

## Killing of Chronic Lymphocytic Leukemia by the Combination of Fludarabine and Oxaliplatin Is Dependent on the Activity of XPF Endonuclease

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### Abstract

**Purpose:** Chronic lymphocytic leukemia (CLL) resistant to fludarabine-containing treatments responds to oxaliplatin-based therapy that contains fludarabine. We postulated that a mechanism for this activity is the incorporation of fludarabine into DNA during nucleotide excision repair (NER) stimulated by oxaliplatin adducts.

**Experimental Design:** We analyzed CLL cell viability, DNA damage, and signaling pathways in response to treatment by fludarabine, oxaliplatin, or the combination. The dependency of the combination on oxaliplatin-induced DNA repair was investigated using siRNA in CLL cells or cell line models of NER deficiency.

**Results:** Synergistic apoptotic killing was observed in CLL cells after exposure to the combination *in vitro*. Oxaliplatin induced DNA synthesis in CLL cells, which was inhibited by fludarabine and was eliminated by knockdown of XPF, the NER 5'-endonuclease. Wild-type Chinese hamster ovarian cells showed synergistic killing after combination treatment, whereas only additive killing was observed in cells lacking XPF. Inhibition of repair by fludarabine in CLL cells was accompanied by DNA single-strand break formation. CLL cells initiated both intrinsic and extrinsic apoptotic pathways as evidenced by the loss of mitochondrial outer membrane potential and partial inhibition of cell death upon incubation with FasL antibody.

**Conclusions:** The synergistic cell killing is caused by a mechanistic interaction that requires the initiation of XPF-dependent excision repair in response to oxaliplatin adducts, and the inhibition of that process by fludarabine incorporation into the repair patch. This combination strategy may be useful against other malignancies. *Clin Cancer Res*; 17(14); 1–11. ©2011 AACR.

### Introduction

Introduction of fludarabine and other purine nucleoside analogues in the treatment of chronic lymphocytic leukemia (CLL) generated a significant improvement in patient response (1). These proved more effective than the use of alkylating agents alone (2–4). Fludarabine is incorporated into the DNA by DNA polymerases, inhibiting replication (5). The incorporated analogue is not a substrate for ligation (6) and attempts of DNA polymerase  $\epsilon$  to excise it inactivate the polymerase (7). The great majority of CLL cells are not cycling; thus, fludarabine incorporation into

DNA is at a low level, likely representing endogenous repair synthesis (8).

Induction of excision repair of DNA damage is a potential mechanism to increase fludarabine incorporation. Irradiation with UV light, which induces nucleotide excision repair (NER), led to the dose-dependent incorporation of fludarabine into the DNA of quiescent lymphocytes and inhibition of 60% to 70% of repair (9, 10). Similar results were observed in response to fludarabine or clofarabine and cyclophosphamide in CLL cells (11). The greater-than-additive killing of cells provided rationale for the design of combinations in clinical trials. Subsequently, the clinical activity of combinations of these 2 classes of drugs, in particular fludarabine and cyclophosphamide, was proved superior to that of single-agent fludarabine (12–14). Recently, strategies to include antibody therapy have given substantial increases in the complete response rate for CLL patients (14–19) and an indication of increased overall survival in response to fludarabine–cytoxan–rituximab therapy (20). Nevertheless, relapses remain problematic and development of drug resistance continues to be a major challenge in CLL treatment (19), suggesting the need for new effective drugs.

Although these drug combinations have shown activity in earlier studies, the toxicity associated with the fludarabine-and-cisplatin combination with or without

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### Translational Relevance

The combination of fludarabine, cyclophosphamide, and rituximab is now standard therapy for most previously untreated chronic lymphocytic leukemia (CLL). However, it was remarkable that patients who failed to maintain a response to this therapy subsequently responded to a phase I–II trial of oxaliplatin-based therapy that also included fludarabine. We hypothesized that the mechanism of action for this combination was dependent on the ability of cells to excise the oxaliplatin adduct and initiate DNA-repair synthesis. Experiments that examined the fludarabine–oxaliplatin interactions showed synergistic killing of CLL cells that was dependent on the activity of the nucleotide excision repair endonuclease, XPF. Fludarabine blocked XPF-dependent DNA resynthesis in this repair mechanism. Treatment of CLL cells with the combination also caused accumulation of DNA damage and initiated DNA repair signaling followed by activation of apoptotic pathways. This understanding of the mechanistic interaction of fludarabine and oxaliplatin predicts that this combination may be equally, if not more, effective than the current standard of care. This suggests further mechanistic investigations and encourages expansion of clinical trials to previously untreated patients and other indolent B-cell malignancies.

cytarabine has limited further clinical investigations (21, 22). Oxaliplatin is a third-generation platinum compound. It has a different toxicity profile from that of cisplatin, and it induces distinct cell responses from its predecessor (23, 24). Oxaliplatin forms a variety of DNA crosslinks and monoadducts (25–27), which are excised by NER (28). Components of NER sense bulky adducts or distortions in the DNA helix (29) and recruit helicases, which unwind the region around the adduct. This process allows the endonucleases XPF-ERCC1 and XPG to make incisions 5' and 3' of the adduct, respectively (30, 31). Subsequently, the single-stranded 27- to 29-nucleotide oligomer with the adduct is removed during the gap-filling resynthesis of DNA (32, 33). This resynthesis provides an opportunity for incorporation of fludarabine nucleotide, which terminates further resynthesis, an action that presumably leaves a single-stranded gap in the DNA repair patch (34). The observations that oxaliplatin with fludarabine elicits greater-than-additive killing of CLL cells *in vitro* (27) provided the rationale for inclusion of this combination as a part of a phase I–II clinical trial in relapsed/refractory CLL (35) in which oxaliplatin might serve as the initiator of DNA repair and synthesis. The level of cytarabine in this combination treatment has been recently reduced to minimize myelosuppression (36). The therapy retained its effectiveness suggesting that the combination could be successful without the need for a secondary analog.

