DNA Repair Initiated in Chronic Lymphocytic Leukemia Lymphocytes by 4-Hydroperoxycyclophosphamide Is Inhibited by Fludarabine and Clofarabine

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3-The abbreviations used are: CLL, chronic lymphocytic leukemia; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoro adenine; fludarabine, 9-β-D-arabinofuranosyl-2-fluoroadenine-5'-monophosphate; F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoroadenine-5'-trimonophosphate; 4-HC, 4-hydroperoxycyclophosphamide; FITC, fluorescein isothiocyanate; clofarabine, Cl-F-ara-A, 2-chloro-9-(2-deoxy-2-fluoro-β-D-
arabinofuranosyl)adenine; Cl-F-ara-ATP, 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine -5'-triphosphate.
Abstract

**Purpose.** Chronic lymphocytic leukemia (CLL) lymphocytes respond to DNA alkylation by excision repair, with the extent of repair increasing as the cells acquire resistance to alkylating agents. Because incorporation of nucleotide analogues into the repair patches elicits death signals in quiescent cells, the increased capacity for excision repair in alkylator-resistant cells could facilitate incorporation of nucleotide analogues. We hypothesized that the mechanism-based interaction of nucleoside analogues with alkylating agents could elicit greater than additive killing of CLL cells.

**Experimental Design.** Lymphocytes from 50 patients with CLL that were not refractory to alkylators were treated *in vitro* with 4-hydroperoxycyclophosphamide (4-HC) with or without prior incubation with fludarabine nucleoside (F-ara-A) or with clofarabine (Cl-F-ara-A). DNA damage repair kinetics were determined by the single-cell gel electrophoresis (comet) assay. Cytotoxicity was assessed by the staining with annexin V.

**Results.** CLL lymphocytes promptly initiated and completed excision repair in response to 4-HC. A 2-h pre-incubation with 10 μM F-ara-A or 10 μM Cl-F-ara-A inhibited the
repair initiated by 4-HC, with inhibition peaking at the intracellular concentrations of 50 μM F-ara-ATP or 5 μM Cl-F-ara-ATP. Combining 4-HC with either F-ara-A or Cl-F-ara-A produced more than additive apoptotic cell death than the sum of each alone. The increase in cytotoxicity was proportional to the initial magnitude of the DNA incision and to the extent of repair inhibition by the nucleoside analogues, suggesting close correlation between the repair inhibition and induction of cell death.

**Conclusions.** DNA repair, which is active in CLL lymphocytes, may be a biologic target for facilitating the incorporation of nucleoside analogues and increasing their cytotoxicity. Thus, the increased repair capacity associated with resistant disease may be manipulated to therapeutic advantage.
Introduction

Two classes of anticancer agents important in the treatment of CLL are alkylating agents and nucleoside analogues (1-3). Alkylating agents have long been the mainstay in the conventional treatment of CLL needing therapy. Between 60 to 80% of patients respond to initial therapy based on alkylating agents such as chlorambucil or cyclophosphamide, but remissions are usually incomplete, and eventually all patients experience progressive disease that is frequently accompanied by drug resistance (4, 5).

Recently, purine nucleoside analogues have been shown to be efficacious in the therapy of CLL and other indolent B-cell malignancies. One such agent, the deoxyadenosine nucleoside analogue monophosphate fludarabine produced a high response rate, with a substantial complete remission rate both as an initial treatment and for disease that has become refractory to other drugs (2, 3, 6, 7). Nevertheless, fludarabine alone is still considered unlikely to improve long-term survival (2, 3, 6) and rationales for combinations are being sought.

With regard to its mechanism of action, fludarabine is a potent inhibitor of cellular DNA synthesis. After being incorporated into DNA, the drug terminates DNA
elongation (8) and inhibits ligation of DNA strands (9). Thus, the major cytotoxic effect of fludarabine is specific to S-phase in growing cells (10-11). In indolent diseases such as CLL, the cytotoxic action of fludarabine may be limited because the cells are usually quiescent (12, 13). Thus, we have focused on the DNA excision repair responses to DNA damage as a means of increasing the population of CLL lymphocytes that are synthesizing DNA (14, 15). DNA alkylation initiates base excision, nucleotide excision, and mismatch repair systems, as well as recombinational repair (4, 5, 16). These repair mechanisms direct the removal of damaged nucleotides followed by DNA resynthesis (16-18); a process that allows the incorporation of nucleoside analogues into the DNA repair patch (15). Studies in model systems have demonstrated that incorporation of fludarabine nucleotide during nucleotide excision repair is inhibitory to this process (15,19, 20), and is associated with increased cytotoxicity in UV-irradiated lymphocytes (15).

Although CLL lymphocytes can initiate excision repair processes of damage induced in DNA by UV or alkylation, the repair capacity varies widely and is usually less in CLL lymphocytes than in normal lymphocytes (4, 5, 21-23). However, an increased
capacity for nucleotide excision repair was reported to be one of the major mechanisms for resistance to alkylating agents in CLL (4, 21, 23, 24). If so, the increased repair capacity initiated by alkylating agents in resistant CLL lymphocytes could facilitate the incorporation of nucleoside analogues, which in turn would inhibit DNA repair and could contribute to overcoming such a resistance mechanism. In this sense, DNA repair may be viewed as a biologic target in CLL, particularly in the context of drug resistance, and the combination of alkylating agents and nucleoside analogues could enhance cytotoxicity and overcome cellular resistance by a mechanism that is different from that of each agent alone (14, 15).

