Synergistic Growth Inhibitory and Differentiating Effects of Trimidox and Tiazofurin in Human Promyelocytic Leukemia HL-60 Cells

By Thomas Szekeres, Monika Fritzter, Herbert Strobil, Kamran Gharehbaghi, Gabriele Findeniog, Howard L. Elford, Christian Lhotka, Hans J. Schoen, and Hiremagalur N. Jayaram

Increased ribonucleotide reductase (RR) activity has been linked with malignant transformation and tumor cell growth. Therefore, this enzyme is considered to be an excellent target for cancer chemotherapy. We have examined the effects of a newly patented RR inhibitor, trimidox (3,4,5-trihydroxybenzohydroxamidoxime). Trimidox inhibited the growth of human promyelocytic leukemia HL-60 cells with an IC_{50} of 35 μmol/L. Incubation of HL-60 cells with 50 μmol/L trimidox for 24 hours decreased deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) pools to 24% and 39% of control values, respectively. Incubation of HL-60 cells with 20 to 80 μmol/L trimidox even up to a period of 4 days did not alter the distribution of cells in different phases of cell cycle. Sequential incubation of HL-60 cells with trimidox (25 μmol/L) for 24 hours and then with 10 μmol/L tiazofurin (an inhibitor of inosine monophosphate dehydrogenase) for 4 days produced synergistic growth inhibitory activity, and the cell number decreased to 16% of untreated controls. When differentiation-linked cell surface marker expressions were determined in cells treated with trimidox and tiazofurin, a significantly increased fluorescence intensity was observed for the CD 11b (2.9-fold), CD 33 (1.9-fold), and HLA-D cell surface antigens. Expression of the transferrin receptor (CD71) increased 7.3-fold in cells treated with both agents, compared with untreated controls. Our results suggest that trimidox in combination with tiazofurin might be useful in the treatment of leukemia.

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We examined the growth inhibitory and cytotoxic effects of trimidox in human promyelocytic leukemia HL-60 cells and tested the effects of trimidox on ribo- and deoxyribonucleoside triphosphate concentrations. In addition, we have studied the effects of trimidox on the cell cycle distribution of HL-60 cells to evaluate its specificity on different phases of cell cycle.

Tiazofurin, an S-phase specific agent, acts through its metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD), an analog of NAD, which inhibits inosine 5’-monophosphate (IMP) dehydrogenase activity resulting in depletion of guanylates, including guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP). Tiazofurin down regulates c-myc and Ha-ras oncogene expression and causes differentiation of human leukemia cells. Tiazofurin was shown to be effective in the treatment of patients with refractory chronic granulocytic leukemia in blast crisis. Both trimidox and tiazofurin are capable of lowering intracellular deoxyribonucleotide levels, although acting through different mechanisms. We have now examined whether a combination of these agents exhibits synergistic growth inhibitory and cell differentiation properties.

MATERIALS AND METHODS

Drugs. Didox and Trimidox were synthesized as described, and tiazofurin was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

Cell culture. Human promyelocytic leukemia HL-60 cell line was a gift from Dr R.C. Gallo (National Cancer Institute). CCRF-CEM and Reh human lymphocytic leukemia cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS; Grand Island Biological Co, Grand Island, NY) and with 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO2. Cell counts were determined using the microcell counter CC-108 (Sysmex, Kobe, Japan). Cells growing in logarithmic phase of growth were used for all the studies described below.

Growth inhibition assay. HL-60 cells were seeded in 25-cm2 flasks at a cell density of 1 × 10^5 cells per milliliter and incubated with various drug concentrations (1-100 μmol/L). Cells were counted and IC_{50} concentrations were determined 4 days later.

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SYNERGISTIC ACTIVITY OF TRIMIDOX AND TIAZOFURIN

Chemosensitivity assay (MTT-assay). HL-60 cells were seeded at a concentration of $2 \times 10^4$ cells per milliliter in 96-well microtiter plates and incubated with various concentrations (1-1000 mmol/L) of trimidox for 48 hours in supplemented RPMI 1640 medium. The reduction of the tetrazolium compound (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide; MTT) was determined with an assay kit (Promega, Madison, WI) according to the supplier's manual. The absorbance was recorded using a Beckman Biomek 1000 Work Station (Beckman, St. Louis, MO).

High performance liquid chromatography (HPLC) determination of ribonucleotides. HL-60 cells ($1 \times 10^6$) in culture were treated with 100 mmol/L trimidox for 24 hours. Cells were collected by centrifugation for 5 minutes at 400g, extracted with cold 10% trichloroacetic acid, and neutralized with 0.5 mol/L tri-n-octylamine in freon, and the ribonucleotide concentrations were determined as described.

