity of Aurora kinase inhibitors in AML cell lines and patient samples though, in many cases, the mechanism of action remains unclear.
- Around 30% of AML patients express a mutant form of Flt-3, manifested as either internal tandem domain repeats (ITD) or activating point mutations, and are associated with a poor prognosis. Activating mutations in c-kit have been identified in 17-46% of AML patients and both receptors have been studied intensively as potential therapeutic targets in AML. Furthermore, constitutive activation of these signalling pathways is achieved through Ras mutation in 19% of AML cases.
- Here we investigate the relative contributions of AT9283's kinase inhibitory activities to its cytotoxicity profile in AML. We show that AT9283 is a potent inhibitor of AML cell growth both *in vitro* and in patient samples.
- In a panel of AML cell lines treated with AT9283 we observed 2 main phenotypes; 1) Accumulation of cells in the G₂/M phase of the cell cycle followed by apoptosis, or 2) Accumulation of cells with >4N DNA (polyploid) followed by apoptosis.
- In general cell lines that exhibit phenotype 1 are those driven by mutation in an oncogenic kinase. We demonstrate inhibition of Auroras A and B, Flt-3 and c-kit at concentrations consistent with the cell cycle inhibitory effects in relevant cell lines and suggest reasons for the difference in response of these cell lines to AT9283.

COMPOUND PROFILE

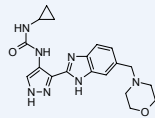


Figure 1: Chemical Structure of AT9283

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Protein Kinase	IC ₅₀ (nM)
Aurora A	52% @ 3nM
Aurora B	58% @ 3nM
JAK2	1.2
JAK3	1.1
T315I ABL	4
Flt3	57% @ 15 nM
c-kit	46% @ 250 nM
RSK-1	37
Lck	63
Src	97
c-abl	110

Table 1: AT9283 *in vitro* Kinase Inhibition

- AT9283 (Figure 1) was tested against a panel of kinases in a biochemical assay (Table 1).
- The inhibitory activity of AT9283 against a panel of AML cell lines harbouring a range of molecular defects and cytogenetic abnormalities was tested (Table 2). Cell lines were obtained from DSMZ.
- Cells were exposed to AT9283 for 72hr. Cell viability was determined using an Alamar BlueTM assay. IC₅₀ values were determined where possible. In some cases biphasic dose response curves were obtained, indicative of a dominating polyploid phenotype. Here the lowest dose at which polyploidy was observed is quoted. p53 functionality was determined by cell cycle arrest in response to topoisomerase II inhibition.
- AT9283 induced 2 predominant cellular responses in sensitive AML lines; either cell death by 72hrs at low nanomolar concentrations (e.g. MV4-11) (phenotype 1) or endoreduplication and polyploidy (e.g. PL-21) (phenotype 2), at concentrations around 30nM.

RESULTS

Cell Line	FAB Classification	Characteristics	Functional p53 pathway	IC ₅₀ (nM)
MUTZ-2	M2	Growth factor dependent	n.d.	12
MV4-11	M5	Flt-3-ITD	Functional	18
Kasumi-1	M2	c-kit ^{WT/WT}	Non-Functional	18
MOLM-13	M5a	Flt-3-ITD	Functional	32
CMK	M7	JAK3 ^{WT/WT}	n.d.	66
TF-1	M6	Growth factor dependent	n.d.	110
HEL	M6	JAK2 ^{WT/WT}	n.d.	150
MOLM-16	M5a	Growth factor dependent	n.d.	220
ML-2	M4	carries t(8;11)(q27;q23) (MLL-AF9 fusion gene)	Functional	p/ploidy @ 0.03µM
HL-60	M2	mut Ras, amplified c-Myc	n.d.	p/ploidy @ 0.03µM
THP-1	M5	carries t(8;11)(p21;q23) (MLL-AF9 fusion gene)	n.d.	p/ploidy @ 0.03µM
SKM-1	M5	Complex Karyotype	Functional	p/ploidy @ 0.03µM
PL-21	M3	Complex Karyotype	Functional	p/ploidy @ 0.03µM
ME-1	M4	Carries t(9;11)(p13;q22) (CBFB-MYH11 fusion gene)	n.d.	>1µM
F-36P	M6	Growth factor dependent	n.d.	>1µM
GF-D8	M1	Growth factor dependent	n.d.	>1µM

