Selection of Myeloid Progenitors Lacking BCR/ABL mRNA in Chronic Myelogenous Leukemia Patients After In Vitro Treatment With the Tyrosine Kinase Inhibitor Genistein

By Carmelo Carlo-Stella, Gianpietro Dotti, Lina Mangoni, Ester Regazzi, Daniela Garau, Antonio Bonati, Camillo Almici, Gabriella Sammarcelli, Barbara Savoldo, Maria Teresa Rizzo, and Vittorio Rizzoli

Chronic myelogenous leukemia (CML) is a clonal disorder of the hematopoietic stem cell characterized by a chimeric BCR/ABL gene giving rise to a 210-kD fusion protein with dysregulated tyrosine kinase activity. We investigated the in vitro growth of CML and normal marrow-derived multipotent (colony-forming unit-mix [CFU-Mix]), erythroid (burst-forming unit-erythroid [BFU-E]), and granulocytemacrophage (colony-forming unit–granulocyte-macrophage [CFU-GM]) hematopoietic progenitors. Continuous exposure of CML and normal marrow to genistein induced a statistically significant and dose-dependent suppression of colony formation. Genistein doses causing 50% inhibition of CML and normal progenitors were not significantly different for CFU-Mix (27 μmol/L v 23 μmol/L), BFU-E (31 μmol/L v 29 μmol/L), and CFU-GM (40 μmol/L v 32 μmol/L). Preincubation of CML and normal marrow with genistein (200 μmol/L for 1 to 18 hours) induced a time-dependent suppression of progenitor cell growth, while sparing a substantial proportion of long-term culture-initiating colonies (LTC-IC) from CML (range, 91% ± 9% to 32% ± 3%) and normal marrow (range, 85% ± 8% to 38% ± 9%). Analysis of individual CML colonies for the presence of the hybrid BCR/ABL mRNA by reverse transcription-polymerase chain reaction (RT-PCR) showed that genistein treatment significantly reduced the mean ± SD percentage of marrow BCR/ABL+ progenitors both by continuous exposure (76% ± 18% v 24% ± 12%, P ≤ .004) or preincubation (75% ± 16% v 21% ± 10%, P ≤ .002) experiments. Preincubation with genistein reduced the percentage of leukemic LTC-IC from 87% ± 12% to 37% ± 12% (P ≤ .003). Analysis of individual colonies by cytogenetics and RT-PCR confirmed that genistein-induced increase in the percentage of nonleukemic progenitors was not due to suppression of BCR/ABL transcription. Analysis of nuclear DNA fragmentation by DNA gel electrophoresis and terminal deoxynucleotidyl transferase assay showed that preincubation of CML mononuclear and CD34+ cells with genistein induced significant evidence of apoptosis. These observations show that genistein is capable of (1) exerting a strong antiproliferative effect on CFU-Mix, BFU-E, and CFU-GM while sparing multipotent LTC-IC and (2) selecting benign hematopoietic progenitors from CML marrow, probably through an apoptotic mechanism.

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In the past decade, several natural or synthetic compounds able to inhibit the signaling cascades triggered by protein tyrosine kinases (PTKs) have been generated. Inhibitors of PTK catalytic activity that exhibit rather broad specificity in the micromolar range include genistein, quercetin, herbimycin A, and tyrphostins. Genistein, a naturally occurring PTK inhibitor, has been shown to exhibit specific inhibitory activity against receptor and cytoplasmic tyrosine kinases, including epidermal growth factor receptor, pp60c-src, pp110alpha, platelet-derived growth factor receptor, and c-kit. Genistein induces differentiation and apoptosis of HL-60 and K562 cells and may also inhibit topoisomerases I and II. Using established leukemic cell lines, it has been shown that genistein, herbimycin A, and tyrphostin inhibit p21OBCwABL tyrosine kinase activity. However, the effect of PTK inhibitors on primary CML cells has not been extensively investigated.

