A Novel Celecoxib Derivative, OSU03012, Induces Cytotoxicity in Primary CLL Cells and Transformed B-cell Lymphoma via a Caspase and Bcl-2 Independent Mechanism

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Abstract

Chronic lymphocytic leukemia (CLL) is an incurable adult leukemia characterized by disrupted apoptosis. OSU03012 is a bioavailable third generation celecoxib derivative devoid of cyclooxygenase-2 inhibitory activity that potently induces apoptosis in prostate cancer cell lines and is being developed as an anti-cancer therapy in the NCI Rapid Access to Intervention Therapy (RAID). We assessed the ability of OSU03012 to induce apoptosis in primary CLL cells and the mechanism by which this occurs. The LC_{50} (lethal concentration 50%) of OSU03012 at 24 hours was 7.1 µM and this decreased to 5.5 µM at 72 hours. Additionally, we have demonstrated that OSU03012 mediates apoptosis by activation of the intrinsic, mitochondrial pathway of apoptosis but also activates alternative cell death pathways that are caspase independent. The early activation of both caspase dependent and independent pathways of apoptosis is novel to OSU03012 and suggests it has great potential promise for the treatment of CLL. Moreover, unlike the great majority of therapeutic agents utilized to treat leukemia or other forms of cancer, OSU03012 induces cell death entirely independent of bcl-2 expression. Overall, these data provide justification for further pre-clinical development of OSU03012 as a potential therapeutic agent for CLL.
Introduction

CLL is one of the most common types of adult leukemia in the United States.\textsuperscript{1} CLL is diagnosed on the basis of the malignant B-cell phenotype similar to normal B1 lymphocytes that co-express CD19/CD5 with dim surface immunoglobulin. The clonal B-cell of CLL has a low proliferation rate and disrupted apoptotic mechanism caused both by primary tumor features and interactions with co-dependent stromal elements.\textsuperscript{2,3} While the majority of patients with CLL are asymptomatic at diagnosis, most progress and require therapy. Therapy for CLL has advanced with the introduction of fludarabine, which three randomized trials has demonstrated improves response and progression-free survival over alkylator-based therapy.\textsuperscript{4-6} Furthermore, recent studies adding rituximab to fludarabine demonstrate promising results for the treatment of symptomatic CLL.\textsuperscript{7,8} Nonetheless, these therapies are not curative for CLL. Identification of new molecular targets and relevant therapeutic agents is therefore a high priority for the treatment of CLL.

Recent studies in prostate cancer cell lines demonstrate that celecoxib induced rapid apoptosis at a concentration range of 25-100 \(\mu\)M.\textsuperscript{9} Unfortunately, such steady state concentrations of celecoxib are not obtainable \textit{in vivo}. Structural modifications of celecoxib were carried out with the goal of dissociating COX-2 inhibitory and apoptosis-inducing activities.\textsuperscript{10} Accordingly, a series of derivatives with different substituents at the terminal phenyl ring were synthesized to examine respective apoptosis-inducing potencies.\textsuperscript{11} Furthermore, the mechanism by which these compounds induce apoptosis appears the same as that of celecoxib \textsuperscript{12}, i.e., facilitating the dephosphorylation of AKT and ERK2. Significant tumor growth inhibition has also been noted with the second \textsuperscript{11}
generation compounds following daily oral lavage administration in vivo in a prostate cancer cell xenograft model. Further synthesis has yielded other celecoxib derivatives with increased ability to induce apoptosis in the 1-10 µM range in prostate cancer cells, a similar proposed mechanism of action, and increased in vivo activity in the same prostate cancer cell xenograft model (personal communication Ching-Shih Chen). Based upon these data, a Rapid Access to Intervention Development proposal is currently underway to generate sufficient OSU03012 for early clinical studies in prostate cancer. Based upon these results, we examined the biologic effects of these new compounds in primary CLL cells and lymphoblastic lymphoma cell lines, demonstrating a potential novel mechanism of cell killing independent of caspase activation and bcl-2 overexpression, the latter which serves as a critical component to drug, antibody, and radiation mediated therapy resistance.