In the present investigation, we hypothesized that the DNA excision repair processes initiated by alkylating agents would allow nucleoside analogues to be incorporated into repairing DNA, thereby inhibiting the repair and enhancing cytotoxicity in quiescent CLL lymphocytes. For this study, CLL lymphocytes were stimulated to undergo DNA repair initiated by 4-HC alone or in the presence of the nucleoside of fludarabine F-ara-A, in vitro. DNA damage repair kinetics were evaluated as DNA strand breaks associated with the repair process using the alkaline single-cell gel.
electrophoresis (comet) assay (25-27). Intracellular levels of the triphosphate drug forms, the active metabolites of the nucleoside analogues, were also measured and compared to the repair response. The subsequent effect on cell viability was determined as externalization of phosphatidylserine by FITC-conjugated annexin V (28).

We compared these results with those generated by a new purine nucleoside analogue, clofarabine (Cl-F-ara-A) (Fig.1). Cl-F-ara-A has a chlorine atom at C-2 in the purine ring that confers resistance to deamination and a fluorine at the C-2’ position in the arabino configuration that decreases the susceptibility of Cl-F-ara-A to phosphorolytic cleavage by bacterial purine nucleoside phosphorylase. As with F-ara-A, the incorporation of the Cl-F-ara-A monophosphate into DNA is critical for the cytotoxicity of this compound (29, 30). Cl-F-ara-A has shown potent cytotoxicity in human cell lines such as CEM, K562, and Hep2, and in the murine leukemia cell line L1210 (31-33) as well as therapeutic activity in murine tumor models (34). In phase I clinical trials, Cl-F-ara-A produced a marked decrease in leukemic cell counts in patients with CLL (35). Thus, we compared the actions of this agent directly with F-ara-A in the same experimental settings.
Materials and Methods

Chemicals and reagents. The cyclophosphamide prodrug 4-HC was kindly provided by Dr. M. Colvin and Dr. S. Ludeman (Duke Univ., Durham, NC). This compound is metabolized to phosphoramid mustard, an active bifunctional alkylator (36). F-ara-A was synthesized by the dephosphorylation of fludarabine with alkaline phosphatase. Cl-F-ara-A was supplied by Dr. John A. Secrist, III (Southern Research Institute, Birmingham, AL). [8-3H]F-ara-A (specific activity, 11.8 Ci/mmol) and [8-3H]Cl-F-ara-A (specific activity, 4 Ci/mmol) were prepared by Moravek Biochemicals, Inc. (Brea, CA). [methyl-1’, 2’,3H]Thymidine (123 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). FITC-conjugated annexin V was purchased from Becton Dickinson (San Jose, CA).

CLL lymphocyte preparation. Leukemic lymphocytes from 50 patients with CLL that were not refractory to alkylating agents were used. Whole blood was drawn into heparinized tubes, diluted 1:3 with cold phosphate-buffered saline, layered over Fico/Lite LymphoH (specific gravity, 1.077) (Atlanta Biologicals, Norcross, GA), and
centrifuged at 1,500 rpm for 20 min. CLL lymphocytes were harvested from the interphase, washed once, counted and sized with a Z2 Coulter particle counter and size analyzer (Coulter Corporation, Miami, FL). The lymphocytes were resuspended at 1 x 10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY) and 2 mM L-glutamine, and incubated in a 5% CO2 humidified atmosphere at 37 °C overnight.

**Evaluation of DNA damage repair response by the alkaline single-cell gel electrophoresis (comet) assay.** To evaluate DNA damage repair kinetics, we used the alkaline comet assay as described by Singh et al (25). For this assay, approximately 5,000 CLL lymphocytes after the treatment were mixed with 40 µl of 0.5% low melting point agarose in phosphate-buffered saline at 37 °C. The mixture was layered onto a fully frosted microscope slide (Fisher Scientific, Pittsburgh, PA) previously coated with 100 µl of 0.65% normal agarose in phosphate-buffered saline, followed by a top layer of 80 µl of low melting point agarose. After solidification, the slides were left in a lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM ethylenediamine tetraacetic acid, 10% dimethylsulfoxide, 1% Triton X100, pH 10) at 4 °C for 1 h. The slides were then placed in
electrophoretic buffer (1 mM ethylenediamine tetraacetic acid, 300 mM NaOH, pH 13) for 20 min at 4 °C to allow unwinding of DNA. Electrophoresis was conducted for the next 20 min at 35 V (0.7 V/cm) and 300 mA. After the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris, pH 7.5) and stained with 25 µl of 20 µg/ml ethidium bromide. Cells, 100 per treatment condition, were analyzed at 200x magnification with an epifluorescence microscope (LABOPHOTO-2, NIKON, Japan) with a 515- to 560-nm excitation filter, and a 590-nm barrier filter connected through a black and white charge-coupled device video camera to a computer-based image analysis system (Kinetic Imaging Komet system, Version 4.0, Liverpool, UK). Cellular responses to DNA damage were expressed as the “tail moment,” which combines a measurement of the length of the DNA migration and the relative DNA content therein (37).

