Table 3 Prevalence of risk factors of transfusion and proportions of patients transfused during hospitalization

Factor	*n*	Prevalence (1000 admissions)	Proportion of patients with transfusion: RBC transfusion (%)	PLT transfusion (%)
Age				
20–64	259588	442·2	3·5	1·0
65–79	220247	375·2	6·5	1·5
80+	107210	182·6	9·0	1·1
Blood disorders				
Acute leukaemia	1986	3·4	59	60
Chronic leukaemia	476	0·8	28	16
Malignant lymphoma	1625	2·8	36	17
Multiple myeloma	6032	10·3	14	11
Aplastic anaemia	940	1·6	58	34
MDS	1476	2·5	63	37
DIC	4372	7·4	43	26
Cancer				
Without chemotherapy	93103	158·6	8·3	0·8
With chemotherapy	42088	71·7	4·7	1·0
Surgery				
Cardiovascular surgery				
Without CPB	2205	3·8	50	18
With CPB	2629	4·5	73	43
Hip fracture surgery	7457	12·7	28	0·9
Severe trauma				
Obstetric bleeding	823	1·4	10	1·5
GI bleeding	19808	33·7	34	2·5
Chronic renal failure	24691	42·1	17	2·1

GI, gastrointestinal; DIC, disseminated intravascular coagulation; CPB, cardiopulmonary bypass; MDS. Myelodysplastic syndrome; RBC, red blood cell.

risk factors for RBC transfusion were cardiovascular surgery with cardiopulmonary bypass (CPB) (OR = 106·9), followed by acute leukaemia (OR = 46·9), MDS (OR = 39·2), cardiovascular surgery without CPB (OR = 35·0), gastrointestinal haemorrhage (OR = 13·2), and hip fracture surgery (OR = 11·0). Risk of RBC transfusion increased with patient age.

Greatest risk factors for platelet transfusion were acute leukaemia (OR = 310) and cardiovascular surgery with CPB (OR = 185), followed by MDS (OR = 78·9), cardiovascular surgery without CPB (OR = 46·5), disseminated intravascular coagulation (DIC) (OR = 43·2), multiple myeloma (OR = 39·5) and aplastic anaemia (OR = 33·6). No association was observed between patient age and risk of platelet transfusion.

These logistic regression models showed good prediction performances. Areas under ROC curves for proportions of patients with RBC transfusions and platelet transfusions were 0·83 and 0·90, respectively.

Case-mix adjusted models predicting total units of blood products used

Figure 1 shows expected vs. observed use of RBCs and platelets at each hospital. The goodness-of-fit of this model, assessed using r^2 for linear regression, was 0·88 for RBC use and 0·57 for platelet use. Therefore, use of blood products at each hospital was successfully predicted by the case-mix adjusted model.

Table 5 compares results of the medical chart review (percentage of appropriateness) and O/E ratios calculated using the two prediction models. In general, proportions of appropriate transfusions were higher for RBC than for platelet transfusions. Also, Hospital B showed better performance in terms of appropriate transfusions than did Hospital A. Both models successfully assessed the proportion of appropriate transfusions.

Discussion

Due to critical situations and complex clinical conditions underlying the use of blood products, it is often difficult for physicians to make appropriate decisions about blood transfusions. Surveys of blood product usage for specific diagnoses have demonstrated wide variation in comparable patients at different institutions [8,26–29]. Some studies have shown that a system approach is effective for improving transfusion practices. System approaches include implementation of institutional guidelines, education, prospective audits and approval of transfusion orders [12]. Of these approaches, 'clinical audits' with feedback can effectively decrease the amount of blood product used [28–35]. However, these effects on blood product use are often temporary, and continuous efforts are required for maintenance of good transfusion practices [30,36]. Moreover, audits require considerable labour and cost. Therefore, it is difficult to implement audits for routine and continuous evaluations. If another method were available for routine evaluations, then it would contribute greatly to comparison of transfusion performance between hospitals and resultant improvement in transfusion practices at hospitals.

We proposed methods which utilizing administrative data to evaluate hospital-wide use of blood products in Japan. Few studies have employed administrative data to perform such comparisons. Comparison of blood usage across hospitals usually is difficult given that risks of transfusion differ between patients and between hospitals. On the contrary, some studies have demonstrated that a patient classification

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Table 4 Logistic regression models for predicting transfusion of red blood cell and platelet

Factor	RBC Regression coefficient	RBC Odds ratio	RBC 95% CI	PLT Regression coefficient	PLT Odds ratio	PLT 95% CI
Age*						
65–74	0·40	1·5	1·4–1·5	0·11	1·1	1·0–1·2
80+	0·75	2·1	2·1–2·2	−0·06	0·9	0·9–1·0
Female	−0·12	0·89	0·86–0·91	0·06	1·1	1·0–1·1
Haematopoietic disorders						
Acute leukaemia	3·85	46·9	42·4–51·8	5·74	310	278–346
Chronic leukaemia	2·33	10·3	8·1–12·9	3·26	26·2	19·2–35·6
Aplastic anaemia	3·12	22·7	19·4–26·6	3·52	33·6	27·3–41·4
Multiple myeloma	2·84	17·2	15·4–19·2	3·68	39·5	33·8–46·1
Malignant lymphoma	1·51	4·5	4·1–4·9	3·10	22·2	20·0–24·7
MDS	3·67	39·2	34·9–44·1	4·37	78·9	68·6–90·7
DIC	2·44	11·4	10·6–12·3	3·77	43·2	39·3–47·5
Obstetric bleeding	1·82	6·2	4·9–7·8	0·99	2·7	1·4–5·0
Severe trauma	2·65	14·1	12–16·6	2·08	8·0	5·9–11
Cardiovascular surgery						
Without CPB	3·56	35·0	32·0–38·3	3·84	46·5	40·8–52·9
With CPB	4·67	106·9	97·7–117	5·22	185	169–203
Malignant tumour						
Without chemotherapy	0·93	2·5	2·5–2·6	0·32	1·4	1·3–1·5
With chemotherapy	0·66	1·9	1·8–2	0·81	2·2	2·0–2·5
GI bleeding	2·58	13·2	12·8–13·7			
Hip fracture surgery	2·40	11·0	10·4–11·6			
Chronic renal failure	1·59	4·90	4·7–5·1			
Liver cirrhosis				1·25	3·5	3·0–4·0
Constant	−4·10			−5·78		

GI, gastrointestinal; DIC, disseminated intravascular coagulation; CPB, cardiopulmonary bypass; MDS. Myelodysplastic syndrome; RBC, red blood cell 95% CI, 95% confidence intervals for odds ratios.
*Patients aged below 65 years were used as reference.

system such as DRG can provide a means for inter-institutional benchmarking and cost comparison [14–16,37]. DRG is widely used for healthcare payments and analyses of hospital activities. The basic concept underlying the employment of DRG for reimbursement is that 'treatments for similar patients consume a similar degree of medical resources'. Therefore, patients within the same DRG are considered to have similar resource (e.g., blood product) utilization.

By the use of a patient classification system and identification of risk factors of transfusion, we can properly compare blood product use between different healthcare providers. Furthermore, such a method should be useful for identifying providers with extremely high levels of blood product use. If a simple risk adjustment model could be developed by analyzing large healthcare databases representing information obtainable with little labour and at low

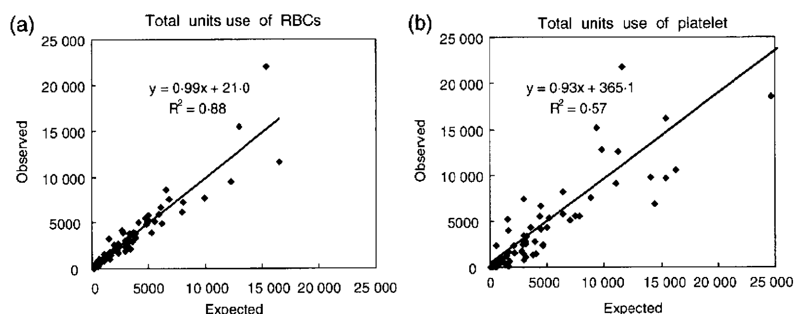

Fig. 1 The multiple linear regression model to predict total unit use of RBCs and platelets transfused at each hospital

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Table 5 Results of the medical chart review (percentage of appropriateness) and O/E ratios calculated using the two prediction models

		Red blood cell			Platelet		
Patient	Hospital	% of appropriateness	O/E (Model 1)	O/E (Model 2)	% of appropriateness	O/E (Model 1)	O/E (Model 2)
Overall	A	72	1·09 (1·02–1·18)	1·11 (1·05–1·16)	35	1·54 (1·34–1·81)	1·60 (1·41–1·83)
	B	81	0·93 (0·89–0·93)	0·78 (0·75–0·81)	64	1·13 (1·03–1·25)	0·78 (0·75–0·89)
Haematopoietic malignancy	A	71	1·35 (1·11–1·72)	1·61 (1·40–1·90)	25	1·50 (1·19–2·00)	2·30 (1·91–2·89)
	B	91	1·09 (0·97–1·25)	0·90 (0·82–0·99)	78	1·17 (1·02–1·36)	0·78 (0·75–0·89)
Cardiovascular surgery	A	62	1·25 (1·08–1·48)	1·73 (1·52–2·01)	17	1·76 (1·35–2·53)	1·23 (1·04–1·52)
	B	71	0·96 (0·88–1·05)	0·86 (0·79–0·93)	83	0·99 (0·85–1·18)	0·95 (0·86–1·06)

cost, it would be possible to evaluate the frequency of blood product use by various healthcare providers. It should be noted, however, that such a method is not designed to replace the detailed analyses conducted by auditing committees. Instead of pinpoint evaluations on blood product utilization at the patient level, the purpose of our method is to provide continuous surveillance and routine general evaluations on a larger scale that allows for multi-institutional comparisons with a satisfactory level of accuracy.

