Quercetin Inhibits the Growth of Leukemic Progenitors and Induces the Expression of Transforming Growth Factor-β1 in These Cells

By Luigi M. Larocca, Luciana Teofili, Simona Sica, Mauro Piantelli, Nicola Maggiano, Giuseppe Leone, and Franco O. Ranelletti

We previously showed that quercetin (3,3′,4′,5,7-pentahydroxyflavone) inhibits in a dose-dependent manner the growth of acute leukemias and is able to enhance the antiproliferative activity of cytosine arabinoside. We show here that quercetin inhibits the clonogenic activity of 20 of 22 acute leukemias (AL; 4 M1-AML, 3 M2-AML, 2 M3-AML, 3 M4-AML, 3 M5-AML, and 7 ALL). In the present report, we show that the induction of transforming growth factor-β1 (TGF-β1) in leukemic blasts is one of the growth-inhibitory mechanisms of quercetin in these cells. This observation was supported by the following data. (1) Quercetin-sensitive leukemic blasts, when treated with quercetin, secrete large amounts of TGF-β1 in the medium and show positivity for TGF-β1-immunoreactive material in the cytoplasm. (2) At a concentration of 8 μmol/L, antiserum TGF-β1 oligonucleotides prevent the growth-inhibitory action of quercetin. (3) Anti-TGF-β1 neutralizing monoclonal antibodies can prevent almost completely the growth-inhibitory activity of quercetin. The analysis of quercetin-resistant cases confirmed as well the central role of TGF-β1 in the growth-inhibitory activity of quercetin. In conclusion, quercetin can act as a cytostatic agent for leukemic cells by modulating the production of TGF-β1.

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MATERIALS AND METHODS

Isolation of leukemic blasts and clonogenic assay. Fresh leukemic blasts were isolated from heparinized bone marrow aspirates obtained from 20 leukemic patients (14 AML and 6 ALL) at diagnosis and from 2 patients in relapse (1 AML and 1 ALL). All samples were obtained after informed consent was obtained. Diagnoses were established according to the French-American-British recommendations after cytologic examinations and cytochemical assays (peroxidase and naphthyl acetate esterase). Immunologic phenotype was defined in ALL patients. Aspirates diluted with Hank’s Balanced Salt Solution (HBSS) were layered onto Ficoll-Hypaque density gradient (1.077 g/mL, pH 7.6, 292 mOsml/L) and centrifuged at 400g for 30 minutes. Light-density mononuclear cells contained more than 70% blasts cells. In AML and in non-T-ALL, T lymphocytes were removed by rosetting with neuraminidase-treated sheep erythrocytes. After T-cell depletion, the percentage of blasts in the resulting cell population was greater than 90%, as assessed by morphologic, cytoenzymatic, and immunophenotypic analysis. Blasts were suspended at 1 × 10⁵ cells/mL in Iscove’s modified Dulbecco’s medium (IMDM; Flow Laboratories, Irvine, UK) containing 0.9% methylcellulose (Sigma, St Louis, MO), 2 × 10⁻⁶ mol/L 2-mercaptoethanol (Sigma), 20% heat-inactivated fetal calf serum (H1-FCS; Flow Labs), and 10% phytoternagglutinin (PHA)–leukocyte-conditioned medium, prepared according to the method of Aye et al.⑦ Aliquots of 0.1 mL were plated in 96-microwell flat-bottom plates (Becton Dickinson, Lincoln Park, NJ). All plates were incubated at 37°C in a fully humidified 5% CO₂, 95% air atmosphere. Quercetin (Aldrich, Steinheim, Germany) was added at the indicated concentration at the start of cultures and then every 2 days from an absolute ethanol stock solution. Ethanol (vehicle) concentration never exceeded 1% (vol/vol) in untreated and treated cultures. Each sample was cultured in quadruplicate. After 6 to 10 days, aggregates consisting of 10 or more cells were counted as colony-forming unit-leukemic (CFU-L). Result are expressed as the number of colonies from ALL patients were stained with Wright-Giemsa. Quercetin (Aldrich, Steinheim, Germany) was added at the indicated concentration at the start of cultures and then every 2 days from an absolute ethanol stock solution. Ethanol (vehicle) concentration never exceeded 1% (vol/vol) in untreated and treated cultures. Each sample was cultured in quadruplicate. After 6 to 10 days, aggregates consisting of 10 or more cells were counted as colony-forming unit-leukemic (CFU-L). Result are expressed as the number of colonies from ALL patients were stained with Wright-Giemsa.