Therefore, it was the aim of the present study to evaluate the effect of genistein on the in vitro growth of marrow-derived CML and normal progenitors grown in methylcellulose in the continuous presence or after a transient exposure to genistein. In addition, the capability of genistein to select colony-forming units (CFU) was investigated by detecting the BCR/ABL mRNA on single progenitors by reverse transcription-polymerase chain reaction (RT-PCR).

### MATERIALS AND METHODS

**Patients.** Seven patients with Ph+ CML were included in this study (Table 1). Three patients (nos. 1, 3, and 6) were evaluated at diagnosis and before any treatment; the others had been diagnosed 3 to 55 months before the study and had received prior treatment with hydroxyurea, interferon-a, or mitosanfamide-purged ABMT (no. 5). At the time of the study, all patients were in the chronic phase.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Clinical Status</th>
<th>WBC (x10^9/L)</th>
<th>Pit (x10^9/L)</th>
<th>Previous Therapy</th>
<th>Time From Diagnosis (mo)</th>
<th>Ph+/Total Metaphases*</th>
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Abbreviations: WBC, white blood cells; Pit, platelet counts; HU, hydroxyurea; IFNox, interferon a; ABMT, autologous bone marrow transplantation.

*Number and type of bone marrow metaphases obtained after routine cytogenetic analysis of freshly aspirated bone marrow at time of study.

<table>
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<tr>
<th>Table 1. Clinical Characteristics of the Patients at the Time of the Study</th>
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Abbreviations: WBC, white blood cells; Pit, platelet counts; HU, hydroxyurea; IFNox, interferon a; ABMT, autologous bone marrow transplantation.

*Number and type of bone marrow metaphases obtained after routine cytogenetic analysis of freshly aspirated bone marrow at time of study.
colonies, individual colonies were aspirated, divided into two aliquots, and analyzed both at the cytogenetic and molecular level. Briefly, colcemid (1 μg/mL; 100 μL/dish) was added to cultures by using a 26-gauge syringe needle 3 to 4 hours before the end of the incubation period. Colonies were individually removed, transferred into a 96-well tissue culture plate containing 40 μL KCl (0.075 mol/L), and dispersed by gently pipetting up and down. Half of the cell suspension was then aspirated and processed by RT-PCR for BCR/ABL mRNA detection, whereas the remaining cells were processed for cytogenetic analysis according to a previously described technique.

