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CDDO-imidazolid mediated inhibition of malignant cell growth in Waldenström macroglobulinemia

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Abstract

Waldenström macroglobulinemia (WM) is a B-cell malignancy that remains incurable. Synthetic triterpenoids (ST), 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), its methyl ester derivative (CDDO-Me) and imidazolid derivative (CDDO-Im) induce cell death and inhibit growth of various malignancies and hold promise as treatment for cancer patients. We examined the therapeutic potential of these compounds in WM. All three forms of CDDO induced equal toxicity in BCWM.1 cells. In malignant B cells from WM patients, CDDO-Im induced the greatest toxicity. CDDO-Im inhibited proliferation at nanomolar concentrations and arrested the cells in G0/G1. CDDO-Im induced apoptotic cell death that was partially abolished in the presence of caspase inhibitor. CDDO-Im also inhibited survival pathways that have been shown to be important in WM. Overall, our data suggest that ST are likely to provide therapeutic efficacy for WM patients.

Keywords

Waldenström macroglobulinemia; CDDO; CDDO-Im; Non-Hodgkin lymphoma; Therapy

1. Introduction

As in other indolent lymphoproliferative disorders, Waldenström macroglobulinemia (WM) remains an incurable disease. WM is a B-cell disorder with a highly variable clinical outcome. Clinical symptoms vary and include the infiltration of lymphoplasmacytic cells into the bone marrow, the production of a monoclonal IgM protein, and associated symptoms such as anemia, lymphadenopathy and serum hyperviscosity [1-5]. Treatment of WM patients is determined by the presence of disease related symptoms. The most recent recommendations for therapy include the use of combination therapy with nucleoside analogues and alkylating agents, rituximab with nucleoside analogues or combination therapy such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) [2,3,6]. However, even though

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Conflict of interest

MK and MA have stocks and consulting agreements with Reata Pharmaceuticals, Inc.

patients may initially respond to treatment, they invariably relapse and identification of novel therapeutic agents is clearly needed.

2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its methyl ester derivative (CDDO-Me) and imidazolidine derivative (CDDO-Im) are synthetic triterpenoids (ST) derived from oleanolic acid. Studies have shown that CDDO-Im is more potent than its parent compound, CDDO, against murine melanoma and leukemic cells [7]. ST have been shown to provide effective control of cell growth of several tumor cell types [8] including breast cancer [9], lung cancer [10], ovarian cancer [11], melanoma [7], osteosarcoma [12], leukemia [13-17] and multiple myeloma cells [18,19]. Both CDDO and CDDO-Me are currently in Phase I clinical trials, with CDDO-Me being used in an oral form, highlighting the potential importance of these drugs as anti-cancer agents.

At the molecular level, ST have been shown to induce apoptosis of cancer cells by both caspase-dependent and caspase-independent mechanisms [16]. They have also been shown to bind to and induce cell death through the nuclear receptor peroxisome proliferator activator receptor- γ (PPAR γ) [20].

Despite the efficacy of ST in various malignancies, the biological effect of these compounds in WM has not been studied and their effect on survival pathways in WM has not been defined. The goal of the present study was therefore, to evaluate the efficacy of ST as a therapeutic option for WM patients. We analyzed the effects of CDDO, CDDO-Me and CDDO-Im on a cell line derived from a WM patient (BCWM.1) and on cells from WM patients. We demonstrate that CDDO-Me and CDDO-Im induced significant toxicity in WM cells with CDDO-Im having the greatest effect on cell viability. Furthermore, CDDO-Im significantly inhibited WM cell growth. We also demonstrate that CDDO-Im induced apoptosis and inhibited survival pathways in WM cells, supporting the potential use of these compounds as anti-cancer agents for Waldenström macroglobulinemia patients.

2. Materials and methods

2.1. Cells and reagents

The BCWM.1 cell line [21] was a kind gift from Dr. Steven Treon (Dana Farber Cancer Institute, Boston, MA). Bone marrow and tissue biopsy mononuclear cells were isolated as previously described [22] from patients with WM, who provided written informed consent. This study was approved by the Mayo Clinic Foundation Institutional Review Board. CDDO, CDDO-Me and CDDO-Im were kindly provided by Dr. Edward Sausville (NCI) under the RAID program and by Dr. Michael Sporn (Dartmouth Medical College, Hanover NH).