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Table 1. Quercetin Inhibitory Capacity of Colony Formation and Clonogenic Efficiency in Acute Leukemic Subtypes

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Leukemic Cell Subtype</th>
<th>ID 50%* (μmol/L)</th>
<th>Clonogenic Efficiency†</th>
</tr>
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<tr>
<td>1</td>
<td>M1</td>
<td>8.0</td>
<td>91</td>
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<tr>
<td>2</td>
<td>M1</td>
<td>30.0</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>M1</td>
<td>4.0</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>M1</td>
<td>Resistant</td>
<td>186</td>
</tr>
<tr>
<td>5</td>
<td>M2</td>
<td>10.0</td>
<td>154</td>
</tr>
<tr>
<td>6</td>
<td>M2</td>
<td>Resistant</td>
<td>128</td>
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<tr>
<td>7</td>
<td>M2</td>
<td>3.6</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>M2</td>
<td>0.3</td>
<td>132</td>
</tr>
<tr>
<td>9</td>
<td>M3</td>
<td>0.5</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>M4</td>
<td>0.6</td>
<td>46</td>
</tr>
<tr>
<td>11</td>
<td>M4</td>
<td>0.1</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>M4</td>
<td>0.06</td>
<td>6</td>
</tr>
<tr>
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<td>M6</td>
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<td>18</td>
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<tr>
<td>14</td>
<td>M5</td>
<td>0.08</td>
<td>12</td>
</tr>
<tr>
<td>15</td>
<td>M5</td>
<td>0.8</td>
<td>41</td>
</tr>
<tr>
<td>16</td>
<td>ALL</td>
<td>0.01</td>
<td>23</td>
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<td>17</td>
<td>ALL</td>
<td>0.04</td>
<td>15</td>
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<tr>
<td>18</td>
<td>ALL</td>
<td>0.07</td>
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<tr>
<td>19</td>
<td>ALL</td>
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<td>80</td>
</tr>
<tr>
<td>20</td>
<td>ALL</td>
<td>0.03</td>
<td>12</td>
</tr>
<tr>
<td>21</td>
<td>ALL</td>
<td>0.01</td>
<td>21</td>
</tr>
</tbody>
</table>

* The ID 50% was calculated from the analysis of the dose-response curves.
† Number of CFU-L per 10⁴ cells plated. Results shown are the mean of quadruplicate cultures in the presence of vehicle alone (1% ethanol, vol/vol). The standard deviation for each value was less than 10% and was omitted.

debri, and then stored at ~80°C until use. To evaluate the concentration of TGF-β1 and TGF-β2 in conditioned medium, we used a sensitive radioimmunooassay (New England Nuclear Research products, Dupont, Boston, MA) and an immunoenzymatic method (R & D System, Minneapolis, MN) strictly specific for TGF-β1 and TGF-β2 detection, respectively. Each assay was performed according to the instructions of the manufacturer. To accurately quantify TGF-β1, samples were activated using a two-step acidification/neutralization method. Briefly, 100 μL samples were incubated in the presence of 10 μL 1.2 N HCl for 15 minutes at room temperature after vortexing. Then, 20 μL of 0.5 mol/L HEPES/0.72 mol/L NaOH was added to neutralize. The measured TGF-β1 value was multiplied by 1.3 to correct for the dilution. This procedure allows the quantitation of total (active + latent) TGF-β1.