Detection of BCR/ABL mRNA in individual progenitors. BCR/ABL mRNA was detected by RT-PCR. Colonies were individually removed under an inverted microscope using micropipette tips and transferred into microcentrifuge tubes containing 40 μL phosphate-buffered saline (PBS). After adding guanidinium thiocyanate (40 μL), colonies were frozen at -70°C until nested RT-PCR was performed. Total RNA was extracted from thawed colonies according to the method of Chomczynski and Sacchi, with slight modifications. Briefly, 500 μL of TRIzol (GIBCO) and 5 μg of MS2 phage RNA (Boehringer Mannheim, Mannheim, Germany) as a carrier were added to each tube. After incubation for 5 minutes at room temperature, 100 μL of chloroform was added and each tube was vigorously shaken and centrifuged (12,000g for 15 minutes at 4°C). Finally, the upper aqueous phase was aspirated and RNA was precipitated with isopropanol, centrifuged (12,000g for 10 minutes at 4°C), washed with ethanol (75%), dried (room temperature), and resuspended in RNAase-free water (55°C to 60°C for 10 minutes). Total RNA from each colony was reverse transcribed to cDNA in a final volume of 40 μL, using 1.1 nmol of hexa random primer, 10 mmol/L dithiothreitol (DTT), 0.125 mmol/L dNTPs (GIBCO), 40 μL of RNAase Inhibitor (Boehringer Mannheim), and 200 U of murine leukemia virus (MLV) reverse transcriptase (GIBCO) in PCR buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, and 3 mmol/L MgCl2). The mixture was incubated at 37°C for 1 hour and quickly chilled on ice. Total cDNA from each colony was divided into two aliquots for detection of the BCR/ABL rearrangement and the internal ABLC sequence, respectively. The first PCR amplification was performed in a final volume of 45 μL using 10 μL of the reverse transcription mixture, 0.25 mmol/L dNTP, 0.5 μmol/L of each primer, and 2 U of Taq polymerase (GIBCO) in PCR buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl, and 1.5 mmol/L MgCl2). Forty-five cycles, each consisting of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, were performed using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR was performed using the primers of Martiat et al. The BCR/ABL primer sequences were 5'-GAA GAA GTG TTT CAG AAG CCT TTC CC-3' (sense) and 5'-GAC CCG GAG CTT TTC ACC ATT AGT T-3' (antisense). The ABL primer sequences were 5'-TTC TCC AGC AGG CAG TAG CAT CTG ACT T-3' (sense) and 5'-GAC CCG GAG CTT TTC ACC TTT AGT T-3' (antisense). Reamplification of 1 μL of the first PCR product was performed for BCR/ABL under slightly modified conditions (35 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C) using oligoimucogene primers internal to the original primers. The BCR/ABL internal nested primer sequences were 5'-GTT AAA CTC CAG ACT GCT CAC AGC A-3' (sense) and 5'-TCC ACT GGC CAC AAA ATC ATC ATA CAGT-3' (antisense). The reaction products were electrophoretically separated through a 2% agarose gel and stained with ethidium bromide. The expected products generated by PCR were: for BCR/ABL, 272 bp and 197 bp, depending on the position of junction point within M-BCR. The expected product generated by PCR for ABL was 185 bp. Two negative controls consisting of RNA isolated from normal marrow CFU-GM and of sterile diethylpyrocarbonate (DEPC) water (Sigma, St Louis, MO), including all reagents without RNA, were performed in each experiment. As a positive control, RNA extracted from single K562 colonies was used. To ensure that RNA could be reverse transcribed and subsequently amplified, control amplification using exon 2-exon 3 ABL sequence-specific primers was performed in all samples. Only colonies with an internal positive control were considered evaluable. Because ABL was already detectable after the first PCR amplification (on average, 90% of the colonies gave rise to an ABL signal of good quality after a one-step RT-PCR), the first PCR product of ABL was not reamplified. After a one-step PCR, using a 5' primer on ABL exon 2 and a 3' primer on ABL exon 3, amplification of genomic DNA contaminating RNA preparations is expected to give rise to a PCR product with a higher molecular weight as compared with the product generated by cDNA. However, to exclude that the amplification of genomic DNA, in the absence of cDNA, could produce an ABL-positive signal, each ABL detection was controlled by amplifying a sample that had not reverse transcribed. Such samples failed to show any ABL amplification.

Nuclear DNA fragmentation. Nuclear DNA fragmentation was detected by DNA gel electrophoresis and terminal deoxynucleotidyl transferase (TdT) assay. To perform DNA gel electrophoresis, untreated and genistein-treated MNC-AC- CD34+ cells were collected for centrifugation; washed in PBS; resuspended in 20 μL of 5% (vol/vol) sodium lauryl sarcosinate and 0.5 mg/mL proteinase K (Boehringer Mannheim); and incubated at 50°C for 1 hour. After the addition of 10 μL RNAase A (0.5 mg/mL), incubation at 50°C was continued for 1 hour. Samples were heated to 70°C, and 10 μL RNAase-free water (55°C to 60°C for 10 minutes). After the addition of 10 μL DNAse I (0.5 mg/mL), samples were heated to 70°C, and 10 μL of 1,000-fold concentrated solutions that were used at a final concentration of 0.1% (vol/vol) to obtain the appropriate concentrations in culture. The effect of genistein was evaluated by continuous exposure and preincubation experiments. For continuous exposure experiments, MNC-AC- (50,000/mL) were exposed to 10 μL (100 μmol/L) of genistein (1 to 100 μmol/L). For each experiment, appropriate controls with vehicle alone (dimethyl sulfoxide [DMSO]; 1 μL/dish) were set up. For preincubation experiments, MNC-AC- (1 × 106/mL) were exposed (1 to 18 hours at 37°C and 5% CO2) to either control medium (IMDM and 10% FBS) or medium containing genistein (50 to 200 μmol/L). At the end of the incubation period, the cells were washed three times and cultured to quantitate CFU-C, CFU-GM, and LTC-IC.