We selected two indicators to evaluate hospital-wide use of blood products in Japan: (i) proportion of patients that received a blood transfusion and (ii) total amount (number of units) of blood products used. These are typical indicators used for assessment of hospital-level blood usage [9,16,25,26]. We developed two regression models to calculate risk-adjusted blood use. A logistic regression model was used to predict the percentage of transfused patients in each hospital. A multiple linear regression model was used to predict hospital-wide total units of RBCs or platelets transfused.

With a limited number of variables, the logistic regression model could effectively predict proportions of patients that received a blood transfusion. Our predictive model consisted of 19 variables that were easily collected from healthcare data in Japan. In the database of approximately 587 000 cases used for the study, the amount of blood products used for patients with any of these risk factors represented 80–90% of the total amount of blood products used. The multiple linear regression model used distribution of diagnostic groups to predict total unit use of RBCs and platelets transfused at each hospital. Although both models showed good prediction abilities, the logistic regression model better predicted RBC use than platelet use. Also, the multiple linear regression model better predicted RBC use than platelet use (Fig. 1).

The following issues should be noted when evaluating blood product use with O/E ratios. First, the mean value of the group is used as the reference value (expected value) when performing evaluations with O/E ratios, thus the evaluation is relative. For instance, if blood product use in the entire group is excessive, there is a possibility that the O/E ratio will be <1 in hospitals with high blood product use. Second, when O/E ratios are low, it is difficult to distinguish whether this is a result of appropriate blood product use, or under use. However, with blood transfusions, over use and misuse of blood product have been more of a problem than under use. Thus, when use is higher than average, there is a high likelihood that there has been inappropriate blood product use. On the contrary, when use is lower than average, there is a possibility that the healthcare provider is transfusing the bare minimum required.

Actually, O/E ratios calculated by use of the two models were very relevant to proportions of appropriate blood use (Table 5). Larger O/E ratios were associated with a smaller proportion of appropriate transfusions as judged by medical chart reviews. No particular difference was seen between the O/E ratio for proportion of patients receiving transfusions and that for the total amount (number of units) of RBC use. Both methods were considered to have successfully evaluated appropriate blood use.

Based on these findings, we conclude that the assessment of blood product use employing O/E ratios can be used, not only as an index for valid and appropriate transfusions but also as an index for blood product use that takes patient risk into consideration. Additionally, our research strongly indicates that valid comparisons may be made across hospitals in Japan.

Evaluation of blood product use at the hospital level is important in several ways. Wide variation in blood product use exists among hospitals. By comparing blood product use in different hospitals, risk-adjusted assessment of blood product use has the potential to contribute towards appropriate use of blood products [7,10,25]. Because of the labour and cost involved in gathering data on blood product use, however, almost no previous attempts had been made to collect and analyse data from many hospitals in Japan. DPC data are advantageous, as it gathers into a unified format the clinical information and data on

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treatment procedures for all hospitalized patients. Therefore, it is possible to use a shared assessment standard to compare conditions for blood product use between hospitals, and to engage in discussion about clinical standards.

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GUIDELINE

Guidelines for safety management of granulocyte transfusion in Japan

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Abstract Granulocyte transfusion (GTX) has recently been revived by the ability to stimulate granulocyte donors with granulocyte colony-stimulating factor (G-CSF), resulting in a greatly increased number of cells that can be collected. However, there is a paucity of guidelines for assessing the appropriateness and safety management of GTX. The objective of this study was to establish guidelines for the safety management of GTX appropriate for the clinical situation in Japan. The Japan Society of Transfusion Medicine and Cell Therapy, Granulocyte Transfusion Task Force issued the first version of guidelines for GTX considering the safety management of both granulocyte donors and patients who receive GTX therapy. The current guidelines cover issues concerning: (1) the appropriateness of medical institutions, (2) management of granulocyte donors, (3) quality assurance of granulocyte concentrates, (4) administration of granulocyte concentrates, (5) evaluation of the effectiveness of GTX therapy, and (6) complications of GTX therapy. The simple 'bag separation method' without apheresis may be recommended for granulocyte collection in pediatric patients. The first version of guidelines for GTX therapy has been

The Japan Society of Transfusion Medicine and Cell Therapy, Granulocyte Transfusion Task Force.

Although the recommendation and information are believed to be true and accurate at the time of preparation of the guidelines, neither the authors nor the Japan Society of Transfusion Medicine and Cell Therapy accept any legal responsibility for the content of current guidelines.

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established, which may be appropriate for the clinical situation in Japan. Care should be taken to perform the safety management of both granulocyte donors and patients who receive GTX therapy.

Keywords Granulocyte transfusion · Guidelines · Granulocyte colony-stimulating factor · Safety management

1 Introduction

Neutrophils play an essential role in the body's first line of defense against bacterial and fungal infections, and severe neutropenia, defined as an absolute neutrophil count (ANC) of less than 500/μl, is a well-recognized factor predisposing patients to these infections [1]. A direct correlation between the depth and duration of neutropenia and the risk of infection was demonstrated [2]. Because febrile neutropenia (FN), defined as a fever ≥38.3°C (101°F) with severe neutropenia, is associated with potentially life-threatening infection, patients with FN require treatment with broad-spectrum antibiotics as soon as possible without waiting for the results of blood cultures or other studies [3]. In spite of modern antimicrobials and supportive therapy, infections associated with severe neutropenia have been a major cause of morbidity and mortality in patients undergoing aggressive cancer chemotherapy and hematopoietic stem cell transplantation (HSCT) [4]. Granulocyte colony-stimulating factor (G-CSF) stimulates the proliferation of granulocytic precursors, reduces the transit time through the granulocytic compartment, and potently stimulates neutrophil release from the bone marrow [5]. G-CSF also activates neutrophils to enhance their phagocytic function, including respiratory burst activity and surface CD11b/CD18 antigen expression in vitro and in vivo [6, 7]. G-CSF is widely employed in the clinical setting to treat or prevent neutropenia attributable to hematological disorders, myelosuppressive chemotherapy, or HSCT. In addition, the use of G-CSF for the mobilization of peripheral blood progenitor cells (PBSC) has been adopted as an international standard of care [8].

When infections occur in severe neutropenic patients who do not respond to G-CSF therapy, providing the patient with normally functioning neutrophils seems to be logical. Traditional granulocyte transfusion (GTX) therapy showed marginal efficacy, mainly attributable to the inadequacy of the cell dose ordinarily provided [9]. In the G-CSF era, G-CSF stimulation with or without corticosteroids of healthy individuals is well tolerated and allows the collection of large numbers of neutrophils [10, 11]. Although the evidence for the clinical efficacy of GTX therapy is less clear, many single case reports and small cohort studies have been published. The objective of this study was to establish guidelines for GTX therapy considering the safety management of both granulocyte donors and patients, being appropriate for the clinical situation in Japan.

2 Text of the guidelines

2.1 Purpose of the guidelines

This document sets out guidelines specifically addressing the issues regarding GTX therapy, especially the safety management of both granulocyte donors and patients who receive GTX therapy. These guidelines include: (1) the appropriateness of medical institutions, (2) management of granulocyte donors, (3) quality assurance of granulocyte concentrates, (4) administration of granulocyte concentrates, (5) evaluation of the effectiveness of GTX therapy, and (6) complications of GTX therapy.

2.2 Indications for GTX therapy

A good indication for GTX therapy is prolonged 'reversible' neutropenia with an ANC of less than 500/μl, which is refractory to G-CSF therapy and is associated with severe uncontrolled infection (e.g., sepsis including suspicious cases, abscess in the liver or spleen, cellulites, and marrow myelitis). The cause of neutropenia is typically HSCT or aggressive cancer chemotherapy-induced bone marrow failure that is expected to recover. Because the underlying disease process is the main determinant of the outcome in neutropenic patients, the indication for GTX therapy in hematologic disorders may be limited to patients who have received HSCT or aggressive cancer chemotherapy. Patients with congenital neutrophil dysfunction, such as chronic granulomatous disease and leukocyte adhesion deficiency, may also be indicated for GTX therapy when severe uncontrolled infection is accompanied.

2.3 Appropriateness of medical institutions

2.3.1 Transfusion service

Although blood components are administered to patients in most large-scale community and university hospitals in Japan, some hospitals neither have transfusion services nor employ laboratory technologists licensed by the Japan Society of Transfusion Medicine and Cell Therapy. Because granulocyte concentrates are not supplied from branches of the Japanese Red Cross Blood Center (JRCBC), unlike other allogeneic blood components, they need to be collected from granulocyte donors in hospitals. Thus, the hospital where GTX therapy is performed should have a transfusion service or appropriate system, approved

by the hospital transfusion committee, as described below. In particular, the hospital should appoint a professional medical doctor(s) responsible for managing the overall safety of GTX therapy. In the case of granulocyte collection by employing the apheresis method, the hospital is encouraged to employ a professional medical technologist(s) practicing apheresis therapy.