Materials and Methods

Patients, Cell Separation, and Culture Conditions: Written, informed consent was obtained to procure cells from patients with previously diagnosed CLL as defined by the modified NCI criteria. All of the CLL patients had been without prior therapy for a minimum of two months. Clinical data provided in Table 1 include modified Rai stage, karyotypes (described according to the International System for Human Cytogenetic Nomenclature), interphase cytogenetics, and number of previous treatments at the time of cell acquisition. Patients were considered to have active disease if they required initiation of therapy within two months of donating cells. Mononuclear cells were isolated and placed in culture as previously described by our group. Briefly, cells were maintained in culture media containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml
penicillin, and 100µg/ml streptomycin. The third generation PDK1/AKT inhibitor OSU03012 was synthesized as previously described. Experiments examining for caspase dependent apoptosis included addition of 100 µM of Z-VAD-fmk (benzyloxy carbonyl valine-alanine-asparagine-fluoromethyl ketone, (ICN Biomedicals Inc., Aurora, OH) for 30 minutes prior to addition of OSU03012. 697 cell lines stably transfected with retrovirus vector containing recombinant Bcl-2 or empty vector (neo) were obtained from Reed and Kitada at UCSD. The parent cell line was established from bone marrow of child with acute lymphoblastic leukemia. The cell line is CD19+, CD20+, CD3-, CD10+, CD13-, CD34-, CD37-, CD79a-, CD80-, CD138+, HLA-DR+ and EBV-.

LC_{50} determination by MTT assay: MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assays were performed as described previously. Briefly, 1 x 10^6 CLL patient peripheral blood mononuclear cells (PBMC) were incubated for 24 or 72 hours in OSU03012 or vehicle control. MTT reagent (Sigma, St. Louis, MO) was then added and plates were incubated for an additional 24 hours before washing with protamine sulfate in phosphate-buffered saline and measuring by spectrophotometry at 540 nm in a Labsystems 96-well plate reader (Fisher Scientific, Pittsburgh, PA). Data were plotted and values calculated using GraphPad software (San Diego, CA). For the 697 lymphoblastoid cell lines, 5 x 10^5 cells were incubated with media or OSU03012 at concentrations of 1, 5, 10, 50, and 100 µM. Cells were incubated for specified times (4, 24, and 72 hours).

Apoptosis and Flow Cytometric Studies: After exposure to drugs, cells were washed with PBS and resuspended in binding buffer containing annexin V-FITC and propidium
iodide according to the supplier’s instructions (BD Biosciences, San Diego, CA) and assessed by flow cytometry. Rhodamine-123 was used to monitor the integrity of mitochondria following drug treatment. OSU03012 treated cells were washed once in RPMI 1640 media and then incubated in RPMI 1640 media containing 50 ng/ml rhodamine-123 (Molecular Probes Inc., Eugene, OR) for 30 minutes at 37°C. Stained cells were washed once in RPMI 1640 media, placed on ice, and analyzed by flow cytometry. Experiments examining for caspase dependent apoptosis included addition of 100 µM of Z-VAD-fmk 30 minutes prior to addition of OSU03012. For all flow cytometry experiments, FACS analysis was performed using a Beckman-Coulter model EPICS XL cytometer (Beckman-Coulter, Miami, FL). Fluorophores were excited at 488 nm. Fluorescence was measured using channel FL1 for annexin V-FITC and channel FL3 for propidium iodide and rhodamine-123. Data was analyzed with the System II software package (Beckman-Coulter). At least 10,000 cells were counted for each treated sample. Each sample was run in duplicate.

**Western Blot Analysis:** Whole cellular lysates were prepared as previously described by our group with the addition of the phosphatase inhibitors sodium orthovanadate (1 mM) and microcystin LR (1 µM) (both Sigma, St. Louis, MO) to the lysis buffer. Phosphatase inhibitors are used in our lysate preparation to avoid any degradation that could occur with dephosphorylation of proteins in the sample. Antibodies used included caspase-3 and capase-8 (monoclonal antibody gift of Kitada and Reed), caspase-9 (rabbit polyclonal antibody Ab-1, Oncogene Research Products, San Diego, CA), and PARP (monoclonal antibody C-2-10, Oncogene Research Products, San Diego, CA) and GAPDH (Chemicon International Inc., Temecula, CA) and were analyzed by Western blot SDS-PAGE.
Equivalent protein samples (50 µg/lane) were separated on 8-14% polyacrylamide gels, and transferred onto 0.2 µm nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Following antibody incubation, the proteins were detected with chemiluminescent substrate (SuperSignal, Pierce Inc). Protein bands were quantified by integration of the chemiluminescence signals on a ChemiDoc system with Quantity One software (Bio-Rad Laboratories, Hercules, CA) using the rectangular integration tool after background subtraction.