Statistical analysis. Four plates were scored for each data point per experiment and the results were expressed as the mean ± 1 standard error of the mean (SEM). Statistical analysis was performed with the statistical package Statview (BrainPower Inc, Calabasas, CA) run on a Macintosh LCII personal computer (Apple Computer Inc, Cupertino, CA). The Student t-test for paired data (two-tail) or the Wilcoxon matched pairs test was used for nonparametric data.
the Wilcoxon signed-rank test were used where appropriate to test the probability of significant differences between samples. Genistein concentrations resulting in 50% inhibition (ID_{50}) of colony formation were calculated for each experiment by extrapolating from a least-square linear regression line relating genistein concentration to the percentage of colony growth inhibition.

**RESULTS**

*Effect of genistein on CML and normal progenitors.* As shown in Fig 1A, when CML (n = 5) MNC-AC<sup>-</sup> were exposed throughout the entire culture period to genistein, a statistically significant (CFU-Mix: P < .01 at 5 μmol/L; BFU-E: P < .01 at 5 μmol/L; CFU-GM: P < .007 at 5 μmol/L) dose-dependent suppression of colony growth from multipotent and lineage-restricted progenitors was seen. Regression analysis showed that inhibition was linearly related (CFU-Mix: r = .84, P = .03; BFU-E: r = .88, P < .01; CFU-GM: r = .95, P < .003) to genistein concentration over the range tested (1 to 100 μmol/L). Similarly (Fig 1B), continuous exposure of normal (n = 4) MNC-AC<sup>-</sup> to genistein induced a statistically significant (CFU-Mix: P < .02 at 5 μmol/L; BFU-E: P < .004 at 5 μmol/L; CFU-GM: P < .007 at 5 μmol/L) dose-dependent suppression of colony formation, with an inhibitory pattern that was linearly related (CFU-Mix: r = .79, P = .05; BFU-E: r = .85, P = .03; CFU-GM: r = .86, P = .02) to genistein concentrations. Genistein doses causing 50% inhibition of CML and normal progenitors were not significantly different for CFU-Mix (27 μmol/L v 23 μmol/L), BFU-E (31 μmol/L v 29 μmol/L), and CFU-GM (40 μmol/L v 32 μmol/L). Individual marrow samples exhibited variable sensitivity to the inhibitory effects of genistein, but no one failed to respond to this agent. The degree of colony suppression was not related to colony number in control cultures.

As shown in Fig 2, transient exposures (1 to 18 hours) of CML (n = 5) and normal (n = 5) marrow to genistein (200 μmol/L) followed by washing showed a time-dependent suppression of CFU-Mix, BFU-E, and CFU-GM growth, suggesting that the effect of the drug does involve toxicity. Under the experimental conditions used in this study, no differential toxic effect was evident between CML and normal progenitors.

To investigate the effect of genistein on primitive progenitors, LTC-IC growth was also evaluated. As shown in Fig 2A, the percentages of CML-derived (n = 5) LTC-IC surviving after a transient exposure to genistein (200 μmol/L) for 1 (91% ± 9%), 2 (88% ± 6%), and 18 (32% ± 3%) hours were significantly higher (P < .05) than those of CML-derived CFU-Mix, BFU-E, and CFU-GM. Similarly, normal LTC-IC were suppressed to a significantly lower degree than were normal CFU-Mix, BFU-E, and CFU-GM (Fig 2B). Again, no difference in the inhibitory pattern was detected by comparing CML and normal LTC-IC.

