Synergistic Cytotoxic Effect of Azidothymidine and Recombinant Interferon Alpha on Normal Human Bone Marrow Progenitor Cells

By Ellin Berman, Rosemarie Duigou-Osterndorf, Susan E. Krown, Michael P. Fanucchi, Joseph Chou, Martin S. Hirsch, Bayard D. Clarkson, and Ting-Chao Chou

Azidothymidine (AZT) and interferon alpha (IFN-α) are among the drugs showing strong in vitro activity against the human immunodeficiency virus type-1 (HIV-1). Each drug, however, has significant toxicity against normal marrow progenitor cells that frequently proves dose-limiting in patients. In this study, AZT and recombinant IFN-α2a (rIFN-α2a) were tested as single agents and in combination against normal myeloid (CFU-GM) and erythroid (BFU-E) colony forming cells in a standard methylcellulose culture assay. The data were analyzed using a quantitative computerized analysis based on the median-effect principle and the isobologram equation as described by Chou and Talalay (Adv Enz Regul 22:27, 1984). The ED₅₀ for BFU-E and CFU-GM inhibition was then compared with previously measured in vivo plasma levels of each drug and the ED₅₀ for the anti-HIV-1 effect in vitro. We demonstrate that (a) the drugs are strongly synergistic in inhibiting marrow progenitor cell growth and that this synergism occurs at drug levels that are within the range of measured plasma levels in phase I clinical trials, (b) BFU-E are more sensitive than CFU-GM to the inhibiting effects of AZT, rIFN-α2a or both drugs in combination, (c) the drug concentrations in combination that synergistically inhibit bone marrow progenitors are much higher than those required to inhibit HIV-1 replication in vitro, and (d) the anti-HIV-1 effect for the combination of AZT and rIFN-α2a was clearly superior to the effect of AZT or rIFN-α2a alone as indicated by the combination index and the dose-reduction index. These data suggest that substantially lower doses of AZT and rIFN-α than those currently being tested in clinical trials might not only maintain a strong synergistic anti-HIV-1 effect but might also avoid significant hematologic toxicity.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Hematopoietic progenitor cell assay. Bone marrow samples were obtained from normal volunteers after informed consent was obtained. Marrow was diluted with sterile McCoy’s media (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), and the mononuclear cell population separated by Ficoll-Hypaque gradient (1.07 g/mL) centrifugation. Mononuclear cells were incubated with the selected drug combination and the limiting plasma levels in phase I clinical trials.

From the Memorial Sloan-Kettering Cancer Center, New York; Harvard College, Cambridge, MA; and Massachusetts General Hospital, Harvard Medical School, Boston.

Address reprint requests to Ellin Berman, MD, Leukemia Service, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.
plated in triplicate at a concentration of 2 × 10^6 cells/mL. Cell cultures consisted of 1 mL of Iscove's modified Dulbecco's medium (IMDM) (GIBCO) containing 24% FCS, 0.8% deionized bovine serum albumin (Sigma Chemical, St Louis), 10^-4 mol/L 2-mercaptoethanol (Sigma), 10% conditioned medium (prepared from the Mo T lymphoblast cell line),^13 0.2 μmol/L hemin (ferric chloride protoporphyrin 1×) (Sigma), and methylcellulose at a final concentration of 1.3% in 35-mm Lux tissue culture dishes (Miles Scientific, Naperville, IL). One unit of partially purified human urinary erythropoietin (specific activity 63 U/mg, Toyobo Biochemical, New York) was added at the initiation of culture. Culture plates were incubated in a humidified atmosphere of 5% CO2 in air for 14 days and scored using an inverted microscope. Aggregates ≥40 cells were considered colonies; those containing hemoglobinized cells were counted as BFU-E.

**Drugs.** AZT was supplied by the Burroughs Wellcome Co (Research Triangle Park, NC) and reconstituted in NaCl 0.9% prior to these experiments. Human rIFN-α2a (specific activity 2 × 10^8 U/mg protein) was supplied by Hoffmann La-Roche Inc (Nutley, NJ) and reconstituted in NaCl 0.9%.