In seeking a mechanistic explanation for this activity, we hypothesized that NER is required for the synergistic

activity of fludarabine and oxaliplatin in CLL cells. In support of this postulate, removal of XPF endonuclease, an essential component of the excision repair process, eliminated greater-than-additive effect of the fludarabine and oxaliplatin combination. We conclude from this work that NER of oxaliplatin DNA adducts is essential for the mechanism of action of the fludarabine and oxaliplatin chemotherapeutic combination.

### Materials and Methods

#### Isolation of CLL cells

CLL samples used were from patients who signed a written informed consent to participate in the laboratory protocol, which was approved by The University of Texas MD Anderson Cancer Center Institutional Review Board. Whole blood was collected in heparinized tubes, diluted 1:4 with PBS, layered onto Fico/Lite LymphoH (specific gravity, 1.077; Atlanta Biologicals) and centrifuged for 20 minutes at 1,500 rpm. Cells were collected from the interface, washed twice in PBS, and counted using a Z-2 Coulter particle counter. The cells were incubated at  $1 \times 10^7$ /mL in RPMI 1640 supplemented with 10% autologous serum, at 37°C and 5% CO<sub>2</sub>.

#### Chemicals and reagents

Fludarabine and oxaliplatin were from Berlex Biosciences and LKT Laboratories, respectively. The inhibitors of ATM (KU-55933) and caspases [zVAD(OMe)-FMK] were purchased from Calbiochem and MP Biomedicals, respectively. KuDOS Pharmaceuticals supplied the DNA-PKcs inhibitor, NU7441. Hydroxyurea and DiOC<sub>6</sub> were obtained from Sigma-Aldrich and Invitrogen.

#### Exposure

CLL samples were incubated with the designated concentration of fludarabine for 2 hours, followed by the addition of oxaliplatin. Where indicated, samples were pretreated with 10 μmol/L KU-55933 or NU7441 for 1 hour prior to the addition of fludarabine and/or oxaliplatin, or exposure to 5 Gy ionizing radiation (Nasatron). Where indicated, cells were also incubated with 30 μmol/L zVAD or 8 μg/mL of FasL antibody (NOK-1, Santa Cruz) for 1 hour prior to treatment.

#### Analysis of DNA repair resynthesis

DNA repair synthesis was quantified by incorporation of [<sup>3</sup>H]dThd (Moravek Biochemicals, 81.1 Ci/mmol; ref. 37). Cells were preincubated with hydroxyurea (3 mmol/L) for 30 minutes prior to the start of each experiment. Each patient sample was assayed in triplicate ( $n = 5$ ).

#### Single-cell gel electrophoresis (comet) assay

Samples were exposed as noted, washed in PBS, and 1,000 cells were mixed with 200 μL of LMA agarose (Trevigen). Immediately, 75 μL of the volume was added onto each comet slide (2 per slide). The slides were kept at 4°C, in, for 30 minutes. Lysis buffer (Trevigen) with

dimethyl sulfoxide was added for 1 hour. For alkaline assay, fresh sodium hydroxide solution (pH > 12) was added to the slides for 1 hour (room temperature, dark). Slides were transferred to the electrophoresis apparatus, at 15 V for 15 minutes, in the alkaline buffer. For neutral assays, slides were washed once in PBS, then transferred to the electrophoresis apparatus (Tris-borate EDTA buffer, pH 6.8), at 15 V for 15 minutes. Slides were fixed in 70% ethanol for 5 minutes. Cells were stained with propidium iodide (PI; Sigma-Aldrich) solution and analyzed by using a Nikon EFD3 microscope and Komet 5.5 software.

### Apoptosis

Apoptotic cell death was determined by flow cytometry with the use of Annexin V-FITC (BD Biosciences) or DiOC<sub>6</sub> (Invitrogen) and PI (Sigma-Aldrich). Cells were centrifuged at 1,500 rpm and incubated with Annexin V and PI or DiOC<sub>6</sub> and PI for 20 minutes before analysis.

### Immunoblot

Cells were lysed in ice-cold lysis buffer (50 mmol/L Tris pH 8, 250 mmol/L NaCl, 1% NP40, 0.1% SDS, 5 mmol/L EDTA, 2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/L Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 10 mmol/L NaF) freshly supplemented with complete protease inhibitor mixture (Roche) and phenylmethylsulfonyl fluoride (1 μmol/L). Protein concentrations were determined with the use of a detergent-compatible protein assay kit (Bio-Rad) and samples were loaded onto 4% to 12% SDS-polyacrylamide gels (Bio-Rad). The proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad) overnight at 25 V and blocked with 10% milk. Primary antibodies were incubated for 4 hours followed by 1 hour of secondary antibody incubation. Blots were visualized with the Li-Cor Odyssey Imager (Li-Cor Biosciences) and quantified using ImageJ. Antibodies were from the following companies: monoclonal antibody against total DNA-PKcs and XPF (Neomarkers); polyclonal DNA-PKcs Ser2056, polyclonal total ATM, polyclonal Ser957 SMC1, monoclonal total SMC1, and monoclonal against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam); monoclonal Ser1981 ATM and monoclonal γ-H2AX (Upstate); monoclonal tubulin (Santa Cruz); monoclonal actin (Sigma-Aldrich); polyclonal caspase-9 (Cell Signaling); polyclonal caspase-8 (GeneTex); polyclonal PUMA (ProSci).

### siRNA electroporation

XPF siRNA and scramble siRNA SMARTpool were from Dharmacon, Inc. CLL cells were transfected using electroporation (Lonza Cell Line Nucleofector Kit V, Nucleofector I). Briefly, cells were resuspended in 100 μL of supplemented buffer V (8 million cells per transfection). For each sample, 32 million cells were transfected with XPF or with scramble siRNA. Transfection efficiency was tested with siGLO (Dharmacon, Inc.) and was determined to be at 60%. After transfection, cells were incubated for 72 hours with cell death determined by Annexin V and PI staining immediately after transfection, 24, 48, 72, and 96 hours.

Portions of the transfected samples were lysed and analyzed by immunoblot as described.

