Imatinib mesylate inhibits T-cell proliferation in vitro and delayed-type hypersensitivity in vivo

Allan B. Dietz, Lina Souan, Gaylord J. Knutson, Peggy A. Bulur, Mark R. Litzow, and Stanimir Vuk-Pavlović

Imatinib mesylate (STI571, imatinib) inhibited DNA synthesis in primary human T cells stimulated with allogeneic mature dendritic cells or phytohemagglutinin (PHA) but did not induce apoptosis. The values for the concentration that inhibits 50% (IC50) of T-cell proliferation stimulated by dendritic cells and PHA were 3.9 μM and 2.9 μM, respectively, that is, within the concentration range found in patients treated with imatinib mesylate. Interestingly, imatinib mesylate did not inhibit expression of T-cell activation markers CD25 and CD69, although it reduced the levels of activated nuclear factor-κB (NF-κB) and changed phosphorylation or protein levels of Lck, ERK1/2, retinoblastoma protein, and cyclin D3. When T cells were washed free of imatinib mesylate, they proliferated in response to PHA, demonstrating that inhibition is reversible. Treatment with imatinib mesylate led to accumulation of the cells in G0/G1 phase of the cell cycle. The in vitro observations were confirmed in vivo in a murine model of delayed-type hypersensitivity (DTH). In mice treated with imatinib mesylate, DTH was reduced in comparison to sham-injected controls. However, the number of splenic T cells was not reduced showing that, similarly to in vitro observations, imatinib mesylate inhibited T-cell response, but did not cause apoptosis. These findings indicate that long-term administration of high-dose imatinib mesylate might affect immunity.

© 2004 by The American Society of Hematology

Materials and methods

Cells and reagents

T cells were isolated by negative immunoadsorption (Pan T kit; Miltenyi Biotec, Auburn, CA), pooled from 3 or more normal donors and cryopreserved until use. On thawing, the cells were incubated in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with 1.0% human AB serum (Sigma, St Louis, MO), in a humidified atmosphere of 5.0% CO2 at 37°C. For experiments longer than 1 day, the medium contained 1.0% penicillin/streptomycin solution (Sigma) and 10% FBS. For experiments longer than 1 day, the medium contained 1.0% penicillin/streptomycin solution (Sigma) as well. Human acute T-cell leukemia cells Jurkat (ATCC TIB-152), acute T lymphoblastic leukemia cells CCRF-CEM (ATCC CCL-119), and hematopoietic malignant K-562 cells (ATCC CCL-243), were obtained from American Type Culture Collection (Manassas, VA) and cultured under the same conditions. Allogeneic DCs were derived from CD14+ cells and cultured with T cells as previously described.16 Imatinib mesylate (Novartis) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM. The stock solution was stored at –20°C until use, diluted to the final concentration in X-VIVO 15 medium, and added to cells immediately.

Cell proliferation assays

T cells, 1 × 10⁶ in 200 μL/well of 96-well microtiter plates (Corning, Corning, NY), were stimulated with DCs or phytohemagglutinin M

From the Stem Cell Laboratory, Mayo Clinic Cancer Center, Rochester, MN; Division of Hematology, Department of Internal Medicine; Division of Transfusion Medicine, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN; and Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN.


Supported by National Institutes of Health grant R01CA-84368 and Mayo Clinic Comprehensive Cancer Center Support grant CA-15083. Stem Cell Laboratory has been supported by Mrs Adelyn L. Luther, Singer Island, FL; and Commonwealth Cancer Foundation for Research, Richmond, VA. L.S. is a scholar of the Glen and Florence Voyles Foundation, Terre Haute, IN.

