In Vitro Potency of Inhibition by Antiviral Drugs of Hematopoietic Progenitor Colony Formation Correlates with Exposure at Hemotoxic Levels in Human Immunodeficiency Virus-Positive Humans

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Inhibition of in vitro colony formation of human hematopoietic progenitors (CFU-granulocyte-macrophage, burst-forming unit-erythroid) by the antiviral nucleoside drugs alovudine, zalcitabine, zidovudine, ganciclovir, stavudine, didanosine, lamivudine, and acyclovir was measured. Significant correlations between in vitro 50% inhibitory concentrations and the daily human exposures (area under the concentration-time curve from 0 to 24 h; in micromolar·hour) of these chronically administered drugs in human immunodeficiency virus-positive patients that induced neutropenia or anemia were demonstrated by both linear regression and Spearman rank-order analyses. These quantitative correlations allow estimation of the exposure at which bone marrow toxicity may occur with candidate compounds.

The results of in vitro assays are used to understand and predict in vivo observations. Candidate antiviral compounds are typically evaluated in vitro for both antiviral activity and cellular toxicity to identify selective compounds (33). Bone marrow toxicity is the principal dose-limiting toxicity associated with a number of antiviral drugs (30, 49). Thus, candidate antiviral compounds have been tested in colony-forming assays for human marrow progenitors as an in vitro model for hematologic toxicity in humans (32, 38, 54).

Colony-forming assays for bone marrow progenitors are useful for measuring in vivo hematopoietic responses to both disease and chemotherapeutic agents and serve as key in vitro model systems in experimental hematology (4, 6, 36). Parallels between in vitro and in vivo bone marrow toxicity have been documented for individual chemotherapeutic agents, including antiviral drugs (12, 29, 43, 54). Quantitative relationships between in vitro progenitor toxicity and in vivo plasma drug levels associated with decreased numbers of peripheral blood cells with certain therapeutic agents have also been postulated (28, 32, 37, 44, 56, 58). However, no study which has demonstrated a statistical correlation between in vitro and in vivo bone marrow toxicity with a survey of drug has been reported. In this report, we present a comprehensive analysis of quantitative correlations between in vitro and in vivo hematopoietic toxicity for clinically significant antiviral drugs.

In vitro bone marrow progenitor colony-forming assay. The in vitro toxicities of antiviral drugs to human bone marrow progenitors were assessed in a standardized colony-forming assay by previously published methods (15). Zidovudine (AZT) samples at 10, 1, and 0.1 μM were included as standards in every experiment. CFU-granulocyte-macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) colonies were counted at ×40 and ×100 magnifications, respectively, on a phase-contrast, inverted microscope on days 11 to 13 of the assay (the setup day was day 0). Average colony numbers in phosphate-buffered saline-treated controls from 142 experiments with samples from 61 different donors were 33 ± 14 (standard deviation) per well for CFU-GM and 77 ± 29 per well for BFU-E (from experiments cited for AZT; Table 1).

Antiviral drugs were obtained through the Wellcome compound registry. Inhibition of colony growth from bone marrow progenitors by each compound was determined over an appropriate range of concentrations. Each drug was evaluated in a minimum of four experiments with a minimum of four different marrow donors (Table 1). The percentage of control values was calculated by dividing the average of the colony counts from drug-exposed samples (n = 2 wells) by the average colony counts from control samples (n = 6 wells, saline solvent) in each independent experiment. The percentage of control values for each drug at each concentration was averaged across independent experiments. Dose-response curves were derived from these data.

Calculation of IC₅₀ and IC₉₀ of antiviral drugs. The 50% inhibitory concentrations (IC₅₀) were calculated from the dose-response curves for each drug. IC₉₀ was determined by either the log drug concentration-versus-percent inhibition algorithm or the Hill equation (15, 40). The error associated with the estimation of the IC₅₀ is ± the standard error (i.e., the fit of data to the line; Table 1). Estimates of the IC₅₀ for a particular drug varied less than twofold when the drugs were tested in separate replicate experiment sets (i.e., interassay variation).

