Fludarabine increases oxaliplatin cytotoxicity in normal and chronic lymphocytic leukemia lymphocytes by suppressing interstrand DNA crosslink removal

Short title: Fludarabine-oxaliplatin interactions in CLL

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Supported in part by grant CA81534 and Cancer Center support grant CA16672 from the National Cancer Institute, Department of Health and Human Services.

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Abstract word count: 191
Total text word count: 4039
Scientific heading: Neoplasia
ABSTRACT

Oxaliplatin and fludarabine have different but potentially complementary mechanisms of action. Previous studies have demonstrated that DNA repair is a major target for fludarabine. We postulate that potentiation of oxaliplatin toxicity by fludarabine maybe due to the inhibition by fludarabine of the activity of the DNA excision repair pathways activated by oxaliplatin adducts. To test this, we investigated the cytotoxic interactions between the two drugs in normal and CLL lymphocytes. In each population, the combination resulted in greater than additive killing. Analysis of oxaliplatin damage revealed that fludarabine enhanced accumulation of interstrand crosslinks (ICLs) in specific regions of the genome in both populations, but to a lesser extent in normal lymphocytes. The action of fludarabine on the removal of oxaliplatin ICLs was explored to investigate the mechanism by which oxaliplatin toxicity was increased by fludarabine. Lymphocytes from CLL patients have a greater capacity for ICL unhooking compared to normal lymphocytes. In the presence of fludarabine the extent of repair was significantly reduced in both populations, more so in CLL. Our findings support a role of fludarabine-mediated DNA repair inhibition as a mechanism critical for the cytotoxic synergy of the two drugs.
INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia in Western countries and it accounts for approximately 25% of all leukemias\(^1\). While at the present there is no curative treatment, combinations of cytotoxic agents, and of immunotherapies that generate high complete remission rates hold promise for altering the natural history of this disease\(^2-4\). Alkylating agents and nucleoside analogues are two classes of anticancer agents important in the treatment of chronic lymphocytic leukemia. Indolent B-cell malignancies such as CLL exhibit a high DNA damage repair capacity\(^5\). This biological property provides a rationale for therapeutic strategies that combine DNA-damaging cytotoxic agents, such as oxaliplatin, with an inhibitor of DNA repair such as fludarabine.

Fludarabine (9-beta-D-arabinofuranosyl-2-fluoroadenine 5’-phosphate) is the most effective purine nucleoside analogue for the treatment of indolent lymphoproliferative disorders including CLL, low-grade lymphoma, and prolymphocytic leukemia\(^6,7\). Upon infusion into the blood stream fludarabine is dephosphorylated to the respective nucleoside (F-ara-A) which is a substrate for transporters\(^8\). Upon entering the cell, fludarabine is anabolized to its triphosphate form, its major intracellular metabolite\(^9\). The triphosphate can then become incorporated into DNA or RNA, actions which block further synthesis\(^10\). Its inhibition of ribonucleotide reductase, an action that depletes the deoxynucleotide pools required for DNA repair and replication, may favor its increased incorporation into newly synthesized DNA\(^11\). Further, its inhibitory actions against both DNA ligase\(^12\) and DNA primase\(^13\) are likely to antagonize the consequences of its
incorporation. The finding that it is resistant to excision\textsuperscript{14} is likely to contribute to its effective inhibition of DNA synthesis. Finally, \textit{in vitro} studies have shown that the loss of cell viability correlates directly with the level of incorporation of the analogue into cellular DNA.

Oxaliplatin (DACH oxalate-platinum(II); Eloxatin) is a third generation platinum compound with a different spectrum of activity and low cross-resistance with cisplatin\textsuperscript{15}. It is currently indicated for use with fluorouracil and leucovorin for the treatment of advanced cancer of the colon or rectum\textsuperscript{16}. Oxaliplatin has demonstrated in vitro and in vivo supra-additive effects in combination with several other antitumor agents, including 5-fluorouracil (5-FU), topoisomerase I inhibitors, thymidylate synthase inhibitors, paclitaxel, cisplatin, and carboplatin \textsuperscript{17,15}. The mechanism of action of oxaliplatin is mediated by the formation of DNA adducts. The main types of DNA lesions induced by oxaliplatin are intrastrand crosslinks covalently binding the platinum compound to guanine residues\textsuperscript{18}. The other type of lesions include DNA interstrand crosslinks (ICLs), and DNA protein crosslinks\textsuperscript{19}. Oxaliplatin-DNA adducts are suggested to exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis.

