Induction of apoptosis in rat peripheral blood lymphocytes by the anticancer drug CI-994 (acetyldinaline)*

Michael J. Graziano,† Teresa A. Spoon,§ Erin A. Cockrell,§ Paul E. Rowe, and Andrea J. Gonzales

Drug Safety Evaluation, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, Michigan 48105, USA

CI-994 (acetyldinaline) is an investigational anticancer drug currently in clinical trials. In preclinical safety studies in rats and dogs, CI-994 resulted in significant toxicity to bone marrow and lymphoid tissue. To determine if apoptosis was involved in CI-994 toxicity, peripheral blood lymphocytes were isolated from untreated male Wistar rats and exposed to CI-994 (1, 3, 10, or 30 µM) in vitro for up to 24 hours. Morphological and biochemical features of apoptosis were evaluated using several techniques, and lactate dehydrogenase (LDH) release was measured as an indicator of cell necrosis. No evidence of apoptosis or necrosis was detected in lymphocytes exposed to CI-994 for 4 hours. After 24 hours, concentration-dependent increases in apoptosis characterized by DNA condensation, DNA fragmentation, and/or externalization of phosphatidyl serine were seen at CI-994 concentrations as low as 1 µM and were statistically significant beginning at 10 µM. Ultrastructural analysis confirmed the presence of DNA condensation, DNA fragmentation, cell shrinkage, and membrane blebbing in cells exposed to 30 µM CI-994. After 24 hours, the percent of maximum LDH release from lymphocytes treated with 10 and 30 µM CI-994 was 7% and 15%, respectively, compared with 0% in the controls. In comparison, morphological changes of apoptosis detected by fluorescent microscopy were observed in 79% of the lymphocytes at these two concentrations. Additionally, apoptosis was seen in more than 24% of lymphocytes exposed to 1 and 3 µM CI-994, whereas maximum LDH release was less than or equal to 1% at these concentrations. These results show that apoptosis is the primary mode of cell death in rat lymphocytes exposed to CI-994 in vitro.

INTRODUCTION

CI-994 (acetyldinaline; Figure 1) is an investigational anticancer drug with activity in a broad spectrum of mouse, rat, and human tumor models [1–5]. CI-994 can also potentiate the activity of other standard chemotherapeutic agents. For example, simultaneous administration of CI-994 and gemcitabine results in greater than additive cell death in a tumor xenograft model of squamous cell lung carcinoma [6]. Although the mechanism of action of CI-994 is unknown, it appears to be novel when compared with other existing anticancer agents. CI-994 is an antimitabolite, does not covalently bind to or intercalate DNA, and does not affect microtubule synthesis [7]. Studies have shown that CI-994 can inhibit a 16 kDa nuclear phosphoprotein and increase histone acetylation [8, 9]. Currently, CI-994 is in phase II clinical trials.

Despite an unknown mechanism of action, the toxicity of CI-994 is similar to many other cytotoxic anticancer drugs in that it primarily involves effects on bone marrow, lymphoid tissue, and testes [10–13]. When CI-994 was administered orally to Wistar rats or beagle dogs once daily for two weeks, toxicity in both species included neutropenia, lymphocytopenia, thrombocytopenia, lymphoid depletion, bone marrow hypoacellularity, and testicular degeneration [10]. Lymphocytopenia and depletion of lymphoid tissue were also noted in rats within 24 hours of a single oral dose of CI-994 [11]. In mice, neutropenia, lymphocytopenia, and thrombocytopenia were observed when CI-994 was administered orally for 14 days [12]. Neutropenia and thrombocytopenia were also the dose-limiting toxicities of CI-994 in a phase I clinical trial [13]. Collectively, these results demonstrate that peripheral blood lymphocytes represent a toxicologically relevant model for studying the mechanism of CI-994 toxicity in vitro.

Since myelosuppression was observed in all species and was the dose-limiting toxicity in humans, characterizing CI-994 toxicity in hematopoietic cells is of great interest. Toxicity can lead to cell death by two distinct processes: necrosis or apoptosis [14–18]. Necrosis is a more passive form of cell death that is characterized by metabolic collapse resulting from severe pathologic- or chemical-induced injury. During necrosis, cells lose their ability to maintain osmotic balance.