2.3.2 Role of the hospital transfusion committee

Every hospital where GTX therapy is performed should have a multidisciplinary hospital transfusion committee to oversee the provision of safe and appropriate transfusion support. The hospital transfusion committee may comprise doctors and nurses from clinical departments where blood administrations are frequently required, pharmacists, laboratory technologists, as well as representatives of the hospital. The practice of GTX therapy should be approved by the committee.

2.3.3 Area for collection of granulocyte concentrates

Blood collection from granulocyte donors should be carried out in a well-cleaned room, and it is recommended to use a reclining phlebotomy seat. In addition, there should be emergency kits including oxygen inhalation for resuscitation if the conditions of donors deteriorate. As described below, granulocyte concentrates should be irradiated before administration to the patient to prevent transfusion-associated graft-versus-host disease (TA-GVHD). Thus, the hospital should have an exclusive irradiation apparatus or an alternative way to irradiate blood components.

2.4 Management of granulocyte donors

2.4.1 Selection of granulocyte donors

A phase I/II trial of GTX therapy employing donors selected from pools of community apheresis donors has been reported [12]. Because the JRCBC does not participate in the collection of granulocyte concentrates for GTX therapy, granulocyte donors may be selected from family members or friends of the patient undergoing GTX therapy. The current guidelines do not positively recommend non-family members for granulocyte donors at present, unlike allogeneic HSCT.

2.4.2 Age of granulocyte donors

The criteria for granulocyte donor selection should be broadly inline with those used for other blood donations. The age of granulocyte donors should be from 19 to 54 years old, in accordance with the standard for platelet apheresis donors of the JRCBC.

2.4.3 Blood group of granulocyte donors

Granulocyte donors should be ABO- and Rh(D)-compatible with the patient, because a relatively large number of red blood cells (RBCs) are contained in a typical granulocyte concentrate. If the hospital has a transfusion service, where the plasma fraction can be removed from granulocyte concentrates in the case of 'the bag separation method' as described below, granulocyte donors with minor incompatibility may also be selected.

2.4.4 Collection from the same donor

Granulocyte concentrates may be collected from the same donor in the case of a limited number of available granulocyte donors. Granulocyte collection from the same donor should be conducted on two consecutive days in the case of apheresis donation, but repeated collections from the same donor are not prohibited in the presence of an intermission.

2.4.5 Cytomegalovirus (CMV) serology

If the patient is CMV-seronegative, granulocyte donors should also be CMV-seronegative except for life-threatening situations, because most patients who receive GTX therapy are in a patient population that requires CMV-safe components.

2.4.6 Alloimmunization

In the case of alloimmunized patients, granulocyte concentrates may be collected from either HLA-matched donors or donors who are selected by leukoagglutination crossmatching, although the best method to accurately assess donor and leukocyte compatibility has yet to be determined [13]. Considering life-threatening situations, granulocyte concentrates may also be collected from an HLA-mismatched donor for GTX therapy for the patient with anti-HLA antibody.

2.4.7 Medical examinations and laboratory testing

A doctor responsible for GTX therapy should fully interview granulocyte donors regarding episodes of suspected infectious disease transmission and conduct physical examinations before granulocyte collection. The timing of medical examinations may be optimal at the time of G-CSF administration 12–18 h before granulocyte collection. Laboratory tests for granulocyte donors should be as consistent as possible with those for any allogeneic blood

components supplied from branches of the JRCBC, including blood group ABO and Rh(D); serum antibody screening; infectious disease screening of hepatitis B virus (HBs-Ag and Hbc-Ab), hepatitis C virus (HCV-Ab), human immunodeficiency virus (HIV-1/-2-Ab), human T cell lymphotropic virus type I (HTLV-I-Ab), and syphilis (TPHA); complete blood count; and biochemical analysis (e.g., alanine aminotransferase). In the case of infectious disease screening, the current guidelines recommend performing the tests as many as possible in the hospital, although the results of tests will not immediately be obtained.

2.4.8 Informed consent

Informed consent should always be obtained from the granulocyte donor for: (a) granulocyte collection, (b) collection procedures, (c) the administration of G-CSF with or without corticosteroids, (d) use of RBC-sedimenting agents (when employed), and (e) any possible short- and long-term consequences of granulocyte collection. There should always be an opportunity for the donor to reconsider granulocyte donation in the light of a response or lack or response.

2.4.9 Post-donation care

Considering the administration of G-CSF to healthy individuals and its potential long-term adverse effects, as described below, a record of granulocyte donors regarding any post-donation complications should be made. Care of granulocyte donors should include observations in the immediate post-apheresis period to minimize the occurrence of delayed complications (e.g., thrombocytopenia). The current guidelines recommend the establishment of a donor registry to collect the necessary data on short- and long-term side effects of G-CSF administration to normal donors [14, 15]. Comprehensive, prospectively obtained registration data are needed to fully evaluate long-term safety concerns among healthy individuals who receive G-CSF.

2.5 Quality assurance of granulocyte concentrates

2.5.1 Collection of granulocyte concentrates

2.5.1.1 G-CSF For granulocyte mobilization, donors may receive recombinant human G-CSF (non-glycosylated G-CSF [Filgrastim] or glycosylated G-CSF [Lenograstim]) with or without corticosteroid administration. It has been reported that optimal granulocyte mobilization can be achieved in normal donors with a combined regimen of subcutaneous G-CSF at 450 μg and oral dexamethasone (DEX) at 8 mg in a single-dose format designed for clinical GTX therapy [10]. Although the daily administration of G-CSF (e.g., 5 consecutive days) results in higher yields of granulocytes, the current guidelines recommend a single subcutaneous dose of G-CSF (5–10 μg/kg) 12–18 h before each granulocyte collection. As described above, granulocyte collection from the same donor on consecutive days is recommended over 2 days, but repeated collections from the same donor are not prohibited in the presence of an intermission.

2.5.1.2 Corticosteroids To maximize the number of granulocytes obtained, corticosteroids have been administered to mobilize granulocytes from the marrow storage pool and to increase circulating granulocyte counts [10, 11]. Usually, DEX at 8 mg is orally administered once 12 h before granulocyte collection. On frequent collection from the same donor, the medical doctor in charge should monitor the donor regarding corticosteroid-induced adverse events, as discussed below.

2.5.1.3 RBC-sedimenting agent The RBC-sedimenting agent, traditionally hydroxyethyl starch (HES), may be continuously added to the donor's blood during an apheresis procedure to achieve an adequate separation of granulocytes from RBCs. It has been shown that high-molecular weight (MW) HES resulted in a significantly higher yield compared with low-MW HES [16]. However, high-MW HES products have, at present, not been approved in Japan. In the case of using a high-MW HES for granulocyte collection, it should be approved by the Ethics Committee of the hospital.

2.5.2 Methods of granulocyte collection

2.5.2.1 Bag separation method The simple 'bag separation method' without apheresis may be recommended for granulocyte collection in pediatric patients [17]. In brief, whole blood (200 or 400 ml) is drawn into the main bag of a triple-collection bag [200- or 400-ml capacity containing 34 or 68 ml, respectively, of citrate–phosphate–dextrose (CPD) solution] employing the gravity-flow principle. After centrifugation at 640*g* for 15 min at 20°C, the plasma layer is separated into the first sub-bag. The buffy-coat layer and the upper one-third of the RBC layer, both of which are rich in granulocytes, are collected into the second sub-bag by applying pressure on the main bag. The remaining RBC and plasma components are returned to the donor using a sterile-connecting device. This process is repeated two or three times, when necessary. It is noteworthy that the bag separation method does not require the use of an RBC-sedimenting agent, such as high-MW HES, which reduces the burden on the donor [17, 18].

2.5.2.2 Apheresis method Granulocyte collection may usually be performed on various blood cell separators using the white blood cell cytapheresis set and an exclusive program of the separator's software. It was reported that the use of higher interface offset settings (35 vs. 15) resulted in a significant increase in the granulocyte collection efficiency [19]. Because interface offset settings are dependent on the apheresis systems used, the relevant setting should be evaluated and used for achieving a maximal granulocyte yield in the hospital. The required apheresis procedure for granulocyte collection would present a potential clinical risk for cardiac or cerebrovascular events in donors with preexisting inflammatory or vascular disease, and, as such, should be avoided in these subjects.

2.5.3 Preparation of granulocyte concentrates

2.5.3.1 Gamma irradiation Granulocyte concentrates contain significant amount of donor lymphocytes and are frequently transfused to immunocompromised patients with neutropenia [20]. Currently, the gamma irradiation of blood components is the only proven effective method for TA-GVHD prevention [20]. The AABB Standards recommend a minimum 25 Gy dose of gamma irradiation to the central portion of the container, with no less than 15 Gy delivered to any part of the bag [21]. 'HLA one-way match' results in the inability to reject donor lymphocytes even if the recipient is immunocompetent, and it occurs at a rather high frequency, one in several hundred blood transfusions from unrelated donors in Japan [22]. The JRCBC disseminated transfusion information regarding TA-GVHD to most Japanese hospitals in December 1999, in which the administration of irradiated blood components except for fresh-frozen plasma is recommended for preventing TA-GVHD. Most Japanese hospitals are generally supplied with 15-Gy (or more)-irradiated blood components from branches of the JRCBC. If hospitals have an exclusive gamma-irradiation apparatus for blood, non-irradiated components are supplied and irradiated at a dose between 15 and 50 Gy in transfusion services [23]. Thus, granulocyte concentrates should be irradiated before administration to the patient at a dose between 15 and 50 Gy. Recent studies have demonstrated that the irradiation of neutrophils did not affect their in vitro functions, including respiratory burst activity and phagocytosis [24].