Immunohistochemical analysis. Primary leukemic blasts (2 × 10⁶ cells/mL) were cultured for 12 hours in 35 × 10 mm Petri dishes (Falcon; Becton Dickinson) in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, and 2% HI-FCS at 37°C in a fully humidified 5% CO₂/95% air atmosphere in the presence of 1 μmol/L quercetin. An equivalent amount of ethanol was added to control cells. At the end of culture, cells were incubated for 10 minutes in Ca²⁺/Mg²⁺-free cold PBS containing 0.02% ethylenediaminetetraacetic acid, harvested by gently pipetting, and washed three times with phosphate-buffered saline (PBS) supplemented with 2 mg/mL of bovine serum albumin (Ficol Labs). Cytospins prepared with Shandon Cytospin (Shandon, Cheshire, UK) were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature (RT) and permeabilized with cold (~20°C) methanol for 10 minutes. Cells were then washed with PBS and incubated for 1 hour at RT with an anti-TGF-β1 mouse monoclonal antibody (MoAb; R & D System; clone TB 21; 10 μg/mL) or with an unrelated mouse MoAb isotype-matched control. Hydrogen peroxide, normal goat blocking serum, biotinylated IgGs, avidin-biotin complex, and 3-amino-9-ethylcarbazole substrate solutions were used according to the manufacturer (ABC ELITE detection system; Vector, Burlingame, CA). Cells were lightly counterstained with Mayer's hematoxylin and mounted with CrystalMount (Biomeda, Foster City, CA). In the negative controls, no immunostaining was detectable. The positivity of the reactions were independently assessed in blind fashion by two pathologists; 100 consecutive cells in three or more fields were counted. The overall intensity of the staining was arbitrarily scored as negative or positive. The results were expressed as the percentage of positively stained cells regardless of intensity.

Inhibition of TGF-β1 synthesis by antisense oligonucleotide. The sequences of the phosphorothioate oligonucleotides were as follows: TGF-β1 antisense, 5'-CCCGGAGGGCWCGGGGGA-3'; TGF-β1 sense, 5'-TCCGACGAGGCGAGGCATGGGGGA-3'; TGF-β1 antisense, 5'-GGCGAGCGAGGTAGGCGCGG-3'M (ATG ini-
tiation codon or its complement underlined in the sense and antisense sequences). Phosphorothioate oligonucleotides were purchased high performance liquid chromatography (HPLC)-purified from Med-Probe (Oslo, Norway). Cells were incubated in the presence of oligonucleotides at the final concentration of 8 μmol/L, essentially as described by others for evaluation of different genes expression. Oligonucleotides or control medium were added to the cells in IMDM in the absence of serum. Two hours later, 10% HI-FCS was added to the liquid culture. After 15 to 17 hours, oligonucleotides or control medium were added again and cells were plated as described above in a semisolid culture medium containing quercetin at various concentrations (0.001 to 10 μmol/L) or vehicle alone. CFU-Ls were scored after 6 to 10 days.

RESULTS

Inhibition of CFU-L by quercetin. As shown in Table 1, the colony formation by leukemic cells was inhibited by quercetin in all but 2 cases (patients no. 4 and 6) that were resistant to quercetin at a concentration up to 100 μmol/L. Confirming previous reports, blasts with high clonogenic efficiency were less sensitive to quercetin than those with a low clonogenic capacity. In fact, the quercetin concentration inhibiting growth by 50% (ID 50%) positively correlated with the clonogenic efficiency of leukemic cells (r = .81; n = 19; P < .001). AML-M3, -M4, and -M5 and common-ALL were highly sensitive to quercetin, with an ID 50% ranging from 0.1 to 0.01 μmol/L. AML-M1 and -M2 were less sensitive to quercetin, including the 2 resistant cases.

Secretion of TGF-β1 by leukemic blasts treated with quercetin. To test the prevention of the growth inhibitory effect of quercetin by a monoclonal anti-TGF-β1, -β2, -β3 neutralizing antibody (anti-TGF-β MoAb; Genzyme), leukemic blasts were plated, as reported above in semisolid culture medium containing the anti-TGF-β MoAb and/or quercetin at the indicated final concentrations. Matched control cultures contained unrelated MoAb. CFU-Ls were scored after 6 to 10 days.