Pharmacokinetic data. Daily drug exposure (area under the concentration-time curve [AUC] from 0 to 24 h [AUC₀–₂₄]) data were derived from clinical pharmacokinetic assessments in human immunodeficiency virus (HIV)-infected patients (5, 13, 20, 24, 31, 34, 35, 48). The methods used by the original investigators to calculate AUC varied, but most included accepted applications of trapezoidal methods. AUC₀–₂₄ values were calculated from available pharmacokinetic data to match as closely as possible the dosage regimen used in trials that reported hematologic toxicity (see legend to Fig. 1). When available, daily exposure was estimated from the sustained exposure at steady state from multiple dosings. If only single-dose exposure data were available, the AUC₀–₂₄ was estimated by

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multiplying the AUC from time zero to infinity (AUC<sub>0–</sub>∞) for a single dose by the number of doses that the patients received per day. Estimates were made from data in which the dose linearity and proportionality of each drug for the dose calculated were confirmed. Estimates were also confirmed by comparison with available pharmacokinetic data for non-HIV-infected patients with normal renal and liver functions for each drug (data not shown). The standard of 70 kg for human body mass was used for conversion purposes in our pharmacokinetic calculations. For our analysis, we assumed that the ratio of the drug concentration in bone marrow to that in plasma was the same for all drugs. The AUC was expressed as micromolar hour.

Hematologic toxicity of antiviral drugs in HIV-positive humans from clinical studies. Hematologic toxicity has been documented in clinical trials with the antiviral drugs analyzed in our study (1, 9, 24, 41, 46–48, 63). Assessment of hematologic toxicity caused by a particular antiviral drug, however, is complicated by the multiple factors that induce bone marrow suppression in HIV-infected patients (14, 16). The largest population allowing estimation of a background rate of hematologic dysfunction was a group of 107 placebo-dosed, HIV-infected patients with advanced symptoms from early trials with AZT (47). On the basis of the data from this group of AIDS patients, we used 10% neutropenia (defined as &lt;1,000 cells per mm<sup>3</sup>) and 5% anemia (defined as &lt;7.5 g of hemoglobin per dl or transfusions) as background levels of hematologic suppression. Only percentages greater than these were considered attributable to drug.

Because the present study is in part a retrospective analysis of published clinical trials, a number of parameters were not controllable. For example, both the HIV disease stage and the absolute number of patients evaluated in a particular study differed; thus, the relative percentage of patients affected with hematologic toxicity varied proportionally. For this reason, we matched clinical parameters as closely as possible in our analysis, including the criteria used to define anemia or neutropenia.

The percentages of patients affected with neutropenia or anemia varied among the different drugs and exposures; for this reason original AUC<sub>0–24</sub> data were analyzed and compared with AUC<sub>0–24</sub> data adjusted to approximate a 50% incidence of the corresponding level of cytopenia for each drug at each exposure. A logistic equation based on biochemical binding curve kinetics was used to determine a theoretical 50% incidence of cytopenia at a particular level of exposure (40, 61). The adjusted AUC<sub>0–24</sub> values are based on the assumption that an increase or a decrease in drug exposure is proportional to the increase or decrease in toxicity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of ext</th>
<th>No. of donors</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alovudine</td>
<td>0.60 ± 0.3</td>
<td>4</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>ddC</td>
<td>1.6 ± 0.3</td>
<td>11</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>AZT</td>
<td>7 ± 6</td>
<td>142</td>
<td>61</td>
<td>15, 59</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>15 ± 3</td>
<td>18</td>
<td>15</td>
<td>7, 59</td>
</tr>
<tr>
<td>Stavudine</td>
<td>70 ± 10</td>
<td>30 ± 10</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Didanosine</td>
<td>&gt;400&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 ± 4</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>250 ± 8</td>
<td>180 ± 2</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>Ayclovir ACV</td>
<td>220 ± 50</td>
<td>280 ± 50</td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± standard errors of the means.
<sup>b</sup> Depicted and analyzed as equal to 400 μM in Fig. 1A and B and Table 2.

The adjusted data provided a theoretical exposure equal to a 50% effect, thus equalizing the in vivo data among all the drugs and permitting analysis with in vitro IC<sub>50</sub>.

For comparative purposes, the data are also presented for the anti-herpes simplex virus drug acyclovir, which at elevated doses does not induce hematologic toxicity (64). No clinically significant changes in hematologic parameters were reported in the previous study (64), and the patients on acyclovir had a survival benefit. In another study with the same dose of acyclovir used in combination with AZT (10), data averaged from patients with AIDS and AIDS-related complex resulted in less than background level increases in hematologic toxicity over the percentages observed in patients treated with AZT alone (&lt;10% increase in neutropenia; &lt;5% increase in anemia). In addition, the pharmacokinetic data available for acyclovir in HIV-infected patients cited here were from a study in which acyclovir was administered in combination with AZT (13).

Regression and rank-order analyses. Linear regression and Spearman rank-order correlation analyses were performed by using the STATVIEW II program (Abacus Concepts, Inc., Berkeley, Calif.) (Table 2). Statistical analyses were also performed on AUC<sub>0–24</sub> data adjusted to approximate a 50% incidence of the corresponding level of cytopenia for each drug at each exposure (see equation above). Because of the large range of potencies of these compounds, all in vitro IC<sub>50</sub>s and AUC<sub>0–24</sub> data were log transformed prior to statistical correlation analyses.