**Results**

**OSU03012 Is Cytotoxic Toward CLL Cells *in vitro***

To determine the potential *in vitro* activity of OSU03012 against CLL cells, PBMCs from eleven CLL patients were incubated in various concentrations of OSU03012 for 24 or 72 hours. These results summarized in Figure 1 demonstrate that OSU03012 is highly cytotoxic toward primary B-cell CLL cells *in vitro*. A dose and time dependent loss of viability is observed in CLL cells treated with OSU03012 as compared to vehicle control. The LC$_{50}$ of OSU03012 at 24 hours was 7.12 +/- 0.48 µM and this decreased to 5.45 +/- 0.42 µM at 72 hours. Incubation with serum free media demonstrated greater toxicity in five patients examined as compared to the same primary tumor cells treated with 10% FBS. Minimal viability was observed in any of the patient samples at OSU03012 concentrations of 10 µM or greater. These data overall suggest OSU03012 is highly cytotoxic toward CLL cells *in vitro* at concentrations well below those attainable for prolonged periods of time without appreciable toxicity in the murine xenograft model of prostate cancer previously described.$^{11}$
**OSU03012 Induces Cytotoxicity Toward CLL via Apoptosis**

Cell death induced by therapeutic agents can occur through caspase dependent or independent apoptosis or by necrosis. To assess if apoptosis was contributing to the cytotoxic effects of OSU03012 we utilized annexin-V/PI surface staining following treatment with OSU03012 (10 $\mu$M) or media alone as shown in Figure 2. Specifically, we demonstrate both early (annexin-V positive only) and late (annexin-V/PI both positive) apoptosis (Figure 2A) concurrent with loss of mitochondrial membrane potential (Figure 2B) typical of apoptosis. These results provide support that cytotoxicity induced by OSU03012 occurs in part through induction of apoptosis and corroborate our MTT data in Figure 1 regarding the significant *in vitro* activity of OSU03012 against CLL cells.

**OSU03012 Induces Cell death Through Caspase Activation in CLL Cells**

Apoptosis with other cytotoxic agents in CLL can utilize caspase dependent and independent pathways. In an attempt to determine if the cytotoxicity induced by OSU03012 was due to an increase in caspase dependent apoptosis, mononuclear cells from eight CLL patients were incubated in medium or 10 $\mu$M of OSU03012 for 24 hours after which assessment of active p20 caspase-3 cleavage product and Poly(ADP-ribose) polymerase (PARP). Figure 3A and 3B demonstrates one such representative patient demonstrating a dose dependent increase in active p20 caspase-3 concurrent with a decrease in the pro-form 32 kD form. The active p20 caspase-3 identified in Figure 3A occurred concurrently with the appearance of the 85 kD cleaved product of PARP that is a down stream target of activated caspase-3 that is typically observed in the setting of caspase mediated apoptosis. These data support the conclusion that OSU03012 is
inducing cytotoxicity at least in part through activation of effector caspase-3 mediated apoptosis.

**OSU03012 Induces Activation of the Intrinsic Pathway of Apoptosis**

Caspase-3 mediated apoptosis can occur both through activation of the tumor necrosis receptor family members via caspase-8 cleavage (extrinsic pathway) or through the mitochondria (intrinsic pathway) that involves activation of caspase-9. We sought to determine which pathway of apoptosis was activated by OSU03012. CLL cells from eight patients were incubated with OSU03012 (10 µM) or medium for 24 hours and examined for processing of caspase-8 and caspase-9, both initiating caspases in either the extrinsic or intrinsic pathway of apoptosis. Figure 4A demonstrates a representative study of eight different patients following treatment with OSU03012 demonstrating no change in the level of the pro-form, inactive caspase-8. In contrast to the studies observed with caspase-8, Figure 4B demonstrates evidence of processing of caspase-9 with the appearance of the active cleaved form concurrent with caspase-3 and PARP cleavage and as outlined above. These data suggest that OSU03012 in part utilizes the intrinsic pathway of apoptosis to promote cell death of CLL cells.

