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RESEARCH ARTICLE

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

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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 activity in a preclinical 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 not an antimetabolite, 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 hypocellularity, 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*.

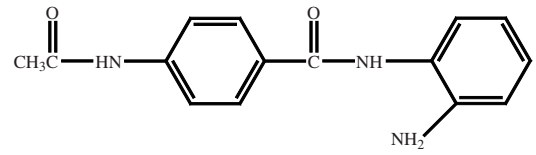

Figure 1: Chemical structure of CI-994.

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

due to a depletion of ATP. This, in turn, leads to swelling of organelles and lysis of the plasma membrane. Necrosis is often associated with extensive tissue damage and an intense inflammatory response. Apoptosis, on the other hand, is an active process that can occur under normal physiological conditions as well as in response to chemical injury. Apoptosis involves the activation of various cell signaling cascades which results in characteristic morphological and biochemical changes such as cell shrinkage, membrane blebbing, DNA condensation, and fragmentation. The cell is eventually broken down into smaller membrane-bound vesicles termed apoptotic bodies that become engulfed by surrounding cells without initiating an inflammatory response.

The objective of the present study was to investigate whether CI-994 induced apoptosis in peripheral blood lymphocytes, one of the cell types depleted during CI-994 treatment *in vivo*. Specifically, peripheral blood lymphocytes were isolated from male Wistar rats and morphological and biochemical features of apoptosis were assessed following incubation with CI-994 for up to 24 hours. Multiple techniques were used in this study since it is well recognized that no single assay is sufficient for unambiguous classification of apoptotic cells [19]. LDH release (indicating altered membrane permeability) was also measured as an indicator of necrosis. The results showed that apoptosis was the primary mode of cell death in rat lymphocytes exposed to CI-994 *in vitro*.

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 test concentrations. The intended drug concentration of each solution was confirmed analytically. Solutions were stored at room temperature. All other chemicals, unless noted, were purchased from Sigma Chemical Co. (St. Louis, MO).

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 heparinized tubes by cardiac puncture. Blood was diluted in supplemented Hank's Balanced Salt Solution (Gibco BRL, 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 diatrizoate) and centrifuged at $425 \times g$ for 30 minutes at room temperature. The mononuclear cell layer was removed and washed three times with supplemented Hank's Balanced Salt Solution ($300 \times g$, 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 1×10^6 cells/ml. A fixed number of cells were added to 96-well microtiter plates (2×10^5 cells) or 6-well microtiter plates (2×10^6 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% CO_2. 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.

Lymphocyte proliferation

Lymphocyte proliferation was determined by the addition of 0.5 μCi of [^{3}H]thymidine to each well in a 96-well plate according to the assay conditions described above. [^{3}H]Thymidine was added at the time of assay initiation. The amount of [^{3}H]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 at $40 \times$ using FITC filter sets to excite (488 nm) and visualize (530 nm) stains. Cells containing stained pycnotic nuclei (DNA condensation) were considered apoptotic. Staining and scoring procedures followed those described in McGahon *et al.* [21]. Two to four hundred cells were counted per treatment, and measurements were obtained from at least four independent experiments. For image capture, cells were stained for DNA using Hoechst 33342 (Molecular Probes, Eugene, OR) and mounted on standard microscope slides. Images were captured using a $100 \times$ objective with a high-resolution, liquid-cooled CCD camera (Quantix, RS-Photometrics,

Tucson, AZ) mounted on an inverted fluorescence microscope (Axiovert 135, Zeiss, New York, NY). Illumination was provided by a 100 watt mercury lamp using a DAPI dichroic filter set (Chroma Technology, Brattleboro, VT).