### Colony formation

Chinese hamster ovarian (CHO) cell lines, AA8 (wild-type) and its derivatives UV41 (XPF deficient) and UV135 (XPG deficient), were purchased from American Type Culture Collection and authenticated by their sensitivity to UV and by cytogenetic analysis. Due to higher sensitivity of UV41 and UV135 to oxaliplatin (Supplementary Fig. S1), the cells were exposed to equitoxic doses (AA8 5 μmol/L; UV41 0.05 μmol/L; UV135 0.5 μmol/L). The cell line passage numbers ranged from 8 to 14. Cells were seeded at density of 400 cells per well, on 6-well dishes. Cells were allowed to attach overnight, then incubated with 0.5% FBS (Atlanta Biologicals) MEM media (Mediatech) for 24 hours, followed by exposure to specified concentrations. After treatment, samples were washed once with PBS and 10% FBS supplemented MEM alpha media was added. Ten days after exposure, cells were fixed with 100% methanol and stained with Giemsa.

### Statistical analysis

We used ANOVA for the analysis shown in Table 1. The analysis presented in the figures was done using the paired Student *t* test. Analysis of the combinational effect for oxaliplatin and fludarabine exposure (Fig. 1A) was done by using CalcuSyn (Biosoft), based on the median-effect method created by Chou and Talalay (38). Calculated combination index less than 1 indicates synergistic, equal to 1 indicates additive, and more than 1 indicates antagonistic relationship between agents.

### Results

To understand the mechanism of fludarabine and oxaliplatin activity in CLL cells, we compared the cytotoxicity that the 2 drugs produce alone and in combination in 20 CLL samples. Clinical characteristics of the samples used in this study can be found in Supplementary Table S1. Preliminary investigations showed that a 24-hour incubation with either drug at 5 μmol/L evoked minimal cell death as determined by the lack of PI and Annexin V staining (data not shown). Under these conditions, incubation of CLL cells with both drugs gave a significant decrease in live cells relative to the sum of killing by each alone (Table 1, predicted vs. observed, *P* < 0.01). There was no significant difference in response within samples treated with either oxaliplatin or fludarabine prior to the addition of the second drug (Supplementary Table S2). Thus, these minimally toxic concentrations, which are clinically achievable, induced greater-than-additive cell killing in combination.

Synergy between 2 drugs is best observed upon the analysis of a wide-dose range for both drugs alone and in combination. The dose range tested for our studies was 0.5 to 17 μmol/L. Analysis of cell survival using CalcuSyn showed the combination index for oxaliplatin

**Table 1.** Percentage of cells negative for Annexin V and PI

	Oxaliplatin	Fludarabine	Oxaliplatin → Fludarabine	
			Predicted	Observed
CLL 1	99.3	89.1	88.5	63.6
CLL 2	96.7	77.9	75.3	62.6
CLL 3	99.7	97.5	97.2	79.7
CLL 4	86.7	92.1	79.6	66.5
CLL 5	99.6	85.6	85.3	74.7
	Oxaliplatin	Fludarabine	Fludarabine → Oxaliplatin	
	Oxaliplatin	Fludarabine	Predicted	Observed
CLL 6	99.3	91.6	90.9	83.9
CLL 7	93.7	74.9	70.2	47.2
CLL 8	97.4	93.8	91.4	55.2
CLL 9	96.4	98.9	95.3	65.7
CLL 10	95.1	78.1	74.3	23.4
CLL 11	98.4	90.6	89.2	58.5
CLL 12	99.9	87.4	87.3	63.5
CLL 13	92.1	69.9	64.4	46.8
CLL 14	96.8	86.3	83.5	49.1
CLL 15	100.6	99.7	100	80.7
CLL 16	99.8	94.8	94.6	69.2
CLL 17	96.7	90.8	87.8	77.5
CLL 18	87.7	93.1	81.6	60.9
CLL 19	96.5	95.7	92.3	78.2
CLL 20	96.2	99.5	95.7	89.2

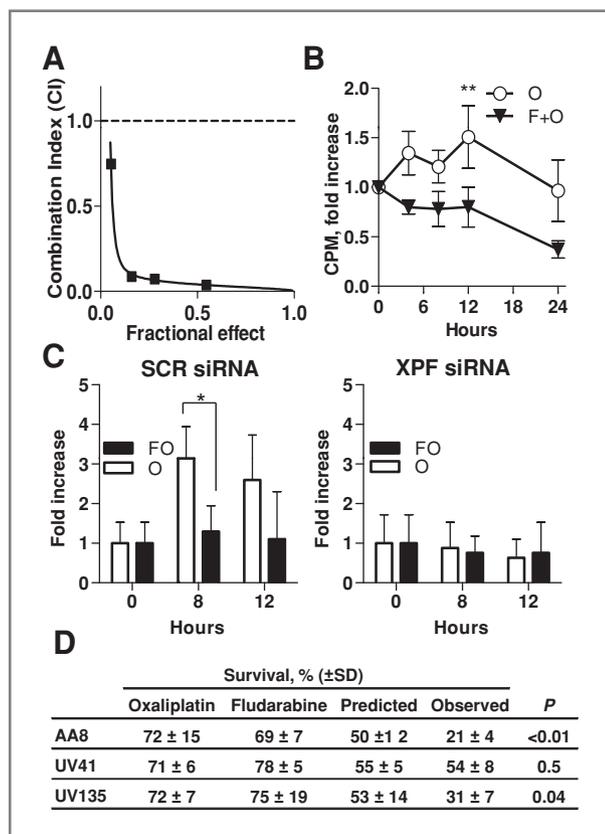
and fludarabine to be less than 1 (Fig. 1A), signifying synergism in CLL cells by the combination.

