Effects of the novel aurora kinase/JAK inhibitor, AT9283 and imatinib on Philadelphia positive cells *in vitro*

To the Editor

Despite the clinical success of targeted tyrosine kinase inhibitors (TKI) such as imatinib (IM; Glivec®, Novartis) in chronic myeloid leukaemia (CML), leukaemic stem cell persistence *in vivo* necessitates the search for novel targets for alternative therapeutic intervention. Several novel investigational medicinal products aimed at hitting cellular survival signalling pathways that are independent of BCRABL in the hope of increasing efficacy of TKI monotherapy in CML patients are in clinical development. One such target maybe Janus Kinase (JAK) 2 that plays a role in transducing growth factor mediated signalling through STAT5. In CML, JAK2 can be directly phosphorylated by BCRABL kinase, the causative oncoprotein in CML, and in turn phosphorylates tyrosine 177 of BCRABL that appears necessary for its stabilisation and activation. However, JAK2 can also directly and independently lead to activation of survival/anti-apoptotic signals via growth factor receptors even when BCRABL kinase activity is inhibited [1,2]. JAK2, as a result, warrants further investigation as a potential therapeutic target in CML.

We planned to reveal the relevance of JAK2 kinase in survival and proliferation of the human CML cell line, KCL22 using the novel multi-targeted kinase inhibitor AT9283 (1-cyclopropyl–3[5-morpholin-4yl methyl-1H-benzomidazol-2-yl]-urea) (Astex Pharmaceuticals Ltd.) [3] that has already reached clinical development in solid tumours [4]. AT9283 has *in vitro* activity against multiple kinases but is active at low

(nanomolar) concentrations particularly against JAK2 and aurora kinases. KCL22 is a human BCRABL positive cell line established from the pleural effusion of a CML blast crisis patient [5] displaying human hyperdiploid karyotype with 3.3% polyploidy as described in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ -German Collection of Microorganisms and Cell Cultures) (DSMZ # ACC 519).

In order to investigate AT9283 and its effects in conjunction with IM on CML cells *in vitro*, we first assayed the effect of AT9283 at varying concentrations over 24 to 96h to establish its IC_{50} (a measure of effectiveness of a compound in inhibiting biological or biochemical functions, here KCL22 cell proliferation). We analysed the individual and combined effects of AT9283 with IM, at fixed concentration, on apoptosis of KCL22 cells by flow cytometry (Annexin V and Via-Probe™ staining).

AT9283 was significantly more potent (IC_{50} 15 ± 0.2 nM, n = 3, Figure 1A) than IM (IC_{50} is in the micromolar range), with respect to surviving cell number enumerated by trypan blue dye exclusion method. Total cell number counted by a dye exclusion method does not discriminate which cells are undergoing apoptosis as cells are scored as 'live' or 'dead'. Bright 'live' cells under phase contrast microscopy are a mix of the most viable cells as well as those initiating programmed cell death. We therefore next assayed apoptosis in response to AT9283 using a standard Annexin V / DNA staining protocol. AT9283 committed cells to apoptosis at a level above background (i.e. in the No Drug Control) at concentrations of 30nM and above at 72h (Figure 1B). When used in combination, an enhanced apoptotic response was found compared with either agent alone at 72h (Figure 1D). Thus, even when BCRABL had been maximally

inhibited with 2μM IM, a deeper apoptotic response could be detected in KCL22 when AT9283 was present suggesting that AT9283 can act independently of BCRABL to effect cell kill. AT9283 with its multitargeted profile of kinase inhibition, is a potent inhibitor of serine/threonine kinases targeting aurora family kinases as well as tyrosine kinases including JAK and ABL. It is therefore likely that aurora kinase or JAK inhibition is mediating the drug effect seen here under conditions of maximal ABL/BCRABL kinase inhibition. Being nuclear proteins, aurora kinases are unlikely to interact with BCRABL. However, it is theoretically possible for both JAK2 [6] and BCRABL [7] to shuttle into the nucleus, although nuclear import of the latter occurs when the kinase is inactive [7] and to the best of our knowledge, there is no known direct interaction between aurora kinases and ABL/BCRABL or JAK.