2.2. Assessment of cell viability by Annexin-V and Propidium Iodide (PI) double-staining

To assess cell viability, we used Annexin V/Propidium Iodide double staining as previously described [23]. Bone marrow cells from WM patients were sorted into CD19⁺ CD138⁺ cells using CD19 and CD138 double sorting with RoboSep beads (StemCell Technologies Inc., Vancouver, BC) and resuspended at a concentration of 0.5×10^6 cells/ml. Cells were cultured in 48-well plates (0.5 ml/well) in RPMI supplemented with 10% FBS in the presence or absence of the indicated doses of CDDO, CDDO-Me or CDDO-Im. A DMSO control treatment was also applied in parallel. At the indicated times, cells were stained with 1 μ g Annexin-V-FITC (Caltag, Burlingame, CA) for 20 min at 4 °C and washed in Annexin-V binding buffer. Cells were then stained with 0.5 μ g PI and immediately analyzed by flow cytometry. Data acquisition was done on a FACSCalibur and analysis was done using FlowJo software (Tree Star, Ashland, OR). Viability was determined as cells that were negative for Annexin-V and PI as a percentage of total cells.

2.3. Proliferation assay

Cell proliferation was assessed by thymidine incorporation as previously described [23]. BCWM.1 cells were cultured for 3 days in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at a density of 0.25×10^5 cells/well in the presence of the indicated treatments. Cultures were pulsed with 1 μ Ci (0.037 MBq) tritiated thymidine (^{3} H-TdR; 5.0 Ci/mmol [185 GBq/mmol] (Amersham, Piscataway, NJ) for 18 h prior to harvesting and ^{3} H-TdR incorporation levels were determined using a Beckman scintillation counter (GMI, Inc., Ramsey, MN).

2.4. Cell cycle analysis

To analyze cell cycle, BCWM.1 cells were treated with CDDO-Im for 24 h. Cells were then stained with BrdU Flowstaining kit (BD Pharmingen) following protocol provided by manufacturer. Briefly, CDDO-Im treated cells were stained with BrdU for 30 min at 37 °C and then fixed and permeabilized. Following DNase treatment, cells were stained with anti-BrdU-APC for 20 min then washed. Cells were then resuspended in FACS buffer containing 7-amino-actinomycinD(7AAD) and data was acquired using a FACSCalibur. Data analysis was done using FlowJo software (Tree Star).

2.5. Immunoblotting

To assess the mechanism of CDDO-Im activity, 3×10^6 BCWM.1 cells were treated as indicated and lysed in 150 μ l RIPA lysis buffer (150mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50mM Tris-Cl) and analyzed by SDS-PAGE. Z-VAD-FMK caspase inhibitor was purchased from (R&D Systems, Minneapolis, MN), anti-phospho (p)-Akt, Akt, pErk1/2, Erk, anti-Caspase-3, anti-Caspase-8, and anti-Caspase-9 (Cell Signaling Technology, Inc., MA), PARP (Santa Cruz Biotechnology, CA), and α -actin (Novus Biologicals, CO).

2.6. Statistical analysis

Comparisons between groups were based on χ^2 tests for nominal variables; the Wilcoxon rank-sum test or the Kruskal–Wallis test was used for continuous variables. For all statistical tests $p < 0.05$ was considered significant. Analysis was performed on Statview software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Synthetic triterpenoids decrease viability of WM cells

To evaluate the anti-tumor effects of ST in WM, we first assessed the sensitivity of BCWM.1 cells and freshly isolated CD19⁺ CD138⁺ cells from WM patients to CDDO, CDDO-Me and CDDO-Im. All 3 molecules induced equal toxicity on BCWM.1 cells after 24 h of culture (Fig. 1A). We found no difference in the relative viability of BCWM.1 cells at day 1 through day 4 of culture (data not shown) and therefore we used the 24 h time point for the remainder of the experiments. We tested the effect of CDDO, CDDO-Me and CDDO-Im on freshly isolated CD19⁺ CD138⁺ cells from WM patients. At the concentrations used, CDDO (Fig. 1B) had minimal effect on cell viability, while CDDO-Me (Fig. 1C) and CDDO-Im (Fig. 1D) had significant effects on viability. Since CDDO-Im appeared to induce the greatest toxicity on patient cells, we used CDDO-Im for the remainder of the experiments. Cell death as a result of CDDO-Im treatment was a very early event as BCWM.1 cells stained positive for Annexin as early as 4 h after culture with CDDO-Im and more than 50% of the cells were annexin positive at 6 h post CDDO-Im treatment (Fig. 1E). We compared the viability of B lineage cells (CD19⁺ CD138⁺) from WM patients to that of peripheral blood (PB) and bone marrow (NBM) obtained from healthy donors (Fig. 1F). We found a significant difference in the