Electron microscopy

Approximately $1–2 \times 10^6$ 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 10 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 [23–25]. 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 180 bp was also observed in rat lymphocytes exposed to CI-994 for 24 hours when analyzed by gel electrophoresis

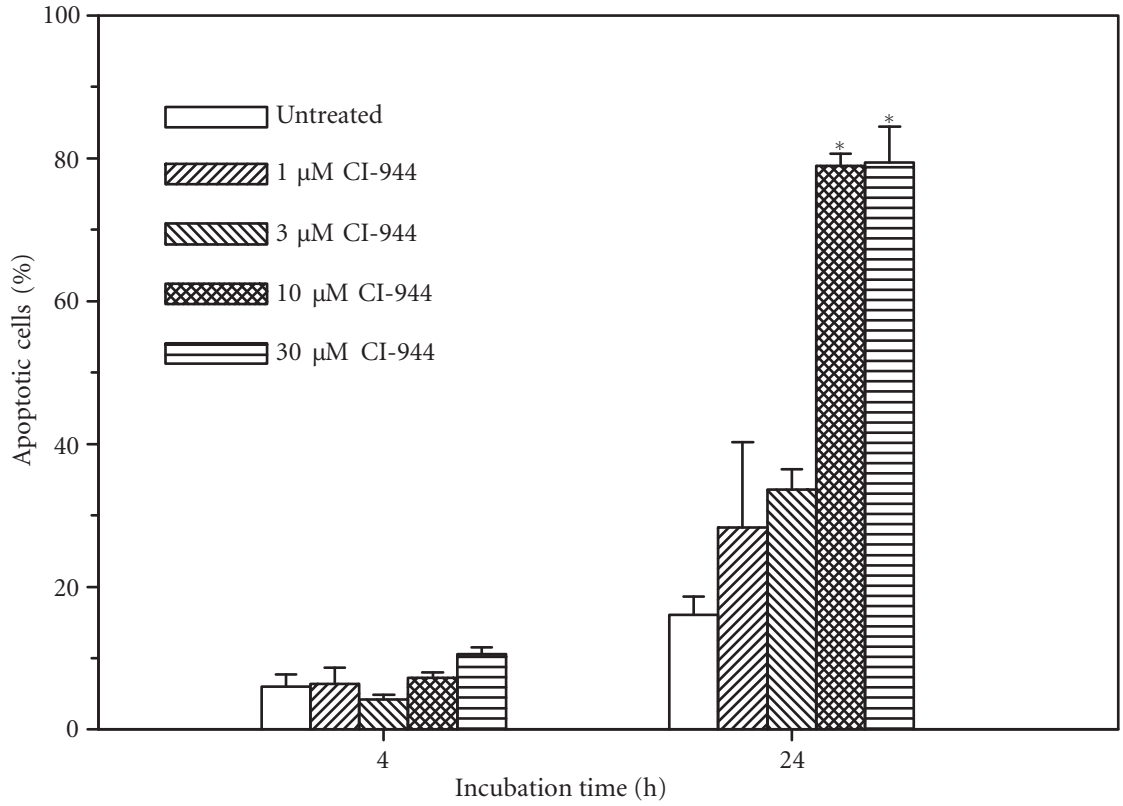

Figure 2: Apoptosis in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. Apoptotic cells characterized by condensed DNA were visualized by fluorescent microscopy and acridine orange/ethidium bromide 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 ($p < 0.05$).

(Figure 6). DNA fragmentation was evident at CI-994 concentrations as low as 3 μM with a dose-related increase in band intensity.

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.

Treatment group	Percent of maximum LDH release[a]	
	4 h incubation	24 h incubation
Untreated	0.0 ± 0.0	0.0 ± 0.0
1 μM CI-994	0.0 ± 1.0	0.0 ± 1.3
3 μM CI-994	0.0 ± 0.4	0.4 ± 1.9
10 μM CI-994	1.6 ± 0.9	7.0 ± 2.2*
30 μM CI-994	2.0 ± 0.7	15.1 ± 3.5*
Positive control[b]	111.5 ± 13.6*	129.9 ± 14.8*

[a]Values are means ± SE, $n = 3$.
[b]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 ($p < 0.05$).