**Data analysis.** The median-effect principle and isobologram equation described by Chou and Talalay^13,14^ were used to analyze combined drug effects. Using this method, the dose-effect curve was plotted for each agent and for multiple dilutions of a fixed-ratio combination by using the median-effect equation:

\[ f_i/f_a = (D/D_a)^m \]

where \( D \) was dose, \( D_a \) was the dose required for 50% effect (eg, 50% inhibition of growth), \( f_i \) was the fraction affected by dose D (eg, 0.9 if growth activity was inhibited by 90%), \( f_a \) was the unaffected fraction, and \( m \) was an index of the dose-effect curve. The dose-effect curve was plotted by using a logarithmic conversion of this equation which determined the m and \( D_a \) values. The dose (\( D_m \)) required for a given degree of effect (\( f_i \)) was then calculated by

\[ D_m = D_a [f_i/(1 - f_i)]^{1/m} \]

A combination index (CI) for drugs with different modes of action was then determined with the conservative isobologram equation:

\[ CI = \left( \frac{(D_1)}{(D_2)} \right)^{1/d} \]

where \( D_1 \) was the dose of agent 1 required to produce x percent effect alone, and \( D_2 \) was the dose of agent 1 required to produce the same x percent effect in combination with \( D_3 \). Similarly, \( D_2 \) was the dose of agent 2 required to produce the same effect alone. Different values of CI were obtained by solving the equation for different values of \( f \) (eg, different degrees of inhibition of growth). CI values of < 1 indicate synergism, values > 1 indicate antagonism, and values equal to 1 indicate additive effects.\(^{13,14}\) Computer programs\(^{13,14}\) based on the median effect plot and the CI equation were used for data analysis.

The same data for combination studies can also be analyzed using the classical isobologram equation for drugs assumed to have similar or mutually exclusive modes of action in which the above CI equation will be the sum of the first two terms (\( \alpha = 0 \); the third term is dropped). The CI values obtained from both classical (\( \alpha = 0 \)) and conservative (\( \alpha = 1 \)) isobologram equations are presented in this report.

**Calculation of selectivity (therapeutic) index.** The selectivity index indicates the degree of inhibition of one assay relative to another. This is calculated by dividing the ED_{50} of the first assay by ED_{50} of the second.

**RESULTS**

Suppressive effects of AZT and rIFN-α2a as single agents on normal marrow BFU-E and CFU-GM. In this assay system, we chose concentrations of AZT that correspond to plasma levels achievable in vivo that have been reported to be in the range of 0.5 to 4 μmol/L.\(^{5,17}\) We therefore tested 0.5, 1.0, 2.0, and 4.0 μmol/L in vitro. Serum interferon levels are more difficult to interpret because of the variability noted among different patients treated at the same dose level.\(^{18,19}\) We therefore chose test concentrations of 100, 200, 500, and 2,000 U/mL, which are within a broad range of peak serum interferon levels reported with doses between 3 to 36 × 10^5 U.\(^{18,19}\)

Table 1 shows the percent inhibition of BFU-E and CFU-GM from two (BFU-E) or three (CFU-GM) different experiments in which the marrow was incubated with different concentrations of AZT, rIFN-α2a, and the two drugs in combination. AZT had approximately equal inhibitory activity against BFU-E and CFU-GM. At higher concentrations of rIFN-α2a, however, BFU-E were inhibited to a greater degree than CFU-GM.

AZT and rIFN-α2a were then combined at an ED_{50} equipotency ratio of 1:50 and serially diluted. At the lowest concentration tested (0.2 μmol/L AZT plus 10 U/mL rIFN-α2a), approximately 50% of BFU-E colony growth was inhibited. Increasing the concentration of both drugs at a constant ratio demonstrated a dose-dependent suppression of both BFU-E and CFU-GM formation.

**Quantitative analysis of drug interactions.** We chose to analyze the pattern of inhibition observed with each drug alone and in combination using the median effect principle described by Chou and Talalay.\(^{13,14}\) The parameters required for calculating the CI described in Materials and Methods are shown in Table 2. Both the classical isobologram equation (\( \alpha = 0 \)) and conservative isobologram equation (\( \alpha = 1 \)) were used in these calculations.

CI values < 1 (drug synergism) were noted for BFU-E at 50% and 75% levels of inhibition (CI values, 0.83 and 0.29, respectively). At the ED_{50} dose level, moderate antagonism was indicated (CI value, 1.157). CI values were < 1 (eg, 0.146 to 0.308) for all levels of CFU-GM inhibition calculated.