Reprints: Allan B. Dietz or Stanimir Vuk-Pavlović, Mayo Clinic, 200 First St SW, Rochester, MN 55905; e-mail: dietz.allan@mayo.edu or vuk@mayo.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology
For 5 minutes. 19 Lysate aliquots, each equivalent to 0.6 μL of the cell suspension per well was plated in 6-well tissue culture plates. At ed by Quantity One 1-D analysis system (Bio-Rad Laboratories) and quan

ci antibodies speci

from Cell Signaling Technology we obtained immunopuri

c for individual bands on the

antibody for 1 hour at room temperature, and washed again. Thereafter, the

membrane was washed with TBS/T and incubated with the primary antibody at

5.0% nonfat powdered milk in 0.1 M NaCl, 20 mM proteines were electroblotted onto Trans-Blot nitrocellulose transfer membranes

(right footpad and of 25 g/mL, Sigma) or PDGF or both (R&D Systems, Minneapo-

saline (PBS), pH 7.8, at 7.0 g/100 mL. Six days after priming, mice received

injections of the challenge dose of 25 μL NP-O-Su (2.0 g/100 mL) into the right footpad and of 25 μL PBS into the left. Footpad thickness was measured 24 hours later by a digital micrometer.

We prepared single-cell suspensions from spleens of these mice by meshing individual spleens through metal sieves and lysing red blood cells in lysis buffer (1.0 mL/spleen; Sigma) for 5 minutes at room temperature. Thereafter, cells were incubated with anti-CD3 and anti-CD8 antibody (BD Biosciences, Mountain View, CA) for 20 minutes at 4 °C, washed, and fixed with 1.0% paraformaldehyde. Flow cytometry was performed with a FACScan flow cytometer and CellQuest software (both BD Biosciences).

Statistics

All experiments were repeated 2 to 5 times with qualitatively the same outcome. The probability that the mean values of 2 experimental groups were identical was tested by 2-tailed Student t test for paired samples. The level of significance was set at a P value of .05. Where applicable, data are reported as the mean ± SD.

Results

Imatinib mesylate inhibits proliferation of human primary T cells and T-cell lines

To determine if imatinib mesylate affects T-cell proliferation, we stimulated the cells with allogeneic mature DCs or PHA in the presence of imatinib mesylate. The drug inhibited T-cell proliferation as a function of concentration (Figure 1A). The effects were signi

cant at 0.5 μM imatinib mesylate for the cells stimulated by DCs (P = .002) and at 1.0 μM imatinib mesylate for the cells stimulated with PHA (P = .00004). The IC50 values for imatinib mesylate–inhibited T-cell proliferation stimulated by DCs and PHA were 3.9 μM and 2.9 μM, respectively. Thus, imatinib mesylate arrested T-cell proliferation in a dose-dependent manner at concentrations akin to those achieved in the serum of patients receiving standard imatinib mesylate therapy of 400 mg daily.1 Because the effects of PHA and DCs were similar, for further experiments we selected PHA to simplify interpretation of the results obtained by electrophoresis and Western blotting.

We quantified the effects of imatinib mesylate on proliferation of transformed human CD3 + T cell–derived lymphoblastoid line CCL119,
Imatinib mesylate neither reduces T-cell viability nor stimulates apoptosis

Imatinib mesylate toxicity might provide an explanation for inhibited T-cell proliferation. Therefore, we incubated the cells with imatinib mesylate for 72 hours and quantified apoptosis by determining the fraction of cells demonstrating activated caspase 3\(^24\) and the fraction that bound annexin V.\(^{25,26}\) Frequency of cells positive for activated caspase 3 and binding of annexin V were low in the absence of PHA (Figure 2). Stimulation by PHA increased the frequency of cells exhibiting either marker (\(P < .001\) for both). Imatinib mesylate–treated and PHA-activated T cells did not change their annexin V binding (\(P = .10\)), indicating that the drug did not induce apoptosis under these conditions. Caspase 3 activation can be used as a marker for apoptosis, but the levels of enzyme are increased in T-cell stimulation.\(^{27,28}\) PHA treatment activated caspase 3, but imatinib mesylate inhibited this effect (\(P = .03\)), similarly as it inhibited T-cell proliferation. Thus, the changes induced by PHA and imatinib mesylate in caspase 3 activation may reflect changes in T-cell stimulation, rather than enhanced apoptosis. Consequently, under these conditions imatinib mesylate was cytostatic, but not cytotoxic.