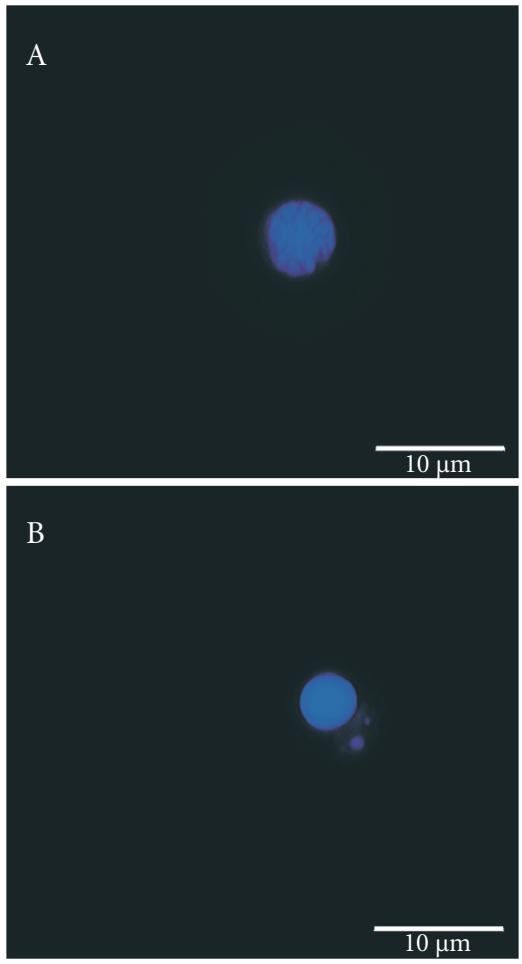

FIGURE 3: Fluorescent staining of (A) untreated rat peripheral blood lymphocyte and (B) rat peripheral blood lymphocyte exposed to 30 μM CI-994 for 24 hours. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. Morphological changes typical of apoptosis including condensed chromatin, apoptotic body, and membrane blebbing are apparent in treated lymphocyte.

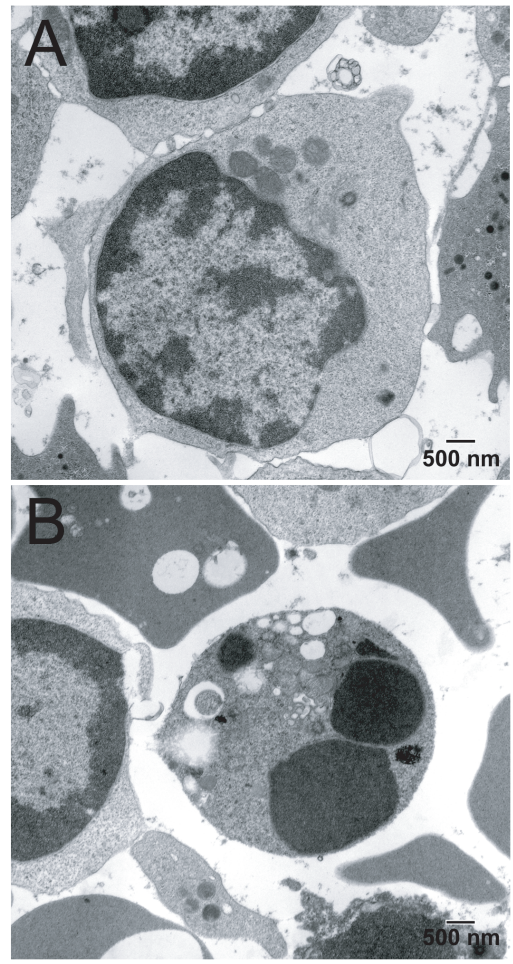

FIGURE 4: Electron micrograph of (A) untreated rat peripheral blood lymphocyte and (B) rat peripheral blood lymphocyte exposed to 30 μM CI-994 for 24 hours. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. Treated lymphocyte shows morphological changes of apoptosis including chromatin condensation and fragmentation, membrane blebbing, and cell shrinkage.