Measurement of intracellular F-ara-ATP and Cl-F-ara-ATP. Intracellular concentrations of the triphosphate forms, the crucial metabolites for cytotoxicity of nucleoside analogues, were measured and compared with the DNA damage repair response. After CLL lymphocytes were incubated with [8-³H]F-ara-A or [8-³H]Cl-F-ara-A, the nucleotides were extracted with 0.4 N perchloric acid and applied to a high-
performance liquid chromatographic analysis. Naturally occurring triphosphates and F-ara-ATP were separated using a strong anion-exchange column, Partisil 10-SAX (Whatman, Clifton, NJ), and gradient curve #9 run over 50 min at 1.5 ml/min, starting with 60% buffer A (0.005 M NH₄H₂PO₄, pH 2.8) and concluding with 100% buffer B (0.75 M NH₄H₂PO₄, pH 3.6) (15). Cl-F-ara-ATP was separated with the use of the same column and a linear gradient over 60 min at 1.5 ml/min, starting at 100% Buffer A and concluding at 100% Buffer B (30). The eluting radioactive nucleotides were mixed with scintillation fluid (Ultima Flo AP; Packard Instrument Co., Meriden, CT) at a ratio of 1:3 and detected by a flow-through scintillation counter (Model A250; Packard Instrument Co.) connected in series to the high-performance liquid chromatography system.

**Evaluation of DNA resynthesis.** DNA synthesis during the repair process was evaluated by assessing the incorporation of tritiated thymidine (15). CLL lymphocytes (2 x 10⁶ cells) having previously been treated and washed into fresh media were incubated with tritiated thymidine (6 μCi) for the indicated periods (0, 1, 2, 4 and 6 h). The lymphocytes were then collected, centrifuged, and resuspended in 500 μl of 0.4 N perchloric acid. The sample was mixed, centrifuged, and resuspended again in
perchloric acid. After another mixing and centrifugation, the pellet was resuspended in 1 ml of 0.5 N KOH and incubated at 45 °C overnight to dissolve the pellets. Radioactivity was counted with a liquid scintillation counter (Packard Instrument Co.).

**Quantitation of apoptotic cell death by flow cytometry of FITC-conjugated annexin V and propidium iodide double staining.** To evaluate cytotoxicity, apoptotic cell death was determined as phosphatidylserine externalization (28). At 24 h after treatment, CLL lymphocytes were collected by centrifugation (1500 rpm, 5 min) and resuspended in 200 µl of binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl$_2$) and 10 µl of FITC-conjugated annexin V. The sample was incubated for 15 min at room temperature and centrifuged again. The pellet was resuspended with 500 µl of the same buffer and with 10 µl of 50 µg/ml propidium iodide. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson). Cells positive for annexin V were considered apoptotic.

**Statistical analysis.** All statistical analyses were performed with Microsoft Excel (Redmond, WA). Cytotoxic effects were evaluated by the paired t tests between
the sum of death due to 4-HC and either nucleoside analogue, and the death observed following the combination. All graphs, linear regression lines, and curves were generated with GraphPad, Prism software (GraphPad Software, Inc. San Diego, CA).

Results

**DNA repair response initiated by 4-HC.** Our first objective was to define the dose and time response of CLL lymphocytes to treatment with 4-HC. When CLL lymphocytes were incubated for 30 min with 4-HC doses ranging from 20 to 100 µM, a dose-dependent increase in the tail-moment response appeared that was linear between 20 and 80 µM (Fig. 2A). This is indicative of the initiation of the incision aspect of the repair processes. Although there was substantial heterogeneity among samples, the maximum response was generally observed between 45 and 60 min (Fig. 2B). The decline in response at later times is due to completion of the repair and inactivation of 4-HC in media. To determine the time course of the repair response to the 4-HC treatment, CLL lymphocytes were pulsed with 60 µM 4-HC for 30 min, followed immediately by washing the drug from the samples. The tail moment in each cell
sample was greatest at the end of the incubation (Fig. 2C), but the absolute values varied among individuals. The rapid decrease in the tail-moment value after removal of 4-HC represents the rate of completion of the repair process with DNA ligation. These rates appeared to decrease to half the peak value by 2 h after removal of 4-HC (Fig. 2C). On the bases of these results, DNA repair responses in subsequent studies were initiated by the incubation with 60 μM 4-HC for 30 min.