Table 2: Growth Inhibitory Effect of AT9283 in AML Cell Lines

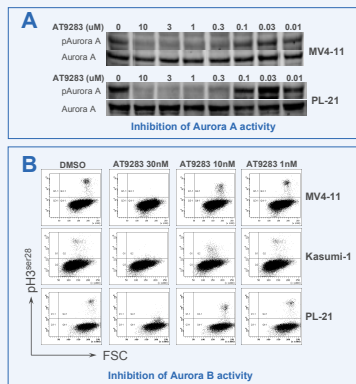


Figure 2: AT9283 Potently Inhibits Aurora A & B Activity in AML Cell Lines

RESULTS

- To investigate the mechanism underlying the two different responses to AT9283, the level of Aurora A and B inhibition was measured in cell lines from each of the two phenotype groups, MV4-11 and Kasumi-1 for phenotype 1 and PL-21 for phenotype 2, treated with AT9283 for 4hrs prior to analysis (Figure 2).
- Inhibition of Aurora A was determined by measuring levels of phospho-Aurora A^{Thr288} by western blotting (Figure 2A), and Aurora B inhibition was assessed by measuring of phospho-histone H3^{ser28} by flow cytometry (Figure 2B).
- The expression of phospho-Aurora A^{Thr288} was inhibited to an equal degree in all AML cell lines tested with IC₅₀ values of 100-300 nM (Figure 2A and data not shown). Similarly, we found no difference in Aurora B inhibition by AT9283 between cell lines of the two groups, with complete inhibition of pHH3^{ser28} at 30nM in all cell lines tested (Figure 2B and data not shown).
- Given that we found no difference in the degree of inhibition of the Auroras to determine if the two phenotypes observed were a result of differential expression within the cell line panel (Figure 3).

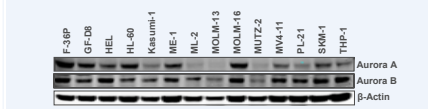


Figure 3: Relative Expression Levels of the Aurora Kinases in AML Cell Lines

- Cell lysates from 14 of the AML cell lines included in the proliferation panel (Table 1) were generated from untreated cells in log phase growth and the expression of Aurora A and B was determined in each by western blotting (Figure 3).
- We could find no correlation between the relative expression of either of the Auroras kinases and the resulting phenotype following AT9283 exposure.

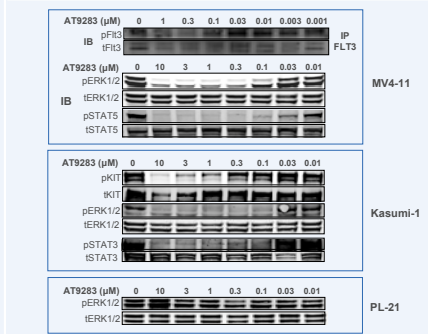


Figure 4: AT9283 Inhibits oncogenic kinases and their downstream pathways in AML cells

- Since AT9283 is a multi-targeted kinase inhibitor and inhibits a number of kinases important in AML, including Flt-3 and c-kit, we measured the inhibition of these and downstream pathways by AT9283 (Figure 4).
- Cells were treated with AT9283 for 4hrs and western blotting performed for Flt-3, c-kit and downstream signalling pathways.

RESULTS

- Treatment of MV4-11 and Kasumi-1 cells (phenotype 1 cells) with AT9283, reduced phosphorylation of Flt-3 and c-kit (Figure 4) at concentrations of 100-300nM and 300-1000nM, respectively.
- Signalling pathways downstream of these oncogenic kinases (STAT5 and ERK1/2) were also potentially inhibited by AT9283 concentrations consistent with inhibition of these primary targets (Figure 4).
- AT9283 had no effect on these signalling pathways in cell lines not driven by oncogenic kinases, such as PL-21 cells (phenotype 2 cells).
- These data suggested that AT9283 may inhibit growth via two distinct mechanisms; 1) where present, via direct inhibition of an oncogenic kinase activity (phenotype 1 cells); or 2) via inhibition of the Auroras kinases where oncogenic kinases are not present (phenotype 2 cells).
- If inhibition of the oncogenic kinases is the predominant mechanism by which AT9283 induces cell death in phenotype 1 cells, G₂ arrest should occur prior to apoptosis. We therefore determined the cell cycle profiles of cells from each of the two phenotype groups following AT9283 exposure. The Flt-3 and c-kit inhibitor Sunitinib[®] was used as a control (Figure 5).