**Imatinib mesylate–treated T cells accumulate in the G0/G1 phase of the cell cycle**

Because imatinib mesylate was not cytotoxic, we postulated that the decrease in DNA synthesis (Figure 1) results from the effects of imatinib mesylate on cell cycle progression. To test this hypothesis, we stimulated imatinib mesylate–treated cells with PHA alone or with the combination of PHA (10 \(\mu\)g/mL) and IL-2 (500 U/mL) that powerfully stimulates T-cell proliferation.\(^{29}\) We measured DNA content 72 hours later. Data in Figure 3A show that essentially all unstimulated (control) T cells were in the G0/G1 phase of the cycle. PHA stimulated DNA synthesis and progression into the S phase (\(P < .001\) relative to resting cells), an effect inhibited by imatinib mesylate (\(P < .001\) relative to PHA-stimulated cells). Imatinib mesylate inhibited T-cell cycle progression even in the presence of PHA plus IL-2 (\(P < .01\) relative to the cells stimulated by both PHA and IL-2).

A prerequisite for entry of cells into the S phase is phosphorylation of the retinoblastoma protein (Rb) that is regulated by the cyclin D3/cdk4 complex\(^{30,31}\) (also reviewed by Olashaw and Pledger\(^{32}\)). We quantified the levels of cyclin D3 protein and Rb phosphorylation in the presence of imatinib mesylate by Western blotting. At 12, 16, and 24 hours after initiation of a typical experiment the amount of Rb phosphoprotein was reduced to 19%, 39%, and 77% of control, respectively; the amount of cyclin D was reduced to 28%, 32%, and 59% of control, respectively (data not shown; effects at later times might have been artificially reduced by the film saturation at the darker control bands). These effects are consistent with imatinib mesylate–induced arrest of the cell cycle before the S phase.

**Imatinib mesylate does not inhibit expression of T-cell activation markers CD25 and CD69**

Membrane molecules CD25 and CD69 are expressed as a result of T-cell stimulation, but their expression is not coupled to proliferation.\(^{33}\) Hence, these molecules can be viewed as evidence of early T cell activation.\(^{33}\) We measured the levels of CD25 and CD69 in...
imatinib mesylate–treated T cells activated by PHA. After an 18-hour incubation with PHA, the frequency of cells expressing CD25 and CD69 markedly increased (Figure 3B). Imatinib mesylate–treated T cells demonstrated a similar increase in CD25 and CD69 levels characteristic of activated T cells, rather than of resting T cells. Thus, the combined data in Figures 1-3 show that imatinib mesylate selectively inhibits cellular targets leading to proliferation without affecting T cell activation or apoptosis.

**Imatinib mesylate can terminate already initiated proliferation signals, but its effects are reversible**

Because the mechanisms initiating T-cell proliferation and activation are uncoupled, in agreement with the data in Figure 3A-B, it is of interest to determine if imatinib mesylate can terminate proliferation signals after they have been initiated. We plated cells without imatinib mesylate in the presence of PHA as in Figure 2. Imatinib mesylate was added at increasing intervals from initiation of culture (Figure 4A) and DNA synthesis was determined. We found that imatinib mesylate could inhibit proliferation of T cells stimulated even 48 hours before introduction of the drug (Figure 4A). Thus, imatinib mesylate could terminate already initiated intracellular signaling in the pathways leading to proliferation.

If imatinib mesylate inhibits proliferation but does not induce apoptosis, the cells will resume proliferation on removal of the drug. To test this hypothesis, we incubated T cells with imatinib mesylate, stimulated one half of the wells with PHA, and incubated all cells for 24 hours. Then we washed the cells, treated one half of the wells from each sample with PHA and incubated all for additional 96 hours when we measured thymidine incorporation. We found the cells unstimulated in both incubations did not proliferate (Figure 4B). The cells stimulated by PHA in the first incubation proliferated without it in the second. The cells stimulated in the second incubation proliferated to the same extent irrespective of whether they were stimulated in the first. These observations demonstrate that imatinib mesylate did not affect the proliferation potential of T cells and that the effects of the drug are reversible.