Six antiviral drugs inducing severe neutropenia in chronically dosed HIV-positive patients were analyzed: alovudine, zalcitabine (ddC), stavudine, and lamivudine at elevated doses; AZT at elevated and current doses; and ganciclovir at currently recommended doses. All six drugs induce neutropenia in 13 to 60% of patients, an incidence up to sixfold above the background incidence. The in vitro IC<sub>50</sub>s for inhibition of CFU-GM bone marrow progenitors were compared with the AUC<sub>0–24</sub> values at which neutropenias were observed (Fig. 1A; Table 1). The percentage of patients affected with neutropenia upon chronic dosing with each drug at the associated level of exposure is included parenthetically in Fig. 1A. Regression analyses were performed for original and adjusted data (Fig. 1A; Table 2). The regression analyses described highly significant positive linear correlations between in vitro toxicity to CFU-GM progenitors and the drug exposure inducing neutropenia in patients dosed chronically with these drugs (Fig. 1A; Table 2). In addition, Spearman rank-order analysis of these same data confirmed the statistical significance of this correlation (Table 2).

We performed similar analyses for four antiviral drugs which induce anemia when dosed chronically (Fig. 1B): alovudine,
stavudine, and didanosine at elevated doses and AZT at both elevated doses and the currently recommended dose (2, 3, 21, 23, 47, 49). These drugs induce moderate to severe anemia in 15 to 75% of patients tested, which is 3- to 15-fold the background of 5% anemia observed in placebo-dosed AIDS patients. The regression analyses for the erythroid data, both with original data and with adjusted data, yielded linear correlations which were statistically significant (Fig. 1B; Table 2). Spear-
Table 2. Correlation analyses of in vitro to in vivo hematologic toxicity and in vitro IC₅₀₅ from independent laboratories

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Linear regression analysis</th>
<th>Spearman rank-order analysis</th>
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<tr>
<td></td>
<td>r</td>
<td>r²</td>
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<tr>
<td>CFU-GM IC₅₀₅ versus neutropenia (Fig. 1A)</td>
<td></td>
<td></td>
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<tr>
<td>Data (n = 9; six different drugs)</td>
<td>0.913</td>
<td>0.834</td>
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<tr>
<td>Adjusted data</td>
<td>0.938</td>
<td>0.880</td>
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<tr>
<td>BFU-E IC₅₀₅ versus anemia (Fig. 1B)</td>
<td></td>
<td></td>
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<tr>
<td>Data (n = 8; four different drugs)</td>
<td>0.775</td>
<td>0.601</td>
</tr>
<tr>
<td>Adjusted data</td>
<td>0.833</td>
<td>0.694</td>
</tr>
<tr>
<td>Dornsife et al. (see Table 1) versus Sommadossi et al. (27, 50, 54–56)</td>
<td></td>
<td></td>
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<tr>
<td>Drugs that induced neutropenia (n = 6)</td>
<td>0.930</td>
<td>0.866</td>
</tr>
<tr>
<td>All drugs reported (n = 8)</td>
<td>0.952</td>
<td>0.906</td>
</tr>
<tr>
<td>Dornsife et al. (see Table 1) versus Sommadossi et al. (27, 50, 54–56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E IC₅₀₅</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs that induced anemia (n = 4)</td>
<td>0.920</td>
<td>0.846</td>
</tr>
<tr>
<td>All drugs reported (n = 7, except acyclovir)</td>
<td>0.845</td>
<td>0.714</td>
</tr>
</tbody>
</table>

- r is the correlation coefficient. P is the associated probability for correlation if the null hypothesis (no correlation between the two variables) is rejected at the indicated significance level (P ≤ 0.05 or 0.01). Confidence intervals for the mean and the slope of a regression line allow estimation of the likelihood that a true value would lie in the selected range on the basis of the original samples and a chosen level of probability (e.g., 90%; see insets in Fig. 1A and B). Sets of quantitative data are expressed in terms of rank order for Spearman correlation analyses (57). For the Spearman rank-order analysis, P is a two-tailed probability.

- Significant at the level of P ≤ 0.01.
- Significant at the level of P ≤ 0.05.

Man rank-order analyses of these same data confirmed this result (Table 2).