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

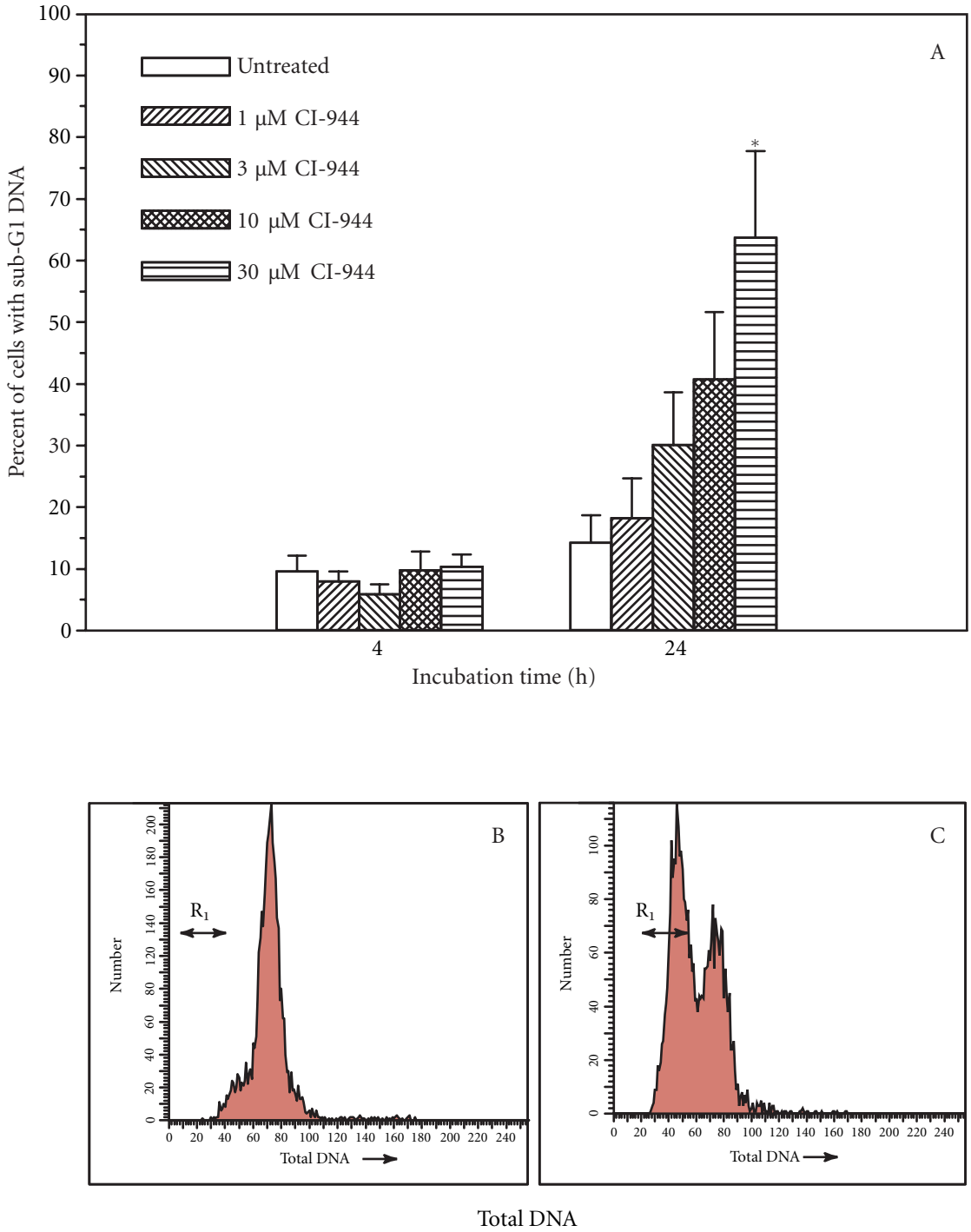

FIGURE 5: Sub-G1 DNA content in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. (A) Sub-G1 DNA content quantitated by flow cytometry and Hoechst 33342 stain. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. Data represent the mean ± SE of at 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. R_1 = gated sub-G1 DNA population.