**Caspase Inhibitor z-VAD-fmk Does Not Prevent OSU03012 Mediated Cell Death**

To determine if alternative mechanisms of apoptosis not involving caspase-8, caspase-9, or caspase-3 activation also might also be responsible for apoptosis induced by OSU03012, we exposed CLL cells to OSU03012 in the presence or absence of the pan-caspase inhibitor z-VAD-fmk and assessed for apoptosis (n=5). As shown in Figures 5A and 5B, apoptosis and cell death was not diminished by the pan-caspase inhibitor z-VAD-fmk at the 24-hour time point.
We next examined if z-VAD-fmk was truly inhibiting OSU03012 activation of caspase-3 as measured by processing of the pro-form and down stream cleavage of PARP that is characteristic of caspase dependent apoptosis in primary CLL cells. We exposed CLL cells to OSU03012 in the presence or absence of the pan-caspase inhibitor z-VAD-fmk and made cell lysates (n=4). As shown in Figure 5C and 5D, z-VAD-fmk greatly diminished processing of cleavage of PARP and caspase-3 despite not preventing cell death. These data together demonstrate that OSU03012 induces apoptosis independent of caspase activation in CLL.

**Bcl-2 Expression Does Not Protect against OSU03012-Mediated Cytotoxicity**

The anti-apoptotic protein Bcl-2 plays an important protective role in intrinsic and caspase dependent apoptosis through stabilization of the mitochondrial membrane. The bcl-2 protein is over-expressed in the majority of CLL patients at baseline and increased levels of this and other bcl-2 family members such as mcl-1 are associated with both drug resistance and inferior survival. We used the 697 lymphoblastoid cell line over-expressing bcl-2 to examine the role of this protein in OSU03012 mediated cytotoxicity. The 697 cell line was stably transfected with vectors encoding a neomycin-resistance gene, either empty or containing the \textit{bcl-2} gene. Lysates were assessed for bcl-2 protein levels to verify the protein was actively expressed. As can be seen in figure 6A, the \textit{bcl-2}-containing cell line (697-Bcl-2) expresses Bcl-2 protein at ten-fold higher levels than the empty control vector (697-Neo) line. These cell lines were then exposed to varying concentrations of OSU03012, 2-F-ara-A, and celecoxib after which proliferation and viability assessed. As shown in figure 6B, the growth inhibition between the \textit{bcl-2} over-expressing cell line and neo-line was markedly different. Specifically, both fludarabine
and celecoxib demonstrated potent inhibition of the neo-line, but not the bcl-2 transfected cell line. In contrast, equivalent inhibition was observed with OSU03012 between the neo and bcl-2 over-expressing cell line. These growth inhibitory findings are likely reflective of loss of viability as apoptosis is also diminished with 2-F-ara-A but equivalently observed with OSU03012 as demonstrated in Figure 6C. Here we see that bcl-2 over-expression greatly diminishes the apoptosis observed with both 2-F-ara-A, and celecoxib, but potent apoptosis is equally observed with OSU03012. Overall, these results demonstrate that OSU03012 mediates cell death through a pathway independent of bcl-2 over-expression.

**Bcl-2 Over-Expression Alters Pathway of Apoptosis Utilized**

Similar growth inhibition and apoptosis was noted between the neo and bcl-2 over-expressing 697 cell line despite the known stabilizing effect of bcl-2 on the mitochondrial membrane. To determine if truly caspase independent mechanisms were being activated in this cell line highly resistant to intrinsic pathway apoptosis, the neo and bcl-2 transfected 697 cell lines were treated with OSU03012 and assessed for caspase-3 and PARP processing characteristic of apoptosis. As shown in Figure 7A and 7B, the neo-697 cell line which has modest bcl-2 expression undergoes apoptosis with 2-F-ara-A and OSU03012 with demonstration of caspase-3 and PARP cleavage as shown previously with primary CLL cells. Similar to that observed in primary CLL cells, caspase-9 but not caspase-8 processing was observed in the 697 lymphoblastic cell line (data not shown). However, forced over-expression of bcl-2 results in disrupted apoptosis and lack of PARP cleavage with 2-F-ara-A. Contrasting with this, OSU03012 treatment results in marked apoptosis but an absence of PARP and caspase-3 processing.
This was not dependent upon time as no caspase processing was noted for the bcl-2 over-expressed cell lines at all examined time points whereas caspase activation was noted in the neo-697 cell line beginning as early as four hours. These studies further support the novel caspase independent mechanism of action of OSU03012 as compared to 2-F-ara-A and other traditional therapies such as fludarabine utilized in, the active metabolite of fludarabine that is a commonly utilized therapy for the treatment of CLL.