**Imatinib mesylate reduces levels of phosphorylated Lck and ERK1/2 and of activated NF-κB**

Abelson kinase (and its constitutively activated mutant BCR-ABL), c-KIT protein, and PDGF-R are well-documented cellular targets of imatinib mesylate. PDGF, common in human serum, suppresses T-cell activation. Monoclonal antibodies specific for the extracellular component of c-KIT did not recognize T cells (data not shown), suggesting that the full-length c-KIT is absent in T cells. Indeed, we detected no c-KIT and no imatinib mesylate–associated change in the levels of c-ABL phosphorylation by Western blotting (data not shown). Similarly, by functional assays we found that exogenous PDGF did not affect T cells (data not shown). Apparently c-ABL, c-KIT, and PDGF-R do not play a manifest role in inhibition of T-cell proliferation by imatinib mesylate.
Thus, T cells must contain other imatinib mesylate targets. In an attempt to identify some imatinib mesylate–sensitive intracellular signaling pathways we measured the levels of phosphorylation of Lck molecule and ERK1/2, both associated with T-cell receptor (TCR)–mediated signaling and of activated NF-κB, a transcription factor activated by numerous pathways including the one initiated at the TCR.35 We exposed PHA-stimulated T cells to imatinib mesylate and determined the relative levels of Lck protein and phosphorylation at its Tyr505 as a function of time. We found no apparent difference between drug-treated and control cells in the levels of Lck protein, but the drug reduced Tyr505 phosphorylation (Figure 5). This effect was apparent at the first time point measured at 15 minutes after stimulation. Similarly, we noticed that imatinib mesylate inhibited phosphorylation of ERK1/2 (Figure 5). In addition, we determined the level of activated NF-κB in nuclear extracts of T cells activated by PHA in the presence of increasing imatinib mesylate concentrations and expressed the results as percentage of PHA-stimulated increase in levels of activated NK-κB. At 4.4 μM imatinib mesylate, the amount of activated NK-κB was reduced to 58.7% ± 1.1% (P = .003) and decreased to 38.4% ± 0.9% at 17.6 μM. These effects of imatinib mesylate on phosphorylation of Lck and ERK1/2 and activation of NF-κB are compatible with the concentration dependence of the observed inhibition of T-cell proliferation and function.

Imatinib mesylate inhibits DTH in mice

To determine if imatinib mesylate affects T cells in vivo similarly to the effects in vitro, we treated mice with the drug daily throughout the experiment.36,37 After 10 days of treatment, we immunized mice with NP-O-Su and challenged 6 days later with a subcutaneous injection of the same agent into one footpad and of PBS into the other. Twenty-four hours later we quantified the extent of DTH by measuring the thickness of the footpads. Footpads of control animals (no imatinib mesylate) thickened considerably on the challenge with NP-O-Su (Table 1). Systemic treatment with imatinib mesylate abolished this effect (Table 1). In contrast, imatinib mesylate had no effect on the total splenocyte number (P = .25) and numbers of CD3+ cells (P = .52) and CD8+ cells (P = .16; data not shown). Taken together, these data show that the reduced DTH might result from a systemic inhibition of the T-cell response, rather than from the diminished number of T cells.

### Table 1. Imatinib mesylate suppresses DTH in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Footpad thickness, mm, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham injected, not primed</td>
<td>2.74 ± 0.11</td>
</tr>
<tr>
<td>2. Sham injected, primed</td>
<td>3.55 ± 0.13</td>
</tr>
<tr>
<td>3. Imatinib mesylate-treated, not primed</td>
<td>2.80 ± 0.33</td>
</tr>
<tr>
<td>4. Imatinib mesylate-treated, primed</td>
<td>2.88 ± 0.29</td>
</tr>
</tbody>
</table>

The mean ± SD values refer to the thickness of the footpad of mice, which did not receive (groups 1 and 2) or did receive imatinib mesylate (groups 3 and 4) and were primed (ie, immunized; groups 2 and 4) or not primed by NP-O-Su. All groups were challenged by NP-O-Su 1 day before measurement of the footpad thickness. A 2-tailed t-test comparison for independent groups yielded the following P values: for group 1 versus 2, P = .00006; for group 3 versus 4, P = .21; for group 1 versus 3, P = .702; and for group 2 versus 4, P = .004.