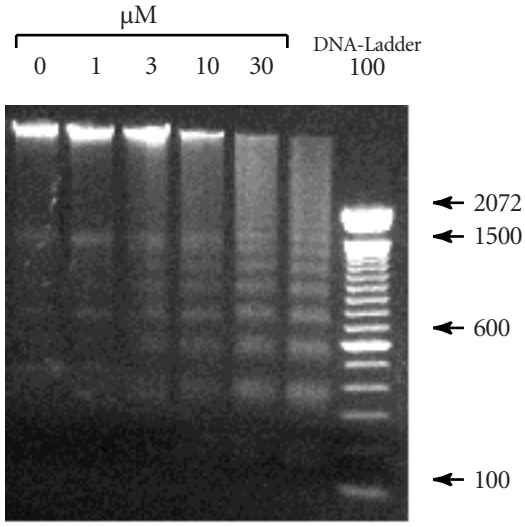

Figure 6: DNA fragmentation in rat peripheral blood lymphocytes exposed to CI-994 for 24 hours. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures at time zero. DNA (0.6 μg) was separated on a 1% agarose gel stained with ethidium bromide. Arrows indicate molecular weight markers. Positive control = 1 μM staurosporine (lane 6).

that peripheral rat blood lymphocytes represent a toxicologically relevant model for studying the mechanism of CI-994 toxicity *in vitro*.

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. However, even with the addition of concanavalin A, little if any proliferation occurred over 24 hours ($\leq$4%). This observation is consistent with a reported 24–28 hours delay in cell division in rat lymphocytes in culture with concanavalin A [34], and suggests that the induction of apoptosis by CI-994 occurred independently of cell proliferation. The ability of CI-994 to induce apoptosis in nonproliferating lymphocytes was confirmed by the results of several supplemental assays in which mitogen was omitted from the incubation mixture. The apoptotic effects of CI-994 on nonproliferating lymphocytes is similar to that of topoisomerase inhibitors and opposite of cisplatin and 2-chloro-2′-deoxyadenosine, the latter of which appear to only induce apoptosis in dividing cells [35–38]. The effect of CI-994 on nonproliferating cells is also consistent with the rapid loss of peripheral blood lymphocytes following CI-994 administration *in vivo* [11]. The majority of circulating lymphocytes are considered long-lived and do not proliferate in the absence of antigenic stimulation [39–41]. Therefore, apoptotic cell death is one possible mechanism by which circulating lymphocytes may be depleted during *in vivo* exposure to CI-994.

Apoptosis induced by CI-994 does not occur as rapidly as with other cytotoxic anticancer drugs. In the present study, no apoptosis was observed in lymphocytes after 4 hours of 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 cytotoxicity *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 effect macromolecular synthesis. However, inhibition of CI-994 and dinaline on DNA synthesis measured by [^{3}H]thymidine incorporation has been observed in rat peripheral blood lymphocytes and human colon carcinoma SW707 cells [11, 52].

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*.

