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, 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 increases the antitumor activity of gemcitabine in tumors [6–9]. Currently, CI-994 is in phase II clinical trials. 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.
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 solution. The amount of ethanol in the CI-994 solutions was less than 1% (v/v). 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 × 10^5 cells/ml. A fixed number of cells were added to 96-well microtiter plates (2 × 10^6 cells) or 6-well microtiter plates (2 × 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.

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 AX-50 microscope at 40 × 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 McGahan 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 × objective with a high-resolution, liquid-cooled CCD camera (Quantix, RS-Photometrics, Inc).
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.

**Electron microscopy**

Approximately 1–2 × 10⁶ 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.

**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).
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* |
|-----------------|---------------------------------|
|                 | 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 ± 1.4       |
| 3 µM CI-994     | 0.0 ± 0.4      | 0.4 ± 1.0       |
| 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| 111.5 ± 13.6*  | 129.0 ± 14.8*   |

*Values are means ± SE, n = 3.
*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.
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 (≤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 camptothecin, which can induce apoptosis in dividing cells [35–38]. The effect of CI-994 on nonproliferating cells is also consistent with the rapid loss of apoptosis in dividing cells [35–38]. The effect of CI-994 on deoxyadenosine, the latter of which appear to only induce apoptosis in dividing cells [33]. 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 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.
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REFERENCES

[1] Berger MR, Richter H, Seelig MH, Eibl H, Schmähl D. New cytostatics-more activity and less toxicity. Cancer Treat Rev. 1990;17:143–154.

[2] El-Beltagi HM, Martens ACM, Dahab GM, Hagenbeek A. Efficacy of acetyldinaline for treatment of minimal residual disease (MRD): preclinical studies in the BNML rat model for human acute myeloid leukemia. Leukemia. 1993;7:1795–1800.

[3] El-Beltagi HM, Martens ACM, Lieleveld P, Haroun EA, Hagenbeek A. Acetyldinaline: a new oral cytostatic drug with impressive differential activity against leukemic cells and normal stem cells-preclinical studies in a relevant rat model for human acute myeloid leukemia. Cancer Res. 1995;55:3008–3014.

[4] Howard CT, Roberts BJ, Vincent PW, Elliot WL. Acetyldinaline: a small molecule with relevant rat model for human acute myelocytic leukemia. Cancer Res. 1995;53:3008–3014.

[5] LoRusso PM, Demchik L, Foster B, et al. Preclinical antitumor activity of CI-994. Invest New Drugs. 1996;14:349–356.

[6] Howard CT, Giusbert EA, Schafer JS, Merriman RL. In vivo antitumor activity of CI-994 (N-acetyl-dinaline, GOE 5549) alone and in combination with gemcitabine against LCL1 squamous cell lung carcinoma. Proc Am Assoc Cancer Res. 1999;40:590.

[7] Grabow D, Graziano M, Grove W, Merriman R, Mosley S, Olson S. Investigator’s Brochure: CI-994. Parke-Davis Pharmaceutical Research, Division of Warner-Lambert/PPD; 1999.

[8] Bummel SA, Kraker AJ, Steinkampf RW, Hook KE, Kohls WD. Role of a small molecular weight phosphoprotein in the mechanism of action of CI-994 (N-acetyl-dinaline). Int J Cancer. 1995;62:636–642.

[9] Kraker AJ, Hartl B, Main J, Merriman RL. Effect of CI-994 (N-acetyldinaline or 4-acetyllamine)-N(2-amino phenyl) benzamide on histone acetylation and differentiation in HCT-8 colon carcinoma cells. Proc Am Assoc Cancer Res. 1999;40:120.

[10] Graziano MJ, Pilcher GD, Walsh KM, Kasali OB, Radulovic L. Preclinical toxicity of a new oral anticancer drug, CI-994 (acetyldinaline), in rats and dogs. Invest New Drugs. 1997;15:295–310.

[11] Graziano MJ, Galan AJ, Walsh KM. Immunotoxicity of the anticancer drug CI-994 in rats: effects on lymphoid tissue. Arch Toxicol. 1999;73:168–174.

[12] Foster BJ, Jones L, Wiegard R, LoRusso PM, Corbett TH. Preclinical pharmacokinetic, antitumor and toxicity studies with CI-994 (N-acetyldinaline). Invest New Drugs. 1997;15:187–194.

[13] LoRusso PM, Wozniak A, Foster B, et al. Phase I clinical trial of CI-994. Proc Am Soc Clin Oncol. 1997;16:213a.

[14] Alison MR, Sarraf CE. Apoptosis: regulation and relevance to toxicology. Hum Exp Toxicol. 1995;14:234–247.

[15] Corcoran GR, De L, Jones DF, Molten MT, Nicotera P, Oberhammer FA, Buttyan R. Apoptosis: molecular control point in toxicity. Toxicol Appl Pharmacol. 1994;128:169–181.

[16] Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wylie AH. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochim Biophys Acta. 1992;1133:275–285.