**Comparison of plasma drug levels, marrow inhibition, and inhibition of HIV-1 replication.** The plasma levels of each drug achievable in patients, the concentration of each drug alone and in combination required for 50%, 75%, and 90% inhibition of HIV-1 replication,\(^{8}\) and the concentration of each drug in combination required to suppress 50%, 75%, 90%, and 95% of normal marrow BFU-E and CFU-GM are compared in Table 3. In our study the peak plasma levels of AZT occurred at one hour and were in the range of 1.68 μmol/L; higher levels up to 4 to 6 μmol/L have been
SYNERGISTIC EFFECT: AZIDOTHYMIDINE AND INTERFERON

Afl (zmoI/L) when it was combined with AZT (8.15 rIFN-a2a(U) (zmol/L) rIFN-a2a(U) AZT a2a).

hours and were in the range of 20 to 283 U/mL (median, 167 intramuscular injection of 9 x 106 U occurred at about four reported by others.5 Peak plasma levels of rIFN-α2a after an approximate 18-fold reduction in AZT concentration and 70-fold reduction in rIFN-α2a concentration, levels that should have minimal effects on myeloid and erythroid precursors.

Similarly, the same experiment (day 14) indicated that AZT 0.012 μmol/L plus rIFN-α2a 19 U/mL inhibited HIV-1 replication by 95% which represents a ninefold and 56-fold dose-reduction, respectively, when compared with the percent inhibition produced by each drug alone. The peak plasma and serum levels, respectively, for both AZT (1.68 μmol/L) and rIFN-α2a (167 U/mL) were 140-fold and nine-fold, respectively, higher than those required for inhibiting HIV-1 replication by 95% in vitro in combination after a 14-day exposure.

The strong degree of synergism of these drugs in combination against HIV replication is outlined in Table 3. In the day 14 HIV-1 replication experiment, 0.08 μmol/L of AZT was required to inhibit 90% of viral growth (ED90). Similarly, 517 U/mL of rIFN-α2a was required for 90% viral growth inhibition. When the two drugs were combined, only 0.0045 μmol/L of AZT and 7.27 U/mL of rIFN-α2a were necessary to inhibit 90% of viral replication. This represents an approximately 18-fold reduction in AZT concentration and 70-fold reduction in rIFN-α2a concentration, levels that should have minimal effects on myeloid and erythroid precursors.

Table 1. Dose-Effect of AZT, rIFN-α2a, and Combinations on Normal Marrow BFU-E and CFU-GM Expressed as Percent Inhibition

<table>
<thead>
<tr>
<th>Drug</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>EXP 1</th>
<th>EXP 2</th>
<th>EXP 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Colonies</td>
<td>% Inhibition</td>
<td>No. of Colonies</td>
<td>% Inhibition</td>
<td>No. of Colonies</td>
</tr>
<tr>
<td>AZT (μmol/L)</td>
<td>108 ± 2</td>
<td>63 ± 3</td>
<td>35 ± 3</td>
<td>94 ± 5</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>0.5</td>
<td>86 ± 4</td>
<td>20</td>
<td>47 ± 1</td>
<td>25</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>1.0</td>
<td>72 ± 2</td>
<td>33</td>
<td>43 ± 3</td>
<td>32</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>2.0</td>
<td>50 ± 4</td>
<td>54</td>
<td>37 ± 3</td>
<td>41</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>4.0</td>
<td>56 ± 3</td>
<td>48</td>
<td>37 ± 3</td>
<td>41</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>rIFN-α2a(U)</td>
<td>100</td>
<td>71 ± 4</td>
<td>48 ± 2</td>
<td>24</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>500</td>
<td>54 ± 4</td>
<td>50</td>
<td>40 ± 4</td>
<td>37</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>2,000</td>
<td>18 ± 1</td>
<td>83</td>
<td>16 ± 2</td>
<td>75</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

Abbreviation: EXP, experiment.

*Data expressed as mean ± SEM of triplicate plates for each experiment. Low density bone marrow cells were plated at a concentration of 2 x 10⁴ cells/mL and scored on day 14 as described in Materials and Methods.

Table 2. Synergistic Effects of AZT and rIFN-α2a on Normal Human Bone Marrow Progenitor Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>EXP 1</th>
<th>EXP 2</th>
<th>EXP 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dₘₘ</td>
<td>m</td>
<td>r</td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>AZT</td>
<td>4.638 μmol/L</td>
<td>0.489</td>
<td>0.968</td>
<td>(0.085)</td>
<td>(0.310)</td>
</tr>
<tr>
<td>rIFN-α2a</td>
<td>0.215 μmol/L + 10.7 U/mL</td>
<td>0.395</td>
<td>0.978</td>
<td>0.083</td>
<td>0.290</td>
</tr>
<tr>
<td>AZT + rIFN-α2a</td>
<td>(1:50)</td>
<td>0.0045</td>
<td>0.994</td>
<td>(0.085)</td>
<td>(0.310)</td>
</tr>
</tbody>
</table>

Abbreviation: EXP, experiment.