The absolute number of viable cells was calculated as cell number per mL (live trypan blue count) x % Annexin V / Via-Probe™ double negative (quadrant 4 in Figure 1C). It can be seen that at IC_{50} (15nM; as calculated by dye exclusion method), the absolute number of viable cells at 24h is not only less than half the count in the No Drug Control but less than input (2×10^5 in 1mL; Figure 1E), indicating that the drug is more cytotoxic than cytostatic. The cell counts are not reduced with increasing concentration of AT9283 up to 10nM at 72h compared to No Drug Control suggesting either that there is a threshold of drug concentration below which the cells can recover or that the drug is chemically unstable in culture over extended time frame losing its potency. Nonetheless, given enough of the compound (here above 15nM), there is a diminution in absolute number of viable cells to less than input by 72h with AT9283 alone.

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We explored the potential of the novel aurora kinase / JAK inhibitor, AT9283 for its effects on blast crisis CML cells *in vitro*, in combination with a TKI. We chose IM, a rationally designed TKI that blocks the ATP-binding site of BCRABL so suppressing downstream signalling, as it is currently recommended as the first-line therapy for CML in chronic phase by the National Comprehensive Cancer Network (NCCN) and European LeukemiaNet (ELN) and is the current standard of care [8]. In our investigation, we found that AT9283 effectively blocked CML blast crisis cell proliferation and was more potent than IM. More significantly, a greater degree of apoptosis was seen at all time intervals (24 to 72h) in response to AT9283 than IM. Response to IM was typically found to be slow only peaking at 72h; it was neither as fast nor as effective as AT9283. The IC_{50} of AT9283 was significantly lower being in the nanomolar range clearly indicating the potency of the drug. Importantly, the combination of AT9283 (15nM) and IM (2μM) proved to be more effective than either drug acting alone in increasing apoptosis and controlling cell number at 72h [Figure 1D&E]. We chose to use IM at 2μM at which concentration BCRABL kinase is maximally inhibited but death is not necessarily elicited in the inherently TKI resistant, KCL22. We therefore could anticipate seeing additional biological effects owing to the activity of AT9283 in the combination. Of note, the cells were found to undergo apoptosis in response to AT9283 in combination with IM suggesting that the former also had some drug activity that was BCRABL independent [9].

We and others have shown that despite TKI driven inhibition of BCRABL activity, CML cells can survive in culture leading us to believe that BCRABL dependent signalling pathways are not the only survival signals active in CML [10,11]. This has

been indirectly confirmed in the laboratory based investigation reported here by the increased efficacy of combining aurora kinase / JAK2 with BCRABL inhibition in our Ph^+ cell model. In summary, the results generated from this project emphasise the therapeutic potential of drugs like AT9283 in combination with TKI and potentially identify the JAK family as a valid target for further study in treatment of CML.