The non-overlapping side effect profiles of oxaliplatin and fludarabine and their different but potentially complementary mechanisms of action provide a basis for investigation of the activity of the drugs in combination. The rationale for combining oxaliplatin with fludarabine is based on preclinical data demonstrating synergistic cytotoxicity between
cisplatin in combination with the nucleoside analogues ara-C \textsuperscript{20}, gemcitabine \textsuperscript{21} \textsuperscript{22}, clofarabine\textsuperscript{23} and fludarabine \textsuperscript{24} \textsuperscript{25} \textsuperscript{26}.

It has been suggested previously that enhanced crosslink repair is a primary mechanism of resistance to nitrogen mustards in B-CLL\textsuperscript{27}. As such, we hypothesize that fludarabine may modulate oxaliplatin sensitivity by suppressing ICL removal, thereby resulting in synergistic cytotoxicity in CLL. Modulation of DNA repair represents one strategy to overcome cellular drug resistance to platinum compounds and achieve therapeutic gain. Such approaches focus on inhibiting the repair processes, thereby either subjecting the cells to the full consequence of the damage to DNA, or by creating a new type of damage that the cell is incapable of repairing. In CLL, DNA repair has been suggested as the mechanism underlying drug resistance. It has been reported that CLL cells resistant to alkylating agents exhibit an increased nucleotide excision repair (NER) activity measured using the comet assay \textsuperscript{28} \textsuperscript{29}. We have hypothesized that this increased capacity for repair could favorably facilitate incorporation of fludarabine into the DNA repair patch\textsuperscript{23}. To that end, we have demonstrated the synergistic cytotoxic interaction between oxaliplatin and fludarabine in normal and chronic lymphocytic leukaemia lymphocytes, and investigated the mechanistic basis for this finding.

**Materials and Methods**

**Drugs and solvents**

To facilitate investigations, fludarabine (Berlex Biosciences, Richmond, CA) which is impermeable to cells, was dephosphorylated with alkaline phosphatase to generate the cell-permeable nucleoside which was used in these \textit{in vitro} studies. It was dissolved in
phosphate-buffered saline to a concentration of 2 mM, and stored at -20 °C. Oxaliplatin, provided by Sanofi-Synthelabo Research (Great Valley, PA), was dissolved in phosphate-buffered saline to a concentration of 1 or 5 mM. Drug solutions were prepared fresh for every experiment. All other reagents were of analytical grade and commercially available, and solutions were prepared using H₂O from a MilliQ academic purification system (Millipore, MA).

**Preparation of normal and leukemic lymphocytes**

A total of 39 samples were collected for this study, 15 from healthy individuals and 24 from CLL patients who had been selected for early stage disease (Rai stage: 0-1, 14; stage 2, 4; stage 4, 4). Only two patients had received prior fludarabine treatment. The median age was 62 years (range 33-90 years). The median absolute lymphocyte count was 40,755/µL (range, 3,828-226,080/µL), while the median percentage of lymphocytes in the white blood cell count was 86% (range, 6%-96%) with the median percentage of CD19/CD5 co-expressing lymphocytes being 89.9 (range, 8-98%). Because of the labor-intensive nature of the procedures use in these investigations, we generally used separate cohorts of samples for the various experiments. We assumed that samples were derived from reasonably similar individuals because the disease of most patients was had not progressed to a state that required treatment, and volunteers were generally under age 25 years. Whole blood was drawn into heparinized tubes, diluted 1:5 with cold PBS and layered over Fico/Lite LymphoH (specific gravity, 1.077; Atlanta Biologicals, Norcross, GA). The mixtures was then centrifuged at 430 x g for 20 min. Mononuclear cells were removed from the interphase, washed twice with cold PBS and resuspended in 10 ml of
RPMI 1640 medium containing 10% fetal bovine serum (FBS). The cells were then counted and their median volume was determined using a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). Cells were diluted to a concentration of 2 x 10^6 cells/ml, and incubated in plastic plates in a 5% CO₂ humidified atmosphere at 37°C for 4 hr to remove the adherent monocytes and acclimatize the cells to culture conditions. The lymphocytes were then carefully collected, counted and resuspended at a concentration of 2 x 10^6 cells/ml. All donors provided written informed consent approved by the MD Anderson Cancer Center review board.