### Discussion

We found that imatinib mesylate inhibits T-cell proliferation at concentrations similar to those found in patients treated for CML and gastrointestinal stromal tumors.3 The effect is manifest both in T cells nonspecifically stimulated by PHA and in those stimulated by DCs. Imatinib mesylate neither prevented activation of T cells nor killed them, but it inhibited cell cycle progression. In an attempt to identify the pertinent intracellular pathways targeted by imatinib mesylate, we found reduced phosphorylation or protein levels of all molecules we selected for study. This observation could explain the attenuation of T-cell function, but it was not helpful in the identification of critical imatinib mesylate targets. Possibly, T-cell function is impaired by the cumulative effect of partial inhibition of numerous phosphonucleotide-binding molecules rather than by the definitive inhibition of few key molecules (eg, BCR-ABL or c-KIT). A similar conclusion has been reached recently in a study of differentiation of CD34+ hematopoietic cells into DCs where imatinib mesylate suppressed phosphorylation of molecules participating in numerous intracellular signaling pathways that are independent of c-ABL, c-KIT, and PDGF-R.35

We did detect the presence of c-ABL in T cells and cannot thus completely rule out its role in imatinib mesylate effects. However, there is little evidence for such a role of c-ABL in signal transduction leading to T-cell cycle triggering, although the molecule generally does participate in regulation of later stages of cell cycle progression.39 Our data are compatible with such a role for c-ABL because imatinib mesylate inhibited T-cell cycle progression, but did not affect T-cell activation. This observation is fully in line with the recent evidence for such uncoupling of activation and cell cycle progression in human T cells.31 In addition, these data demonstrate that imatinib mesylate can be used as a tool in studies of (un)coupling of T-cell activation and proliferation.

We found that imatinib mesylate was immunosuppressive in an in vivo model of DTH. The clinical relevance of this finding is unclear. Because the effects of the drug are rapidly reversed on its removal, these effects are likely to be sensitive to imatinib mesylate pharmacokinetics. It is possible, in fact, that the key determinant of the extent of immunosuppression is the nadir in fluctuations of levels of imatinib mesylate. Thus, sustaining therapeutically adequate imatinib mesylate levels may impede acute inflammatory responses or undermine control of subclinical infections. Nonetheless, information about the effects on imatinib mesylate on immunity is scarce and circumstantial. Several mostly anecdotal observations of secondary effects of imatinib mesylate in patients with CML support the notion that imatinib mesylate suppresses immunity in vivo. For example, patients treated with imatinib
borne immunosuppression could explain the surprising complications receded after reduction in the imatinib mesylate dose or complete discontinuation of the drug. Similarly, imatinib mesylate–borne immunosuppression could explain the surprising remission of rheumatoid arthritis in a patient with CML 2 months after initiation of imatinib mesylate therapy. Recent attempts to overcome resistance to imatinib mesylate by higher doses of the drug may provide data for further unraveling of the relationship of imatinib mesylate and immunity. A more definitive establishment of immunosuppressive effects of imatinib mesylate may add this well-tolerated drug to the list of clinically useful agents for control of T-cell malignancies and autoimmunity.

Acknowledgments
We thank Mr Troy Voeltz for technical help and Dr Frank Prendergast for continuing interest and support.

References
7. McLaughlin ME, Robson CD, Kieran MW, et al. Commitment to T-cell malignancies and autoimmunity. A more definitive establishment of immunosuppressive effects of imatinib mesylate may add this well-tolerated drug to the list of clinically useful agents for control of T-cell malignancies and autoimmunity.

From www.bloodjournal.org by guest on November 6, 2017. For personal use only.
Imatinib mesylate inhibits T-cell proliferation in vitro and delayed-type hypersensitivity in vivo

Allan B. Dietz, Lina Souan, Gaylord J. Knutson, Peggy A. Bulur, Mark R. Litzow and Stanimir Vuk-Pavlovic