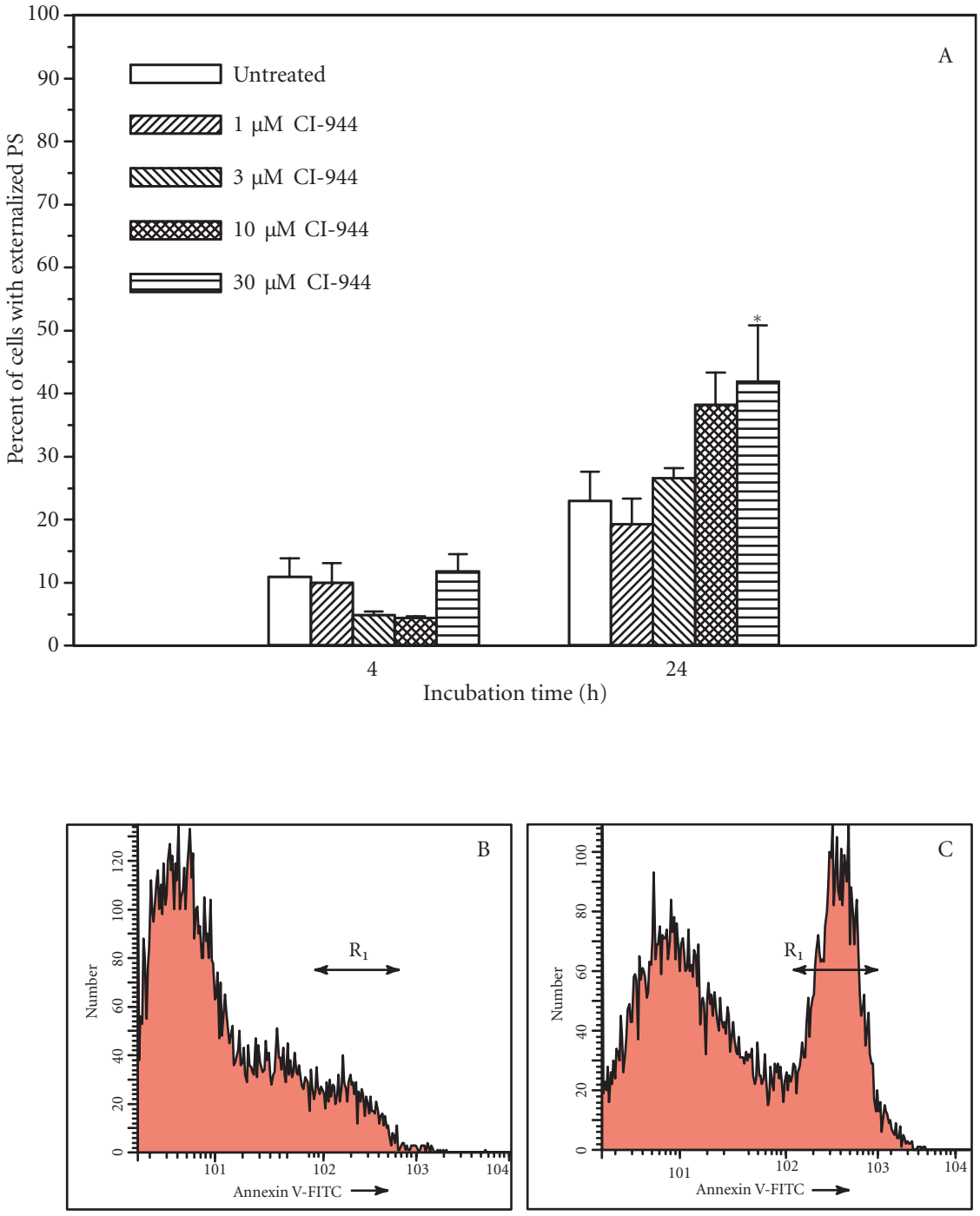

FIGURE 7: Externalization of phosphatidyl serine (PS) in rat peripheral blood lymphocytes exposed to CI-994 for 4 or 24 hours. (A) Externalized PS in the cellular membrane was detected by flow cytometry and annexin V-FITC stain. Mitogen (concanavalin A, 0.63 μg/ml) was added to all lymphocyte cultures 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. R_1 = gated annexin V-FITC population.

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