*PII: S1110-7243(01)00014-6
Induction of apoptosis in rat lymphocytes by CI-994

MATERIALS AND METHODS

Chemicals

CI-994 (97.6% active) was synthesized at Goedecke AG Research and Development (Freiburg, Germany). Stock solutions of CI-994 were prepared in 100% ethanol and further diluted with sterile water to achieve the appropriate concentrations. The intended drug concentration of each solution was significantly reducing the spontaneous incidence of apoptosis. Supplemental assays were also performed to determine the effect of CI-994 on nonproliferating lymphocytes. For these latter assays, lymphocytes were incubated with CI-994 (1 and 30 μM) for 24 hours without mitogen.

Lymphocyte proliferation

Lymphocyte proliferation was determined by the addition of 0.5 μCi of [3H]thymidine to each well in a 96-well plate according to the assay conditions described above. [3H]Thymidine was added at the time of assay initiation. The amount of [3H]thymidine incorporated into lymphocyte DNA was counted by liquid scintillation spectroscopy. Four separate lymphocyte proliferation assays were performed.

Fluorescent microscopy

Lymphocytes were collected and immediately incubated with 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (in PBS) to visualize apoptotic cells. For quantitative analysis, cells were viewed with an Olympus AH-3 microscope equipped with a 40 x objective and a high-resolution, liquid-cooled CCD camera (Quantix R8-Photometrics, Grand Island, NY) containing 5% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 0.1% streptomycin (Gibco BRL) to yield a final volume of 24.5 ml. The diluted blood samples were layered on top of Histopaque 1077 (Ficoll/sodium dextran) and centrifuged at 425 x g for 30 minutes at room temperature. The mononuclear cell layer was removed and washed three times with supplemented Hank’s Balanced Salt Solution (300 x g, 10 minutes). Following the last wash, cells were counted and resuspended in supplemented RPMI-1640 media (Gibco BRL), pH 6.8, containing 25 mM Hepes, 10% FBS, 100 U/ml penicillin, and 0.1% streptomycin to give a final concentration of 1 x 10^6 cells/ml. A fixed number of cells were added to 96-well microtiter plates (2 x 10^4 cells) or 6-well microtiter plates (2 x 10^5 cells) and immediately treated with mitogen (concanavalin A; 0.63 μg/mL) and CI-994 (1, 3, 10, or 30 μM). The cells were then incubated for 4 or 24 hours at 37°C in 5% CO2. An equivalent volume of vehicle (distilled water) was added to the untreated (control) lymphocytes. The amount of ethanol in the CI-994 solutions was less than 1% (v/v). Concanavalin A was added to the culture media in the present study since preliminary experiments showed that it had a protective effect on untreated lymphocytes by significantly reducing the spontaneous incidence of apoptosis. Supplemental assays were also performed to determine the effect of CI-994 on nonproliferating lymphocytes. For these latter assays, lymphocytes were incubated with CI-994 (1 and 30 μM) for 24 hours without mitogen.

Isolation and treatment of peripheral blood lymphocytes

Random-bred, male Wistar (Crl:WI)BR rats were obtained from Charles River Laboratories (Portage, MI). Animals were housed individually in stainless steel cages and were maintained in environmentally controlled rooms with a 12 h light/dark cycle. Powdered Purina Certified Rodent Chow 5002® (Ralston Purina, St. Louis, MO) and tap water were provided ad libitum. Animals were acclimated to the laboratory environment for a minimum of 7 days prior to use on study. This study was conducted according to current guidelines for the care and use of laboratory animals [20] and was approved by the Pfizer Animal Care and Use Committee. Clinically acceptable rats weighing more than 170 g were used on study. Animals were euthanized by carbon dioxide inhalation and blood samples were collected in hepari-
and data were analyzed and quantitated using Modfit software (Verity Software House, Inc.). Cells with sub-G1 DNA content were considered to be apoptotic. At least four independent experiments were performed.

Electron microscopy
Approximately 1–2 × 10^6 cells were fixed in 2.5% glutaraldehyde (Polysciences, Warrington, PA) for 1 h. Cells were then collected by centrifugation and rinsed three times in 0.1 M cacodylate buffer, pH 7.4. Cells were osmicated for approximately 1 h, rinsed three times in cacodylate buffer, then dehydrated and embedded in epoxy resin. Ultrathin sections were examined using an FEI Philips CM100 BioTWIN transmission electron microscope.