**BCR/ABL mRNA expression on single colonies.** The quantitatively similar inhibition of CML and normal progenitors argues for an antiproliferative effect of genistein that is not specifically related to BCR/ABL inhibition. Several pieces of evidence suggest that BCR/ABL<sup>+</sup> and BCR/ABL<sup>-</sup> progenitors coexisting in CML marrow have different biologic characteristics. Therefore, we hypothesized that genistein could affect CML colony formation not only quantitatively, but also qualitatively. To test this hypothesis, CML colonies were individually harvested and analyzed by RT-PCR for the expression of hybrid BCR/ABL mRNA. At the
time of the study, all of the patients but 1 were 100% Ph+ by standard cytogenetics (Table 1).

Table 2 shows that, as compared with control cultures, continuous exposure to genistein (10 to 50 μmol/L) significantly reduced the mean ± SD percentage of BCR/ABL+ progenitors (76% ± 18% vs 24% ± 12%, P ≤ .004). Figure 3 shows PCR analysis of single CFU-GM from patient no. 5 grown in the absence or continuous presence of genistein. This treatment reduced the percentage of BCR/ABL+ progenitors from 65% to 10%. As shown in Table 3, preincubation of marrow cells with genistein resulted in a significant reduction of progenitors expressing BCR/ABL mRNA (75% ± 16% vs 21% ± 10%, P ≤ .002).

The capability of genistein to reduce the percentage of leukemic progenitors was also evaluated at the level of the primitive LTC-IC. In 2 of 5 cases (no. 4 and 5), molecular analysis of CFU-GM produced by LTC-IC showed a high percentage of BCR/ABL+ progenitors; therefore, the effect of genistein could be evaluated only in 3 cases (no. 3, 6, and 7). As shown in Table 4, in these patients, genistein treatment resulted in a marked reduction of BCR/ABL+ LTC-IC (87% ± 12% vs 37% ± 12%, P ≤ .003). PCR analysis of individual CFU-GM present in marrow from patient no. 3 and produced by LTC-IC after 5 weeks in long-term culture is shown in Fig 4. In this case, genistein treatment could reduce BCR/ABL+ LTC-IC from 80% to 35%.

To rule out the possibility that genistein could affect BCR/ABL transcription, thus inducing the growth of Ph+ progenitors with nonfunctional BCR/ABL gene (BCR/ABL-), in two separate experiments colonies were individually harvested and split into two aliquots, one for cytogenetics and the other for RT-PCR. In control cultures, only 1 of 70 Ph+ colonies (1.4%) did not transcribe the BCR/ABL gene (Table 5). In genistein-treated cultures, 13 of 60 colonies were found to be Ph+ and 12 of 13 (92%) transcribed the BCR/ABL gene (Table 5). These findings confirm that genistein-induced increase in the percentage of nonleukemic progenitors is not related to suppression of BCR/ABL transcription.

**Nuclear DNA fragmentation.** To examine whether apoptosis is the mechanism responsible for genistein-induced inhibition of progenitor cell growth, CML-derived MNC-AC- were treated with genistein (200 μmol/L for 18 hours) and analyzed for the presence of intracellular DNA fragmentation using TdT assay. A significant portion of genistein-treated cells (38% ± 11% of the live gated population) was found to be in a progressive stage of apoptosis, whereas virtually no apoptotic cells were detected in the control samples (3% ± 2%). In addition, genistein-treated samples showed evidence of DNA fragmentation by DNA gel elec-

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**Table 2. PCR Analysis of Individual Bone Marrow Colonies After Continuous Exposure to Genistein**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Colony Type</th>
<th>Ununtreated Progenitors %</th>
<th>Genistein- Treated Progenitors %</th>
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<tr>
<td>3</td>
<td>CFU-GM</td>
<td>20/20</td>
<td>6/20</td>
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<tr>
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<td>CFU-GM</td>
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<tr>
<td>7</td>
<td>CFU-GM</td>
<td>25/31</td>
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</tbody>
</table>

* Bone marrow cells were continuously exposed to genistein (10 to 50 μmol/L) added in methylcellulose culture on day 0.