sensitivity of malignant WM cells to CDDO-Im at 62.5 nM and 125 nM when compared to NBM and PB ($p < 0.05$).

3.2. CDDO-Im inhibits the proliferation of WM cells

To further characterize the role of CDDO-Im in WM, we tested whether CDDO-Im had any effect on WM cell growth. BCWM.1 cells were cultured for 3 days in the presence of either media alone or DMSO control or the indicated doses of CDDO-Im (range 3.9–500 nM) and cell proliferation was assessed by thymidine incorporation (Fig. 2A). There was a dose-dependent decrease in the proliferation of BCWM.1 cells in the presence of CDDO-Im after 3 days in culture. Furthermore, the decrease in cell proliferation was seen with the lowest dose of CDDO-Im (3.9 nM) further supporting the finding that WM B cells are sensitive to CDDO-Im mediated effects. Of note, cell proliferation was assessed at day 1 through day 3 and there was no difference in the relative proliferation of the cells in the presence or absence of CDDO-Im (data not shown). Furthermore, lower doses of CDDO-Im had no effect on cell viability (data not shown) and at 62.5 nM, approximately 90% of the cells remained viable (Fig. 1D). Cell cycle analysis was done using BCWM.1 cells treated for 24 h in the presence CDDO-Im. CDDO-Im treated cells were incubated with the thymidine analogue, BrdU, and stained with anti-BrdU-APC antibody and 7AAD, a fluorescent dye that incorporates into DNA and is a measure of total DNA content. Flow cytometric analysis of cells stained with anti-BrdU and 7AAD allows the discrimination of cell subsets that reside in G0/G1 (Fig. 2C, lower left gate), S (top gate), or G2 + M (lower right gate) phases of the cell cycle and had recently synthesized DNA. Our analysis indicates that the cells were arrested in the G0/G1 phase (Fig. 2B and C). Taken together, these data suggest that in addition to its effect on cell viability, CDDO-Im also inhibits WM cell proliferation.

3.3. CDDO-Im induces apoptosis of WM B cells

Synthetic triterpenoids have been shown to induce cell death by both caspase-dependent and caspase-independent mechanisms. To determine the mechanism of CDDO-Im mediated cell death in WM, BCWM.1 cells were cultured in the presence or absence of the indicated doses of CDDO-Im. Since our annexin/PI studies indicated that at 6 h, >50% of cells stain positive for annexin (Fig. 1E), we used the 6 h time point for our signaling studies. CDDO-Im induced caspase-3 activation and PARP degradation at 500 nM (Fig. 3A), suggesting CDDO-Im effects are caspase mediated. To determine the involvement of the intrinsic and/or extrinsic apoptotic pathways, we tested for activation of caspase-8 and caspase-9 and found cleavage of both caspase-8 and caspase-9 in cells treated with 500 nM CDDO-Im (Fig. 3B), suggesting the involvement of both the intrinsic and extrinsic apoptotic pathways in CDDO-Im mediated effects in WM. Consistent with our Annexin/PI staining (Fig. 1E), CDDO-Im mediated signaling was evident at early times points as BCWM.1 cells treated with 500 nM CDDO-Im showed Caspase-3 activation and PARP degradation at 4 h post treatment (Fig. 3C). Both intrinsic and extrinsic apoptotic cell death were also evident at 4 h post CDDO-Im treatment (Fig. 3D).