**Effect of nucleoside analogues on DNA resynthesis initiated by 4-HC.** To evaluate the DNA resynthesis step in the repair process, we measured the incorporation of tritiated thymidine into DNA. The incorporation of thymidine initiated by 4-HC increased time-dependently, but appeared to reach a plateau at 4 h, suggesting completion of the repair process. (Fig. 3) Pre-treatment with either nucleoside analogue reduced the extent of the 4-HC-induced thymidine incorporation by half ($P < 0.001$ between 4-HC alone and 4-HC with either nucleoside analogue at 6 h, by paired t test). The magnitude of the reduction of thymidine incorporation was not different between the 2 analogues.
Cellular pharmacology of F-ara-A and Cl-F-ara-A. The ability of CLL lymphocytes to accumulate the triphosphates of F-ara-A or Cl-F-ara-A was determined by incubating cells with escalating concentrations of either tritiated nucleoside, followed by extraction of the analogue metabolites and analysis by high-performance liquid chromatography (Figs. 4A & B). A dose response was evident between exogenous nucleoside and the cellular concentration of the respective triphosphate forms in each sample, although the absolute values varied among individuals. The relative ability of CLL cells to accumulate each nucleotide analogue was compared in a larger cohort of CLL lymphocyte samples. There was a close correlation between the relative accumulation of F-ara-ATP and Cl-F-ara-ATP among the population (R = 0.72), but cells appeared to accumulate F-ara-ATP to approximately 6-fold greater concentrations than Cl-F-ara-ATP (Fig. 4C). This may be due to the differences in the cellular metabolism of the two analogues. For instance, the major intracellular metabolite of Cl-F-ara-A in CLL cells is the monophosphate form (the mean ratio of mono- to tri-phosphate was 3.61), whereas F-ara-ATP is the major cellular metabolite of F-ara-A in CLL cells (the ratio of mono- to tri-phosphate was 0.28) (data not shown) (38, 39).
Inhibition of DNA repair by F-ara-ATP and Cl-F-ara-ATP. To evaluate the action of the nucleoside analogues on the DNA repair process, CLL lymphocytes were pre-loaded with F-ara-ATP or Cl-F-ara-ATP by incubation with various concentrations of tritiated F-ara-A or Cl-F-ara-A, followed by 4-HC treatment to initiate the repair response. To relate the ability of cells to complete the DNA repair process to the cellular concentrations of the triphosphates, samples were taken 2 h after washing cells into fresh media and then analyzed by the comet assay. In this experimental design, an increase in the tail-moment value represents a combination of on-going incision and inhibition in the ability of cells to complete the repair process. The pattern of response to the nucleotide analogues was remarkably similar with respect to the F-ara-ATP (Fig. 5A) and Cl-F-ara-ATP (Fig. 5B). The concentration-dependent increase in the tail-moment values indicated that the ability of CLL lymphocytes to complete the repair process declined in proportion to the cellular concentration of the analogue triphosphates. This relationship reached a plateau in all samples. However, the magnitude of the response varied substantially among samples, representing inter-patient variability in repair capacity. Interestingly, although the pattern of response to the two analogues was
similar within patients, a nearly 10-fold difference was found in the cellular triphosphate concentrations that produced the same response. Neither F-ara-ATP levels greater than 50 μM nor Cl-F-ara-ATP concentrations in excess of 5 μM appeared to increase tail-moment values. This disparity between analogues suggest that Cl-F-ara-A nucleotide is the more potent inhibitor of repair, which may be attributed to differences in the affinity of DNA repair enzymes for the two analogues. Treatment with nucleoside analogues alone did not evoke a DNA damage and repair response detectable by the comet assay (data not shown).

To relate the analogue triphosphate concentration to the inhibition of DNA repair among the samples, CLL lymphocytes were incubated with or without 10 μM F-ara-A or 10 μM Cl-F-ara-A, followed by 4-HC. Triphosphate concentrations were determined immediately after 4-HC was washed from the samples and tail moments were quantitated 2 h later. Inhibition of repair processes, expressed as the ratio of the tail moment values in the presence and absence of the nucleosides, was related to the cellular concentration of the respective triphosphate (Figs.5C & D). There appeared to be a dose response at the lower concentrations of each nucleotide, but generally, no
further effect on DNA repair capability was discernable at F-ara-ATP concentrations greater than 50 µM or at Cl-F-ara-ATP levels in excess of 5 µM. This finding suggests target concentrations for achieving maximum inhibition in the design of clinical investigations.

**Effect of nucleoside analogues on DNA repair responses initiated by 4-HC.**

To characterize the range of capabilities in CLL lymphocytes to initiate and conduct incision repair, cells were incubated with 4-HC and the repair response was evaluated by the tail moment at 0 h and 2 h after the drug was washed from the samples. The magnitude of the incision response of cells to DNA damage initiated by 4-HC varied among 50 patients (median tail moment value at 0 h, 5.7; range, 1.5-20.0) (Fig. 6A). When compared with values obtained 2 h after removal of the drug, the tail moment decreased in all but 2 samples (median, 2.6; range, 0.5-8.8), suggesting the re-sealing of DNA that had been incised. Again, there was variation among samples with regard to the rate of this process; the coefficient of variation was 30% in samples, demonstrating substantial heterogeneity of DNA repair capacity. The initial tail-moment value generated by 4-HC treatment (5.1 ± 3.8, mean ± SD) was not changed by pre-incubating
the samples with nucleoside analogues (5.6 ± 3.9 for F-ara-A, 5.4 ± 3.4 for Cl-F-ara-A), suggesting that these analogues have no effects on the incision steps of repair that are discernable by the comet assay (data not shown).