Because CLL cells have low replicative capacity, the combination strategy postulates that excision of oxaliplatin adducts by NER would provide an opportunity for the incorporation of fludarabine into the DNA repair patch. As a measure of this process, CLL cells were first incubated with oxaliplatin alone; pulses with [<sup>3</sup>H]dThd at times thereafter revealed a 50% increase in DNA synthesis, consistent with activation of the NER process (Fig. 1B). A portion of each of these samples was also preincubated with fludarabine 2 hours before addition of oxaliplatin. The results indicate a significant inhibition of repair synthesis 12 hours after oxaliplatin addition. Therefore, even though the increase of DNA synthesis measurable after oxaliplatin was small, it represents a level of excision repair that in combination with fludarabine was adequate to induce greater than additive cell death (Table 1).

The working hypothesis states that the mechanism of fludarabine and oxaliplatin combination is dependent on the excision of oxaliplatin adducts by NER. XPF endonuclease incises 5' of the DNA adduct and is an essential component of the NER process. CLL cells were incubated with scrambled or XPF siRNA to create a CLL model of NER deficiency (Supplementary Fig. S2). In samples incubated with scrambled siRNA, oxaliplatin induced a 3-fold

increase in thymidine incorporation at 8 hours (Fig. 1C, left) indicating an increase in DNA synthesis. Fludarabine pretreatment blocked this effect, a response similar to that shown in Fig. 1B. In contrast, XPF siRNA samples showed no increase in thymidine incorporation after oxaliplatin; pretreatment of samples with fludarabine lacked effect (Fig. 1C, right). These results are consistent with the hypothesis that the thymidine incorporation after oxaliplatin is a consequence of NER-mediated excision repair of DNA adducts.

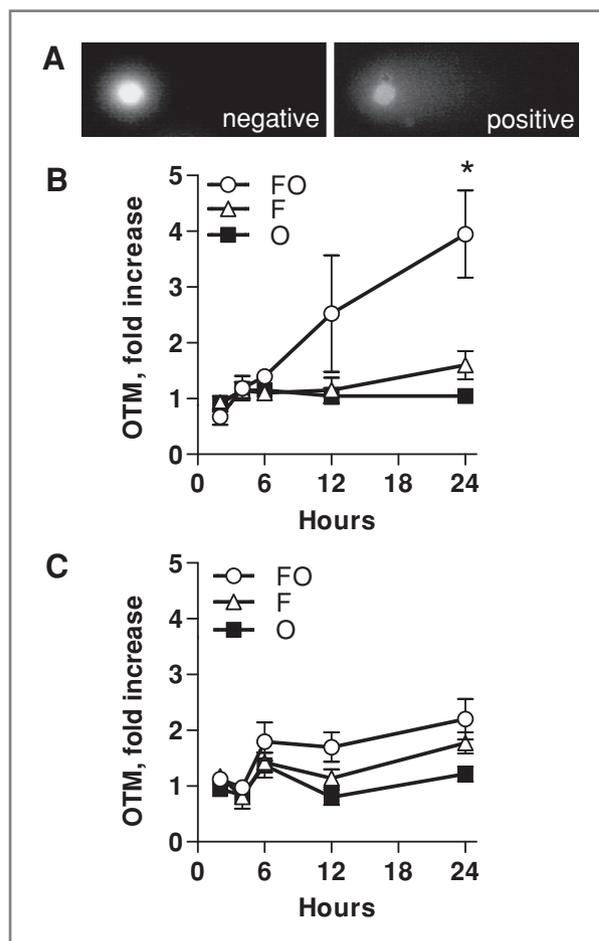
Results shown in Fig. 1C suggested that NER is essential for synergistic activity of fludarabine and oxaliplatin. Unfortunately, the toxicity of the electroporation procedure limited the usefulness of CLL cell survival assays. Therefore, we examined the CHO cell culture model of XPF deficiency with equitoxic doses of oxaliplatin and fludarabine. The observed survival for wild-type AA8 cells was only 21%, which was significantly less than predicted (50%,  $P < 0.01$ ), confirming that the combination of the 2 agents results in greater-than-additive cell death (Fig. 1D). In contrast, the survival rate of XPF mutant UV41 cells was 54%, which was not significantly different from the predicted survival rate of 56%. A second AA8 cell line derivative, UV135, lacks XPG activity, the NER endonuclease that incises 3' of the lesion. The predicted additive survival rate of UV135 cells after combination treatment was 52%, and



**Figure 1.** Fludarabine blocks NER-dependent DNA synthesis. A, synergistic interaction of oxaliplatin (O) and fludarabine (F) in 5 CLL samples as determined by CalcuSyn. B,  $[^3\text{H}]\text{dThd}$  incorporation was used to measure DNA-repair synthesis after 5  $\mu\text{mol/L}$  oxaliplatin alone or in combination with 5  $\mu\text{mol/L}$  fludarabine pretreatment ( $n = 5$ ; \*\*,  $P < 0.01$ ). Scrambled siRNA (C, left) and XPF siRNA (C, right) samples were exposed to oxaliplatin or fludarabine and oxaliplatin, prior to  $[^3\text{H}]\text{dThd}$  pulses ( $n = 3$ , mean  $\pm$  SD; \*,  $P = 0.04$ ). D, survival of wild-type (AA8: 5  $\mu\text{mol/L}$  F, 5  $\mu\text{mol/L}$  O), XPF mutant (UV41: 5  $\mu\text{mol/L}$  F, 0.05  $\mu\text{mol/L}$  O) and XPG mutant (UV135: 5  $\mu\text{mol/L}$  fludarabine, 0.5  $\mu\text{mol/L}$  O) cells as determined by colony formation assays ( $n = 6$ , mean  $\pm$  SD).

the observed survival rate was 31% (Fig. 1D). Thus, NER is needed for the greater-than-additive killing by the combination, but the contribution of XPF is more substantial than that of XPG.