[17] Fawthrop DJ, Boobis AR, Davies DS. Mechanisms of cell death. Arch Toxicol. 1991;65:237–444.

[18] Kerr JFR, Wylie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26:239–257.

[19] Renouvé C, Biola A, Pallardy M, Bréard J. Apoptosis: identification of dying cells. Cell Biol Toxicol. 1998;14:111–120.

[20] National Research Council. Guide for the care and use of laboratory animals. National Academy Press; 1996.

[21] McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shy, Moshfegh RJ, Nishikawa WK, Green DR. The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol. 1995;46:153–185.

[22] Darzynkiewicz Z, Li X, Gong J. Assays of cell viability: discrimination of cells dying by apoptosis. Methods Cell Biol. 1994;41:15–38.

[23] Afanasyev VN, Korol VA, Matveichik NP, Pechatnikov VA, Umanovsky SR. The use of flow cytometry for the investigation of cell death. Cytometry. 1993;14:663–669.

[24] Darzynkiewicz Z, Bruno S, Del Bino G, et al. Features of apoptotic cells measured by flow cytometry. Cytometry. 1992;13:795–808.

[25] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods. 1991;139:271–279.

[26] Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992;148:2207–2216.

[27] Martin SJ, Reutelingperger CPM, McGahon AJ, Radar JA, van Schie BCAA, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995;182:1545–1556.

[28] Koopman G, Reutelingperger CPM, Kuijten GAM, Keenhuis RMJ, Pals ST, van Oers MHH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994;84:1415–1420.
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[29] Compton MM. A biochemical hallmark of apoptotic internucleosomal degradation of the genome. Cancer Metastasis Rev. 1992;11:105–119.

[30] Eastman A. Apoptosis: a product of programmed and unprogrammed cell death. Toxicol Appl Pharmacol. 1993;121:160–164.

[31] Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev. 1992;11:121–139.

[32] Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol. 1984;142:67–77.

[33] Riva L, Blaney SM, McGuffey SM, Berg SL. Pharmacokinetics and cerebrospinal fluid penetration of CI-994 (N-acetyldinaline) in the nonhuman primate. Proc Am Assoc Cancer Res. 1999;40:388.

[34] Guzzie PJ, Oshiro Y. An optimized procedure for culturing rat lymphocytes for in vitro cytogenetic screening. Environ Mol Mutagenesis. 1989;14(suppl 15):80.

[35] Evans DL, Dive C. Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res. 1993;53:2133–2139.

[36] Evans DL, Tilley M, Dive C. Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat thymocytes is independent of the levels of drug accumulation and DNA adduct formation. Cancer Res. 1994;54:1596–1603.

[37] Lassota P, Kazimierzczuk Z, Darzynkiewicz Z. Apoptotic death of lymphocytes upon treatment with 2-chloro-2′- deoxyadenosine (2-Cda). Arch Immunol Ther Exp. 1994;42:17–23.

[38] Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF, Lassota P, Kazimierzczuk Z, Darzynkiewicz Z. Apoptotic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. 1989;49:5870–5878.

[39] Onishi Y, Aruma Y, Sato Y, Mizuno Y, Tadakuma T, Kazuki H. Topoisomerase inhibitors induce apoptosis in thymocytes. Biochim Biophys Acta. 1993;1175:147–154.

[40] Sun XM, Snowden RT, Dinsdale D, Ormerod MG, Cohen GM. Changes in nuclear chromatin precede internucleosomal DNA cleavage in the induction of apoptosis by etoposide. Biochem Pharmacol. 1994;47:187–195.

[41] Kramer AJ, Wolven A, Fry DW, Kloos WD. Cell cycle and nucleotide effects of 4-(acetylamino)-N-(2-aminophenyl)benzamide (PD 123654, Goe 5549) in HCT-8 cells. Proc Am Assoc Cancer Res. 1991;32:96.

[42] Safdarian SH, Cockrell EA, Gonzales AJ, Graziano MJ. Characterization of CI-994 induced apoptosis in human leukemia (HL60) cells. Toxicol Sci. 1999;48(1-S):152.

[43] Benjamin RC, Gill DM. Poly(ADP-ribose) synthesis in vitro programmed by damaged DNA. J Biol Chem. 1980;255:10502–10508.

[44] Kaufman SH, Desnoyers S, Ottaviano S, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res. 1993;53:3976–3985.

[45] Simbulan-Rosenthal CM, Rosenthal DS, Iyer SI, Boulahes AH, Smulson ME. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. J Biol Chem. 1998;273:3703–3712.

[46] Leopold WR, Hooke KE, Fry DW. Activity and biochemical properties of Goe 1734 (PD 104208), an anticancer agent with a novel mechanism of activity. Proc Am Assoc Cancer Res. 1987;28:302.

[47] Schaider H, Haberkorn U, Stohr M, Berger MR. Dinaline inhibits amino acid transport and proliferation of colon carcinoma cells in vitro. Anticancer Res. 1995;15:2501–2510.

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