The action of the nucleoside analogues on the rate of DNA repair, defined as the ratio of the tail moments at 0 h and 2 h, was compared (Fig. 6B). The rate of DNA repair initiated by 4-HC (2.4 ± 0.7) was significantly decreased by a pre-incubation with either F-ara-A (1.2 ± 0.3, P < 0.0001) or Cl-F-ara-A (1.1 ± 0.4, P < 0.0001), suggesting interference in the repair process by these analogues. The symmetry in the plot in Fig. 6B indicates that the two nucleoside analogues had similar effects (P = 0.35).

**Optimal schedule for the combination of 4-HC with nucleoside analogues.**

To evaluate the importance of scheduling nucleoside analogues and an alkylating agent, CLL lymphocytes from 2 patients were incubated with F-ara-A or Cl-F-ara-A to load cells with the respective triphosphates either before 4-HC addition or at various times thereafter. Tail moments were quantitated 2 h after the addition of the nucleosides (Fig. 7). The tail moment values, and hence inhibition of repair, were highest when either triphosphate was present in the cell prior to 4-HC addition. There was a marked
decrease in the effect of the nucleosides relative to that of 4-HC alone as the time between the administration of the alkylating agent and the addition of the nucleoside was increased. This suggests that the time during which the repair processes initiated by 4-HC are sensitive to the action of the nucleoside analogues is brief, and that the maximum effect will be obtained by administration of the nucleoside analogues immediately prior to the alkylating agent.

**Increase in 4-HC-induced cytotoxicity by the addition of nucleoside analogues.** To evaluate the cytotoxicity of such combination, CLL lymphocytes were treated with either nucleoside analogue, or 4-HC, or these agents in combination. At 24 h after the treatment, apoptotic cell death was detected by FITC-conjugated annexin V staining. The combination treatment produced more than additive apoptotic cell death than the sum of the nucleoside analogue and 4-HC alone ($P = 0.01$ for F-ara-A, $P = 0.001$ for Cl-F-ara-A) (Fig. 8). Cl-F-ara-A induced greater cytotoxicity than equal concentrations of F-ara-A ($P = 0.01$). Consequently, the combination of 4-HC with Cl-F-ara-A provided the greater cytotoxicity than that with F-ara-A ($P = 0.01$).
Correlation between increased cytotoxicity and inhibition of DNA repair.  

To link the mechanism-based interaction between nucleoside analogues and 4-HC to the cytotoxic effect, DNA repair and apoptotic cell death were evaluated in the same CLL samples. The increase in the apoptotic cell death, expressed as the ratio of the apoptotic cell death in the presence of the nucleoside analogues relative to that observed in their absence, was proportional to the inhibition of DNA repair, the parameter used in Figs. 5C &D (Figs. 9A & B). This finding suggests that inhibition of DNA repair by nucleoside analogues was directly associated with cytotoxicity. The increase in apoptotic cell death was also proportional to the initial tail-moment value of the 4-HC treatment, suggesting the existence of proportionality among DNA incision, nucleotide analogue incorporation, and cytotoxicity (Figs. 9C & D).

Discussion

In previous studies, DNA excision repair initiated by UV or 4-HC in quiescent normal lymphocytes allowed the nucleoside analogue F-ara-A to be incorporated into the repair patch (15, 40), an action that was associated with the inhibition of the repair
processes. In both, such mechanism-based interactions enhanced cytotoxicity. The present study extended this approach to CLL lymphocytes. Although the magnitude of the repair response was heterogeneous in these primary tumor cells, DNA repair was initiated promptly and completed rapidly in most cases. Treating cells with F-ara-A or Cl-F-ara-A before the addition of 4-HC maximized the repair inhibition with the greatest effect at their intracellular triphosphate concentrations at 50 μM or 5 μM, respectively. The combinations produced more than additive apoptotic cell death than the sum of each agent alone. This increase in cytotoxicity was proportional to the extent of repair inhibition by these nucleoside analogues, or to the initial tail-moment value generated by 4-HC.

Phosphoramidate mustard, the active compound of cyclophosphamide, forms approximately 67% phosphotriester monoadducts, 26% N7 guanine monoadducts, and 6.7% N7 guanine - N7 guanine diadducts (16, 36, 41). The monoadducts are usually repaired by base excision repair and nucleotide excision repair. The diadducts are eliminated by the combined actions of nucleotide excision repair and the recombination system (16). Thus, single strand breaks are generated as the result of incision
processes of all these repair mechanisms, which may be detected by the comet assay.