† Values represent the number of PCR+ colonies/number of colonies tested.
Fig 3. Example of an ethidium-bromide-stained agarose gel electrophoresis analysis of RT-PCR products, including negative and positive controls, from a single CFU-GM from patient no. 5 grown in a standard methylcellulose assay in the absence or continuous presence of genistein added on day 0 of culture. (1) Untreated progenitors and (2) genistein-treated progenitors. (a) BCR/ABL-derived products and (b) ABL-derived products serving as controls indicating that RNA could be reverse transcribed and subsequently amplified in all samples. After double amplification, the BCR/ABL PCR product consisted of 272 bp. The positive control consisted of single K562-derived colonies with 5 µg MS2 phage RNA as a carrier, resulting in a 272-bp product. Negative controls included sterile DEPC water including all reagents without RNA, single CFU-GM from normal bone marrow with 5 pg MS2 phage RNA as a carrier, and 5 µg MS2 phage RNA only.

Trofophoresis. To evaluate progenitor cell-enriched populations, CD34⁺ cells were also analyzed. In Fig 5, a representative experiment demonstrating that CD34⁺ are induced to apoptosis on genistein treatment is shown.

**DISCUSSION**

Several clinical and experimental studies have shown the coexistence of normal and leukemic progenitors in CML. In vitro studies suggested that incubation of CML marrow cells with mafosfamide or interferon-γ reduces Ph⁺ progenitors while sparing some Ph⁻ progenitors. On average, cytogenetic analysis of MNC- or CD34⁺-derived progenitors results in percentages of Ph⁺ progenitors ranging from 20% to 40%, with 60% of CML patients being responsive to such in vitro treatments. The Vancouver experience with long-term bone marrow culture shows about 50% of CML patients having 100% Ph⁻ LTC-IC. Analysis of CD34⁺ HLA-DR⁺ progenitors showed the possibility of selecting a population of exclusively nonclonal hematopoietic stem cells in a substantial proportion of CML patients.

PTKs play a crucial role in regulating hematopoietic cell proliferation. The increasing knowledge of transmembrane and intracellular signal transduction phenomena now allows us to manipulate cell growth by altering signaling pathways. Because PTKs participate in the establishment and

<table>
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<tr>
<th>Case No.</th>
<th>Colony Type</th>
<th>Untreated Progenitors</th>
<th>PCR⁺ Colonies (%)</th>
<th>Genistein-Treated Progenitors*</th>
<th>PCR⁺ Colonies (%)</th>
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* Bone marrow cells were preincubated with genistein (200 µmol/ L) for 2 or 18 hours.
† Values represent the number of PCR⁺ colonies/number of colonies tested.

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<tr>
<th>Case No.</th>
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* Bone marrow cells were preincubated with genistein (200 µmol/ L for 2 or 18 hours) and subsequently cultured in long-term.
† Values represent the number of PCR⁺ colonies/number of colonies tested.
progression of several malignant diseases, inhibitors of PTKs represent attractive antiproliferative agents. Genistein inhibits tyrosine phosphorylation events both at the membrane level and, distal to membrane-bound growth factor receptors, induces apoptosis and inhibits topoisomerases I and II. By acting as a general inhibitor of tyrosine kinase activity, genistein could prevent the phosphorylation of regulatory proteins such as Grb-2 and Shc that have the potential to stimulate Ras and might play a crucial role in the pathogenesis of CML.

In the present study, we show that genistein strongly inhibits marrow-derived CML as well as normal CFU-Mix, BFU-E, and CFU-GM, while sparing a substantial proportion of LTC-IC. Under the experimental conditions used in this study, both continuous and transient exposure of marrow cells to genistein induced a similar antiproliferative effect on leukemic and normal progenitors. This argues for a non-specific inhibitory effect occurring through a complete shut-off of the PTK signaling pathways rather than a selective inhibition of BCR/ABL tyrosine kinase, as has been shown for other PTK inhibitors. Analysis of MNC-AC and CD34 cells for intracellular DNA fragmentation using DNA gel electrophoresis and TdT assay showed that apoptosis mediates genistein-induced inhibition of progenitor cell growth.