**Quantitation of apoptotic cell death**

Apoptotic cell death in normal or CLL lymphocytes was evaluated by flow cytometry analysis using annexin V and propidium iodide (PI) double staining. Lymphocytes were treated with drugs alone (2.5, 5, 10 μM oxaliplatin; 2.5, 5, 10 μM fludarabine) or in combination simultaneously for 16, 24 or 36 hr. After the treatment, apoptotic cell death was detected by FITC-conjugated annexin V (BD Biosciences) staining. Following treatment, CLL cells were washed twice with PBS, resuspended in 200 μl of binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and incubated with 10 μl annexin V-FITC for 15 min at room temperature. After centrifugation, the pellets were resuspended with 500 μl of binding buffer with 10 μl of 50 mg/ml PI. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson). Cells positive for annexin V were considered apoptotic.
Detection of oxaliplatin DNA damage-DNA probes

Three different probes, all genomic inserts labelled by random priming, were utilized to detect oxaliplatin-induced ICLs in various regions of the genome. The 1.8 kb *Eco*RI fragment containing exons I and II of the DHFR gene was isolated from pBH31R1.8 plasmid probe (Dr V. A. Bohr, National Institute on Aging, NIH, Baltimore, MD), and labelled using \( \alpha\)–\(^{32}\)PdATP. The probe was used to detect a 22-kb fragment of the DHFR gene localized to the 5’ end of the gene. The plasmid pBR322 (Dr V. A. Bohr, National Institute of Aging, NIH, Baltimore, MD) was digested with *Eco*RI and *Bam*HI to generate the 0.8 kb \( \delta\)-globin insert. The human \( \delta\)-globin probe was used to detect the entire 18 kb gene. The plasmid pCRII-HI (Dr C. A. Filburn, National Institute of Aging, NIH, Baltimore, MD) was digested with *Eco*RI to generate a 2,574 bp fragment of the human mitochondrial genome (nucleotides 652-3226). The mitochondrial fragment was used to detect the 16.5 kb human linearized *Bam*HI mitochondrial DNA (mtDNA) genome.

Drug treatment of cells and DNA extraction

Cells were seeded in 15 cm dishes at a density of 5 x 10^6 cells/dish and incubated in the absence or presence of fludarabine for 2 hr before exposure to increasing concentrations of oxaliplatin for 7 hr. For the repair experiments, cells were incubated with oxaliplatin for 5 hr in the absence or presence of fludarabine, to induce damage, followed by treatment with 0.1 M thiourea for 1 hr to block the conversion of monoadduct to an ICL during the post-treatment incubation. The cells were then incubated for up to 8 hr in drug-free medium or in fludarabine-treated medium. Cells were subsequently washed twice
with PBS, trypsinized and pelleted by centrifugation for 5 min at 1,500 rpm. Pellets were stored at -80 °C until required. The cell pellets were then resuspended in 200 µl PBS and total genomic DNA was extracted using the Qiagen QIAamp DNA blood isolation kit using the protocol provided, and the DNA concentration of the samples was determined. All experiments were performed at least three times.

**Detection of gene-specific oxaliplatin interstrand crosslinks**

To study the effect of fludarabine on the accumulation of oxaliplatin-induced ICL in specific DNA genes, DNA (6 µg) from treated cells was restricted with Hind III (to release the 22 kb DHFR fragment and the 18 kb δ-globin gene) whereas 4 µg DNA was digested with BamH1 (to linearize the mitochondrial genome) for 2 hr at 37 °C. The DNA was extracted once with phenol, once with chloroform and precipitated with ethanol. The pellet was resuspended in 15 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and denatured by the addition of an equal volume of 100 mM NaOH and incubated for 20 min at 37 °C. Loading buffer (4 µl of 10x buffer: 10 mM EDTA, 26% Ficoll, 0.25% bromocresol green) was added to each sample and the DNA was then electrophoresed through a 0.5 % agarose gel in TAE buffer at 27 V overnight. The DNA was transferred to nylon (Hybond N+, Amersham), fixed to the membrane by baking for 2 hr at 80 °C, and probed for the gene fragment of interest. Band visualisation and quantitation were performed by PhosphorImager™ (Molecular Dynamics) and Image Quant software (Version 4.2a, Molecular Dynamics). Assuming that crosslink formation is random along the DNA fragments, the Poisson distribution was used to calculate the average number of interstrand crosslinks present in DHFR, δ-globin and mitochondrial DNA fragments.
(normalized to crosslinks/10 kb) using the following formula\textsuperscript{30}, where N is the natural log of the fraction of DNA in the single-strand form:

\[ N = -\ln \left( \frac{\text{ssDNA}}{\text{ssDNA} + \text{dsDNA}} \right) \]

\[ = -\ln \left( \text{fraction of DNA molecules free of interstrand crosslinks} \right) \]

**Statistical analysis**

Statistical significance was determined using Student’s \( t \) test calculated with GraphPad Prism 4 software. A \( P \)-value < 0.05 was considered to be statistically significant.

**Results**

**Toxicity of oxaliplatin and fludarabine to CLL lymphocytes alone and in combination**

The induction of apoptosis by oxaliplatin and fludarabine as single agents was evaluated by flow cytometric analysis of annexin V stained CLL lymphocytes. Cell death was dependent on the concentration of oxaliplatin (Fig. 1A) or fludarabine (Fig. 1B). Induction of apoptosis was also time-dependent, reaching maximum activity at \(~12\) hr for 5 \( \mu \text{M} \) oxaliplatin (Fig. 1C) and 2.5 \( \mu \text{M} \) fludarabine (Fig. 1D). Drug concentrations that induced 15-25\% apoptosis were chosen for combination experiments. We then evaluated the ability of fludarabine and oxaliplatin as single agents or in combination for 24 hr to induce cell death. This is illustrated by the increase in number of cells staining positively for annexin V (Fig. 2A). The actions of oxaliplatin and fludarabine were then evaluated in lymphocytes from healthy donors (Fig. 2B) and from patients with CLL (Fig. 2C).
Alone, each drug induced apoptosis, but treatment with the combination for 24 hr resulted in greater than additive killing in each population (p<0.05). The interaction between fludarabine and oxaliplatin was further investigated by evaluating several concentrations of each drug at 16 hr and 36 hr (Fig. 3). While cell killing by each drug alone was dependent upon time of incubation, this was more evident for fludarabine. No marked differences of the single drugs were seen in the concentration ranges investigated. Again, the combination generated greater than additive killing, which was more pronounced at 36 hr. This range of concentrations are relevant to the clinical use of fludarabine\textsuperscript{31} and oxaliplatin\textsuperscript{32}.

**Fludarabine increases the accumulation of oxaliplatin-induced DNA interstrand crosslinks in both nuclear DNA and mtDNA**

We then sought to investigate the actions of fludarabine on the accumulation of the important oxaliplatin lesion, the ICL. Normal or CLL lymphocytes were incubated in the absence or presence of fludarabine for 2 hr before exposure to several concentrations of oxaliplatin for 7 hr. Total genomic DNA was isolated and analyzed for the presence of the ICLs in a 22 kb fragment of the DHFR gene, the entire 18 kb δ-globin gene as well as the 16.5 kb mtDNA. DNA from control cells incubated in the absence of any drugs was completely denatured under the conditions employed and migrated as single-strand DNA (Fig. 4A). In contrast there was an oxaliplatin concentration-dependent increase in double-strand cross-linked DNA in the DHFR gene from CLL lymphocytes. There was greater crosslinking in DNA from cells that were incubated with fludarabine prior to oxaliplatin addition. Quantitation of ICLs in three separate samples demonstrated a
linear increase as a function of oxaliplatin concentration within the range of 100 to 400 µM, reaching similar levels at 400 µM of 0.6 ICLs/10 kb in normal (Fig. 4B) and CLL lymphocytes (Fig. 4C). Incubation of normal lymphocytes (Fig. 4B) with oxaliplatin in the presence of fludarabine increased ICL levels by 40% \( (P < 0.05) \) at 400 µM. Similar results were obtained following analysis of ICLs in the transcriptionally inactive δ-globin gene and mtDNA. The amount of ICLs in the DHFR gene and the mitochondrial genome was similar, although fewer oxaliplatin lesions were detected in the δ-globin gene. In the CLL lymphocytes, incubation with 400 µM oxaliplatin in the presence of fludarabine nearly doubled ICL levels in the DHFR gene (Fig. 4C). Again, similar findings were observed in both the δ-globin and the mtDNA in which the accumulation of ICLs was also increased by fludarabine (Table 1). These data support an association between the effect of fludarabine on oxaliplatin-induced ICL accumulation and the cytotoxic synergism observed in CLL lymphocytes.