2.5.3.2 Storage There is general agreement that granulocyte concentrates should be administered as soon as possible after collection [21]. The British Committee for Standards in Haematology (BCSH) recommended that granulocytes should be stored in the same donor's citrate-anti-coagulated plasma at room temperature, kept unagitated, and administered within 12 h of preparation [25]. In the case of a limited number of available granulocyte donors, there may be a need for storage of an aliquot of granulocyte concentrates. G-CSF has been shown to inhibit granulocyte apoptosis [26], and may be useful in lengthening the acceptable storage time for granulocyte concentrates and, thereby, improving the logistics of GTX programs [9]. Drewniak and colleagues [27] investigated granulocytes from leukapheresis products mobilized by G-CSF with DEX, where in vitro granulocyte functions were intact at least 24 h. Mochizuki and colleagues [18] also reported the extended storage of granulocyte concentrates mobilized by G-CSF with or without DEX, where in vitro granulocyte functions were maintained for as long as 72 h after collection by the 'bag separation method'. The current guidelines recommend that granulocyte concentrates should be transfused within 48 h after collection.

2.6 Administration of granulocyte concentrates

2.6.1 Infusion of granulocyte concentrates

Granulocyte concentrates should be slowly administered over 1–4 h through a standard transfusion set with a screen filter (170–200 μm) within 6 h after collection. In the case of 200 ml of granulocyte concentrates, it should be administered over 1– 2 h in adults and 2–4 h in pediatric patients. In general, granulocyte concentrates are administered every other day until complete recovery from infection is documented. The BCSH guidelines recommend that all granulocytes should be irradiated for patients of any age and transfused as soon as possible after irradiation [25]. Leukocyte reduction filters must not be used, because it makes no sense to use these filters in GTX. Patients should be monitored by pulse oximetry. The blood pressure should also be measured every 15 min during the infusion of granulocyte concentrates.

2.6.2 Premedication

The administration of antipyretics or corticosteroids (e.g., 100 mg of hydrocortisone) is appropriate for patients who experience symptoms such as chills and fever. Routine prophylaxis with these agents is not necessary [9].

2.7 Evaluation of effectiveness of GTX therapy

2.7.1 Success of GTX therapy

The success of GTX therapy is defined as complete recovery from infection, being documented by: (a) disappearance of clinical symptoms (e.g., fever), (b) negativity of laboratory findings (e.g., C-reactive protein), (c) disappearance or

marked reduction of radiological findings, or (d) negativity of microbiological cultures.

2.7.2 Discontinuation of GTX therapy

In general, GTX therapy is continued daily to maintain an ANC of more than 500/μl until neutrophil recovery, clinical improvement, or stability. However, prolonged GTX therapy may be difficult in cases of a limited number of available granulocyte donors. The current guidelines recommend criteria for the discontinuation of GTX therapy as follows: (a) neutrophil recovery or bone marrow engraftment with an ANC of more than 500/μl in patients who received HSCT, (b) recovery from infection without the need of GTX support, (c) refractoriness to GTX therapy even if continued for 7 consecutive days, or (d) occurrence of an adverse event due to GTX therapy.

2.8 Complications of GTX therapy

2.8.1 Donor-associated side effects

2.8.1.1 G-CSF Short-term side effects: The most commonly reported side effects of G-CSF administration include bone pain, headache, fatigue, nausea, fever (with or without chills and sweats), insomnia, anorexia, and myalgias [28]. All side effects appear to be generally mild and usually resolve after the discontinuation of G-CSF. However, analgesics may be needed for bone pain, which was the most frequent symptom [29, 30]. Suggested contraindications to G-CSF administration in donors include the presence of active inflammatory conditions and hypercoagulable states, with or without previous venous thrombosis and known or suspected atherosclerotic vascular disease [28].

Long-term side effects: The question regarding the long-term safety of G-CSF administration to normal donors, particularly in terms of the leukemogenic potential, has been raised. Theoretically, a prior history of malignancy or a strong family predisposition to acute myeloid leukemia (AML) or myelodysplasia may place individuals at a higher risk of developing hematologic malignancies [14]. There are limited data generated by long-term follow-up studies on normal donors who received G-CSF administration for granulocyte collection. Quillen and colleagues [31] recently reported 2 cases of lymphoid malignancy (one case each of non-Hodgkin's lymphoma and chronic lymphocytic leukemia) in 83 unrelated granulocyte donors who received repeated administrations of both G-CSF and DEX and were followed for a median of 10 years. Although it has been shown that pharmacologic doses of G-CSF affect cytokine production by lymphocytes in vitro and in vivo [32], there is no evidence to date supporting an association between G-CSF and lymphoid malignancy [31]. Bux and colleagues [16] reported on a 2-year follow-up of 183 granulocyte donors, where no severe G-CSF-related adverse events were noted. The Research on Adverse Drug Events and Reports (RADAR) project reviewed clinical literature on adverse events that occur when G-CSF is administered to healthy individuals for PBSC collection [29]. Three PBSC donors were described who developed AML following stem cell mobilization, but the evidence supporting causality is unclear.

2.8.1.2 Corticosteroids It remains controversial whether the administration of corticosteroids along with G-CSF stimulation to granulocyte donors increases the risk of posterior subcapsular cataract [33, 34]. However, the administration of corticosteroids to granulocyte donors, especially in frequent donations, should be used with caution.

2.8.1.3 RBC-sedimenting agent RBC-sedimenting agents, such as high-MW HES, act as a plasma expander and can cause transient hypertension with flushing and headache. Severe itching following the infusion of HES may be observed in a small number of granulocyte donors [16].

2.8.1.4 Apheresis donation During apheresis, anticoagulation is necessary to prevent coagulation and the clumping of collected components. CPD is returned to the donor, and its toxicity occasionally causes symptoms associated with decreased ionized calcium levels (e.g., peri-oral paresthesia). As an antidote to citrate toxicity, calcium prophylaxis may be required during large-volume leukapheresis.

2.8.2 Recipient-associated side effects

2.8.2.1 Transfusion reactions Mild to moderate fever and chills are relatively common, whereby the slowing of administration may be required. These reactions are preventable on subsequent transfusions by treatment with antipyretics or corticosteroids [12]. However, routine prophylaxis with these agents is controversial. More severe reactions may occur in approximately 1–5% of cases of GTX therapy, including hypotension, pulmonary infiltrates, and respiratory distress [35]. In patients who receive repeated GTX therapy, alloimmunization and platelet refractoriness may develop [36]. The rate of leukocyte alloimmunization has been reported to be 24% [16].

2.8.2.2 Concurrent use of Amphotericin B Although an association between pulmonary infiltration and Amphotericin B administration has not been confirmed [37], it is still common practice to separate the administration times if Amphotericin B and granulocyte concentrates are being given concurrently [35]. The current guidelines recommend that granulocyte concentrates should be administered

at least 4 h after stopping Amphotericin B administration in patients who receive both Amphotericin B and granulocyte concentrates.

3 Conclusion

The current guidelines may be appropriate for the clinical situation in Japan, in which granulocyte donors cannot be selected from the community pool of apheresis donors of the JRCBC, and high-MW HES products are not approved. Care should be taken to perform GTX therapy considering the safety management of both granulocyte donors and patients. Future randomized controlled trials are needed to clarify the efficacy of GTX therapy and identify which subgroup of patients benefits the most.

Conflict of interest statement The authors declare no conflicts of interest.

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Activity of the multitargeted kinase inhibitor, AT9283, in imatinib-resistant BCR-ABL–positive leukemic cells

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Despite promising clinical results from imatinib mesylate and second-generation ABL tyrosine kinase inhibitors (TKIs) for most BCR-ABL$^+$ leukemia, BCR-ABL harboring the mutation of threonine 315 to isoleucine (BCR-ABL/T315I) is not targeted by any of these agents. We describe the in vitro and in vivo effects of AT9283 (1-cyclopropyl-3[5-morpholin-4yl methyl-1H-benzomidazol-2-yl]-urea), a potent inhibitor of several protein kinases, including Aurora A, Aurora B, Janus kinase 2 (JAK2), JAK3, and ABL on diverse imatinib-resistant BCR-ABL$^+$ cells. AT9283 showed potent antiproliferative activity on cells transformed by wild-type BCR-ABL and BCR-ABL/T315I. AT9283 inhibited proliferation in a panel of BaF3 and human BCR-ABL$^+$ cell lines both sensitive and resistant to imatinib because of a variety of mechanisms. In BCR-ABL$^+$ cells, we confirmed inhibition of substrates of both BCR-ABL (signal transducer and activator of transcription-5) and Aurora B (histone H3) at physiologically achievable concentrations. The in vivo effects of AT9283 were examined in several mouse models engrafted either subcutaneously or intravenously with BaF3/BCR-ABL, human BCR-ABL$^+$ cell lines, or primary patient samples expressing BCR-ABL/T315I or glutamic acid 255 to lysine, another imatinib-resistant mutation. These data together support further clinical investigation of AT9283 in patients with imatinib- and second-generation ABL TKI-resistant BCR-ABL$^+$ cells, including T315I. (*Blood.* 2010;116(12):2089-2095)