**Discussion**

Herein, we have described a new synthetic celecoxib derivative OSU03012 that lacks COX-2 inhibitory activity, but nonetheless induces potent apoptosis in primary non-proliferating CLL cells. The premise that COX-2 is involved in the pathological process of cancer growth and progression is supported by animal studies indicating that tumorigenesis is inhibited in COX-2 knockout mice. Furthermore, selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells. On the other hand, several lines of evidence argue that the ability of COX-2 inhibitors to promote apoptosis is dissociated from the enzyme activity of COX-2. Much work has been to show that OSU03012 does not affect COX-2 activity, yet induces apoptosis via an AKT/PDK pathway. Blockade of this survival pathway via inhibition of PDK1 has recently been described. The cell death observed with OSU03012 occurs early and at well below concentrations that have been attained in vivo following prolonged oral administration in mice xenograft models (personal communication CS Chen). Additionally, we have demonstrated that OSU03012 mediates apoptosis by activation of the intrinsic, mitochondrial pathway of apoptosis but also activates alternative cell death pathways that are caspase independent. The early activation of both caspase dependent
and independent pathways of apoptosis is novel to OSU03012 and suggests it has great potential promise for the treatment of CLL. Additionally, unlike the great majority of therapeutic agents utilized to treat leukemia or other forms of cancer, OSU03012 induces apoptosis entirely independent of bcl-2 expression. Overall, these data provide justification for further pre-clinical development of OSU03012 as a potential therapeutic agent for CLL.

The pathway of apoptosis utilized by celecoxib has only recently been characterized. Several groups have demonstrated that celecoxib mediates apoptosis through a caspase dependent pathway,\textsuperscript{12,26} but only recently has Jendrossek and colleagues fully investigated this.\textsuperscript{27} In a study utilizing Jurkat cell lines with defective caspase-8 activity, they demonstrated complete independence from the extrinsic pathway of apoptosis. Celecoxib activates the caspase-9 pathway of apoptosis and could be prevented by inactivating this caspase, implicating mitochondria disruption in the celecoxib-mediated cytotoxic effect. In addition, inhibition with the broad caspase inhibitor z-VAD-fmk or a caspase-9 specific inhibitor blocked apoptosis induced by celecoxib. Contrasting with this finding, OSU03012 induced apoptosis independent of caspase activation. This highlights the structural differences between celecoxib and OSU03012 and suggests the latter compound likely utilizes additional alternative mechanisms of action.

Several studies have identified bcl-2 and other bcl-2 family members are important in mediating drug resistance in CLL\textsuperscript{23,25} and over-expression of these proteins in patients with CLL may predict poor survival.\textsuperscript{25} Identifying therapies that mediate apoptosis independent of bcl-2 is therefore of high priority. Studies examining the ability
of celecoxib have suggested that it induces apoptosis independent of bcl-2.\textsuperscript{10,26} These results contrast somewhat with our findings where we observed that in stably transfected bcl-2 697 cell line, celecoxib induced apoptosis was prevented by bcl-2 over-expression. A similar finding was observed with 2-F-ara-A, the active metabolite of fludarabine. This finding may be reflective of relative expression of bcl-2 over-expression in this cell line or alternative mechanisms of resistance activated in this transfected cell line that differ from previous studies done with T-lymphoblasts or prostate cancer cell lines. In contrast with this, OSU03012 induced apoptosis with paradoxical independence on bcl-2 over-expression in the 697 lymphoblastic cell line. This finding emphasizes the superiority of OSU03012 over the parent compound celecoxib against malignant B-cells including those which over-express bcl-2. The difference in apoptotic pathways utilized by the parent compound celecoxib and synthetic derivative OSU03012 might reflect structural modifications that allow inhibition of alternative signaling pathways that facilitates activation of several, as opposed to one pathway of apoptosis. OSU03012 represents one of the first agents utilized by our group to effectively abrogate the potent drug resistance mediated by over-expression of bcl-2. Identifying the pathway(s) by which this occurs represents a high priority that is currently under investigation in our laboratory.