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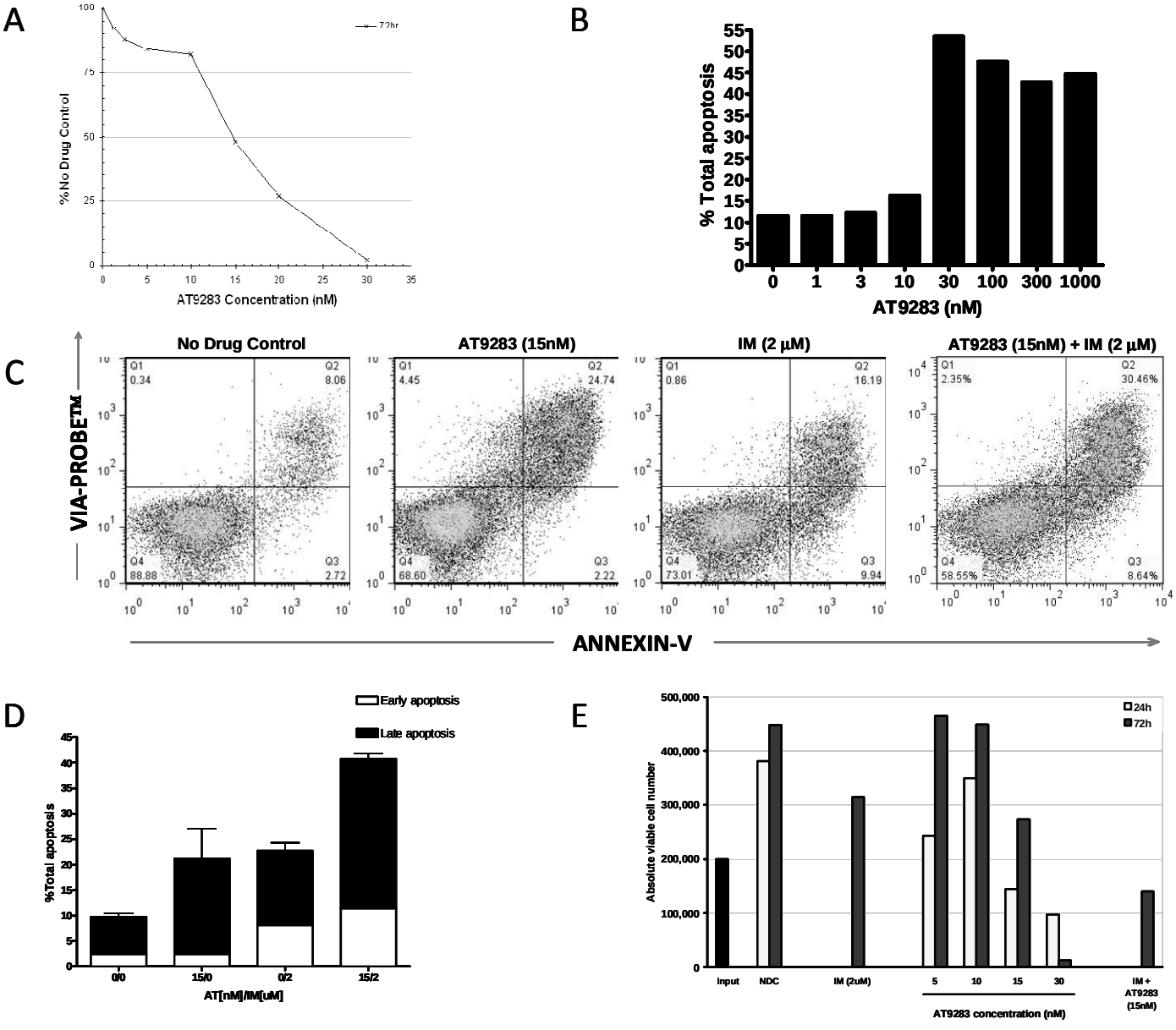
MS was supported by a British Society for Haematology undergraduate medical elective scholarship. PG is a recipient of a Medical Research Council (UK) clinical training fellowship. HJ is supported by Friends of Paul O'Gorman Leukaemia Research Centre, Glasgow.

Figure 1A: Concentration-effect curve of AT9283 on KCL22 over 72h *in vitro.* Surviving cells were counted by trypan blue dye exclusion method after 72h treatment with the aurora kinase / JAK2 inhibitor, AT9283. The number of cells was expressed as a percentage of the No Drug Control and plotted against the drug concentration to determine the IC_{50}. **Figure 1B: Total apoptosis in AT9283 treated KCL22 cells at 72h.** AT9283 committed cells to apoptosis at a level above background (i.e. in the No Drug Control) at concentrations at, or above, 30nM at 72h. **Figure 1C: AT9283 induces apoptosis in Ph^+ blast crisis CML cell line KCL22 *in vitro.*** Representative flow cytometry dot plots of KCL22 cells either untreated (far left panel, No Drug Control), or treated with AT9283 15nM (left panel), or IM 2μM (right panel), or the combination of AT9283 plus IM (far right panel) and stained for apoptosis. Q2 events are both Annexin-V and Via-Probe™ positive and therefore indicative of cells in late apoptosis. Q3 events are Annexin-V positive and Via-Probe™ negative and are indicative of cells undergoing early apoptosis, the most viable cells are in Q4. Total apoptosis would be given as the sum of events in Q2 and Q3. **Figure 1D: Increased apoptotic response combination of IM and AT9283 at 72h**. When combined with IM, AT9283 was more effective in inducing apoptosis in KCL22 cells. First column AT9283 0nM / IM 0μM; second column AT9283 15nM / IM 0μM; third column AT9283 0nM / IM 2μM; fourth column AT9283 15nM / IM 2μM. White boxes represent percentage of cells in early apoptosis, black boxes are late apoptosis. **Figure**