Fig 2. Immunolocalization of TGF-β1 in AML-M5 (A and B) and in ALL (C and D) blasts cultured for 12 hours with (B and D) or without (A and C) 1 μmol/L quercetin. Original magnification x 800.
Table 2. Effect of Quercetin on TGF-β1 Expression in Blast-Enriched Bone Marrow from AML (Case No. 14) and ALL (Case No. 20) Leukemic Patients

<table>
<thead>
<tr>
<th>Cell Lineage</th>
<th>AML-M5 Untreated</th>
<th>Treated</th>
<th>ALL Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytic</td>
<td>51 ± 6†</td>
<td>95 ± 5</td>
<td>60 ± 5</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>Monocytic</td>
<td>—</td>
<td>—</td>
<td>8 ± 3</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>Erythroid</td>
<td>20 ± 4</td>
<td>60 ± 9</td>
<td>36 ± 4</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>Blast</td>
<td>20 ± 4</td>
<td>85 ± 8</td>
<td>0</td>
<td>82 ± 6</td>
</tr>
</tbody>
</table>

† Cells were cultured as outlined in Materials and Methods for 12 hours with vehicle alone (ethanol 0.1%, vol/vol) or 1 μmol/L quercetin.
† Percentage of each cell type as assessed by cytoenzymatic and immunophenotypic analysis.
† Percentage of TGF-β1 immunoreactive cells. Results are expressed as the mean ± SD of two independent quadruplicate counts.

In the presence of 1 μmol/L quercetin, ALL blasts showed a peak of TGF-β1 release after 12 hours of culture (Fig 1B), followed by a decrease at 24 hours to about 50% of the peak value. The number of ALL blasts after 48 hours of culture was 80% of the initial number of cells plated both in quercetin-treated and in control cultures.

The concentration of TGF-β2 was below the detection levels of the enzyme-linked immunosorbent assay (ELISA) sensitivity in all samples tested (data not shown).

Immunolocalization of TGF-β1. To determine if quercetin enhances the intracellular levels of TGF-β1, immunohistochemical assays using an anti–TGF-β1 MoAb were performed on blasts from 1 patient with AML-M5 (patient no. 14) and 1 with ALL (patient no. 20) cultured for 12 hours with or without 1 μmol/L quercetin. As shown in Fig 2, in both cases quercetin enhances the intracellular content of anti–TGF-β1 immunoreactive materials. The immunoreactive product was localized in the cytoplasmic compartment. The blasts of the patient with AML-M5 expressed higher basal levels of TGF-β1 than the blasts of the patient with ALL. The presence of vehicle (ethanol) in the culture medium did not modify the basal level of TGF-β1 expression (data not shown). As shown in Table 2, the percentage of blasts in leukemic bone marrow preparations was ≈95%. With due caution given the low number of cells observed, it could be noted that in the remaining contaminating population (1) most segmented and immature granulocytes showed constitutive expression of TGF-β1 not modified by quercetin treatment and (2) in both monocytic and erythroid cell lineages quercetin increased the percentage of TGF-β1 immunoreactive cells.

Effects of antisense TGF-β1 oligonucleotide and of anti–TGF-β1, β2, β3 neutralizing MoAb on the inhibition effect of CFU-L by quercetin. The aim of our study was to evaluate the role of TGF-β1 in quercetin-dependent inhibition of CFU-L formation. To inhibit TGF-β1 production we used an antisense oligonucleotide. An anti–TGF-β3 MoAb was used to neutralize TGF-β3 produced by leukemic blasts.

Fig 3. CFU-ALL inhibition by quercetin alone (○) or in the presence of 8 μmol/L antisense (△) or missense (□) TGF-β1 oligonucleotides is dependent on the concentration of quercetin. Cells (10⁴ cells) were treated with quercetin and TGF-β1 oligos as reported in Materials and Methods. Results shown are the mean of four replicates for each variable. Standard deviations were less than 10% and were omitted. The number of control CFU-ALL was 41 ± 3 for 10⁴ cells plated.