While OSU03012 represents an entirely new class of drugs devoid of COX-2 inhibitory capacity, the importance of \textit{in vivo} anti-tumor activity is of great importance. Recently, \textit{in vivo} results with a first generation celecoxib derivative (DMC) and celecoxib were reported in Cancer Reserch.\textsuperscript{11} Nude mice were xenografted with a prostate cancer cell line (PC-3). After tumor formation, mice were treated with oral celecoxib or DMC at
two different doses, 100 and 200 mg/kg. Treatment was tolerated without observable toxicities or weight loss. Necropsy after 35 days of treatment yielded no gross pathological abnormalities. The group receiving 200 mg/kg/day DMC displayed a significant effect on the PC-3 tumor growth (p < 0.1). More recently, third generation compounds discussed in this paper have been shown to be active in vivo in the prostate cancer xenograft model. Pharmacokinetic studies indicate that the peak serum concentration of oral OSU03012 at 200 mg/kg exceeded 20 µM. It is noteworthy that after oral administration of OSU03012 at 200 mg/kg for 28 days, the mice did not exhibit observable signs of toxicity. All animals maintained stable body weights throughout the study and lacked gross pathologic abnormalities at necropsy (personal communication, C-S Chen). While it is possible that during the RAID development process an unfavorable toxicity or pharmacologic feature will be identified that prevent full development of this agent. Even if this occurs, the paper described herein provides justification for pursuing alternative derivatives of OSU013012 based upon the novel mechanism of action we have identified that is independent of caspase activation or bcl-2 over-expression.

In summary, OSU03012 is an oral bioavailable therapeutic agent that has potent in vitro activity against primary CLL cells. This cytotoxicity is mediated through both caspase dependent and independent pathways and can over-come over-expression of bcl-2. Based upon these data, future studies investigating both the mechanism of action of OSU03012 and performance of early phase I studies in CLL are warranted.
References