To corroborate these findings on toxicity, we compared our data (Table 1) with the results of a similar assay used by an independent laboratory and analyzed the results for statistical correlations. Representative in vitro IC₅₀₅ of each of the antiviral drugs published by Sommadossi and coworkers were included in the analyses (27, 50, 54, 55, 56). Statistically significant linear and rank-order correlations were found for the CFU-GM data from the two laboratories (Table 2). A correlation for the BFU-E data between the two laboratories was not statistically significant for the drugs that induced anemia, but it was significant for a linear correlation when the available data for all drugs were included. In view of these correlations, it was not surprising that analysis for correlations of the in vitro data of Sommadossi and colleagues with drug exposure and in vivo toxicity findings provided results that closely paralleled those presented above for CFU-GM and neutropenia, whereas the pattern for BFU-E and anemia was generally similar (data not shown).

Relationships between in vitro and in vivo toxicity to bone marrow have been described for anticancer and antiviral drugs (12, 18, 43, 58). Sommadossi and colleagues were among those who initially applied in vitro colony-forming assays to assess the toxicity of antiviral drugs (38, 45, 54, 62). Sommadossi and Carlisle were the first to publish data on AZT (54). These and other investigators have suggested relationships between in vitro potency, peak or sustained concentrations in plasma, and observed levels of cytopenia in humans with individual agents (22, 25, 56, 65). We present here a rigorous quantitative analysis of these hypotheses and test for a statistical correlation between in vitro and in vivo bone marrow toxicity data with a survey of drugs which has not been previously reported. We found relationships between in vitro and in vivo hematologic toxicities that were statistically significant.

The absence of anemia (>5%) reported for both ddC at the elevated dose examined and ganciclovir at the recommended dose suggest that exceptions may exist for this model. Application of 90% confidence intervals to the mean and the slope of the regression (original AUC₀–₂₄ values) suggests that ddC is an exception to the model presented (inset to Fig. 1B). Although both our data and those of other investigators indicate that ddC is a potent inhibitor of BFU-E, no anemias were reported in the patients (n = 5) given ddC at the elevated dosage analyzed (0.54 mg/kg/day – 38 mg/day) (19, 26, 27, 32, 63). Because of the dose-limiting peripheral neuropathy associated with ddC treatment, considerably lower doses (2.25 mg/ day) are currently used in combination therapy with AZT. This dose of ddC, as a monotherapy, is associated with anemia in approximately 5% of patients (51). This level of anemia cannot be distinguished from background incidence levels of anemia in AIDS patients. Therefore, the regression analysis with the adjusted data may more accurately describe the linear correlation for the incidence of severe anemia (Fig. 1B; Table 2).

Comparison of our in vitro IC₅₀₅ with data from an independent laboratory demonstrated both a significant linear correlation and strong to significant rank-order correlations for both progenitors. These results highlight the fact that, despite differences in the absolute IC₅₀₅ of compounds from different laboratories, the data generated in such assays can be highly correlated. Thus, the data from either laboratory can be used to derive similar conclusions on relative toxicity, as was proposed previously (55).

Data on adverse events, including hematologic toxicities, from clinical trials are most often reported as the number of percentage of affected patients in each dose group and the severity of the toxicity. Our analyses with calculated drug exposures and different incidences of cytopenias were also compared with analyses based on theoretical drug exposures for each drug estimated to be equal to a 50% incidence of patients affected by either neutropenia or anemia (adjusted data). This approach was taken in an effort to standardize the percentage of patients receiving the different drugs affected and allow for analysis of correlation with a 50% effect in vitro. Statistically significant linear and rank-order correlations were observed with both the calculated drug exposures and the adjusted data.
drug exposure. However, if the data were available, a 50% decrease in neutrophil numbers or hemoglobin from baseline might provide a more relevant comparison with the in vitro IC_{50} toxicity assessment (48, 58).

The antiviral drugs tested in our study manifest different types of dose-limiting toxicities in humans. In vitro toxicity assessment of a candidate antiviral compound with primary human marrow progenitors cannot predict if marrow toxicity in humans will be the dose-limiting toxicity. However, predictions of sustained drug exposures at which a candidate compound may induce neutropenia or anemia in humans can be made.

The correlation between in vitro and in vivo marrow toxicity observed with these antiviral agents was striking. This finding was especially notable because of the numerous variables involved in in vitro and in vivo hematologic toxicity assessment, the multivariate nature of bone marrow suppression in HIV-infected patients, and the possibly, different mechanisms by which these drugs may exert their toxic effects. Thus, the present analysis confirms the roles of these in vitro assays in assessments of the toxicities of antiviral compounds during preclinical evaluation in selecting compounds with low potential for hematologic toxicity (8, 11, 17, 25, 42).

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REFERENCES