Some care must be exercised to equating nicks measured by the comet assay with excision repair intermediates. Among these adducts, N7 guanine adducts are labile and can produce abasic sites, which might result in a break under alkaline conditions in the comet assay. However, it has been reported that the abasic sites formed by ethyl nitrosourea at concentrations below 2 mM did not produce single strand breaks under alkaline conditions similar to those used in our comet assay (21). Given this background, abasic sites that might have been formed in the present study would be unlikely to produce significant single strand breaks under the alkaline assay conditions. Phosphotriester adducts might also produce nicks during alkali denaturation. However, alkyltriesters are reported to be highly persistent DNA modifications that stay in the DNA for 20 to 50 h (42). Since 4-HC-induced strand breaks detected by the comet assay disappeared almost within 4 h in our present study (Fig. 2C), it would seem unlikely that such alkylphosphotriesters contribute significantly to DNA single strand breaks detected by this assay. Recent reports have also suggested that phosphotriesters do not contribute to comet formation (21, 22, 43). Abasic sites and alkylphosphotriesters may
not be converted into single strand breaks under the alkaline conditions because the alkaline unwinding duration (20 min) in the comet assay used here is very short, compared with the duration for the standard alkaline filter elution procedures (12 to 16 h) that has been used to demonstrate such nick formation. Nevertheless, we have attempted to further minimize this slight possibility by carrying out unwinding denaturations as well as the electrophoresis step at 4°C. More to the point, when we treated normal lymphocytes with 4-HC at 4°C to suppress the repair response, no comet tails were observed (data not shown). This is in contrast to the comet formation and repair over time in these samples, similar to what we have observed in CLL cells. This finding suggests that the chemical sequelae that might occur are unlikely to have a major effect in the formation of comets. Moreover, as interstrand cross-links occupy less than 10% among the adducts produced by phosphoramid musturd, these diadducts would not significantly affect the comet formation. Thus, strand breaks detected by the comet assay are likely to be mainly derived from the incisions during the repair processes. Therefore, it is reasonable to regard the 0-time tail moment as a reflection of
the incision steps of DNA repair processes, whereas the decrease in tail moment thereafter likely reflects the completion of repair processes.

Several DNA repair processes initiated by DNA alkylation involve incision and excision of the oligonucleotides that include the damaged nucleotide. Subsequently, there are gap filling by resynthesis of DNA, and rejoining by ligation (16-18). We used the comet assay to evaluate the kinetics of DNA damage repair because this method measures the initial step of incision and the last step of rejoining. The comet assay in combination with measurement of DNA resynthesis by the incorporation of tritiated thymidine into the DNA allowed us to quantitate the major processes of repair.

Here, we demonstrated that primary CLL lymphocytes could initiate DNA damage repair after treatment with 4-HC (Fig. 2C). The significant increase in the tail moment observed at the end of the incubation period suggested that repair was initiated immediately. Four-HC also initiated the incorporation of tritiated thymidine that represented the gap filling by DNA resynthesis (Fig. 3). This incorporation was time-dependent, but seemed to reach a plateau by 4h, indicating the completion of this process. The steep decrease in the tail moment at subsequent times suggested that
ligation had occurred and that the repair process was generally completed quickly (Fig. 2C). The values for the initial tail moment and the rate of repair (the tail moment ratio between 0 h and 2 h) varied widely (Figs. 6A, & B), suggesting variability in the repair capacity in individuals. This finding also suggests that the clinical response to treatment with alkylating agents might be heterogeneous among patients, although the samples examined here were from the patients with CLL not refractory to alkylators.

Both the cellular response to DNA damage and the rate of repair as determined by the comet assay appears to vary among cell types (26, 27). Lymphocytes, either normal or CLL, appear to be active in this regard. We have already demonstrated that the repair process in normal lymphocytes initiated by 4-HC was completed within 4 h (40). Moreover, despite the large heterogeneity among the samples, the time curves of the tail moment were most linear between 0 h and 2 h (Fig. 2C). Therefore, we used the tail moment values of 0 h and 2 h.

The 4-HC-induced DNA repair was inhibited by pre-incubating the samples with nucleoside analogues, as indicated by the decrease in the rate of repair and inhibition of the thymidine incorporation into DNA. Judging from the thymidine-incorporation results,
the DNA synthesis step was inhibited almost by half after the addition of the either nucleoside analogue (Fig. 3). The rate of repair was also reduced by half by the pre-incubation with analogues (Fig. 6B). No differences were found between the initial tail moments in the presence or absence of nucleoside analogues, which represents the incision step. Thus, the nucleoside analogues seem to have inhibited mainly the DNA resynthesis and the ligation steps of the repair process. This would be in accord with the essential activity of these nucleoside analogues as DNA chain terminators (8, 9).

In our previous studies, we demonstrated that the minimally toxic concentration of F-ara-A inhibited the repair initiated by UV or 4-HC in normal lymphocytes (15, 40). Such inhibitory effects were proportional to the incorporation of F-ara-A nucleotide into DNA, suggesting a mechanism-based specific effect. Cells treated with F-ara-A after completion of the repair processes revealed by the comet assay did not form comets, indicating schedule-dependency (Fig. 7). Similarly, cells did not become annexin-positive when treated with a DNA repair inhibitor, UCN-01, after comet formation had reversed (44). When aphidicolin was used to inhibit DNA repair synthesis in place of F-ara-A, the level on annexin-positive cells generated in combination with UV was less
than additive (unpublished data). Furthermore, concentrations of aphidicolin that block DNA synthesis are antagonistic to the toxicity of F-ara-A (10). We also reported that treatment of normal quiescent lymphocytes with antibodies to fas or fas ligand do not increase the toxicity of UV (45). Therefore, in the present study with CLL lymphocytes, the inhibition of repair would be attributed to the actions resulting from nucleoside analogue incorporation into DNA.