Fig 4. TGF-β1 production by the same ALL blasts shown in Fig 3 cultured for 12 hours in the presence of vehicle alone (none) or 1 μmol/L quercetin (Q) or 1 μmol/L quercetin plus 8 μmol/L antisense TGF-β1 oligos (Q + antisense). At the end of the culture period, the conditioned media were harvested and the amount of total (activated + latent) TGF-β1 was evaluated by a radiolimmunoassay as reported in Materials and Methods.
preliminary dose/response curve showed that oligonucleotide concentrations greater than 10 μmol/L were toxic (data not shown). A final concentration of 8 μmol/L was used in all experiments. To show that antisense TGF-β1 oligonucleotides could inhibit quercetin-induced cytokine production, 1 case of flavonoid-sensitive ALL (ID 50% ~0.4 μmol/L) in which antisense TGF-β1 oligonucleotides prevented the action of quercetin (Fig 3) was analyzed for the production of TGF-β1 in the presence of TGF-β1 antisense oligonucleotides. As shown in Fig 4, TGF-β1 antisense oligonucleotides could prevent the quercetin-induced cytokine production.

Figure 5 shows the effect of TGF-β1 antisense oligonucleotide and neutralizing anti-TGF-β MoAb on quercetin-dependent inhibition of leukemic blasts growth. In 1 AML-M4 (patient no. 10, Fig 5A) and in 1 ALL (patient no. 19, Fig 5B) the addition of antisense but not sense (data not shown) or missense TGF-β1 oligonucleotides prevented the inhibitory action of quercetin. The effect of the TGF-β1 antisense oligonucleotides was proportional to the flavonoid concentrations. The prevention of quercetin effects by anti-TGF-β MoAb was concentration dependent, resulting in a total prevention at an antibody concentration of 100 μg/mL. In the quercetin-resistant AML-M2 case (patient no. 6, Fig 5C), TGF-β1 antisense oligonucleotides produced an inhibition greater than 50% on CFU-L formation that was independent of the quercetin concentration used. Moreover, anti-TGF-β MoAb inhibited in a dose-dependent manner CFU-L formation, in this case with a maximum activity at 100 μg/mL anti-TGF-β MoAb. In the quercetin-resistant AML-M1 case (patient no. 4, Fig 5D), neither TGF-β1 antisense oligonucleotides nor anti-TGF-β MoAb produced any evident effect on CFU-L. An isotype-matched MoAb was used as a control for anti-TGF-β MoAb; no effects on CFU-L formation was observed even at the highest concentration used (100 μg/mL; data not shown).

The addition of TGF-β1 antisense oligonucleotides prevented the inhibitory effect of 1 μmol/L quercetin in all the quercetin-sensitive cases (Fig 6). The extent of the effect of TGF-β1 antisense oligonucleotides varied among the patients and did not seem to be related to the sensitivity of the leukemic blasts to quercetin.

**DISCUSSION**

Although originally described as a negative regulator of normal myeloid progenitor cell growth, recent studies have shown that TGF-β1 is a bifunctional regulator of hematopoietic cells. TGF-β1 can either inhibit or stimulate the growth of murine and human hematopoietic progenitor cells; in particular, TGF-β1 is a potent inhibitor of primitive hematopoietic progenitors, whereas the growth of more committed progenitor cells is either not affected or stimulated by TGF-β1.

Previous studies suggested that the growth of human leukemic cell lines and of the vast majority of cells from AML patients was inhibited by TGF-β1. The data presented here show that the bioflavonoid quercetin inhibits the growth of AML and ALL blasts in vitro by the induction of TGF-β1 production. This finding is based on the following observations: (1) quercetin enhances the intracellular content and the secretion of TGF-β1 by sensitive leukemic blasts; (2) the inhibitory action of quercetin can be partially blocked by TGF-β1 antisense oligonucleotides and can be almost totally abolished by neutralizing anti-TGF-β1, -β2, -β3 MoAb.
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Because it has been reported that retinoic acid, which also induces TGF-β1 production, is able to upregulate TGF-β1 receptor expression on the HL-60 cell line, it seems interesting to investigate whether quercetin can act in a similar fashion.