Determination of deoxyribonucleoside triphosphates. Cells ($5 \times 10^7$) in culture were treated with 25 or 50 mmol/L trimidox for 24 hours, and the cells were centrifuged at 400g, resuspended in 100 mL phosphate-buffered saline (PBS), and extracted with 6 mL of cold 90% (wt/vol) trichloroacetic acid. Samples were allowed to stand on ice for 30 minutes, and then neutralized with 0.5 mol/L tri-n-octylamine in freon, and the deoxyribonucleoside triphosphate concentrations were determined according to the method of Garrett and Santi.

Cell surface phenotyping. A panel of monoclonal antibodies (MoAbs) with the following specifications were used for phenotyping precursor cells: anti--HLA-D (VID-1); granulocytic/monocytic lineage: CD 11b (VIM-12), CD 33 (MY-9), and CD 71 (transferrin receptor). The specificity and reaction pattern of these MoAbs have previously been reported.

The binding of MoAbs was assessed by indirect immunofluorescence staining with fluorescein isothiocyanate (FITC)-conjugated sheep F(ab')2 fragments of anti-mouse IgG and IgM antibodies. Normal mouse ascitic fluid served as control in all experiments. Nonspecific Fc-receptor binding was blocked by adding 1% (final concentration) of human gammaglobulin (Serogam; Serotherapeutics Institut, Vienna, Austria) to the cell suspension. Samples were analyzed with a fluorescence-activated cell sorter (FACS 440; Becton Dickinson, Sunnyvale, CA).

Flow cytometric analysis of total DNA content and incorporation of bromodeoxyuridine. An aliquot of cells ($1 \times 10^6$) was labeled by treatment with 10 mmol/L bromodeoxyuridine (BrdU) for 30 minutes at 37°C. Cells were then centrifuged for 5 minutes at 400g in a Beckman TJ-6 tabletop centrifuge, washed three times with PBS, and fixed in 70% ethanol. Cells were removed from ethanol, resuspended in 1.5 mol/L HCl, incubated at 25°C (room temperature) for 20 minutes, and washed with PBS as described. An anti-BrdU antibody (20 µL; Becton Dickinson) was added, and the mixture was incubated for 30 minutes at 4°C, followed by incubation for another 30 minutes with 20 µL of fluorochrome FITC-conjugated F(ab'); goat anti-mouse IgG. Finally, the cells were resuspended in 1 mL PBS containing 10 µmol/L propidium iodide (Polyscience, Warrington, PA). Flow cytometric analysis was performed using a FACS 440 system (Becton Dickinson), wherein double-labeled cells were excited at 488 nm. The red fluorescence from propidium iodide was collected through a 620-nm pass filter and recorded as a measure of total DNA content, whereas the green fluorescence from FITC was collected through a 530-nm filter and recorded as a measure of incorporated BrdU. The resulting data were accumulated from a bivariate channel distribution showing the distribution of DNA (red fluorescence) and BrdU (green fluorescence) among the cell population. Routinely, 20,000 cells were analyzed, and the cell cycle distribution was calculated by gating analysis of the bivalent histogram.

Statistical analysis of synergism. The calculation of dose response curves and determination of synergism were performed using software designed by Chou and Talalay.

RESULTS

Growth inhibitory and cytotoxic effects of trimidox on HL-60 cells. Human promyelocytic leukemia HL-60 cells were incubated with various concentrations of trimidox, didox,
and hydroxyurea for 4 days, and the effect on cell growth was determined. Trimidox exhibits an IC₅₀ of 35 µmol/L, whereas didox and hydroxyurea inhibited cell proliferation with IC₅₀ concentrations of 54 and 73 µmol/L, respectively (Fig 3). When human leukemia HL-60, CCRF-CEM, or Reh cells were incubated with various concentrations of trimidox for 2 days, IC₅₀ of 61 µmol/L, 89 µmol/L, and 104 µmol/L, respectively, were obtained by the MTT assay (Table 1).

Effect of trimidox on ribonucleoside triphosphate concentration in HL-60 cells. Cells were incubated with 100 µmol/L trimidox for 24 hours, and ribonucleotide levels were analyzed. Trimidox did not significantly alter the concentration of any of the ribonucleotides (data not shown).

Effect of trimidox on deoxyribonucleoside triphosphate levels in HL-60 cells. HL-60 cells were incubated with 25 or 50 µmol/L trimidox for 24 hours, and the intracellular deoxyribonucleoside triphosphate concentrations were measured as described in Materials and Methods. Treatment with 50 µmol/L trimidox resulted in a significant decrease in intracellular concentrations of deoxycytidine triphosphate (dCTP; 39% of control) and dGTP (24% of control; Table 2). The deoxythymidine triphosphate (dTTP) level was not significantly changed, whereas intracellular deoxyadenosine triphosphate (dATP) concentration was increased by 25% and 37% of control after incubation with 25 and 50 µmol/L trimidox, respectively. In cells treated with tiazofurin and trimidox, dCTP and dGTP pools decreased below the level of detectability of this assay.