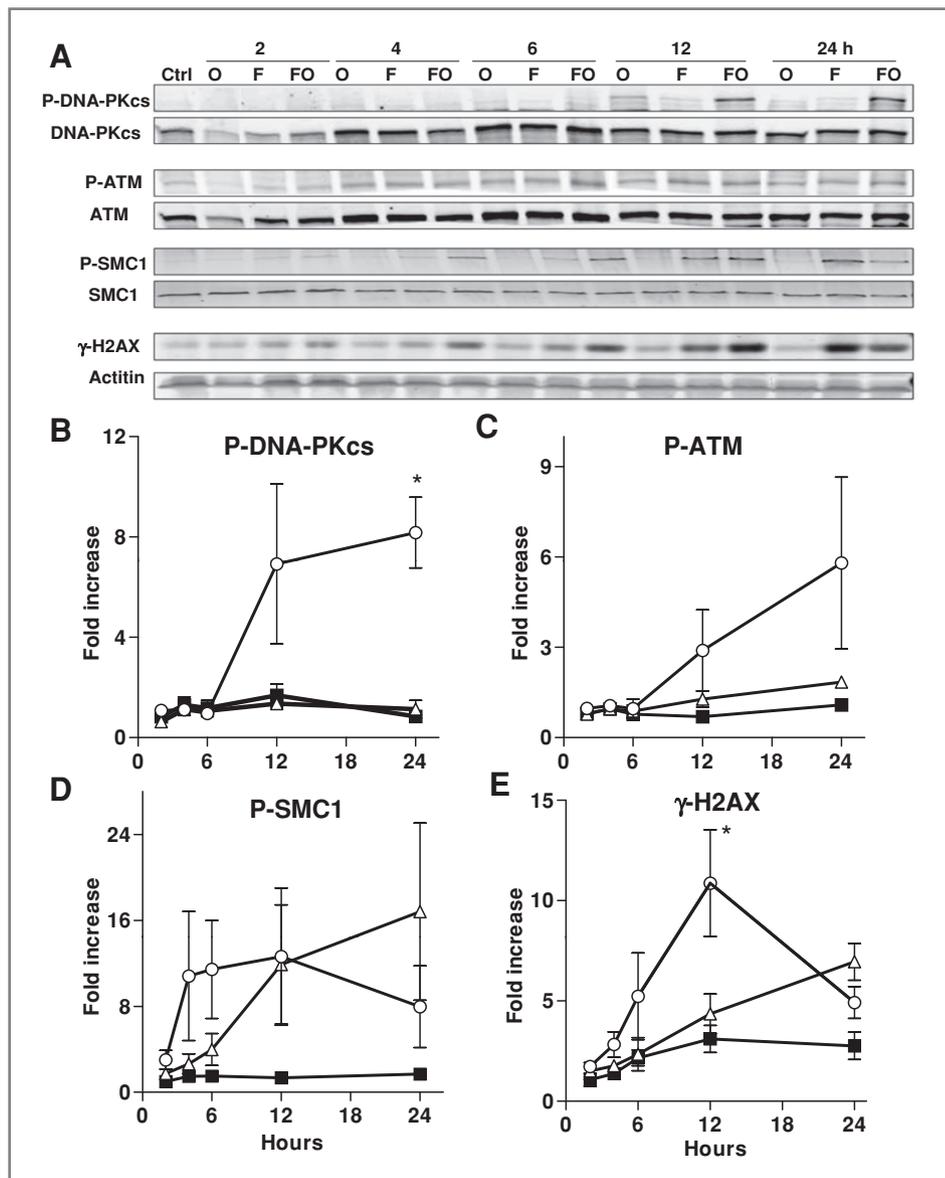
Blocked DNA synthesis likely results in incomplete repair due to termination of the resynthesis step caused by the misincorporation of fludarabine triphosphate. This generates gaps in the DNA that could be detectable by the comet assay (Fig. 2A). Preincubation of samples in an alkaline solution ( $\text{pH} > 12$ ) before electrophoresis unwinds the DNA and allows detection of single- and double-strand breaks. The exposure of samples to both fludarabine and oxaliplatin resulted in a 4-fold increase of the Olive tail moment (OTM) compared with the time-matched controls at 24 hours and when compared with either agent alone (Fig. 2B;  $P = 0.03$ ). Neutral comet assay was used to detect double-strand breaks. Analysis of 4 samples showed that, whereas there was a tendency for an



**Figure 2.** CLL cells accumulate DNA damage after combination treatment compared with each agent alone. PI stained nuclear DNA (A) in untreated CLL cells (negative) and after 24-hour treatment with fludarabine (F) and oxaliplatin (O (positive)). B, fold-increase in Olive tail moment (OTM) during alkaline comet assay (\*,  $P = 0.03$ ;  $n = 4$ ) and (C) neutral comet assay ( $P = 0.13$ ;  $n = 4$ ).

increase in the OTM after the combination, this increase was not statistically significant (Fig. 2C). Thus, the generation of single-strand breaks supports the hypothesized mechanism for the interaction of fludarabine and oxaliplatin in the CLL cell population.

The presence of DNA damage activates DNA repair and signaling complexes that can be detected by phosphorylation of key proteins. DNA-PKcs and ATM are essential components of the nonhomologous end-joining and homologous recombination pathways, respectively, and both are involved in repair of DNA double-strand breaks. SMC1 is part of the chromatid cohesion complex; it is phosphorylated at Ser966 in response to DNA damage (39). Phosphorylation of histone H2AX at Ser139 is a well-studied marker of DNA double-strand breaks and stalled replication forks (40). DNA-PKcs has a low background level of Ser2056 phosphorylation in CLL samples (Fig. 3A), and exposure to the combination increases its