The differences between TGF-β1 antisense oligonucleotides and anti-TGF-βs MoAb in preventing the effect of quercetin could be explained as follows. (1) Neutralizing antibodies but not antisense oligonucleotides can block the effect of TGF-β1 already present in quercetin untreated cells as shown by immunohistochemistry. If this is true, quercetin could stimulate the secretion in addition to the synthesis of TGF-β1. (2) TGF-β1 antisense oligonucleotides are not able to completely inhibit TGF-β1 synthesis, as shown in the case of the ALL presented in Fig 4. (3) The neutralizing anti-TGF-βs MoAb used in this study is able to neutralize TGF-β2 in addiction to TGF-β1. It is then possible that quercetin could stimulate the release of TGF-β3 as well. TGF-β2 is not involved in the quercetin-dependent growth inhibition because it is not produced by quercetin-treated leukemic blasts.

Two patients (nos. 4 and 6) behaved differently from all other patients. Particularly, in both cases leukemic blasts were resistant to the growth-inhibitory effect of quercetin. In patient no. 4, because quercetin induces TGF-β1 production in leukemic blasts (data not shown), this resistance could be dependent on the unresponsiveness of these cells to TGF-β1. The growth of leukemic blasts from patient no. 6 was stimulated by TGF-β1. Actually, antisense oligonucleotides, blocking the synthesis of TGF-β1, induced a reduction of more than 50% of the clonogenic activity in this case. Furthermore, 100 µg/mL of neutralizing anti-TGF-βs MoAb inhibited almost abolished CFU-L activity (Fig 5C). In this case, quercetin did not significantly increase the release of TGF-β1 above background (data not shown) and was unable either to stimulate or inhibit the leukemic cell growth. These data are in accordance with a recent report about heterogeneous responses of leukemic cell lines and primary leukemic blasts to the growth-regulatory action of TGF-β1.

Although the mechanism of the antiproliferative activity of quercetin remains to be fully clarified, there is evidence suggesting that the action of this substance is probably mediated by its interaction with the so-called type II EBS, Indeed, our data indicate that the quercetin ID 50% in sensitive leukemic cells is compatible with the dissociation constant (kd) of type II EBS in leukemic blasts. This possibility is also supported by the observation that the antiestrogen tamoxifen, which binds to type II EBS, induces TGF-β1 production in estrogen-receptor-positive MCF-7 human breast cancer cells as well as in estrogen-receptor-negative

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Fig 6. Antisense TGF-β1 oligonucleotides prevent the inhibitory action of quercetin (1 µmol/L) on CFU-L. Cells were plated at 10⁶ cells/mL, as reported in Materials and Methods, in the presence of quercetin alone (Q) or in combination with 8 µmol/L antisense (aS) or missense (mS) oligonucleotides. The number of control CFU-L were reported in Table 1. Results were expressed as the mean of quadruplicate cultures. Standard deviations were less than 10% and were omitted. Patient nos.: panel M1-M2, (☐) 1, (☐) 2, (☐) 3, (☐) 4, (☐) 5, (☐) 6, (☐) 7; panel M3-M5, (☐) 8, (☐) 9, (☐) 10, (☐) 11, (☐) 12, (☐) 13, (☐) 14, (☐) 15; panel ALL, (☐) 16, (☐) 17, (☐) 18, (☐) 19, (☐) 20, (☐) 21.
human fetal fibroblasts. For these reasons, it seems interesting to investigate whether tamoxifen could inhibit leukemic cell growth as well.

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Quercetin inhibits the growth of leukemic progenitors and induces the expression of transforming growth factor-beta 1 in these cells

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