Because BCR/ABL+ and BCR/ABL- progenitors coexisting within the same CML marrow have different biologic characteristics, it was hypothesized that genistein could affect the growth of leukemic progenitors not only quantitatively, but also qualitatively. Analysis of individual colonies for the expression of hybrid BCR/ABL mRNA showed that genistein-induced growth inhibition was associated with suppression of leukemic CFU-GM and LTC-IC and the reemergence of nonclonal progenitors. Colonies plucked for RT-PCR analysis were compact and contained ~200 cells. A transient exposure to genistein failed to affect the morphology or the size of colonies detected in methylcellulose culture or produced by LTC-IC after 5 weeks in long-term culture (data not shown). Overall, genistein induced a significant increase in the percentage of BCR/ABL- primitive and committed progenitors (Tables 2 through 4), although a complete disappearance of leukemic progenitors was never detected.

Molecular data are not in contrast with the equal sensitivity of CML and normal progenitors to the growth inhibitory effect of genistein. In fact, normal progenitors persisting in
CML patients are biologically and kinetically different from progenitors detectable within normal marrow. We hypothesize that, due to their intrinsic biologic properties, residual BCR/ABL progenitors within CML marrow are capable to escape the inhibitory action of genistein and have the possibility to re-express their proliferative potential. As shown by DNA fragmentation, genistein may favor the re-emergence of nonclonal progenitors by triggering apoptosis of leukemic progenitors. However, the capacity of genistein to preferentially induce apoptosis of leukemic progenitors while sparing the nonleukemic ones remains to be investigated.

In agreement with data from the Vancouver group, we detected a concentration of LTC-IC in CML marrow that was decreased on average to less than 10% of the LTC-IC concentration in normal marrow. To get adequate numbers of colonies from genistein-treated samples, LTC-IC were assayed by setting up three separate cultures on day 0. This allowed us to analyze BCR/ABL expression in an average number of 20 colonies (range, 16 to 30) generated from genistein-treated samples, thus excluding that genistein acts by suppressing BCR/ABL expression. This is further supported by the evidence of Kaur et al., who have shown that preincubation of K562 cells with the tyrphostin AG957 inhibits cell growth, tyrosine kinase activity, and DNA synthesis as early as 2 hours, a time at which RNA and protein synthesis were not affected.
The use of PTK inhibitors for marrow purging has been proposed. The in vitro selection for normal hematopoietic stem and progenitor cells from within CML marrow and the potential for using these cells as leukemia-free autografts has been the topic of increasing discussion. Data reported herein show the possibility of selecting nonclonal marrow donors. As compared with mofosfamide, geinstein appears to exert a stronger antileukemic effect, thus allowing us to predict a better clinical efficacy in the context of marrow purging. The toxicity of geinstein to normal marrow progenitors could prevent its in vivo use for autografting purposes. However, other drugs, such as the cytoplasmatic derivative mofosfamide, which is at least as toxic as geinstein to normal marrow progenitors but, similarly to geinstein, spares LTC-IC, have been extensively used in vivo for autografting without evidence of clinically unacceptable damage of hematopoietic function. An additional problem related to the in vivo use of geinstein is represented by lot-to-lot changes in the biologic activity of the molecule. In this respect, the recently described use of synthetic PTK inhibitors may represent an important alternative.

The availability of several natural and synthetic PTK inhibitors will allow to screen for compounds with a more effective antileukemic action. Further investigations are required to explore the therapeutic potential of PTK inhibitors alone or in combination with other agents, including biologic response modifiers and antisense oligonucleotides, as well as their effects on highly purified hematopoietic cell subpopulations to improve selection of benign progenitors in CML.

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Selection of myeloid progenitors lacking BCR/ABL mRNA in chronic myelogenous leukemia patients after in vitro treatment with the tyrosine kinase inhibitor genistein

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