Escalating concentrations of nucleoside analogues led to the increased accumulation of the triphosphates in CLL lymphocytes (Fig. 4). The ability to yield the triphosphates varied among samples, but in two-thirds of CLL samples, 10 µM nucleosides produced 50 µM F-ara-ATP or 5 µM Cl-F-ara-ATP, the concentrations that maximally inhibited DNA repair (Figs. 5C & D). Clinically, a 30-min infusion of 25-30 mg/m² fludarabine to the patients with CLL produced 2 - 7 µM F-ara-A concentrations in the plasma and 6 - 52 µM F-ara-ATP concentrations in CLL lymphocytes (38). In another study, a 1-h infusion of 15 mg/m² Cl-F-ara-A yielded 0.3 - 19 µM Cl-F-ara-ATP concentrations in CLL lymphocytes in a phase I clinical trial (35). Therefore, the
triphosphate concentrations needed to exert the maximal repair inhibition in vitro can be achieved during therapy.

In this study, F-ara-A and Cl-F-ara-A were similarly potent inhibitors of the DNA repair processes as incubating CLL lymphocytes with 10 μM of either F-ara-A or Cl-F-ara-A led to equal inhibition of the DNA repair. However, viewed from the perspective of intracellular triphosphate concentrations, Cl-F-ara-ATP demonstrated maximal inhibition at one-tenth the concentration of F-ara-ATP (Fig 5). This finding suggests either that DNA repair enzymes have stronger affinity for Cl-F-ara-ATP, or that the incorporated Cl-F-ara-A nucleotide is a more potent inhibitor of repair. Compared with F-ara-A, Cl-F-ara-A also produced a greater apoptotic cell death both as a single treatment and in combination with 4-HC (Fig. 8), suggesting that it is more easily recognized by surveillance systems that initiate apoptotic cascades.

We have previously evaluated the actions of the combination treatment of F-ara-A with 4-HC or UV on the viability of normal lymphocytes (15, 40). It was confirmed as apoptotic cell death by several detection methods such as morphological features, high molecular weight DNA fragmentation, and inter-nucleosomal DNA fragmentation.
Furthermore, we have reported caspase-3 activation and PARP cleavage in lymphocytes treated with F-ara-A and UV (45). Based on these findings we extended the study to CLL lymphocytes here, using flow cytometric analysis with FITC-conjugated annexin. The combination of 4-HC with nucleoside analogues produced more than additive cytotoxicity than the sum of each drug alone (Fig. 8). This enhanced cytotoxicity was proportional to the amount of the incision initiated by 4-HC, or to the inhibition of repair by the nucleoside analogues (Fig. 9). This finding suggests that the greater incorporation of nucleoside analogues or greater amounts of unrepaired DNA damage would increase cytotoxicity. Therefore, this increase in the cytotoxic effect would be brought about from the mechanism different from that of either agent alone.

The damage repair response to the alkylating agent and the inhibition of the repair by nucleoside analogues were both heterogeneous among CLL samples, but they were directly correlated to the induction of cell death. Because the DNA repair processes are completed rapidly, such combinations should be administered sequentially in the clinical setting to enable CLL cells to accumulate maximum triphosphate levels prior to administering an alkylating agent. Clinically, this mechanistic interaction may be more
effective in the treatment of refractory CLL, if the resistance results from an increased
capacity for the repair response to alkylator-induced DNA damage.

The DNA repair processes active in CLL lymphocytes could be considered as a
biologic target in the context of alkylating agents, since the repair process appears to
facilitate the activity of nucleoside analogues. Our results may provide a mechanistic
explanation for the finding that the combination of fludarabine with cyclophosphamide
produced a higher response rate than that expected from the sum of individual agents in
the salvage therapy for CLL patients resistant to both alkylating agents and fludarabine
(46).

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Legends

Fig. 1 Chemical structures of 2’-deoxyadenosine, F-ara-A, and Cl-F-ara-A.

Fig. 2 DNA repair response initiated by 4-HC. CLL lymphocytes were incubated for 30 min with 20, 40, 60, 80, or 100 µM 4-HC (A), or for 15, 30, 45, 60, 90, or 120 min with 60 µM 4-HC (B). The tail-moment values were determined by the comet assay at the end of the incubation. C, CLL lymphocytes were pulsed with 60 µM 4-HC for 30 min and then washed into fresh media. The tail moments were assayed at 0, 0.5, 1, 2, and 4 h after the washing. Each symbol represents samples from one patient.

Fig. 3 Effect of nucleoside analogues on DNA resynthesis initiated by 4-HC. CLL lymphocytes from 8 patients were incubated for 2 h with or without 10 µM F-ara-A or Cl-F-ara-A, followed by a 30-min incubation with 60 µM 4-HC. After being washed into fresh media, the lymphocytes were incubated with tritiated thymidine for the indicated periods (0, 1, 2, 4, and 6 h), and the radioactivity of the DNA was determined. Closed circles, the treatment with 4-HC, open circles, the combination treatment with 4-HC and
F-ara-A, open triangles, the combination treatment with 4-HC and Cl-F-ara-A.