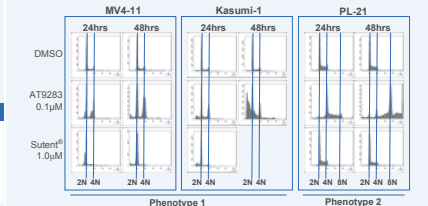


Figure 5: AT9283 Induces G₂/M Arrest and Endoreduplication in AML Cell Lines

- Although AT9283 inhibits both the Auroras kinases (Figure 2) and several signalling pathways aberrant in AML cell lines (Figure 4), we found that the predominant cell cycle phenotype of AML cells following exposure to AT9283 was one consistent with Aurora inhibition.
- Cells either arrested in G₂/M (phenotype 1 cells) or underwent marked endoreduplication (phenotype 2 cells) resulting in >4N DNA (Figure 5). By comparison, the Flt-3/c-kit inhibitor Sunitinib[®] showed marked G₂ arrest in MV4-11 and Kasumi-1 cells. Thus the direct inhibition of oncogenic kinases by AT9283 is unlikely to be sole determinant in the cellular response of AML cells to AT9283.

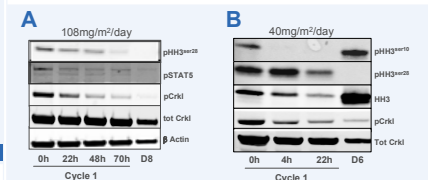


Figure 6: Inhibition of Aurora (pHH3), JAK (pSTAT5) and Abl (Crkl) in Peripheral Blasts From Patients Following Treatment With AT9283

RESULTS

- AT9283 is currently in a Phase I/IIa clinical study in patients with advanced haematological malignancies including AML.
- AT9283 was administered on 2 dose schedules, either as a 72hr i.v. infusion every 3 weeks (Figure 6A) or as a 4 day infusion repeated with the same frequency (Figure 6B).
- Peripheral blasts were isolated by standard density centrifugation methods from patients receiving drug and western blots performed to demonstrate the modulation of biomarkers associated with AT9283 activity.
- AT9283 was found to inhibit biomarkers in these peripheral blasts associated with effects on Aurora and cell signalling pathways associated with the oncogenic kinases investigated *in vitro*.
- All of these activities appeared to occur across similar dose ranges and with similar kinetics implying that AT9283 is achieving pharmacologically active doses against several of its putative targets.

CONCLUSIONS

- AT9283 is a potent inhibitor of AML cell line growth and inhibition of its targets Aurora A and B and the Flt-3 and c-kit signalling pathways is observed at concentrations consistent with its anti-proliferative effects.
- In a panel of AML cell lines two phenotypes were observed. The first, cell cycle arrest at G₂/M. The second endoreduplication followed by apoptosis at later timepoints, the classical Aurora B phenotype.
- Whilst the two phenotypes appeared to segregate based on the cytogenetic profile of the cell lines in question with cells harbouring mutations in the Flt-3 and c-kit signalling pathways forming phenotype 1 and those with a more complex karyotype associated with phenotype 2 the cell cycle profile is inconsistent with AT9283 exerting its effects solely via inhibition of Flt-3 or c-kit signalling in these cell lines.
- Our results suggest that AT9283 probably acts via Aurora A and/or B in all cell lines tested, triggering the post-mitotic checkpoint and inducing apoptosis in cells harbouring mutations in Flt-3 or c-kit possibly via Aurora kinase A inhibition. These AML cases have been shown to be more likely to exhibit normal cytogenetics and this profile may be important in sustaining rapid peripheral blast proliferation typical of this subtype of AML. Cell lines that respond to treatment with AT9283 by becoming polyploid may be manifesting the effect of predominant Aurora kinase B inhibition.
- The outcome of Aurora inhibition following exposure to AT9283 in individual subtypes of AML may be determined by the cytogenetic abnormalities that the cell contains and factors such as p53 pathway functionality, associated with genomic instability, is also likely to play a role.
- Ongoing studies to determine the contribution of both Aurora isoforms to these effects are underway.
- Inhibition of these signalling pathways have been confirmed in AML blasts taken from patients treated with AT9283 in a phase I/IIa study in patients with refractory leukemia. Ongoing studies will attempt to determine whether responses observed in this trial are related to the characteristics of an individual's AML and the relation of this to our *in vitro* observations.

Disclosure Statement

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