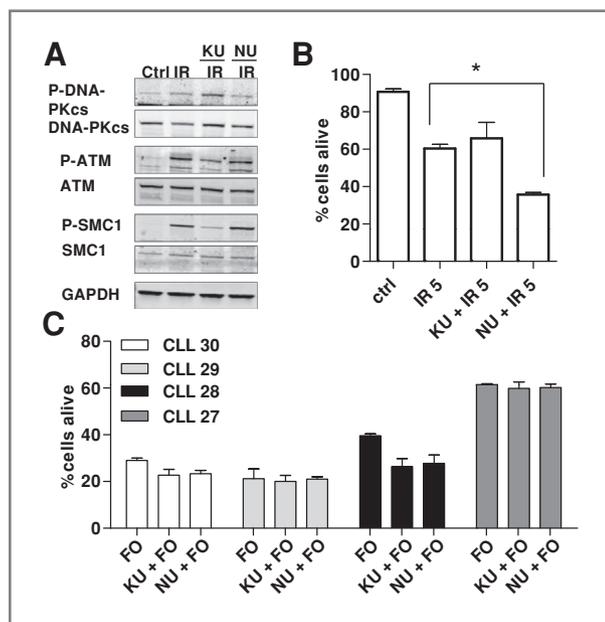


**Figure 3.** Fludarabine (F) and oxaliplatin (O) coexposure results in phosphorylation of DNA-damage sensors. A, immunoblot and time course analysis of DNA-PKcs Ser2056, ATM Ser1981, SMC1 Ser966, and H2AX Ser139 phosphorylation in a representative CLL sample exposed to either oxaliplatin, fludarabine, or the combination. Quantified fold-increase in phosphorylation of DNA-PKcs (B), ATM (C), SMC1 (D), and H2AX (E) relative to control ( $n = 4$ ). All samples were incubated with 30  $\mu\text{mol/L}$  zVAD during the course of drug exposure. Symbols: oxaliplatin (■), fludarabine ( $\Delta$ ), or the combination ( $\circ$ ).

phosphorylation by 7- and 8-fold at 12 and 24 hours (Fig. 3B). Phosphorylation of ATM at Ser1981 was less than DNA-PKcs, increasing 3- to 6-fold at the same times (Fig. 3C). In comparison, the samples showed earlier phosphorylation of SMC1 4 and 6 hours after exposure (Fig. 3D). Although the increase varied from 6- to 20-fold among samples at 6 hours, its time course was consistent, initiated at 4 hours after combination treatment, peaking at 6 and 12 hours, and tapering off by 24. Fludarabine also induced an increase in SMC1 phosphorylation, but this appeared later than with the combination (12 and 24 hours; Fig. 3D). Drug coexposure resulted in 10-fold increase of  $\gamma$ -H2AX at 12 hours (Fig. 3E) and similarly to SMC1,  $\gamma$ -H2AX tapered off by 24 hours (4-fold increase over control). Taken together, the increase in phosphorylation of DNA damage response molecules is consistent with

the conclusion that fludarabine and oxaliplatin combination causes greater-than-additive DNA damage.

Phosphorylation of DNA-PKcs and ATM suggested that the 2 molecules could be participating in repair of DNA damage caused by the drug combination. To assess whether this is the case, samples were preincubated with specific inhibitors of ATM or DNA-PKcs (Fig. 4). Each kinase inhibitor blocked the autophosphorylation of the respective target protein after 5 Gy of radiation (Fig. 4A); the DNA-PKcs inhibitor also decreased cell survival after irradiation (Fig. 4B). The samples were incubated with inhibitors and the combination to test the contribution of ATM or DNA-PKcs to the repair of the resulting DNA damage. The cell survival response was independent of the kinase inhibitor treatment in 4 samples tested (Fig. 4C). Thus, whereas ATM and DNA-PKcs are phosphorylated in



**Figure 4.** DNA-PKcs and ATM kinase activity is not relevant to survival after fludarabine (F) and oxaliplatin (O) combination treatment. A, a CLL sample was pretreated with either 10  $\mu\text{mol/L}$  NU7441 (NU, DNA-PKcs inhibitor) or 10  $\mu\text{mol/L}$  KU55933 (KU, ATM inhibitor) and auto and target phosphorylation was analyzed by Western blot 2 hours after exposure to 5 Gy radiation (IR). B, cell survival was analyzed by negative Annexin V and PI staining in response to NU7441 and KU55933 and 5 Gy radiation ( $n = 3$ ), 24 hours after radiation. \*,  $P < 0.5$ . C, samples were treated with the inhibitors for 1 hour, followed by treatment with fludarabine for 8 and oxaliplatin for 6 hours. Samples were incubated in drug-free media with the inhibitors and analyzed 18 hours later.

response to the combination treatment, it does not seem that the DNA repair processes they mediate contribute substantially to viability of CLL cells.

We investigated signaling pathways that may be involved in the mechanisms of cell death after fludarabine and oxaliplatin exposure. An increase in p53 levels at 6, 12, and 24 hours paralleled the generation of single-strand breaks (Fig. 5A). Analysis of 5 samples showed a general trend for time-dependent increase in p53 levels after exposure to the combination (Fig. 5B, left). Expression of PUMA, the proapoptotic p53 target protein, increased at 12 and 24 hours, although PUMA levels were also induced by fludarabine exposure at 12 hours (Fig. 5A), showing the ability of fludarabine to induce apoptotic signaling alone or in combination. PUMA levels correlated significantly with p53 stabilization (Fig. 5B, right;  $P < 0.01$ ,  $r^2 = 0.70$ ) after the combination exposure.

Treatment of CLL cells with the combination in the presence or absence of the pan-caspase inhibitor (zVAD, 30  $\mu\text{mol/L}$ ) or Fas-L antibody NOK1 showed the contributions of both the extrinsic and intrinsic apoptotic pathways (Fig. 5C). The observed cell death was blocked by inhibition of caspases, revealing that the cell death is caspase dependent. FasL antibody blocked 50% of the observed cell death, indicating partial contribution of the extrinsic pathway. Activation of the intrinsic apoptotic pathway was