Flow cytometric analysis of DNA content
Lymphocytes were collected and fixed in 70% ethanol for at least 30 minutes. Fixed cells were then stained with 5 µg/ml Hoechst 33342 to detect total DNA within cells [22]. Samples were collected on a Coulter Epics Elite flow cytometer, and data were analyzed and quantitated using ModFit LT software (Verity Software House, Inc.). Cells with sub-G1 DNA content were considered to be apoptotic. At least four independent experiments were performed.

DNA fragmentation assay
DNA was isolated from lymphocytes using the apoptotic DNA-Ladder kit (Boehringer Mannheim, Indianapolis, IN). Isolated DNA was quantitated spectrophotometrically using Softmax Pro 2.4.1 software (Molecular Dynamics, Sunnyvale, CA). DNA (0.6 µg) was separated by electrophoresis on a 1% agarose gel stained with ethidium bromide for visualization. Results were confirmed by three independent experiments.

Flow cytometric analysis of phosphatidyl serine externalization
Lymphocytes were collected and immediately incubated with annexin V-FITC, which binds to phosphatidyl serine (PS) present on the outer cellular membrane. Staining was performed using Annexin V-FITC Apoptosis Detection Kit purchased from Oncogene Research Products (Cambridge, MA). Procedures followed the manufacturer’s instructions. Propidium iodide stain was included for differentiating viable and nonviable (late apoptotic/necrotic) cells. Samples were collected using the Coulter Epics Elite flow cytometer, and data were analyzed and quantitated using Winlist 4.0 software (Verity Software House, Inc.). Cells with increased green staining due to the binding of annexin V-FITC to PS were categorized as apoptotic. At least four independent experiments were performed.

Lactate dehydrogenase (LDH) release assay
Lymphocytes were evaluated for the presence of necrotic cell death by measuring LDH release from cells into the culture medium. LDH release was detected using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). Procedures followed manufacturer’s instructions. Data were collected from three independent experiments.

Statistical analysis
Statistical analysis was performed using SigmaStat 2.03 software (SciTech International, Inc., Chicago, IL). Differences with p values less than 0.05 were considered statistically significant.

RESULTS
Lymphocyte proliferation
The number of proliferating lymphocytes in the presence of concanavalin A was 1% and 4% after 4 hours and 24 hours of incubation, respectively.

Morphological changes
There was no significant increase in the number of apoptotic cells detected by fluorescent microscopy after 4 hours of exposure to CI-994 (Figure 2). After 24 hours of exposure, the number of apoptotic lymphocytes at 0 and 30 µM CI-994 was significantly increased relative to the untreated controls: 79% at 10 and 30 µM CI-994 versus 16% in the controls (Figure 2). Although not statistically significant, the percentage of apoptotic cells was also increased at 1 and 3 µM CI-994: 28% and 34%, respectively. For lymphocytes incubated without mitogen, the percentage of apoptotic cells were 12%, 15%, and 51%, at CI-994 concentrations of 0, 1, and 30 µM, respectively. Condensed chromatin, apoptotic bodies, and membrane blebbing were characteristic of apoptotic cells (Figure 3). Ultrastructural examination of rat lymphocytes exposed to 30 µM CI-994 for 24 hours also showed typical morphological features of apoptosis including chromatin condensation and fragmentation, membrane blebbing, and cell shrinkage (Figure 4).

DNA fragmentation
Apoptotic cells fixed and permeabilized in ethanol lose fragmented DNA resulting in DNA histograms with a sub-G1 DNA content [22–25]. No significant increase in sub-G1 cells was detected in rat lymphocytes treated with CI-994 for 4 hours when analyzed by flow cytometry and Hoechst 33352 stain (Figure 5). However, exposure to CI-994 for 24 hours led to a dose-related increase in the number of sub-G1 cells relative to the untreated controls (Figure 5). The increase in sub-G1 cells was statistically significant at 30 µM CI-994. Without mitogen, the percentage of apoptotic cells detected by flow cytometry and Hoechst 33352 stain were 18%, 15%, and 55%, at CI-994 concentrations of 0, 1, and 30 µM, respectively. A distinct DNA fragmentation pattern consisting of multiples of 180bp was also observed in rat lymphocytes exposed to CI-994 for 24 hours when analyzed by gel electrophoresis.
Induction of apoptosis in rat lymphocytes by CI-994

Figure 2: Apoptosis in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. Mitogen (concanavalin A, 0.63 µg/ml) was added to all lymphocyte cultures at time zero. Apoptotic cells characterized by condensed DNA were visualized by fluorescent microscopy and acridine orange/ethidium bromide stain. Data represent the mean ± SE of at least 4 individual experiments. *Significantly different from control using Kruskal-Wallis one way ANOVA on ranked data and Dunn’s post hoc test (p < 0.05).