**Fludarabine retards the removal of oxaliplatin-induced DNA interstrand crosslinks in specific regions of the genome**

The action of fludarabine on the removal of oxaliplatin ICLs was examined to investigate the possibility that the apparent increase in ICL accumulation in the presence of fludarabine was due to suppression of oxaliplatin-induced DNA ICL removal. The recovery of the ability to denature cross-linked DNA, which measures the unhooking of the crosslink, was used to compare the repair proficiency in cells. That fludarabine affects the removal of ICLs can be seen in the analysis of the DHFR gene from normal lymphocytes after oxaliplatin treatment in the absence or presence of fludarabine (Fig.
5A). Lymphocytes from healthy donors (Fig. 5B) and from patients with CLL (Fig. 5C) removed crosslinks with a half-life of about 4 hr. This reaction was retarded in cells that had been first treated with fludarabine.

Consistent with findings above (Fig. 4), the amount of oxaliplatin-induced ICLs does not vary in specific genes between normal and CLL lymphocytes; however the rate of ICL removal was different. Oxaliplatin ICLs were removed efficiently over 8 hr with 68% in normal lymphocytes (Fig. 5B) and 85% in CLL cells (Fig. 5C) crosslinks unhooked in the DHFR gene. Similar findings were observed in the δ-globin gene and the mtDNA (Fig. 6) (Table 2). This suggests that CLL lymphocytes have a greater capacity for DNA crosslink unhooking compared to normal lymphocytes.

In cells treated with oxaliplatin alone and in combination with fludarabine, ICLs were removed from the mtDNA rapidly with few crosslinks remaining after 8 hr (Fig. 6A). Quantitation revealed that CLL cells efficiently repaired oxaliplatin ICL with 75% of damage removed within 8 hr. In the presence of fludarabine however, the extent of repair was reduced to 50% (Fig. 6B). In the presence of fludarabine, the extent of ICL removal in the DHFR gene was reduced to 51% in CLL (40% inhibition) and 48% in normal lymphocytes (29% inhibition) (Table 2). The actions of fludarabine on formation and removal of oxaliplatin ICL in specific genes of normal or CLL lymphocytes are summarized in Table 2. Fludarabine action on the actively transcribed DHFR gene was similar to that observed in the mtDNA in normal or CLL lymphocytes; however it exceeded that on the transcriptionally inactive δ-globin gene. These results suggest that
suppression of the repair of oxaliplatin-induced ICL by fludarabine may vary according to the state of condensation of chromatin.

**Comparison of the actions of fludarabine with clofarabine and cytarabine on accumulation and removal of oxaliplatin ICL in CLL lymphocytes.**

Finally, we extended our investigation to compare the action of cytarabine (ara-C) and clofarabine (Cl-F-araA) relative to fludarabine at equitoxic concentrations (2.5 µM and 10 µM respectively) on ICL accumulation and removal kinetics. Under the same conditions employed above, oxaliplatin ICL accumulation and removal were assessed in the absence or presence of a nucleoside analogue in CLL lymphocytes. Clofarabine, but not cytarabine, significantly increased the level of ICL accumulation (Table 3). Furthermore, in the presence of clofarabine, the extent of ICL removal was retarded by nearly 65% in contrast to only 14% inhibition by cytarabine treatment (Table 3).