*Fig. 4* Cellular pharmacology of F-ara-A and Cl-F-ara-A. CLL lymphocytes were incubated for 2 h with escalating concentrations (1, 3, 5, 10, 20, 50, and 100 µM) of [8-\(^3\)H] F-ara-A (*A*) or [8-\(^3\)H] Cl-F-ara-A (*B*). Intracellular triphosphate levels were determined by high-performance liquid chromatography. Each symbol represents samples from one patient. *C*, CLL lymphocytes from 23 patients were incubated for 2 h with 10 µM [8-\(^3\)H] F-ara-A or 10 µM [8-\(^3\)H] Cl-F-ara-A. The relative accumulation of the 2 analogue triphosphates was compared in each sample. Each point represents samples from one patient.

*Fig. 5* *A, B*, Inhibition of DNA repair by F-ara-ATP and Cl-F-ara-ATP. CLL lymphocytes were incubated for 2 h with 3, 10, 20, or 50 µM [\(^3\)H]F-ara-A (*A*) or [\(^3\)H]Cl-F-ara-A (*B*), followed by the addition of 60 µM 4-HC for 30 min and then washing into fresh media. DNA repair was assayed by the tail moment at 2 h after the washing. Analogue triphosphate levels were determined at the end of the incubation periods. Each symbol
represents samples from an individual patient. C, D, Cellular concentration of nucleoside analogues versus the DNA repair process. CLL lymphocytes were incubated with 10 µM F-ara-A (20 patients) (C) or 10 µM Cl-F-ara-A (17 patients) (D) for 2 h or without nucleosides, followed by a 30-min incubation with 60 µM 4-HC and then washing into fresh media. Triphosphate concentrations were determined after the washing, whereas tail moments were quantitated at 2 h later. Inhibition of DNA repair was expressed as the ratio of the tail moment in the presence or absence of the nucleosides.

Fig. 6 Effect of nucleoside analogues on DNA repair response initiated by 4-HC. A, CLL lymphocytes from 50 patients were incubated for 30 min with 60 µM 4-HC, washed into fresh media, and the repair response was evaluated by the tail moment at 0 h and 2 h after the washing. The connected points represent samples from one patient. B, Effect of the nucleoside analogues on the rate of DNA repair. CLL lymphocytes from 50 patients were incubated with or without 10 µM F-ara-A or 10 µM Cl-F-ara-A for 2 h, followed by a 30-min incubation with 60 µM 4-HC. The tail moment was determined at 0 h and 2 h after 4-HC was washed out. The rate of DNA repair, defined as the ratio of
the tail moment at 0 h and 2 h, was compared in the presence or absence of the nucleoside analogues. The connected points represent samples from one patient.

**Fig. 7** Optimal schedule for the combination of 4-HC with nucleoside analogues. CLL lymphocytes from 2 patients were incubated for 2 h with 10 µM F-ara-A or 10 µM Cl-F-ara-A either before a 30-min incubation with 60 µM 4-HC, or at 0, 2, 4, or 6 h thereafter. Tail moments were quantitated at 2 h after the addition of the nucleosides.

**Fig. 8** Increase in 4-HC-induced cytotoxicity by the addition of nucleoside analogues. CLL lymphocytes from 16 patients were incubated for 2 h with or without 10 µM F-ara-A or 10 µM Cl-F-ara-A, followed by a 30-min incubation with 60 µM 4-HC and washing into fresh media. At 24 h after treatment, the samples were evaluated for apoptotic cell death as phosphatidylserine externalization by the staining with FITC-conjugated annexin V.

**Fig. 9** CLL lymphocytes from 16 patients were incubated for 2 h with or without 10 µM
F-ara-A (A, C) or 10 µM Cl-F-ara-A (B, D), followed by a 30-min incubation with 60 µM 4-HC and washing into fresh media. The tail moments were quantitated at 0 h and 2 h after the drug was washed out. At 24 h after treatment, the samples were assayed by annexin V staining. A, B, Increases in apoptotic cell death versus inhibition of DNA repair. The increase in apoptosis was the ratio of apoptotic cell death in the presence or absence of the nucleoside analogues. The inhibition of DNA repair was the ratio of the tail moment in the presence or absence of the nucleosides at 2 h after the washing, as shown for Fig. 5. C, D, Increases in the apoptotic cell death versus the initial tail moment after 4-HC treatment.
Patient 1

- **F-ara-A**
- **Cl-F-ara-A**

Tail Moment

Time of addition (relative to 4-HC)

Patient 2

- **F-ara-A**
- **Cl-F-ara-A**

Tail Moment

Time of addition (relative to 4-HC)
4-HC F-ara-A Cl-F-ara-A combination

apoptotic cell death (%)
Tail Moment

Increase in apoptosis

Inhibition of DNA repair

P = 0.001
R = 0.72

P = 0.02
R = 0.57

P = 0.05
R = 0.50

P = 0.03
R = 0.52
A

B

C

F-ara-ATP, $\mu$M vs. F-ara-A, $\mu$M

Cl-F-ara-ATP, $\mu$M vs. Cl-F-ara-A, $\mu$M

Cl-F-ara-ATP, $\mu$M vs. F-ara-ATP, $\mu$M

P<0.0001
R=0.72