Introduction

Philadelphia (Ph) chromosome results from a reciprocal translocation between chromosomes 9 and 22 and generates the BCR-ABL chimera protein, the cause of chronic myeloid leukemia (CML) and Ph$^+$ acute lymphoid leukemia (ALL). The ABL tyrosine kinase inhibitor (TKI), imatinib mesylate, has dramatically changed the first-line therapy of CML.[1] Most patients with newly diagnosed CML with chronic phase, when treated with imatinib, achieve durable responses. However, emergence of refractory disease and relapse have frequently been reported, particularly in patients with CML with advanced-stage disease and patients with Ph$^+$ ALL.[2,3] Among several mechanisms of resistance, point mutations within the ABL kinase domain that interfere with imatinib binding are the most critical cause of imatinib resistance.[4,5]

To overcome these imatinib resistance mechanisms, 4 second-generation ABL TKIs have been developed: dasatinib,[6] nilotinib,[7] bosutinib[8] and bafetinib (formerly INNO-406).[9,10] Despite promising clinical results from these second-generation ABL TKIs for most patients with imatinib-resistant BCR-ABL$^+$ leukemia, the mutation of threonine 315 to isoleucine (T315I) confers resistance to all these TKIs.[11,12] Thus, identification of novel agents for the effective treatment of patients with CML with T315I is an important and challenging task.[13] Aurora kinases A and B are a family of serine/threonine kinases involved in many cellular functions.[14-16] Inappropriate expression of these enzymes in certain cancers may result in aneuploidy and carcinogenesis.[17] Consequently, the potential therapeutic value of targeting Aurora kinases has become a focus of anticancer therapy.[14]

Recently, we identified an Aurora kinase inhibitor, AT9283 (1-cyclopropyl-3[5-morpholin-4yl methyl-1H-benzomidazol-2-yl]-urea) by way of structure-based optimization of a ligand-efficient pyrazole-benzimidazole fragment. X-ray crystallographic structures were generated with the use of a novel soakable form of Aurora A and were used to drive the optimization toward potent (half-maximal inhibition constant [IC_{50}] < 3nM) dual Aurora A/B inhibitor. AT9283 that also potently inhibits several kinases, including Janus kinase-2 (JAK2) and JAK3 (1.2 and 1.1nM, respectively), c-ABL (110nM), and ABL/T315I (4nM), is currently under evaluation in phase 1 clinical trials for metastatic solid tumors and hematologic malignancies.[18,19]

Here, we report the putative mechanism by which AT9283 binds to BCR-ABL/T315I and its activity against imatinib-resistant BCR-ABL$^+$ leukemic cells, including those with the T315I mutation.

Methods

Reagent and cell lines

AT9283 was synthesized by Astex Therapeutics Ltd (Figure 1A).[18] Human CML cell lines (K562, MEG-01, BV173, KU812, MYL, KT-1, and

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KBM-5), human acute myeloid leukemia cell lines (HL60, KG1a), human ALL (Jurkat, Nalm6), and mouse pro-B cell line (BaF3) were used. KBM-5/STIR, which was the subclone of KBM-5 with T315I, and BaF3/wild type (wt) BCR-ABL^{p190} were also used. Source of all cell lines used are outlined in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). BaF3/wt-BCR-ABL^{p210} and BaF3 cells harboring imatinib-resistant mutations (glycine 250 to glutamic acid, glutamine 252 to histidine, tyrosine 253 to phenylalanine, glutamic acid 255 to lysine [E255K]), methionine 294 to valine, T315I, threonine 315 to alanine, phenylalanine 317 to leucine, phenylalanine 317 to valine, methionine 351 to threonine, and histidine 396 to proline) in the BCR-ABL^{p210} kinase domain were established as previously described.[9] All cell lines were cultured in RPMI 1640 (Nissui) with 2mM L-glutamine (Nacalai Tesque) and 10% fetal bovine serum (Vitromex) and were maintained at 37°C in a fully humidified atmosphere of 5% CO_2. For the culture of parental BaF3 cells, 1 ng/mL murine interleukin-3 (Sigma-Aldrich) was added to the medium. Cells undergoing exponential growth were used in the experiments.

Bone marrow cells from healthy donors and patients with CML in chronic phase were collected with written informed consents according to the Declaration of Helsinki. Primary leukemic cells were obtained from 2 patients with Ph$^+$ ALL who relapsed after imatinib mesylate treatment because of E255K or T315I.[20] These patients' peripheral blood contained more than 90% leukemic cells. Approval for primary leukemic cells was obtained from the institutional review board at Frankfurt University Hospital.

Docking model of AT9283 bound within the active site of c-ABL/T315I

A model of c-ABL/T315I was prepared in QUANTA (Accelrys Inc) by simple Thr>Ile mutation of residue 315 in an inhouse DFG-in, wt c-ABL structure. Docking of AT9283 into the active site of the c-ABL/T315I model was carried out with the use of a proprietary version of GOLD,[21] using the Chemscore scoring function.[22]

Proliferation assays

Proliferation of the cell lines was determined with a modified 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl-2H tetrazolium bromide assay with the SF reagent (Nacalai Tesque) as previously described.[23] Leukemic cell lines were cultivated in a flat-bottomed 96-well plate (Greiner Labortechnik) at 1×10^4 (BaF3 series), 5×10^4 (BV173, KBM-5, KBM-5/STIR), or 1×10^5 (MEG01, KU812, HL60) cells per well in 100 μL of medium and incubated with various concentrations of AT9283 for 72 hours. The means of 5 data values for each treatment were calculated. IC_{50}s were determined with the use of the nonlinear regression program CalcuSyn (Biosoft).[24]

Western blotting

BCR-ABL$^+$ or BCR-ABL$^-$ cell lines were seeded at a concentration of 1×10^6/mL media onto 6-well tissue culture plates and allowed to recover for 16 hours. AT9283, at the indicated concentration, or vehicle control (0.1% dimethyl sulfoxide) were added for 24 hours. Cells were harvested and lysed in 100 μL of ice-cold Triton lysis buffer (0.1% vol/vol Tx-100). Lysates were cleared by centrifugation, and a sample of the supernatant was removed for protein determination. Equivalent amounts of protein lysate had sodium dodecylsulfate sample buffer added and were boiled for 5 minutes. Samples were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (NuPage system; Invitrogen) and blotted onto polyvinylidene difluoride filters. Immunoblotting was performed with specific antibodies for phospho (p) histone H3 (HH3)$^{(Ser10)}$ and total (t)HH3, p signal transducer and activator of transcription-5 (STAT5)$^{(Tyr694)}$, tSTAT5, pCrkL$^{(Tyr207)}$, tCrkL, p extracellular signal-regulated kinase (ERK)$^{(Thr202/Tyr204)}$, tERK, pAKT$^{(Thr308)}$, tAKT, pBCR-ABL$^{(Tyr177)}$, tBCR-ABL, pAurora A$^{(Thr288)}$, tAurora A, pAuroa B$^{(Thr232)}$, and tAurora B. All antibodies were obtained from Cell Signaling Technology. Detection was achieved with the use of Odyssey Infra Red Imaging, IR Dye secondary antibodies, and a Licor Odyssey Imager (Li-Cor Bioscience Ltd).

In vivo activity of AT9283 in mice bearing BCR-ABL$^+$ leukemic cells

BaF3/wt-BCR-ABL^{p210}, BaF3/T315I, or human CML K562 xenografts used male BALB/c nu/nu mice and were performed according to the United Kingdom Animals (Scientific Procedures) Act 1986. Animals were purchased from Harlan UK Ltd and housed in pathogen-free conditions. Six- to 8-week-old male BALB/c Hsd:athymic nude-*Foxn1*^{nu} mice were implanted subcutaneously with 1×10^7 BaF3/wt-BCR-ABL, 1×10^7 BaF3/T315I, or 1×10^7 K562 cells per mouse into the right flank. Five days after implantation mice were arranged into groups of 8 according to tumor volume with a mean volume of 100 mm^3. AT9283 was prepared in a vehicle of 10% dimethyl sulfoxide, 20% water, and 70% 2-(hydroxypropyl)-beta-cyclodextrin (25% wt/vol). Mice were then dosed according to schedule. Tumor volume was measured every 2 to 3 days. In each case a statistically significant slowing of increase in xenograft volume or regression of tumor volume over time compared with a matched control group was used to characterize efficacy. A complete regression was defined as a decrease in tumor volume to an undetectable size, taken as measurements of less than 3 mm in any dimension. Tolerability was estimated by standard criteria of behavioral observations, body weight loss less than 5%, and survival over the course of the study.