Membrane changes

A biochemical marker of apoptosis that does not involve changes in DNA is redistribution of PS from the inner leaflet to the outer leaflet of the cellular membrane [26, 27]. Using the binding of fluorescein isothiocyanate (FITC)-labeled annexin V to externalized PS, apoptotic cells can be quantitated by flow cytometry [27, 28]. There was no significant increase in the percent of lymphocytes exhibiting externalization of PS after 4 hours of exposure to CI-994 (Figure 7). After 24 hours of incubation, rat lymphocytes treated with 30 µM CI-994 showed a significant increase in externalization of PS relative to untreated controls (Figure 7). Although not statistically significant, the number of lymphocytes exhibiting PS externalization also appeared to be increased at 10 µM CI-994.

Necrotic cell death

No significant increase in LDH release was detected in the culture medium of any of the treatment groups after 4 hours of exposure to CI-994. After 24 hours of incubation, a significant increase in LDH release above control levels was detected in the medium from lymphocytes treated with 10 and 30 µM CI-994 (Table 1). For these two treatments, the percent of maximum LDH release was 7% and 15%, respectively, compared with 0% in the controls.

Table 1: LDH release from rat peripheral blood lymphocytes treated with CI-994.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Percent of maximum LDH release*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4h incubation</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1 µM CI-994</td>
<td>0.0 ± 1.0</td>
</tr>
<tr>
<td>3 µM CI-994</td>
<td>0.0 ± 0.4</td>
</tr>
<tr>
<td>10 µM CI-994</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>30 µM CI-994</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Positive control</td>
<td>111.5 ± 13.6*</td>
</tr>
</tbody>
</table>

*Values are means ± SE, n = 3.
*Positive control = NP40 cell lysis buffer.
*Significantly different from control using Kruskal-Wallis one way ANOVA on ranked data and Dunn’s post hoc test (p < 0.05).
DISCUSSION

Apoptosis is a common mechanism of cell death that is characterized by chromatin condensation, DNA fragmentation, membrane blebbing, and cell shrinkage without altered permeability of the plasma membrane [14–18, 21, 29–32]. In contrast to apoptosis, necrosis is characterized by organelle and cell swelling, loss of membrane integrity, rupture of the plasma membrane, and cell lysis. In the present study, several different techniques were used to assess apoptosis and necrosis in rat peripheral blood lymphocytes exposed to CI-994. Based on changes in cell morphology, a dose-related trend of increased apoptosis was observed at all CI-994 concentrations. The effects at 10 and 30 µM CI-994 were statistically significant. At these latter concentrations, almost 80% of lymphocytes were undergoing apoptosis after 24 hours. In comparison, less than 15% of lymphocytes treated with 30 µM CI-994 were undergoing necrosis based on release of LDH. These results demonstrate that necrosis plays little if any role in the toxicity of CI-994 and that apoptosis is the primary mechanism of cell death.

The ability of CI-994 to induce apoptosis in peripheral blood lymphocytes within 24 hours in vitro is consistent with the in vivo effects produced by this drug. Specifically, administration of CI-994 to male rats caused significant reductions in lymphoid tissue, bone marrow myeloid, and lymphoid cells, and peripheral blood lymphocytes, monocytes, and neutrophils within 24 hours of a single oral dose [11]. Furthermore, CI-994 concentrations used in vitro were in the same range as plasma and cerebrospinal fluid concentrations of CI-994 achieved in vivo in preclinical and clinical studies [10, 13, 33]. Collectively, these results demonstrate
Figure 5: Sub-G1 DNA content in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. (A) Sub-G1 DNA content quantitated by flow cytometry and Hoechst 33342 stain. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. Data represent the mean ± SE of at least 4 individual experiments. *Significantly different from control using Kruskal-Wallis one way ANOVA on ranked data and Dunn's post hoc test (p < 0.05). Representative DNA histograms of (B) untreated rat peripheral blood lymphocytes and (C) lymphocytes treated with 30 μM CI-994 for 24 hours. R₁ = gated sub-G1 DNA population.
In summary, CI-994 induced apoptosis in rat peripheral blood lymphocytes in a concentration-dependent manner. Apoptosis induced by CI-994 does not occur as rapidly as in vivo exposure to CI-994. In contrast, a variety of anticancer drugs, including topoisomerase inhibitors such as etoposide and camptothecin, can induce apoptosis in mouse and rat thymocytes and in human HL60 leukemia cells within 2 to 4 hours of incubation [38, 42–45]. Whether the delayed effects of CI-994 are due to different experimental conditions (e.g., use of rat lymphocytes in the present study) or differences related to the mechanism of action of CI-994 is unknown. Nonetheless, these results correlate with the inhibitory effect of CI-994 on cell cycle progression prior to the appearance of cytoxicity in vitro [46].