**DISCUSSION**

Previous studies have shown that fludarabine is capable of inhibiting DNA repair induced by carboplatin\(^{33}\) or UV\(^{34}\) in quiescent human lymphocytes, that stimulated by 4-hydroperoxy-cyclophosphamide in CLL lymphocytes\(^{23}\), and cisplatin-generated damage in K562 cells\(^{26}\). As an extension of those studies, the present investigations reveal a greater than additive cytotoxic effect of fludarabine in combination with oxaliplatin in both normal lymphocytes and CLL cells. These actions were associated with an inhibition of the rate of ICL removal in the presence of fludarabine, suggesting that this contributes to the increase in the cytotoxicity of the combination.
The action of fludarabine on the accumulation of oxaliplatin-induced DNA ICLs was investigated in specific DNA genes and measured directly using a denaturation/renaturation assay and Southern hybridization technique. This gene-specific assay has been previously used to explore the effects of fludarabine on the repair of cisplatin lesions\textsuperscript{24} and to investigate the actions of gemcitabine on cisplatin damage accumulation and repair\textsuperscript{21}. Although the requirement of quantitating ICLs in single gene DNA requires a relatively high level of ICLs throughout the entire genome, the results demonstrated that fludarabine significantly increased oxaliplatin ICL accumulation in the nuclear DHFR gene, and \(\delta\)-globin gene as well as in the mtDNA in CLL lymphocytes (Table 1).

DNA repair proceeds at different rates throughout the genome depending on the function of the DNA. We selected the DHFR gene as an example of a transcriptionally active gene, in which it is known that repair processes proceed more rapidly than in transcriptionally inactive DNA, an example of which is the \(\delta\)-globin gene\textsuperscript{35}. Mitochondrial DNA is compartmentalized away from the nuclear location of these genes. The formation of ICL in the DHFR gene and the mitochondrial genome was similar, consistent with the findings of others with cisplatin\textsuperscript{21,36,37}. Crosslinking of the \(\delta\)-globin gene was less, possibly because that drug accessibility to the DNA may have been limited. Fludarabine significantly inhibited the unhooking of interstrand crosslinks to similar extents in both the nuclear DNA and mtDNA. This action was greater in CLL cells than in normal lymphocytes, which may reflect the greater capacity for
accumulating fludarabine triphosphate in CLL. Earlier studies demonstrated that fludarabine also inhibited the removal of cisplatin ICL in the total genome and in specific gene fragments of LoVo colon carcinoma cells using the denaturation/renaturation assay\textsuperscript{24}. Since these crosslinking assays detect an early event in the removal of crosslinks (the unhooking of an ICL and not necessarily complete excision of the ICL from both strands), the results indicate that the nucleoside analogue inhibits this step of ICL repair.

Repair of interstrand crosslinks in mammalian cells is not well understood but is suggested to involve the sequential actions of the nucleotide excision repair pathway and of homologous recombination\textsuperscript{38}. Thus, the inhibition of either of these pathways or their interaction with each other by fludarabine could explain the enhanced cytotoxicity with oxaliplatin. Previous investigations have demonstrated that fludarabine triphosphate inhibits incision in the NER pathway, including activity of ERCC1\textsuperscript{25}. The activity of fludarabine may be related to blocking access of ATP to active binding sites on the repair enzymes. In this context, other excision proteins that utilize ATP such as the NER endonuclease ERCC1/XPF may be considered as a potential target for fludarabine. Indeed, it was striking that the purine nucleoside analogue, clofarabine, inhibited ICL unhooking equally as did fludarabine, and to a substantially greater extent than did the pyrimidine analogue, cytarabine (Table 3). Nevertheless, both DNA repair mechanisms require resynthesis of DNA to complete the repair process, so it would be expected that this would be sensitive to both pyrimidine and purine nucleoside analogues.
The use of platinum compounds and nucleoside analogues in cancer therapy is rich in rationale and practice. Indications of synergistic cell killing between cisplatin and cytarabine\(^39\) led to clinical trials in hematologic malignancies\(^40,41\). With the demonstration of the activity of fludarabine in indolent diseases, this analogue was used to augment combinations with cytarabine and cisplatin for the treatment of relapsed/transformed CLL and non-Hodgkin’s lymphoma\(^42-44\). While the latter protocols had activity, they were also associated with myelosuppression. This has been used to advantage in the design of conditioning regimens for marrow transplantation procedures\(^45-47\). Recently, carboplatin was combined with fludarabine and evaluated in the therapy of high-risk acute leukemia\(^48\). Results were judged promising, but marrow suppression was still a problem. Oxaliplatin has a different toxicity profile than either cisplatin or carboplatin with respect to myelosuppression. The development of a model that explains the synergistic cytotoxic interaction between fludarabine and oxaliplatin may aid in the design of more effective nucleoside analogues and novel platinum compounds with fewer side effects leading to better clinical outcomes. Based on this background, we have recently initiated a phase I study of oxaliplatin, fludarabine, cytarabine and rituximab (OFAR) in patients with Richter’s transformation, prolymphocytic leukemia or refractory/relapsed B-CLL\(^49\).