To determine whether caspase activation is necessary for CDDO-Im mediated toxicity, we used a pan caspase inhibitor (Z-VAD-FMK). BCWM.1 cells were pretreated with the caspase inhibitor (I) for one hour and then cells were treated with DMSO control, 125 nM or 250 nM of CDDO-Im for 24 h. Using annexin/PI staining, we found that the presence of caspase inhibitor protected the cells from CDDO-Im mediated killing; however it did not completely abolish CDDO-Im mediated toxicity (Fig. 4A). This suggests that the effect of CDDO-Im in WM is mostly caspase mediated (approximately 20% and 37% protection in the presence of inhibitor (I) at 125 nM and 250 nM CDDO-Im respectively) but not totally caspase dependent. To confirm the inhibition of the caspase pathway, BCWM.1 cells were treated with the caspase inhibitor alone, CDDO-Im alone or both for 6 h and then cell lysates were used to determine

PARP degradation by western blotting (Fig. 4B). We found that pretreatment of BCWM.1 cells with the caspase inhibitor completely abolished PARP degradation verifying the lack of caspase activation. This provides further evidence that although caspases are not activated, BCWM.1 cells may undergo cell death through a caspase independent mechanism.

3.4. CDDO-Im targets survival pathways in WM

Recent studies have identified survival pathways that appear to be important in WM. Genes involved in the MAPK pathway are elevated in WM patients [24]. Recent studies also indicated that protein kinase C beta (PKC β) is upregulated in WM [25]. Furthermore, the Akt pathway has been shown to be upregulated in WM and is an important survival pathway [26]. Previous studies have shown that synthetic triterpenoids inhibit prosurvival pathways including Akt [27] and suppression of MAPK pathways along with the activation of p38 [28]. We therefore sought to determine the effect of CDDO-Im on survival pathways that have been shown to be relevant in WM. BCWM.1 cells were cultured with 500nM CDDO-Im and at various doses, cells were lysed and lysates were used for immunoblotting. We found a decrease in constitutively activated Erk1/2 as early as 2 h post CDDO-Im treatment (Fig. 5A). Since this has been shown to be associated with an increase in p38 activation/phosphorylation in leukemia, we tested for activation of p38 in response to CDDO-Im in WM. There was an increase in the activation of p38 (phosphorylated p38) (Fig. 5A). We also tested the effect of CDDO-Im on the Akt pathway and found a decrease in the levels of constitutively active Akt (Fig. 5B) after treatment with CDDO-Im. In contrast, we found that CDDO-Im did not target the PKC pathway (Fig. 5C). Taken together, these studies show that CDDO-Im inhibits constitutively active proteins involved in the MAPK and Akt pathways, but has no effect on the PKC signaling pathway.

4. Discussion

Although several therapeutic options exist for WM patients, none have been proven effective in providing a cure [6]. Therefore, there is a need for the identification of novel therapeutic agents. Synthetic triterpenoids have been shown to have potent anti-inflammatory and growth-suppressive effects [29,30]. In addition to their role in solid tumors, they have been shown to inhibit growth of cell lines representative of several hematological malignancies [14,16-18, 20,31,32]. Here, we show that CDDO-Me and CDDO-Im can induce death of malignant WM B cells in bone marrow specimens obtained from patients much more effectively than CDDO at nanomolar concentrations (Fig. 1). This is in agreement with other reports showing greater toxicity of CDDO-Im compared to CDDO [7,10,18] and others showing greater effect of CDDO-Im on the intracellular molecular targets compared to CDDO [33,34]. Our results are also consistent with others showing a similar role for synthetic triterpenoids in primary CLL cells [20], primary multiple myeloma cells [18] and primary promyelotic leukemia cells [32].

CDDO mediated cell death occurs in association with down regulation of c-FLIP (caspase-8 homolog Fas-ligand interleukin-1-converting enzyme (FLICE)-inhibitory protein), cleavage of Bid, activation of caspase-8 and caspase-3, release of mitochondrial cytochrome *c*, inhibition of de novo iNOS (nitric oxide synthase), modulation of PPAR- α , and inhibition of NF- κ B activity [12,29,31,35,36]. CDDO-Me has been shown to suppress MAPK pathways in acute myeloid leukemia [28]. In our studies, we provide evidence of caspase-3 activation and PARP degradation (Fig. 3A) suggesting a caspase-mediated mode of action of CDDO-Im in WM. We sought to determine the involvement of the intrinsic (mitochondrial) versus extrinsic (receptor mediated) apoptotic pathways in CDDO-Im mediated apoptosis in WM. Both caspase-8 and caspase-9 are activated in response to CDDO-Im (Fig. 3B) suggesting the involvement of both the mitochondrial and death receptor pathways in WM. Since inhibition of total caspases using a pan caspase inhibitor only partially protected BCWM.1 cells from

CDDO-Im mediated killing (Fig. 4), this suggests that CDDO-Im mediated killing of BCWM.1 cells is caspase mediated, but not totally caspase dependent. Consistent with these results, in acute myelogenous leukemia, CDDO has been found to induce both caspase-dependent and caspase-independent apoptosis [16].