For the experiments to investigate the effects of AT9283 on the primary patient samples with BCR-ABL harboring E255K or T315I, nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were used with the approval from the institutional review board at Kyoto University Hospital. Male NOD/SCID mice 4 to 6 weeks of age (Japan Clea) were individually sublethally irradiated (2 Gy) and inoculated intravenously with 5.0×10^5 primary leukemic cells harboring either BCR-ABL/E255K or T315I as previously described.[20] Briefly, engraftments of inoculated primary human BCR-ABL$^+$ cells at day 7 after transplantation were confirmed by polymerase chain reaction analysis, and treatments with AT9283 were initiated on day 7. Furthermore, the numbers of engrafted BCR-ABL$^+$ cells were monitored by analysis of the percentage of human leukemic cells in mouse peripheral blood by flow cytometry as previously described.[20,23] The mice were randomized into the following groups: E255K [each group, $n = 7$, as follows: (1) untreated mice, (2) mice treated with 6.25 mg/kg AT9283 twice daily] and T315I [each group, $n = 5$, as follows: (1) untreated mice, (2) mice treated with 10 mg/kg AT9283 daily, and (3) mice treated with 15 mg/kg AT9283 daily]. At day 7, treatment was administered intraperitoneally either twice daily 5 days on and 2 days off for 4 weeks at the doses of 6.25 mg/kg (E255K) or daily 4 days on and 3 days off continuously at the doses of 10 mg/kg and 15 mg/kg (T315I). For survival analysis, death was determined either by spontaneous death or elective killing because of pain or suffering according to established criteria.

Results

Identification and in vitro activity of AT9283

By analogy with the binding mode of AT9283 with Aurora A,[18] it is predicted that AT9283 does not form a hydrogen bond with T315 in BCR-ABL in the way that inhibitors, including imatinib, do. Binding of AT9283 is therefore less likely to be affected by the T315I mutation (supplemental Figure 1A). Furthermore, AT9283 does not bind within the selectivity pocket behind the gatekeeper residue, meaning that its potency for ABL kinase is not abrogated by mutation of this residue to the bulkier isoleucine. Imatinib binding is sterically hindered by the presence of isoleucine at amino acid 315, whereas AT9283 is not (Figure 1B).

Cell-based activity of AT9283 in BCR-ABL–dependent cell lines

AT9283 inhibited proliferation of a panel of BaF3 cell lines transformed with either wt-BCR-ABL fusions or a variety of mutant forms, including T315I (Table 1). AT9283 inhibited all lines

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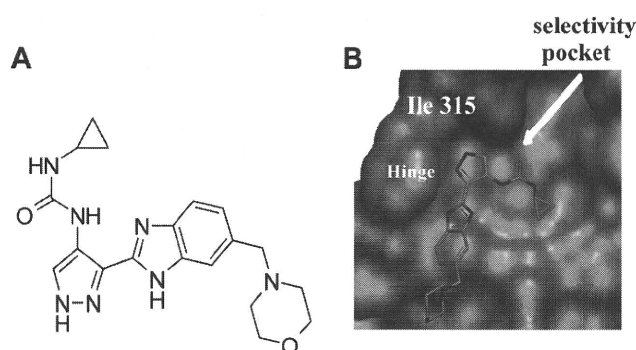

Figure 1. Chemical structure of AT9283 and model of AT9283 bound with ABL. (A) Chemical structure of AT9283. (B) AT9283 does not make a hydrogen bond interaction with T315 in the same way other kinase inhibitors in the class do. The pocket behind the T315 gatekeeper is not occupied by AT9283. Its potency for ABL kinase is not abrogated by mutation of this residue to the bulkier isoleucine (T315I).

with similar potency in the range of 10 to 21nM. The IC_{50} of AT9283 for the BCR-ABL$^-$ parental BaF3 was 103nM, around 5 times higher than that for BaF3 cells harboring wt or mutated BCR-ABL. In addition, we observed that, although multinucleated cells, the hallmark of Aurora B inhibition, were visible microscopically (data not shown), this phenotype did not dominate, and we were able to generate cytotoxic IC_{50} values in the absence of intervention of the polyploid phenotype that maintains cells in a viable state for a longer period of time. These data suggest that additional activities of AT9283, which most likely include BCR-ABL inhibition, are responsible for some of the activity of the compound in vitro.

Similar observations were made in human imatinib-sensitive CML cell lines. These observations are again consistent with the BCR-ABL inhibitory activity of AT9283 contributing to the activity of the compound in CML cell lines harboring the translocation. We observed the dominant Aurora B phenotype of polyploidy in the K562 CML cell line and BCR-ABL$^-$ cell lines such as HL-60, Jurkat, Nalm6, and KG1a. In these cell lines, the dominant phenotype of Aurora B inhibition (polyploidy) was observed in contrast to other CML lines tested. AT9283 was active not only in BaF3/T315I, which was artificially generated, but also in the human CML subclone, KBM-5/STIR, which harbored the T315I mutation (Table 2).

Table 1. Antiproliferative activity of AT9283 in a panel of BaF3 BCR-ABL cell lines

BaF3	AT9283 IC_{50}, nM
Parental (BCR-ABL$^-$)	103
wt p190	16
wt p210	13
Y253F	16
T315I	11
T315A	10
Q252H	21
M351T	18
M294V	18
H396P	21
G250E	12
F317V	14
F317L	15
E255K	13

Y253F indicates tyrosine 253 to phenylalanine; T315A, threonine 315 to alanine; Q252H, glutamine 252 to histidine; M351T, methionine 351 to threonine; M294V, methionine 294 to valine; H396P, histidine 396 to proline; G250E, glycine 250 to glutamic acid; F317V, phenylalanine 317 to valine; and F317L, phenylalanine 317 to leucine.

Table 2. Antiproliferative activity of AT9283 in a panel of human CML cell lines

Cell line	BCR-ABL status	Additional characteristics	IC_{50}, nM
Imatinib sensitive			
BV173	+		55
KU812	+		26
MYL	+		21
KT-1	+		81
KBM-5	+		84
MEG-01	+		31
K562	+		Polyploidy at 100
Imatinib resistant			
KBM-5/STIR	+	T315I mutation	16
BV173/shBim	+	Bim knockdown	12
HL60	−		Polyploidy at 30
Jurkat	−		Polyploidy at 500
Nalm6	−		Polyploidy at 140
KG1a	−		Polyploidy at 55

Induction of apoptosis and alteration of cell cycle by AT9283

Apoptosis was dose-dependently induced in all cell lines tested, including BaF3/wt-BCR-ABL^{p210}, BaF3/T315I, BaF3/E255K, K562, and BV173. AT9283 induced fewer apoptotic cells in K562 than the other 4 CML cell lines (supplemental Figure 2). Exposure of K562 cells to cytotoxic IC_{50} concentrations (100nM) of AT9283 resulted in the appearance of a large multinucleated cell population. Interestingly, exposure of the other 4 cell lines tested to their respective cytotoxic IC_{50} concentrations of AT9283 did not result in the appearance of polyploid cells. The dominant effect in 7 BCR-ABL$^+$ cell lines except K562 was the induction of apoptosis as indicated by a significantly larger increase in subG_1 DNA compared with 3 BCR-ABL$^-$ cell lines at 24 hours and 48 hours (supplemental Figures 3-4). If the concentration of AT9283 was increased to 3 times their respective IC_{50} concentrations, a small polyploid population could be observed in the BaF3 and BV173 cells, but the dominant phenotype remained an increase in the apoptotic population. These observations are consistent with the Aurora inhibitory activity of the compound dominating in BCR-ABL lines and K562 cells, resulting in apoptosis only after an increased exposure time and several rounds of endo-reduplication. In the BaF3/BCR-ABL and in other human CML cell lines, AT9283 results in an early apoptotic response in the absence of the classical Aurora inhibitory phenotype. This response is most likely due to the contribution of ABL inhibition in these cells (supplemental Figures 3-4).

Mechanism of action of AT9283 in BCR-ABL$^+$ cell lines

After 24 hours of incubation of BaF3/BCR-ABL wt or T315I cells with AT9283, inhibition of the signaling pathway downstream of Aurora B and BCR-ABL were examined. Phosphorylation of the Aurora B substrate, pHH3 was inhibited by AT9283 at concentrations greater than 30nM in all cell lines examined (Figure 2). Activity of the BCR-ABL fusion was determined by phosphorylation of the substrate STAT5. AT9283 inhibited phosphorylation of STAT5 at concentrations above 300nM in both BaF3/wt-BCR-ABL^{p210} and BaF3/T315I (Figure 2). Similar data were obtained in the human CML cell lines BV173 and K562 above 300nM and 1000nM, respectively. AT9283 inhibited phosphorylation of the BCR-ABL substrate CrkL only in BV173 at concentrations consistent with inhibition of pSTAT5. Incomplete inhibition of phosphorylation of AKT was observed in all cell lines with the most striking

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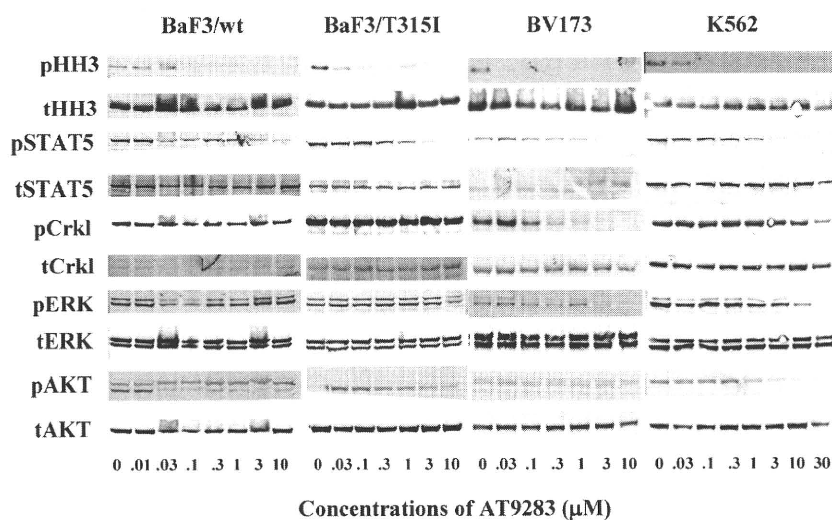