**ACKNOWLEDGEMENT**
The authors are grateful for the dedicated assistance of Min Du and Brenita Tyler in obtaining blood samples and lymphocyte isolation, and to Susan Lerner for providing information on patient characteristics and clinical laboratory evaluations.
REFERENCES


Figure 1. Induction of apoptosis by oxaliplatin and fludarabine in CLL lymphocytes.

CLL lymphocytes were treated with concentrations of oxaliplatin (A) (1, 5, 10 25, 50, 100 μM) or fludarabine (B) (1, 2.5, 5, 10, 25, 50 μM) for 24 hr; or with 5 μM oxaliplatin (C) or 2.5 μM fludarabine (D) for 24 hr. Annexin V positive cells were considered apoptotic, presented as % of apoptotic cells, and expressed as the mean ± SE. The results are the means of three experiments.
Figure 2. Cytotoxic interactions between oxaliplatin and fludarabine.

(A) Apoptosis was compared in untreated CLL lymphocytes (a) or after incubation with 2.5 µM fludarabine (b), 5 µM oxaliplatin (c) or the combination (d) for 24 hr. The apoptotic cell death was measured by annexin V binding. Viable cells not undergoing apoptosis are annexin V-FITC and PI negative; cells undergoing apoptosis are annexin V-FITC positive and PI negative; cells already dead are annexin V-FITC and PI positive.

(B) Lymphocytes from healthy donors were incubated with 2.5 µM fludarabine, 5 µM oxaliplatin, and the combination for 24 hr.

(C) Bar graph showing the percentage of apoptotic cells in untreated, 2.5 µM fludarabine, 5 µM oxaliplatin, and the combination groups.
oxaliplatin, or the combination for 24 hr. (C) Lymphocytes from patients with CLL were treated as in B. Values for annexin V positivity are presented as percentage of apoptotic cell death and expressed as the mean ± SE (n = 3). *, $P < 0.05$ between the combination and the sum of fludarabine and oxaliplatin.

Figure 3. Influence of concentration and time of incubation on cell killing.

CLL lymphocytes were treated with increasing concentrations of fludarabine (2.5, 5, 10 µM), oxaliplatin (2.5, 5, 10 µM) or the combinations (fludarabine 2.5 µM + oxaliplatin 2.5, 5 and 10 µM; fludarabine 5 µM + oxaliplatin 5 and 10 µM; fludarabine 10 µM + oxaliplatin 2.5, 5 and 10 µM,) for 16 hr (A) or 36 hrs (B). The range of concentrations is represented by a wedge. Annexin V positive cells were considered apoptotic, presented as apoptotic cells (% of control). Each point is the mean ± SE of determinations on cell samples from 3 to 6 individuals.
Figure 4. Fludarabine enhances the oxaliplatin-induced DNA interstrand crosslinks in the DHFR gene in normal or CLL lymphocytes

DNA from CLL lymphocytes incubated in absence or presence of fludarabine for 2 hr before exposure to increasing concentrations of oxaliplatin for 7 hr was isolated and analyzed for ICL formation. (A) Representation of Hind III-digested DNA denatured and electrophoresed through a 0.5 % agarose gel, before being transferred to nylon and probed for a 22 kb DHFR gene fragment. DS, double strand DNA; SS single strand DNA. The data shown were based on at least three independent experiments and DNA samples from each biological experiment were subjected to gel electrophoresis and Southern analysis at least twice. Crosslinks in the DHFR gene in normal lymphocytes (B) or CLL lymphocytes (C); this value was normalized to ICLs/10 kb in the absence (■) or presence (●) of 2.5 µM fludarabine, expressed as the mean ± SE (n = 3).
**Table 1** Action of fludarabine on formation of oxaliplatin ICL in specific genes of CLL lymphocytes.

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<th>Gene (size)</th>
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<sup>a</sup> Values represent ICLs/10 kb. Data are from at least 3 blots each of three independent biological experiments. Standard errors were in the range of 0 to 0.09 ICL per fragment.