Although the mechanism of action of CI-994 has not yet been defined, it may be the same in both tumor cells and normal tissue since CI-994 was also shown to induce apoptosis in HL60 leukemia cells [47]. The mechanism of apoptosis was not investigated in this study but previous reports suggest that it may be initiated by either inhibition of a 16 kDa nuclear phosphoprotein or an increase in histone acetylation [8, 9]. In both studies, these targets were modulated within 2 hours of exposure and thus represent the earliest effects detected following CI-994 treatment in vitro. Whether inhibition of the 16 kDa nuclear phosphoprotein or an increase in histone acetylation is the initiating event leading to cell death induced by CI-994 has not been established. In HL60 cells, CI-994-induced apoptosis involves activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase [47]. Since caspase-3 is a downstream effector caspase, it is possible that other caspases may also be involved in CI-994-induced apoptosis.

Levels of PARP, which can recognize and bind to DNA strand breaks [48, 49], appeared to be increased in HL60 cells following exposure to 3 µM CI-994 [47]. It is not known whether this latter observation is indicative of DNA damage at low concentrations of CI-994 or represents a characteristic burst in PARP activity early in the apoptotic process [50]. The precise role of DNA damage in CI-994 cytotoxicity has not been completely investigated but in vitro studies with the deacetylated analogue, dinaline, showed that cytotoxicity in L1210 leukemia cells could not be reversed by purines, pyrimidines, or reduced folates [51]. There were also no direct effects on ribonucleotide phosphate pools or DNA, RNA, or lipid synthesis. Since dinaline and CI-994 have equivalent antitumor activity [1, 4, 46], these results suggest that CI-994 does not act as an antimetabolite, nor does it directly affect macromolecular synthesis. However, inhibition of CI-994 and dinaline on DNA synthesis measured by [3H]thymidine incorporation has been observed in rat peripheral blood lymphocytes and human colon carcinoma SW707 cells [11, 32].

In summary, CI-994 induced apoptosis in rat peripheral blood lymphocytes in a concentration-dependent manner. Apoptosis was confirmed by morphological and biochemical changes including membrane blebbing, chromatin condensation, DNA fragmentation, and externalization of PS. Necrosis was not detected to any significant extent. These results show that apoptosis is the primary mode of cell death in rat lymphocytes exposed to CI-994 in vitro.
Figure 7: Externalization of phosphatidyl serine (PS) in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. (A) Externalized PS in the cellular membrane was detected by flow cytometry and annexin V-FITC stain. Mitogen (concanavalin A, 0.63 µg/ml) was added to all lymphocyte cultures at time zero. Data represent the mean ± SE of at least 4 individual experiments. *Significantly different from control using one way ANOVA and a Tukey post hoc test (p < 0.05). Representative PS histograms of (B) untreated rat peripheral blood lymphocytes and (C) lymphocytes treated with 30 µM CI-994 for 24 hours. R₁ = gated annexin V-FITC population.
ACKNOWLEDGMENTS
The authors thank Gregg Sobocinski for technical assistance with electron microscopy and Dr Jeff Haskins for capturing the fluorescent microscopy images.

REFERENCES


This work was presented in part at the 37th Annual Meeting of the Society for Toxicology, Seattle, WA, March 1998

Corresponding author. E-mail: michael.graziano@pfizer.com. Tel: +1 734 622 5123

Current address: Cancer Research, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI 48105, USA

Current address: Department of Surgery, Section of Urology, University of Michigan Medical Center, Ann Arbor, MI 48109, USA
Submit your manuscripts at http://www.hindawi.com