**Table 1. Cytotoxicity of Trimidox to Human Leukemic Cells**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>IC₅₀ (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>Promyelocytic leukemia</td>
<td>61</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>Lymphocytic leukemia</td>
<td>89</td>
</tr>
<tr>
<td>Reh</td>
<td>Lymphocytic leukemia</td>
<td>104</td>
</tr>
</tbody>
</table>

Cells were exposed to 5-160 µmol/L concentrations of trimidox for 2 days at 37°C in an atmosphere of 95% air and 5% CO₂, MTT assay was then performed according to the method described in Materials and Methods.

**Table 2. Effect of Trimidox on Deoxyribonucleotide Pools in HL-60 Cells**

<table>
<thead>
<tr>
<th>Concentration of Deoxyribonucleotides (µmol/L ± SE)</th>
<th>Control</th>
<th>25 µmol/L</th>
<th>50 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>8.1 ± 0.8</td>
<td>10.1 ± 0.9*</td>
<td>11.1 ± 1.9*</td>
</tr>
<tr>
<td>(100)</td>
<td>(125)</td>
<td>(137)</td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>14.0 ± 2.3</td>
<td>14.0 ± 1.3</td>
<td>5.5 ± 1.2*</td>
</tr>
<tr>
<td>(100)</td>
<td>(100)</td>
<td>(38)</td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td>28.8 ± 1.8</td>
<td>27.8 ± 0.9</td>
<td>28.0 ± 4.7</td>
</tr>
<tr>
<td>(100)</td>
<td>(104)</td>
<td>(104)</td>
<td></td>
</tr>
<tr>
<td>dGTP</td>
<td>9.5 ± 0.6</td>
<td>8.1 ± 0.5*</td>
<td>2.3 ± 0.4*</td>
</tr>
<tr>
<td>(100)</td>
<td>(86)</td>
<td>(24)</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE of four determinations. Cells were treated with 25 or 50 µmol/L trimidox for 24 hours at 37°C and processed, and deoxyribonucleotide levels were determined as described in Materials and Methods. Values in parentheses are percentages of control cells.

* Significantly different from control values (P < .05).

**Effect of trimidox on cell cycle distribution of HL-60 cells.** To examine whether trimidox inhibits cell proliferation by blocking the cell cycle transition, HL-60 cells were seeded at a density of 1.0 × 10⁶ cells per milliliter and incubated with various concentrations (20, 40, or 80 µmol/L) of trimidox. After 4 days, cells were harvested and incubated with BrdU as described in Materials and Methods. Cells were labeled with FITC; and the respective distribution into phases of cell cycle was determined using a FACS-analyzer.

Trimidox incubation had no effect on the distribution of cells into their respective phases of cell cycle (data not shown). As trimidox does not show cell cycle specificity, a combined treatment with cell cycle-specific drugs seemed to be a promising approach. Therefore, we incubated HL-60 cells with trimidox first and then with tiazofurin, an S-phase-specific antimetabolite.²⁴

**Influence of combination of trimidox and tiazofurin on the growth of HL-60 cells.** After incubation of HL-60 cells with 25 µmol/L trimidox for 24 hours, cells were washed in drug-free medium and incubated with 10 µmol/L tiazofurin for 4 days, and then the cell number was quantitated. Trimidox and tiazofurin each decreased cell proliferation to 55% of untreated control cell proliferation. However, after sequential incubation of HL-60 cells with trimidox first and tiazofurin second, the cell number reduced to 16% of untreated control cell number, indicating synergistic cytotoxicity. Similar synergistic effects were observed with different drug concentrations. Combination index was calculated according to the computer program of Chou and Talalay²², and it was below 1 for all combinations tested, indicating synergistic growth inhibition (Table 3).

**Combined effect of trimidox and tiazofurin on cell surface marker expression of HL-60 cells.** We examined the effect of cell surface markers on the expression of cell differentiation (granulocyte/monocyte) CD 33, CD 11b, and HLA-D leukocyte surface antigens, as well as on the expression of the transferrin receptor (CD 71) in control cells and in cells...
treated with either compound alone or in combination. As shown in Fig 4, the expression of cell surface markers significantly increased in cells concurrently incubated with trimidox and tiazofurin. The mean fluorescence intensity of CD 33 increased 1.9-fold, and CD 11b increased in cells concurrently incubated with trimidox and tiazofurin. The mean fluorescence intensity of CD 11b showed a 2.9-fold enhancement of fluorescence in cells incubated with both agents compared with control cells. When HLA-D expression was determined, mean fluorescence intensity increased from 52 in control to 92 in cells incubated with both compounds. However, the expression of the transferrin receptor increased 7.3-fold in HL-60 cells after sequential treatment with trimidox and tiazofurin.