Although the precise mechanism of action of CDDO remains controversial, CDDO and its derivatives have been shown to induce apoptosis through inhibition of pro-survival PI3K/Akt and NF- κ B signaling [18,27,37]. The fact that both of these pathways have been shown to be important in B-cell malignancies, the importance of the Akt pathway in the survival of WM B cells [26], and downregulation of Akt in response to CDDO-Im in WM, may explain the increased sensitivity of WM B cells to CDDO-Im when compared to non-malignant cells (Fig. 1F). This selective effect of CDDO-Im on malignant cells suggests it may have less toxic effects on patients. Additionally, combining CDDO with agents that target NF- κ B, such as bortezomib, has been shown to overcome bortezomib resistance [18] and may have potential success in WM patients. We found that CDDO-Im had no effect on PKC family members (Fig. 5C). Others have shown that the PKC inhibitor enzastaurin, down-regulates PKC family members and induces apoptosis of WM cells [25]. Therefore, the use of CDDO alone or in combination with other agents such as bortezomib or enzastaurin holds promise in WM.

In summary, we show for the first time that synthetic triterpenoids are effective at inhibiting growth and survival of WM cells. We show that ST have pro-apoptotic effects in WM and preferentially target the Akt and MAPK pathways. We also show that CDDO-Im mediated effects in WM occur through both caspase mediated and caspase independent mechanisms. While the precise efficacy of individual CDDO molecules remains to be determined in ongoing clinical trials, our data show that ST are effective in killing WM cells *in vitro*, and suggest that the use of ST therefore holds promise for Waldenström macroglobulinemia patients.

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References

1. Dimopoulos MA, Anagnostopoulos A. Waldenström's macroglobulinemia. *Best Pract Res Clin Haematol* 2005;18:747–65. [PubMed: 16026748]
2. Fonseca R, Hayman S. Waldenström macroglobulinaemia. *Br J Haematol* 2007;138:700–20. [PubMed: 17672883]
3. Vijay A, Gertz MA. Waldenström macroglobulinemia. *Blood* 2007;109:5096–103. [PubMed: 17303694]
4. Kyle RA, Vincent Rajkumar S. Monoclonal gammopathies of undetermined significance. *Best Pract Res Clin Haematol* 2005;18:689–707. [PubMed: 16026745]
5. Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of Waldenström's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenström's Macroglobulinemia. *Semin Oncol* 2003;30:110–5. [PubMed: 12720118]
6. Treon SP, Gertz MA, Dimopoulos M, et al. Update on treatment recommendations from the Third International Workshop on Waldenström's macroglobulinemia. *Blood* 2006;107:3442–6. [PubMed: 16410453]
7. Place AE, Suh N, Williams CR, et al. The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth in vivo. *Clin Cancer Res* 2003;9:2798–806. [PubMed: 12855660]
8. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multi-functional agents for the prevention and treatment of cancer. *Nat Rev Cancer* 2007;7:357–69. [PubMed: 17446857]