*Data expressed as mean ± SEM of triplicate plates for each experiment. Low density bone marrow cells were plated at a concentration of 2 x 10⁴ cells/mL and scored on day 14 as described in Materials and Methods.

*Combination index CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively. CI values given are from classical isobologram equation and those in parentheses are from conservative isobologram equation.
with each drug alone, e.g., for BFU-E ED.<sub>50</sub>, DRI for ability of serum to protect MDBK cells from infection with vesicular stomatitis virus. rIFN-a2a values are expressed as median (range) values. AZT values are mean ± SEM. These Cl values, all < 1, indicate the strong synergistic effect of the combination of AZT and rIFN-a2a against HIV replication.

Table 4 summarizes the selectivity (therapeutic) indices for AZT and for rIFN-α2a at different effect levels. The indices for AZT inhibition of HIV-1 in bone marrow progenitor cells were quite large, in the range of 500 to >10<sup>6</sup>. In the experiments with rIFN-α2a, however, results were not as clear, with selectivity indices generally lower. Two experiments (at 50% and 75% dose-effect levels) gave mixed results with the selectivity indices in experiment A (day 13 reverse transcriptase assay) higher than in experiment B (day 14 reverse transcriptase assay). Nonetheless, for both drugs, BFU-E demonstrated higher sensitivity at a given dose-effect, ie, more inhibition, than CFU-GM.

**DISCUSSION**

The combination of AZT and rIFN-α2a is strongly synergistic in vitro against HIV-1 whether the assay system used is reverse transcriptase activity, p24 antigen production, or virus yield. No clear guidelines exist, however, for the design of clinical trials to evaluate these drugs in combination. One

Table 4. Selectivity Indices for AZT and rIFN-α2a at Different Effect Levels

<table>
<thead>
<tr>
<th>Index Basis</th>
<th>Experiment</th>
<th>Selectivity Index for AZT at Inhibitory Effect of</th>
<th>Selectivity Index for rIFN-α2a at Inhibitory Effect of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>(ED&lt;sub&gt;50&lt;/sub&gt;) BFU-E</td>
<td>Exp A</td>
<td>573</td>
<td>2,576</td>
</tr>
<tr>
<td>(ED&lt;sub&gt;50&lt;/sub&gt;) HIV-1</td>
<td>Exp B</td>
<td>160</td>
<td>913</td>
</tr>
<tr>
<td>(ED&lt;sub&gt;50&lt;/sub&gt;) CFU-GM</td>
<td>Exp A</td>
<td>1,856</td>
<td>&gt;10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>(ED&lt;sub&gt;50&lt;/sub&gt;) HIV-1</td>
<td>Exp B</td>
<td>435</td>
<td>&gt;10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Data in Table 3 were used for calculating selectivity indices.
approach is to examine the in vitro effects of each drug alone and in combination in a standard marrow clonogenic assay and to compare these results with those obtained from anti-HIV-1 studies. The doses required for the different degrees of effect in these test systems can then be compared with the plasma levels of each drug clinically achievable in patients. The median-effect equation and Cl were used to evaluate drug interactions in the present marrow clonogenic studies and previously reported anti-HIV-1 studies. The advantage of this particular method is that it can determine the degree of synergism or antagonism as well as the dose and effect levels required for synergism or antagonism to occur. It also allows the calculation of the degrees of dose reduction in synergistic combinations when compared with the doses required to achieve a given effect by each drug alone.

Our study demonstrates that AZT suppresses more BFU-E growth than CFU-GM growth (ED50: 4.638 μmol/L vs 12.62 μmol/L). Similarly, rIFN-α2a is more suppressive to BFU-E than to CFU-GM (ED50: 292 U/mL vs 1,870 U/mL). Previous studies that have evaluated each of these drugs as single agents in clonogenic marrow assay systems, however, have not all reached the same conclusions. For example, Brommeyer et al evaluated the effect of a highly purified human IFN-α (specific activity 1.4 × 10⁸ U/mg protein) on normal marrow progenitor cells and demonstrated that BFU-E were extremely sensitive to its inhibitory effects while CFU-GM were less so. The ED50 for BFU-E, calculated by the median-effect equation, is approximately 5 U/mL while the ED50 for CFU-GM, similarly calculated, is 252 U/mL. Mamus et al used rIFN-α2b (Schering Corporation) in their normal marrow erythropoietic progenitor cell assays. The ED50 for BFU-E, calculated by the median effect equation, is 3,398 U/mL. Differences in the interferon preparations, purity of the interferons, and differences in assay technique (methyl cellulose vs plasma clot) may account for these discrepancies.