Figure 2. Mechanism of action of AT9283 in BaF3 BCR-ABL cells and human CML cell lines. BaF3/wt-BCR-ABL, BaF3/T315I, BV173, and K562 were incubated with the indicated concentration of AT9283, or vehicle control, for 24 hours before preparation for immunoblotting with the indicated antibodies. The blots shown are representative of at least 2 independent experiments in each cell line.

effects observed in BV173 cells and K562 cells above 100nM. Phospho-ERK inhibition was observed in BV173 and K562 cells but only at concentrations above 3000nM (Figure 2). In addition to the analysis of downstream signaling of AT9283 kinase targets, we also examined the effects of AT9283 on the phosphorylation status of BCR-ABL, Aurora A, and Aurora B themselves. AT9283 inhibited phosphorylation of BCR-ABL, Aurora A, and Aurora B in BV173, BCR-ABL$^+$ cells, at 1.0, 0.1, and 0.03μM, respectively (supplemental Figure 5). In Jurkat, BCR-ABL$^-$ cells, AT9283 also inhibited phosphorylation of Aurora A and Aurora B at 0.3 and 0.3μM, respectively (data not shown). AT9283 inhibited the phosphorylation of HH3 and ERK but AKT in 3 BCR-ABL$^-$ cell lines (supplemental Figure 6). These data suggest that of the signaling pathways studied AT9283 inhibits both the Aurora kinases and BCR-ABL/STAT5 signaling pathways. Inhibition of pCrkL was also observed in the human CML cell line BV173. In other cell lines tested this was less clear and probably reflects the fact that CrkL signaling is regulated by different or additional kinases in BaF3/BCR-ABL lines and in the CML line K562. The latter responds to AT9283 in a manner atypical with respect to the other human CML lines tested, perhaps indicating that it harbors additional mutations or signaling aberrations that impinge on its survival and overall response to AT9283.

Effects of AT9283 in normal hematopoietic progenitors and primary CML cells

The number of colony forming units (CFUs) observed after AT9283 treatment of cells derived from 3 healthy individual donors and 3 patients with CML were examined by colony assay at day 14 to day 16. When normal progenitors were treated with 10, 30, 50, 70, and 100nM AT9283, the CFUs were 0.98% (± 0.06%), 0.62% (± 0.01%), 0.35% (± 0.01%), 0.18% (± 0.01%), 0.11% (± 0.1%), and 0% (± 0%) of the control, respectively. Although when primary CML cells were treated with 10, 30, 50, 70, and 100nM AT9283, the CFUs were 0.45% (± 0.1%), 0.23% (± 0.02%), 0.06% (± 0.02%), 0% (± 0%), 0% (± 0%), and 0% (± 0%) of the control, respectively (supplemental Figure 7). These percentages are the mean plus or minus SE between the 3 persons. These findings indicate that AT9283 was approximately 5 times more effective at inhibiting colony formation with cells derived from patients with CML than from healthy volunteers.

In vivo efficacy of AT9283 in subcutaneous BaF3 BCR-ABL xenograft models

Two cycles of 12.5 mg/kg AT9283 daily for 5 days followed by a 2-day break inhibited tumor growth in subcutaneous xenograft models with BaF3 cells transfected with either BCR-ABL wt (Figure 3A) or T315I (Figure 3B) without obvious adverse effects. Moreover, AT9283 inhibited the growth of human CML cell line K562 xenografts in a dose-dependent fashion after twice daily dosing for 5 days in a 7-day period. Tumor regressions were observed at 12.5 mg/kg, the highest dose, with 4 of 8 mice remaining tumor free at 90 days after initiation of treatment (Figure 3C). This dose schedule was optimized in solid tumor xenograft models.[19]

The activity of AT9283 was investigated in intravenously transplanted models with the use of primary BCR-ABL$^+$ blasts isolated from patients with Ph$^+$ ALL who showed imatinib acquired resistance by harboring 2 of the mutations most resistant to common kinase inhibitor therapies, E255K and T315I. Blasts isolated from patients were injected into the tail vein of NOD/SCID mice and allowed to engraft for a period of 7 days. In the case of the BCR-ABL/E255K model animals were dosed with either vehicle or 6.25 mg/kg AT9283 twice daily for 5 days followed by a 2-day break. This schedule was repeated for 4 cycles and resulted in a significant survival advantage of 17 days over control (Figure 4A). Initially a twice daily schedule was used on the basis of past experience with AT9283 and the optimum schedule in nude mice bearing human tumor xenografts derived from solid tumors. In the course of the studies, we determined that a once daily schedule at the indicated doses allowed indefinite continuous dosing with an improved tolerability profile in NOD/SCID mice and at least as good if not better efficacy in the models described. Thus, for the T315I model, animals were dosed once per day at either 10 or 15 mg/kg. In this case animals continued dosing through to the end of the experiment. In this model a significant survival advantage ($P < .01$) of 17 and 23 days for the 10- and 15-mg/kg groups, respectively, was obtained (Figure 4B). Fluorescence-activated cell sorting analysis, performed on the peripheral blood of mice inoculated with leukemic cells, showed that a number of these cells remained even after dosing with AT9283 (data not shown). The numbers of leukemic cells observed was fewer than that in vehicle-treated mice, consistent with the slower progression of the disease in these animals. In addition, we confirmed the existence of

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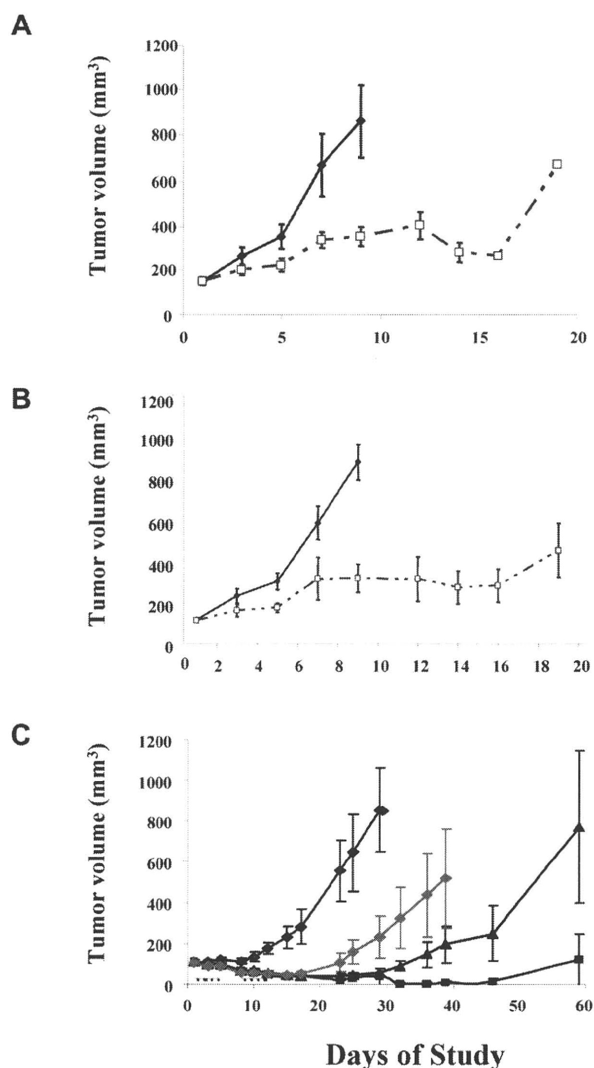

Figure 3. In vivo efficacy of AT9283 in BCR-ABL+ cell line xenograft. Nude mice bearing either BaF3/wt-BCR-ABLp210 (A) or BaF3/T315I (B) xenografts were administered the indicated doses of AT9283 by the intraperitoneal route. Vehicle (♦) and 12.5 mg/kg AT9283 (□) was dosed twice daily for 5 days followed by a 2-day break. The dose cycle was repeated twice in each case. Nude mice bearing human CML cells, K562 (C) xenografts were also administered the indicated doses of AT9283 by the intraperitoneal route. Vehicle (♦), 12.5 mg/kg (■), 10 mg/kg (□), and 7.5 mg/kg (♦) AT9283 was dosed twice daily for 5 days followed by a 2-day break. The dose cycle was repeated twice in each case. Mean growth curves ± SEs are shown for groups of 8 mice in each instance.