DISCUSSION

The activities of ribonucleotide reductase and IMP dehydrogenase were amplified in malignant cells. These enzymes were, therefore, suggested to be excellent targets for chemotherapy.

We have examined the effects of a newly patented compound, trimidox, on deoxyribonucleotide pools and on cell cycle specificity. Trimidox inhibited ribonucleotide reductase activity both in vitro and in vivo in L1210 cells and was active against L1210 leukemia in mice. In this report we demonstrate that trimidox significantly lowers dCTP and dGTP levels in HL-60 cells. Continuous incubation of HL-60 cells with 35 μmol/L trimidox for 4 days inhibited cell growth to 50% of control cell growth, whereas incubation with concentrations up to 80 μmol/L did not have any effect on the cell cycle distribution of HL-60 cells.

Trimidox was earlier shown to have free radical scavenging capacity and was suggested to be an iron chelator. Depletion of intracellular iron increases the number of transferrin receptors, which can explain the upregulation of CD 71 receptor in cells treated with trimidox and tiazofurin. However, addition of 2 mg/mL iron-saturated transferrin could not significantly alter the cytotoxicity of trimidox in HL-60 cells (data not shown), indicating that the iron-chelating capacity may not be mainly responsible for trimidox-exerted growth inhibition of HL-60 cells.

Our finding that trimidox—unlike hydroxyurea—does not show S-phase specificity suggests that trimidox and hydroxyurea should have different mechanisms of action. This is in line with data of Thian et al, who showed that didox (a first generation polyhydroxy-substituted benzohydroxamate, which is active against hydroxyurea-resistant L1210 cells) did not alter the cell cycle phase distribution of L1210 cells. Mann et al demonstrated that the M1 subunit of ribonucleotide reductase was constitutively expressed in rapidly cycling HL-60 cells and was downregulated in quiescent or differentiated cells. However, as the enzyme inhibitory activity of polyhydroxy-substituted benzohydroxamates seems to correlate with their free radical scavenging capacity, future studies should elucidate trimidox's effect on the enzyme subunits.

Lack of significant cell cycle specificity renders trimidox suitable for sequential combination chemotherapy with a number of anticancer drugs. Cancer cells could be pretreated with trimidox and then exposed to cell cycle-specific agents without antagonizing their effect on cell cycle. We have demonstrated synergistic growth inhibition when HL-60 cells were sequentially treated with trimidox first and tiazofurin, an S-phase specific inhibitor of IMP dehydrogenase, second. Figure 2 shows the biochemical actions of trimidox and tiazofurin on deoxyribonucleotide levels. As stated by Jackson, deoxyribonucleotide pools need to be properly balanced to support DNA synthesis, and Cory and Carter have

<p>| Table 3. Growth Inhibitory Effect of Trimidox and Tiazofurin on HL-60 Promyelocytic Leukemia Cells |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (μmol/L)</th>
<th>Cell No. (%) of control</th>
<th>Predicted Value</th>
<th>Combination Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimidox (A)</td>
<td>6</td>
<td>80</td>
<td>38</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>55</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>7</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td>Tiazofurin (B)</td>
<td>10</td>
<td>55</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>48</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>37</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td>Trimidox + tiazofurin</td>
<td>6</td>
<td>31</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>31</td>
<td>38</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>18</td>
<td>30</td>
<td>0.41</td>
</tr>
<tr>
<td>Trimidox + tiazofurin</td>
<td>25</td>
<td>10</td>
<td>30</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16</td>
<td>30</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3</td>
<td>3.5</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Data are means of three or more determinations, and SE values were within 5%. Predicted values are (A × B)/100 (%).

* Combination index was calculated according to the method of Chou and Talalay.
† Synergism: Combination index <1.
suggested that a linear reduction in deoxynucleoside triphosphate levels could produce a sigmoidal decrease in the rate of DNA synthesis. This might explain the synergistic action of trimidox and tiazofurin.

Tiazofurin induces differentiation of human promyelocytic leukemia HL-60 and human myelogenous leukemia K 562 cells; thus, we examined whether the synergistic effects observed with trimidox and tiazofurin might be due to their effect on cell differentiation.1,2,3,4,5 The expression of differentiation-linked cell surface markers was examined in cells treated with trimidox and tiazofurin. We found a significant increase in CD 33, CD 11b, and HLA-D antigen expression in cells incubated with both agents, compared with untreated controls. These results, at least in part, suggest that the combination of these two compounds could lead to differentiation in HL-60 cells.

In summary, treatment with trimidox in combination with tiazofurin might offer an additional promising option in cancer chemotherapy.

REFERENCES

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Synergistic growth inhibitory and differentiating effects of trimidox and tiazofurin in human promyelocytic leukemia HL-60 cells

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