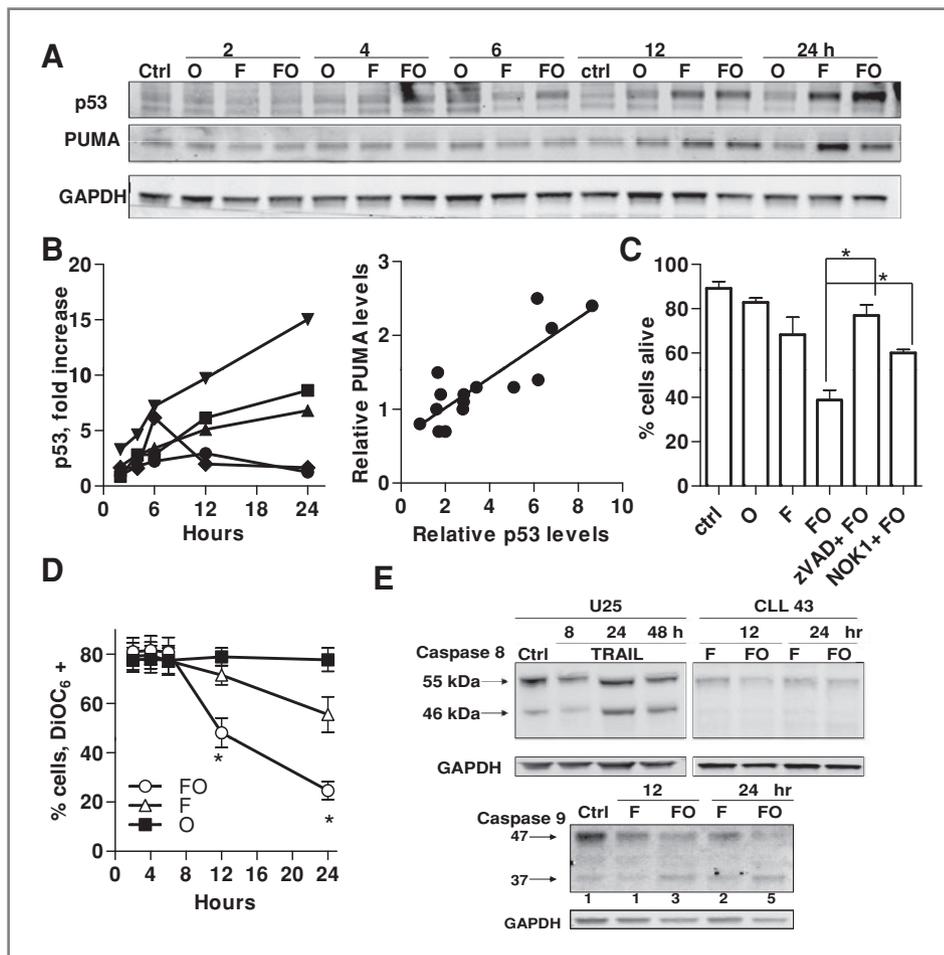
measured by the loss of mitochondrial membrane potential as observed by the decrease in DiOC<sub>6</sub> staining. At early time points, neither single agent nor the combination induced a loss in the mitochondrial potential (Fig. 5D). By 12 hours, 50% of cells had lost DiOC<sub>6</sub> staining with the combination, whereas neither single agent induced any significant decrease over time-matched controls. At 24 hours, 75% of cells lost DiOC<sub>6</sub> staining with the combination, whereas only 40% of cells showed the same effect if treated with fludarabine alone. Increased loss of mitochondrial potential reveals earlier and more extensive activation of the intrinsic apoptotic pathway in CLL samples after the combination treatment.

Mediator caspases-8 and -9 activate the extrinsic and intrinsic apoptotic pathways, respectively. TNF-related apoptosis-inducing ligand (TRAIL)-treated U25 cells died through activation of the extrinsic mechanisms, as can be seen by the cleavage of caspase-8 (Fig. 5E). CLL cells showed low levels of caspase-8 and no detectable cleavage with either fludarabine treatment alone or fludarabine and oxaliplatin in combination (Fig. 5E). Caspase-9 is responsible for the activation of the intrinsic apoptotic pathway. The combination increased the cleavage of caspase-9 five-fold over controls as opposed to fludarabine alone (2-fold) at 24 hours (Fig. 5E). Taken together, the results presented here support the conclusion that the fludarabine and oxaliplatin-induced DNA damage activates both extrinsic and intrinsic pathways of CLL cell death.

## Discussion

Combinations of nucleoside analogues and alkylating agents in CLL treatment were hypothesized to act by the induction of NER, followed by the incorporation of the nucleoside analogue into DNA during the resynthesis of the repair patch (11, 35). It was reasoned that this would block completion of the repair process and induce signaling for cell death (27, 41). As a test of this postulate, knockdown of XPF in CLL cells blocked oxaliplatin-induced DNA synthesis in CLL cells, consistent with the involvement of NER activity. Furthermore, cell lines deficient in XPF failed to exhibit greater-than-additive cell death in a survival assay. Assays determining the downstream mechanism of action showed an induction of single-strand breaks and the activation of DNA damage signaling after exposure to the combination. There was no detectable contribution of DNA repair by the nonhomologous end-joining or homologous recombination complexes. Cells activated the apoptotic response through both extrinsic and intrinsic pathways. The results support the conclusion that the mechanism-based interaction between fludarabine and oxaliplatin depends on the repair of the oxaliplatin-DNA adducts by NER, and that fludarabine blocks the ensuing DNA synthesis resulting in DNA damage and apoptosis.

Oxaliplatin induced DNA synthesis in CLL samples as seen by increased [<sup>3</sup>H]dThd incorporation, and pretreatment of samples with fludarabine blocked this increase



**Figure 5.** Fludarabine (F) and oxaliplatin (O) combination activates both the extrinsic and intrinsic apoptotic pathway. **A**, Western blot analysis of 1 sample after exposure to single agents or the combination. **B** (left), quantified Western blot analysis of p53 levels in 5 CLL samples after the exposure to the combination. **B** (right), correlation between levels of PUMA and p53 measured as an increase over the untreated control ( $n = 3$ ). **C**, cell survival as determined by lack of Annexin V and PI staining after treatment with zVAD or NOK1 FasL antibody ( $n = 3$ ). \*, Both the FO vs. ZVAD + FO and FO vs. NOK1 + FO are with values  $P < 0.05$ . **D**, loss of mitochondrial potential as seen by the loss of the DiOC<sub>6</sub> staining (12 hours, FO vs. O:  $P = 0.02$ , FO vs. F:  $P = 0.03$ ; 24 hours, FO vs. O:  $P < 0.01$ , FO vs. F:  $P = 0.01$ ;  $n = 3$ ). **E**, cleavage of caspase-8 and caspase-9 after exposure to the combination. All immunoblots are representative of at least 3 analyzed samples.