### Table 1: Clinical and Laboratory Features of CLL Patients

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<th>ISCN</th>
<th>Interphase Cytogenetics</th>
<th># Trts</th>
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<th>72 hr</th>
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Table 1. Clinical and laboratory features of CLL patients. Written, informed consent was obtained to procure cells from patients with previously diagnosed CLL as defined by the modified NCI criteria. All of the CLL patients had been without prior therapy for a minimum of two months. ISCN=International System for Human Cytogenetic Nomenclature. # Trts=Number of previous therapies. % Loss of viability was measured by MTT assay.
Figure 1. OSU03012 is cytotoxic toward CLL cells in vitro. PBMCs from eleven CLL patients were incubated in various concentrations (1µM, 5µM, 10µM, and 50µM) of OSU03012 for 24 or 72 hours. MTT reagent was then added and plates were incubated for an additional 24 hours before analysis. Each bar represents the mean of eleven patients. Error bars indicate standard deviation.
Figure 2A. OSU03012 induces cytotoxicity toward CLL via apoptosis. After exposure to 10µM OSU03012 for 24 hours, CLL patient PBMCs were resuspended in binding buffer containing annexin V-FITC and propidium iodide and assessed by flow cytometry. Data is representative of nine patients.
Figure 2B. After treatment with 10μM OSU03012 for 24 hours CLL patient PBMCs were washed once in RPMI 1640 media and then incubated in RPMI 1640 media containing 50 ng/ml rhodamine-123 for 30 minutes at 37°C. Stained cells were washed once in RPMI 1640 media, placed on ice, and analyzed by flow cytometry. Data is representative of four patients.
Figure 3A. OSC03012 Induces Activation of the Intrinsic Pathway of Apoptosis. Protein lysates from cells treated with 4 and 24 hours of 10µM OSC03012 treatment were probed for caspase-3 by immunoblot. M=media, 12= 10µM OSC0312. Positive control is lysate from irradiated Jurkat cells. Data is representative of eight patients.
Figure 3B. Protein lysates from cells treated for 4 and 24 hours with 10µM OSU03012 treatment were probed for PARP by immunoblot. (M=media, 12= 10µM OSU0312) Positive control is lysate from irradiated Jurkat cells. Data is representative of eight patients.
Figure 4A. OSU03012 induces activation of the intrinsic pathway of apoptosis. Protein lysates from cells treated with 4 and 24 hours of 10µM OSU03012 treatment were probed for caspase-8 by immunoblot. (M=media, 12=10µM OSU0312) Positive control is lysate from irradiated Jurkat cells. Data is representative of eight patients.
Figure 4B.  Protein lysates from cells treated with 4 and 24 hours of 10µM OSU03012 treatment were probed for caspase-9 by immunoblot. (M=media, 12= 10µM OSU0312) Positive control is lysate from irradiated Jurkat cells. Data is representative of eight patients.
Figure 5A. The Caspase inhibitor z-VAD-fmk does not prevent OSU03012 mediated cell death. After exposure to 10µM OSU03012 or 5 µM fludarabine for 24 hours, CLL patient PBMCs were resuspended in binding buffer containing annexin V-FITC and propidium iodide and assessed by flow cytometry. Some cells were incubated with 100µM z-VAD-fmk, the cell-permeable pan-caspase inhibitor. Data are representative of five patients.
Figure 5B. **OSU03012 induces apoptosis independent of caspase activation.** CLL cells were incubated in media, 100 µM zVAD-fmk, 10 µM OSU03012, or 100 µM zVAD-fmk and 10 µM OSU03012 followed by assessment of viability by Annexin-V/PI staining 24 hours later. The percent viable cells was not influenced with zVAD-fmk. Data represents mean of five patients. Error bars are standard deviation.
Figure 5C. Protein lysates from cells treated with media (M), 100µM z-VAD-fmk (Z), 10µM OSU03012 (12), or combination were probed for PARP by immunoblot. Positive control is lysate from irradiated Jurkat cells. Blot is representative of four experiments.
Protein lysates from cells treated with media (M), 50µM z-VAD-fmk (Z), 10µM OSU03012 (12), or combination were probed for caspase-3 by immunoblot. Positive control is lysate from irradiated Jurkat cells. Blot is representative of four experiments.

**Figure 5D.** Protein lysates from cells treated with media (M), 50µM z-VAD-fmk (Z), 10µM OSU03012 (12), or combination were probed for caspase-3 by immunoblot. Positive control is lysate from irradiated Jurkat cells. Blot is representative of four experiments.
Figure 6A.  

**Figure 6A. Bcl-2 expression does not protect against OSU03012-mediated cytotoxicity.** To show Bcl-2 overexpression in 697 Bcl-2 stably transfected cell line increasing amounts of protein cell lysates from 697-Neo and 697-Bcl-2 cells lines were probed for Bcl-2 and GAPDH (loading control).
Figure 6B. 697-Neo and 697 cells lines were incubated with increasing doses of OSU03012 for 4, 24, and 72 hours. MTT reagent was then added and plates were incubated for an additional 24 hours before analysis.
Figure 7A. Bcl-2 over-expression alters pathway of apoptosis utilized. Protein lysates were collected from 697-Neo and 697-Bcl-2 cells lines after incubation with media (M), 1μM or 10μM OSU0312, and 25μM 2-F-ara-A for 24 hours. Lysates were probed for PARP and GAPDH by immunoblot. Data is representative of two experiments.
Figure 7B. Bcl-2 over-expression alters pathway of apoptosis utilized. Protein lysates were collected from 697-Neo and 697-Bcl-2 cells lines after incubation with media (M), 1µM or 10µM OSU03012, and 25µM 2-F-ara-A for 24 hours. Lysates were probed for caspase-3 and GAPDH by immunoblot. Data is representative of two experiments.
A novel celecoxib derivative, OSU03012, induces cytotoxicity in primary CLL cells and transformed B-cell lymphoma via a caspase and Bcl-2 independent mechanism

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