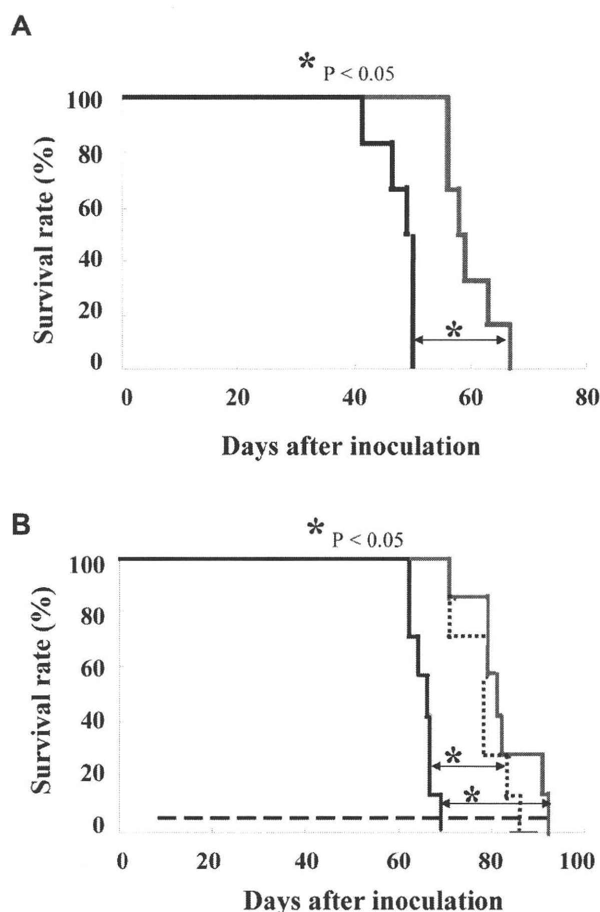

Figure 4. In vivo efficacy of AT9283 in primary samples. Nonlethally irradiated NOD/SCID mice were intravenously inoculated with cells taken from patients with CML harboring BCR-ABL E255K (A) or BCR-ABL T315I (B). Either 6.25 mg/kg AT9283 (gray) or vehicle (black) controlled was administered twice daily (A) on the indicated schedules (- - -). We administered 15 mg/kg AT9283 (gray), 10 mg/kg At9283 (black dot), or vehicle (black) controlled once daily (B) on the indicated schedules (- - -). Kaplan-Meier survival curves show that treatment with 6.25 mg/kg AT9283 twice daily resulted in a significant survival advantage ($P = .008$) over vehicle-treated animals of 17 days in the E255K model. Similarly, in the T315I model 10 mg/kg per day or 15 mg/kg per day AT9283 resulted in a marked survival advantage ($P = .002$).

T315I clones both in peripheral blood of AT9283-treated and vehicle-dosed mice by the modified guanine quenching probe method (supplemental Figure 8). In several mice we performed postmortem examinations for the presence of leukemic cells in liver and spleen with the use of the same method (data not shown). Tissue samples from both vehicle- and AT9283-treated mice were shown to be positive for the T315I clone, showing that, although AT9283 significantly slowed the proliferation of leukemic cells and prolonged the survival of the mice, it could not completely suppress the growth of leukemic cells in this model.

Discussion

To date, at least 90 different point mutations in the BCR-ABL kinase domain have been isolated from patients with BCR-ABL+ leukemia who are resistant to imatinib.[25,26] In addition to imatinib, other novel ABL TKIs are ineffective against the T315I mutant clone. This suggests that more patients with the T315I clone will emerge now that treatment with TKIs has become the standard of care in CML. The observation that T315I mutations were most frequently observed in dasatinib-resistant patients appears to support this observation.[27] Some studies have suggested that patients with T315I have a poor prognosis, with a median survival of 12.6 months from the start of imatinib therapy.[28,29] Therefore, there is much interest in developing novel agents effective against BCR-ABL/T315I clone.

To understand the activity of AT9283 versus BCR-ABL/T315I, we must first understand the mechanisms of resistance to existing agents in the adenosine triphosphate–binding pocket of BCR-ABL/T315I (Figure 1; supplemental Figure 1). As the explanations of resistance of ABL/T315I to imatinib, there are 3 hypotheses such as alteration of the 3-dimensional structure of the adenosine triphosphate pocket,[30,31] the consequence of a conformational readjustment necessary to accommodate the mutant residue,[32] and the breakdown of interactions between imatinib and both E286 and

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M290.[33] In the case of AT9283, the simplest explanation for the tolerance of the T315I is 2-fold: the lack of any hydrogen bond formed by this compound with the side-chain of T315, and the compound's avoidance of the selectivity pocket behind the gatekeeper residue. Put simply, the binding mode and key interactions formed by AT9283 are probably largely independent of the nature of the residue at position 315 (Figure 1B; supplemental Figure 1).

Note that the structure of the kinase domain of c-ABL in complex with a T315I-sensitive Aurora kinase inhibitor, MK-0457 (formerly VX-680), has already been reported.[34] The inhibition by both MK-0457 and PHA-739358 of the T315I mutant also arises because of their particular binding modes, which also avoid the gatekeeper region.[35] MK-0457 was found to be active against BCR-ABL/T315I,[34] and a phase 2 trial on MK-0457 for patients with CML with T315I showed some efficacy.[36] Unfortunately, the development of MK-0457 was halted for commercial reasons. QTc prolongation[37] or any adverse events was not involved in the closing of the phase 2 study, which confirmed significant activity in patients with T315I. PHA-739358 also has been reported to have strong antiproliferative and proapoptotic activity against BCR-ABL including T315I.[38] A phase 2 trial of PHA-739358 in patients with CML who have relapsed after BCR-ABL therapy is ongoing.

Because AT9283 is a multitargeted inhibitor with activities that include ABL and the Aurora kinases, it is important to understand which activities are key in driving the effects in CML cells. AT9283 inhibited both BCR-ABL and Aurora signaling (Figure 2; supplemental Figures 5-6). In addition, cell death observed in BCR-ABL^+ cell lines, treated with AT9283, is consistent with inhibition of BCR-ABL activity and is observed in the absence of the accumulation of large numbers of polyploid cells normally associated with Aurora inhibition in other cell lines. However, we do observe small numbers of multinucleated cells, suggesting that the Aurora activity of AT9283 is manifest in the background (Table 2; supplemental Figures 3-4). AT9283 is multitargeted in nature, and no single activity is the sole driver of its effect. We show potent inhibition of Aurora A and B; however, the BCR-ABL^+ cell lines do exhibit a different phenotype to the classic Aurora phenotype described in our previous study.[14] It is this combinatorial nature of the effect that suggests we may have a beneficial effect in the patient population with T315I for which there is at present no effective treatment.

In vivo models of CML have shown activity of AT9283 in a cell line grown as a subcutaneous xenograft (K562; Figure 3) and in models in which leukemic cells harboring E255K or T315I from patients were intravenously inoculated into NOD/SCID mice (Figure 4). In these models, 2 dosing regimens, twice daily and once daily, were used. In the nude mouse, there was no significant difference between twice daily and once daily dosing on tumor growth inhibition or tolerability (data not shown). In primary BCR-ABL^+ tumor models a larger AT9283 dose administered once daily was tolerated better than a lower dose administered twice daily with no loss of efficacy. This could relate to the mechanism of action of AT9283 in these CML cells, perhaps indicating that transient, yet complete, inhibition of BCR-ABL signaling is sufficient to drive efficacy. Similar observations have been made for dasatinib.[39,40]

Because of the aggressive nature of the cell growth in these animal models, it is usually not possible for any ABL TKI to suppress completely the growth of leukemic cells engrafted in this manner. However, the survival advantage shown here, although small, is significant and offers hope for AT9283 and its therapeutic potential. For example, an ABL/LYN inhibitor INNO-406 (NS-187) prolonged the survival of mice engrafted with BCR-ABL^+ cells in almost the same setting to a similar small yet significant extent showed responses in several imatinib-resistant patients in clinical trials.[41,42] Thus, it is our opinion that the presented survival difference, afterAT9283 treatment in these models, has the potential to reflect clinical benefit.

We show that the colony formation capacity of primary CML cells was inhibited by AT9283 at IC_{50} concentrations that were approximately 5-fold lower than those required to produce the same effect in progenitors from healthy volunteers. In addition, IC_{50} value of AT9283 for parental BaF3 cells was much higher than BCR-ABL^+ BaF3 cells, and IC_{50} values of AT9283 for most human CML cell lines, including imatinib-resistant cell lines. In clinical studies we have defined maximum-tolerated doses in patients with refractory solid tumor and patients with leukemia. Pharmacokinetic studies have shown that concentrations of AT9283 achieved in the plasma are consistent with the concentrations required to inhibit BCR-ABL^+ cell growth in the studies presented here.[43] In addition, biomarker modulations of a number of targets of AT9283 have been shown in samples taken during the course of these studies. These observations suggest that therapeutically relevant concentrations can be achieved at well-tolerated doses.[43]

On the basis of previous preclinical studies,[18,19] we have already treated 2 patients with TKI-refractory CML in accelerated phase as part of a dose-finding study of AT9283. Although the ABL mutational statuses of these patients were unknown, both patients achieved a hematologic response after treatment with AT9283. The present study strongly suggests that AT9283 has the potential to significantly benefit patients with CML or with Ph^+ ALL that is resistant to current forms of therapy. Moreover, AT9283 could be useful for patients bearing T315I clones. Therefore, the efficacy and safety of AT9283 for BCR-ABL^+ leukemias warrants further investigation in a clinical setting.

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Authorship

Contribution: R.T. performed research and analyzed data; M.S.S., and S.K. designed and performed research, and wrote the paper; A.Y., R.N., T.Y., M.T., H.Y., M.R., and E.A. performed research; T.S. designed research; J.F.L., N.T.T., and T.M. designed research and wrote the paper; and O.G.O. collected samples and performed research.

Conflict-of-interest disclosure: M.S.S., M.R., T.S., J.F.L., and N.T.T. are employees of Astex Therapeutics Ltd. The remaining authors declare no competing financial interests.

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