1E: Absolute viable KCL22 cell numbers. Factoring in the apoptotic cells gives a more objective indication of drug activity than a simple cell count by dye exclusion in which cells undergoing programmed cell death may still appear viable. So the absolute number of viable cells (y-axis), calculated as live trypan blue count x % Annexin V / Via-Probe™ double negative, at 24h with 15nM AT9283 was not only less than half the count in the No Drug Control (NDC) but less than input (2×10^5 / mL; 1mL).

Figure 1


A
72hr
% No Drug Control
AT9283 Concentration (nM)
B
% Total apoptosis
AT9283 (nM)
C
No Drug Control
AT9283 (15nM)
IM (2 µM)
AT9283 (15nM) + IM (2 µM)
VIA-PROBE™
ANNEXIN-V
D
Early apoptosis
Late apoptosis
%Total apoptosis
AT[nM]/IM[uM]
E
24h
72h
Absolute viable cell number
Input
NDC
IM (2uM)
AT9283 concentration (nM)
IM + AT9283 (15nM)

References:

[1] Y. Wang, D. Cai, C. Brendel, et al, Adaptive secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) mediates imatinib and nilotinib resistance in BCR/ABL+ progenitors via JAK-2/STAT-5 pathway activation, Blood. 109 (2007) 2147-2155.

[2] D.K. Hiwase, D.L. White, J.A. Powell, et al, Blocking cytokine signaling along with intense Bcr-Abl kinase inhibition induces apoptosis in primary CML progenitors, Leukemia. 24 (2010) 771–778.

[3] S. Kimura, AT-9283, a small-molecule multi-targeted kinase inhibitor for the potential treatment of cancer, Curr. Opin. Investig. Drugs. 11 (2010) 1442-1449.

[4] H.T. Arkenau, R. Plummer, L.R. Molife, et al, A phase I dose escalation study of AT9283, a small molecule inhibitor of aurora kinases, in patients with advanced solid malignancies, Ann. Oncol.; Oct 19 [Epub ahead of print] (2011).

[5] I. Kubonishi and I. Miyoshi, Establishment of a Ph1 chromosome-positive cell line from chronic myelogenous leukemia in blast crisis, Int. J. Cell Cloning. 1 (1983) 105-117.

[6] A. Zouein, R.J. Duhé, G.W. Booz, JAKs go nuclear: Emerging role of nuclear JAK1 and JAK2 in gene expression and cell growth Growth Factors. 29 (2011) 245-252.

[7] P. Vigneri and JY. Wang, Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase, Nat. Med. 7 (2001) 228-234.

[8] M. Baccarani, G. Saglio, J. Goldman, et al, Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet, Blood. 108 (2006) 1809–1820.

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[9] R. Tanaka, M.S. Squires, S. Kimura et al, Activity of the multitargeted kinase inhibitor, AT9283, in imatinib-resistant BCR-ABL-positive leukemic cells, Blood. 116 (2010) 2089-2095.

[10] A. Hamilton, G.V. Helgason, M. Schemionek, et al, Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival, Blood. Dec 19 (2011) [E-pub ahead of print]

[11] A.S. Corbin, A. Agarwal, M. Loriaux, et al, Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity, J Clin Invest. 121 (2011) 396-409.