9. Konopleva M, Zhang W, Shi YX, et al. Synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest in HER2-overexpressing breast cancer cells. *Mol Cancer Ther* 2006;5:317–28. [PubMed: 16505105]
10. Kim KB, Lotan R, Yue P, et al. Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells. *Mol Cancer Ther* 2002;1:177–84. [PubMed: 12467212]
11. Melichar B, Konopleva M, Hu W, Melicharova K, Andreeff M, Freedman RS. Growth-inhibitory effect of a novel synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, on ovarian carcinoma cell lines not dependent on peroxisome proliferator-activated receptor-gamma expression. *Gynecol Oncol* 2004;93:149–54. [PubMed: 15047229]
12. Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S, Kufe D. The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol Pharmacol* 2001;59:1094–9. [PubMed: 11306692]
13. Inoue S, Snowden RT, Dyer MJ, Cohen GM. CDDO induces apoptosis via the intrinsic pathway in lymphoid cells. *Leukemia* 2004;18:948–52. [PubMed: 14990979]
14. Ito Y, Pandey P, Place A, et al. The novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* 2000;11:261–7. [PubMed: 10845427]
15. Suh WS, Kim YS, Schimmer AD, et al. Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. *Leukemia* 2003;17:2122–9. [PubMed: 12931220]
16. Konopleva M, Tsao T, Estrov Z, et al. The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukaemia. *Cancer Res* 2004;64:7927–35. [PubMed: 15520199]
17. Shishodia S, Sethi G, Konopleva M, Andreeff M, Aggarwal BB. A synthetic triterpenoid, CDDO-Me, inhibits IkappaBalpha kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor kappaB-regulated gene products in human leukemic cells. *Clin Cancer Res* 2006;12:1828–38. [PubMed: 16551868]
18. Chauhan D, Li G, Podar K, et al. The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance. *Blood* 2004;103:3158–66. [PubMed: 15070698]
19. Ikeda T, Nakata Y, Kimura F, et al. Induction of redox imbalance and apoptosis in multiple myeloma cells by the novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid. *Mol Cancer Ther* 2004;3:39–45. [PubMed: 14749474]
20. Konopleva M, Elstner E, McQueen TJ, et al. Peroxisome proliferator-activated receptor gamma and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias. *Mol Cancer Ther* 2004;3:1249–62. [PubMed: 15486192]
21. Ditzel Santos D, Ho AW, Tournilhac O, et al. Establishment of BCWM.1 cell line for Waldenström's macroglobulinemia with productive in vivo engraftment in SCID-hu mice. *Exp Hematol* 2007;35:1366–75. [PubMed: 17761288]
22. Novak AJ, Bram RJ, Kay NE, Jelinek DF. Aberrant expression of Blymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. *Blood* 2002;100:2973–9. [PubMed: 12351410]
23. Elsawa SF, Novak AJ, Grote DM, et al. B-lymphocyte stimulator (BLyS) stimulates immunoglobulin production and malignant B-cell growth in Waldenström macroglobulinemia. *Blood* 2006;107:2882–8. [PubMed: 16304043]
24. Chng WJ, Schop RF, Price-Troska T, et al. Gene-expression profiling of Waldenström macroglobulinemia reveals a phenotype more similar to chronic lymphocytic leukemia than multiple myeloma. *Blood* 2006;108:2755–63. [PubMed: 16804116]
25. Moreau AS, Jia X, Ngo HT, et al. Protein kinase C inhibitor enzastaurin induces in vitro and in vivo antitumor activity in Waldenström macroglobulinemia. *Blood* 2007;109:4964–72. [PubMed: 17284528]
26. Leleu X, Jia X, Runnels J, et al. The Akt pathway regulates survival and homing in Waldenström macroglobulinemia. *Blood*. 2007

27. Gao X, Deeb D, Jiang H, Liu Y, Dulchavsky SA, Gautam SC. Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF-kappaB and Notch1 signaling. *J Neurooncol.* 2007
28. Konopleva M, Contractor R, Kurinna SM, Chen W, Andreeff M, Ruvolo PP. The novel triterpenoid CDDO-Me suppresses MAPK pathways and promotes p38 activation in acute myeloid leukemia cells. *Leukemia* 2005;19:1350–4. [PubMed: 15931262]
29. Wang Y, Porter WW, Suh N, et al. A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. *Mol Endocrinol* 2000;14:1550–6. [PubMed: 11043571]
30. Suh N, Wang Y, Honda T, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res* 1999;59:336–41. [PubMed: 9927043]
31. Pedersen IM, Kitada S, Schimmer A, et al. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood* 2002;100:2965–72. [PubMed: 12351409]
32. Ikeda T, Kimura F, Nakata Y, et al. Triterpenoid CDDO-Im down-regulates PML/RARalpha expression in acute promyelocytic leukemia cells. *Cell Death Differ* 2005;12:523–31. [PubMed: 15746941]
33. Suh N, Roberts AB, Birkey Reffey S, et al. Synthetic triterpenoids enhance transforming growth factor beta/Smad signaling. *Cancer Res* 2003;63:1371–6. [PubMed: 12649201]
34. Honda T, Rounds BV, Bore L, et al. Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* 1999;9:3429–34. [PubMed: 10617085]
35. Konopleva M, Tsao T, Ruvolo P, et al. Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* 2002;99:326–35. [PubMed: 11756188]
36. Stadheim TA, Suh N, Ganju N, Sporn MB, Eastman A. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* 2002;277:16448–55. [PubMed: 11880365]
37. Han SS, Peng L, Chung ST, et al. CDDO-Imidazolidine inhibits growth and survival of c-Myc-induced mouse B cell and plasma cell neoplasms. *Mol Cancer* 2006;5:22. [PubMed: 16759389]

Fig. 1.