(Fig 1A). NER is responsible for the removal of oxaliplatin mono-adducts from the DNA (42), and the XPF-ERCC1 complex is an essential part this pathway (32). Cells lacking XPF endonuclease are 100-fold more sensitive to platinum than the wild-type cells (ref. 43; Supplementary Fig. S2). siRNA knockdown of XPF protein in CLL samples showed that the oxaliplatin-induced increase in [3H]dThd incorporation was due to the activity of NER. Exposure of the same samples to fludarabine and oxaliplatin did not affect baseline [3H]dThd incorporation showing that fludarabine blocked DNA synthesis resulting from the NER processing of oxaliplatin-DNA adducts.

Cell death is the most important readout of the combination activity between fludarabine and oxaliplatin. The high toxicity of the electroporation and the long-term incubation necessary to achieve an efficient knockdown rendered the siRNA technique unusable in determining CLL cell death. Therefore, CHO cells with a wild-type and XPF or XPG mutant background were exposed to single agents or the combination. Colony formation assays confirmed that in a wild-type background, cells were more sensitive to combination exposure as opposed to either oxaliplatin or fludarabine alone. Cells with mutant XPF

and XPG were exposed to 100- and 10-fold lower concentrations of oxaliplatin, respectively, to achieve comparable survival response. Without XPF, the effect of oxaliplatin and fludarabine exposure was only additive, suggesting that the mechanistic-based interaction between the 2 agents requires proficient excision of oxaliplatin adducts. In contrast, XPG-deficient cells showed survival similar to that of their wild-type counterparts, signifying that even without XPG, cells are capable of initiating DNA synthesis as a response to oxaliplatin adducts.

There is evidence that XPF is capable of incisions on both 5' and 3' sides of the adduct, as is the case with branched oligonucleotides containing a (psoralen) crosslink (44, 45). If XPF has the 3' endonuclease activity *in vivo*, it might substitute for XPG. If so, XPG-deficient cells would retain partial NER activity allowing for repair of adducts and initiating DNA synthesis after the excision. A second possible explanation for differential survival between XPF and XPG mutant cells after fludarabine and oxaliplatin exposure involves initiation of DNA synthesis without removal of the DNA adduct. Prior to XPF activity, a portion of the DNA would be unwound by XPB and XPD helicases. The XPF incision 5' of the adduct would create a single-strand

patch of DNA and a 3'-DNA flap containing the oxaliplatin adduct. The resulting 3'-terminus of the single-strand patch of the DNA could act as a substrate for DNA synthesis due to its proper orientation, allowing for the incorporation of fludarabine and creation of a single-strand gap. Conversely, in the absence of XPF, XPG would incise 3' of the adduct. Although this oxaliplatin-containing flap could be religated, or extended, the resulting single-strand patch created would not be a substrate for resynthesis. In addition, the adduct would not be removed, raising the possibility of subsequent interstrand crosslink formation which could contribute to the observed lethality.

The proposed mechanism of action suggested that the fludarabine-induced block of DNA synthesis resulting from the removal of oxaliplatin adducts would activate DNA damage sensors. Single-cell electrophoresis in alkaline conditions showed increased DNA breaks and gaps, albeit there was no detection of double-strand DNA damage. DNA damage signaling molecules DNA-PKcs, ATM, SMC1, and H2AX were all phosphorylated in response to the combination exposure with early responses from SMC1 and H2AX. The timing of SMC1 and H2AX phosphorylation coincided with the increase in [3H]dThd incorporation after oxaliplatin exposure, suggesting that the DNA damage correlated with the block in DNA repair synthesis. DNA breaks were undetectable at early times with single-cell electrophoresis, but there was an accumulation of DNA single-strand damage at 12 and 24 hours postexposure also supported by phosphorylation of DNA-PKcs and ATM. This suggests that assays for SMC1 and H2AX phosphorylation may be more sensitive in detecting DNA damage than can be measured with either the comet assay or phosphorylation of the DNA repair kinases. Phosphorylation of DNA-PKcs and ATM suggested active DNA repair after oxaliplatin and fludarabine exposure, similar to their response to ionizing radiation. Inhibition of both DNA-repair kinases produced no effect on cell killing after combination treatment, telling of CLL cell's inability to effectively respond to the type of DNA damaged caused by this specific combination.

Normal lymphocytes induced the extrinsic apoptotic pathway in response to fludarabine and UV exposure (41). In our studies, the combination of clinically relevant concentrations of both drugs induced the stabilization of

p53 protein. This prompted us to ask whether p53 was activating the intrinsic or extrinsic apoptotic pathways. Various tumors, including CLL, evade chemotherapeutic cell killing by inactivating or blocking specific apoptotic pathways. CLL overexpresses the antiapoptotic proteins Mcl-1 and Bcl2 (46), which diminishes activation of the intrinsic apoptosis. Alternatively, colon carcinomas are resistant to TRAIL-induced cell death due to a decrease in cell receptors that allow for activation of extrinsic cell death (47). In this study, incubation of CLL samples with FasL antibody blocked 50% of the observed apoptotic response, though a pan-caspase inhibitor was able to block cell death entirely. This suggested that caspase-8 contributed to but did not account for all of the observed apoptosis. Loss of mitochondrial membrane potential, which coincided with the increased expression of proapoptotic protein PUMA and cleavage of caspase-9, suggested activation of the intrinsic apoptotic pathway. Our findings support the notion that fludarabine and oxaliplatin activate caspase-dependent cell death in CLL cells, by both extrinsic and intrinsic apoptotic pathways. Activation of multiple apoptotic pathways makes this particular combination therapy attractive in tumors as it decreases chances for development of an antiapoptotic advantage.

#### Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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## Killing of Chronic Lymphocytic Leukemia by the Combination of Fludarabine and Oxaliplatin Is Dependent on the Activity of XPF Endonuclease

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