<sup>*</sup> P < 0.05 (Significance was evaluated at 400 µM oxaliplatin)
Figure 5. Fludarabine retards the removal of oxaliplatin-induced DNA interstrand crosslinks in the DHFR gene in normal or CLL lymphocytes

Normal lymphocytes (A) or CLL lymphocytes (B) were incubated with 400 µM oxaliplatin in the absence or presence of 2.5 µM fludarabine for 5 hr, when cells were washed into drug-free medium. DNA from cells allowed to repair for up to 8 hr was analysed for ICL remaining in the 22 kb DHFR gene fragment. (A) Representative ICL blot probed for the DHFR gene in PBMC. The plots represent a quantitation of oxaliplatin ICLs remaining in the DHFR gene of PBMC (B) or CLL lymphocytes (C) as a function of repair time. Crosslinks remaining after repair in the absence (■) or presence (●) of fludarabine were expressed as a % of initial ICL levels.
Table 2 Action of fludarabine on formation and removal of oxaliplatin ICL in specific genes of normal and CLL lymphocytes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal lymphocytes</th>
<th>CLL lymphocytes</th>
<th>Normal lymphocytes</th>
<th>CLL lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repair time, hr</td>
<td>ICL/10kb</td>
<td>Removal (%)</td>
<td>Removal Inhibition (%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>0</td>
<td>0.48</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.29</td>
<td>0.53</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.15</td>
<td>0.41</td>
<td>68</td>
</tr>
<tr>
<td>DHFR</td>
<td>0</td>
<td>0.36</td>
<td>0.47</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.26</td>
<td>0.36</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.2</td>
<td>0.3</td>
<td>45</td>
</tr>
<tr>
<td>δ-globin</td>
<td>0</td>
<td>0.51</td>
<td>0.76</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.34</td>
<td>0.56</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.25</td>
<td>0.49</td>
<td>51</td>
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<tr>
<td>mtDNA</td>
<td>0</td>
<td>0.51</td>
<td>0.76</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.34</td>
<td>0.56</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.25</td>
<td>0.49</td>
<td>51</td>
</tr>
</tbody>
</table>

a Values represent ICL/10 kb. Data are from at least 3 blots each of three independent biological experiments. Standard errors for each group were in the range of 0 to 0.08 ICL per fragment. b Values represent % of ICL removal. c Values represent % inhibition of ICL removal at 4 and 8 hr determined as I = 1 − (%ICL removal + fludarabine)/%ICL removal – fludarabine). * P < 0.05; ** P = 0.07.
Figure 6. Fludarabine impairs the removal of oxaliplatin-induced DNA interstrand crosslinks in the mitochondrial DNA (mtDNA)

CLL lymphocytes were incubated with 400 μM oxaliplatin in the absence or presence of 2.5 μM fludarabine for 5 hr when cells were washed into drug-free medium. DNA from cells allowed to repair for up to 8 hr was analysed for ICL remaining. (A) Representative ICL blot probed for the 16.5 kb mtDNA. (B) Quantitation of oxaliplatin ICLs remaining in the mtDNA as a function of repair time. Crosslinks remaining after repair in the absence (■) or presence (●) of fludarabine were expressed as a % of initial ICL levels.
Table 3  Action of various nucleoside analogues on formation and removal of oxaliplatin ICL in the DHFR gene of CLL lymphocytes.

<table>
<thead>
<tr>
<th>Nucleoside analogs</th>
<th>Fludarabine</th>
<th>Cytarabine</th>
<th>Clofarabine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ICLs/10 kb(^a)</td>
<td>0.62</td>
<td>1.16</td>
<td>0.64</td>
</tr>
<tr>
<td>Removal (%)(^b)</td>
<td>85</td>
<td>51</td>
<td>78</td>
</tr>
<tr>
<td>Removal Inhibition (%)(^c)</td>
<td>40</td>
<td>14</td>
<td>63</td>
</tr>
</tbody>
</table>

\(^a\) Values represent ICLs/10 kb in the DHFR gene from CLL cells incubated with 400 µM oxaliplatin for 7 hr.  
\(^b\) Values represent % of ICL removal at 8 hr.  
\(^c\) Values represent % inhibition of ICL removal at 8 hr.  
Data are from at least 3 blots each of three independent biological experiments.
Fludarabine increases oxaliplatin cytotoxicity in normal and chronic lymphocytic leukemia lymphocytes by suppressing interstrand DNA crosslink removal

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