Synthetic triterpenoids inhibit survival of WM B cells. BCWM.1 cells were cultured 0.5×10^6 cells/ml in duplicate treatments in 24-well tissue culture plates in the presence of either media alone, DMSO control or the indicated doses of CDDO, CDDO-Me or CDDO-Im and viability was assessed using Annexin V/PI staining after 24 h (A). CD19⁺ CD138⁺ cells were positively selected from bone marrow biopsy specimens obtained from WM patients ($n = 4$). Cells were resuspended at 0.5×10^6 cells/ml. 0.5 ml were cultured in duplicate treatments in 48-well tissue culture plates either in the presence of media alone or at the indicated doses of CDDO (B), CDDO-Me (C) or CDDO-Im (D) and viability was assessed using Annexin V/PI staining after 24 h. Lines represent data from individual patients. BCWM.1 cells were treated

with either a DMSO control or 500 nM CDDO-Im and Annexin-V/PI staining was used to determine apoptotic cells (E). Side-by-side comparison of the effects of CDDO-Im on the viability of CD19⁺ CD138⁺ cells from WM patients, bone marrow of healthy donors (NBM) and peripheral blood (PB) (F). Bars represent averages of values from total patients used $\pm$ standard error. * $P < 0.05$, ** $P < 0.01$.

Fig. 2.

CDDO-Im inhibits proliferation of BCWM.1 cells. BCWM.1 cells (0.025×10^6 cells/well) were cultured in 96 well flat-bottom plates in the presence or absence of the indicated doses of CDDO-Im for 3 days at 37 °C in the presence of 5% CO₂. Cell proliferation was assessed using thymidine incorporation (A). Values represent the mean of triplicate values $\pm$ S.D. This experiment was repeated 4 times with similar results. Cell cycle analysis was carried out on BCWM.1 cells treated with the indicated doses of CDDO-Im for 24 h. Cells were harvested and analyzed for BrdU incorporation and then stained with 7-AAD. Bars represent % cells in each phase of cell cycle at the indicated treatments (B). Scatter blots demonstrate % cells gated

based on BrdU and 7AAD staining (C). This experiment was repeated 2 times with similar results.

Fig. 3. CDDO-Im induces apoptosis in WM. BCWM.1 cells were treated with the indicated doses of CDDO-Im and lysates were analyzed by western blotting for Caspase-3 cleavage and PARP degradation (A). To determine the apoptotic pathway involved, BCWM.1 cells were treated as described and lysates were analyzed by western blotting for caspase-8 and caspase-9 activation (B). BCWM.1 cells were treated with 500nM CDDO-Im for various time and cell lysates were analyzed by western blotting to determine the kinetics of CDDO-Im mediated PARP degradation (C) and caspase activation (D). These experiments were repeated at least 3 times with similar results. Arrows indicate cleaved products.

Fig. 4.

Caspase inhibitor protects BCWM.1 cells from CDDO-Im mediated cell death. BCWM.1 cells were pretreated with 100 μ M pan caspase inhibitor Z-VAD-FMK (I) for 1 h. Cells were then treated with either 125nM or 250nM CDDO-Im as indicated and viability was assessed after 24 h using Annexin-V/PI staining (A). BCWM.1 cells were treated as indicated and PARP degradation was assessed by Western blot after 6 h (B). These experiments were repeated 3 times with similar results.

Fig. 5. CDDO-Im inhibits survival pathways in WM. BCWM.1 cells were treated with 500nM CDDO-Im and at various time points, cells were lysed. Cell lysates were used in western blot analysis to determine the ability of CDDO-Im to inhibit MAPK pathway (A), the Akt pathway (B) and the PKC pathway (C).