AZT as a single agent has also inhibited myeloid and erythropoietic progenitor cells in vitro. Sommadossi and Carlisle reported an ED50 of 2.4 μmol/L against BFU-E, which is almost half the value reported in our study (Table 2). CFU-GM in their study also proved more sensitive, with an ED50 of 0.9 μmol/L. Significant variations in culture technique may again account for these differences. For example, Sommadossi and Carlisle used sheep erythropoietin, not purified human erythropoietin, in their assay system and used a different source of colony stimulating factor.

This study is the first to evaluate the drugs in combination against marrow progenitor cells. The data shown in Table 3 suggest that at the dose range currently being used in phase I clinical trials of the combination of AZT and rIFN-α2a (AZT 100 to 200 mg orally every four hours and rIFN-α2a 4.5 to 18 × 10⁶ U/d), clinically achievable drug concentrations are high enough to suppress between 50% and 75% of marrow progenitor growth in vitro. Studies from clinical trials using these drugs either as single agents or in combination appear to corroborate this finding. Richman et al, for example, reported that 45% of patients with AIDS or ARC treated with AZT at a dose of 250 mg orally every four hours had evidence of grade 3 marrow suppression. Approximately one third of their patients required dosage adjustments, often multiple times, and three patients were reported as having persistently hypoplastic marrows 5, 6, and 9 months after cessation of treatment. Similarly, in a phase I trial, Krown et al (unpublished observations) have observed dose-limiting neutropenia in four of 12 patients treated with 100 mg of AZT every four hours and 9 million units of rIFN-α2a daily, and in five of eight patients treated with 200 mg of AZT every four hours and 9 million units of rIFN-α2a daily. In addition, at all dosage levels tested thus far, the combination induced a decrease in mean hemoglobin concentration of 3 g/dL within the first 8 weeks of treatment. These findings are consistent with our in vitro results.

We recognize that significant difficulty may exist in applying these results directly to the clinical setting given such variables as individual viral load and recent evidence demonstrating emergence of HIV-1 isolates with reduced sensitivity to AZT. Nonetheless, preliminary studies by Ruprecht et al using a retrovirus-infected murine model suggest that a significant reduction in the dose of interferon is possible, without toxicity or loss of antiviral activity, when AZT is added at suboptimal doses. rIFN-αA/D administered at a dose of 500,000 units intraperitoneally twice daily to mice infected with Rauscher murine leukemia virus inhibited virus-induced splenomegaly by 94%. Similarly, when AZT was administered at a dose of 1 mg/mL in drinking water, splenomegaly was inhibited by 98% and viremia was completely suppressed. However, all mice developed severe drug related toxicity. At an AZT dose of 0.1 mg/mL there was no toxicity and survival was prolonged but splenomegaly was not prevented, suggesting that this was a suboptimal dose of AZT. When combined with the suboptimal dose of AZT, a 50-fold reduction in the dose of rIFN-αA/D (to 10,000 units) completely prevented clinical and laboratory signs of infection and was nontoxic. It is recognized, however, that significant differences exist between murine retroviruses and HIV-1, and that these results may not be directly applicable to humans infected with HIV-1.

Based on the data presented in the current study, we suggest that it may be possible to adjust the AZT and rIFN-α doses to levels at which >90% of HIV-1 replication would be inhibited while <50% of marrow progenitor growth would be suppressed. Such dosage reductions may permit fewer treatment interruptions, more continuous drug exposure, and allow more successful treatment of HIV-1–related complications without compromise of potential antiviral synergy. We also suggest that the median-effect principle and Cl is a method that can analyze potential drug synergy and antagonism in a manner that can provide useful guidelines for drug dosing in clinical trials.

ACKNOWLEDGMENT

The authors express their grateful acknowledgement to Q.H. Tan for her technical assistance and Duane Jones for his expert assistance in the preparation of this manuscript.
REFERENCES


Synergistic cytotoxic effect of azidothymidine and recombinant interferon alpha on normal human bone marrow progenitor cells

E Berman, R Duigou-Osterndorf, SE Krown, MP Fanucchi, J Chou, MS Hirsch, BD Clarkson and TC Chou

Updated information and services can be found at:
http://www.bloodjournal.org/content/74/4/1281.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml