OSU-DY7, a novel *D*-tyrosinol derivative, mediates cytotoxicity in chronic lymphocytic leukemia and Burkitt lymphoma through p38 mitogen-activated protein kinase pathway

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Running Title:

OSU-DY7 activates p38 MAPK in B-CLL and Burkitt lymphoma

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Summary

Drug resistance and associated immune deregulation limit use of current therapies in chronic lymphocytic leukemia (CLL), thus warranting alternative therapy development. Herein we demonstrate that OSU-DY7, a novel *D*-tyrosinol derivative targeting p38 MAPK, mediates cytotoxicity in lymphocytic cell lines representing CLL (MEC-1), acute lymphoblastic leukemia (697 cells), Burkitt lymphoma (Raji and Ramos) and primary B cells from CLL patients in dose and time dependent manner. The OSU-DY7-induced cytotoxicity is dependent on caspase activation as evidenced by induction of caspase-3 activation and PARP cleavage and rescue of cytotoxicity by Z-VAD-FMK. Interestingly, OSU-DY7-induced cytotoxicity is mediated through activation of p38 MAPK as evidenced by increased phosphorylation of p38 MAPK and downstream target protein MAPKAPK2. Pretreatment of B-CLL cells with SB202190, a specific p38 MAPK inhibitor, results in decreased MAPKAPK2 protein level with concomitant rescue of the cells from OSU-DY7 mediated cytotoxicity. Furthermore, OSU-DY7-induced cytotoxicity is associated with down regulation of p38 MAPK target BIRC5, that is rescued at protein and mRNA levels by SB202190. This study provides an evidence for a role of OSU-DY7 in p38 MAPK activation and BIRC5 down regulation associated with apoptosis in B lymphocytic cells, thus warranting development of this alternative therapy for lymphoid malignancies.

Keywords: *D*-tyrosinol, chronic lymphocytic leukemia, p38 MAPK, apoptosis, BIRC5.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the western world, with an incidence of around 3.5 cases per 100,000 people per year (Dighiero & Hamblin, 2008). While the outcome of patients with CLL vary by many factors, such as age, disease status, associated genetic abnormalities and other co-morbid illnesses, the available therapies including alkylating agents, purine analogues, bendamustine, alemtuzumab, rituximab, and more recently combination therapy with chemoimmunotherapy has shown to be not curative (Montserrat & Moreno, 2008). Similar to chemotherapy combinations in other malignancies, drug resistance often ensues when treatment has been initiated. In particular, patients relapsing after chemoimmunotherapy have a poor outcome with a survival less than 2 years, warranting alternative therapies in CLL.

One alternative to circumvent drug resistance is to utilize agents that act through mechanisms that are different from that of the currently used therapies. The p38 MAPK pathway, initially identified for its role in stress and inflammatory response, was found to have a tumor suppressor function (Nebreda & Porras, 2000; Ono & Han, 2000; Dolado *et al*, 2007; Han & Sun, 2007; Hui *et al*, 2007b; Hui *et al*, 2007a; Kennedy *et al*, 2007). The p38 MAPK pathway has been implicated in negative control of tumorigenesis through cell cycle control (Ambrosino & Nebreda, 2001), cell

differentiation (Puri *et al*, 2000), cell proliferation, oncogene-induced senescence, replicative senescence, contact inhibition, DNA-damage response and induction of apoptosis (Nebreda & Porras, 2000; Bulavin & Fornace, 2004; Wada & Penninger, 2004; Han & Sun, 2007). Some chemotherapeutic drugs have been reported to induce cell apoptosis via p38 MAPK activation, including all-trans retinoic acid in mdulloblastoma cells, vinka alkaloids in HeLa cells, taxol and cisplatin in several non-hematological cell lines (Deacon *et al*, 2003; Hallahan *et al*, 2003; Losa *et al*, 2003; Olson & Hallahan, 2004). Although how p38 MAPK induces cell apoptosis is not fully understood, studies in myeloma and rat pheochromocytoma cells suggest a link between p38 MAPK and the Bcl-2 family protein and mitochondrial pathway (Seo *et al*, 2007; Cai & Xia, 2008). Together, these data suggest a potential use for p38 MAPK targeted therapeutic agents in cancer treatment.

In an attempt to develop a new class of agents targeting p38 MAPK activation, we used the immunosuppressive agent FTY720 as a lead compound to conduct structural optimization, which has been reported to mediate apoptosis in human Jurkat T lymphocytes, in part, via a p38-dependent mechanism (Matsuda *et al*, 1999). Among various derivatives examined, OSU-DY-7 [(R)-2-amino-3-(4-heptyloxy-phenyl)-propan-1-ol] represented the optimal agent, which exhibits unique ability to activate p38 MAPK in a variety of lymphoid

malignancies including primary CLL cells. Here, we demonstrate that OSU-DY7 mediates cytotoxicity in lymphocytic cell lines and primary B-CLL cells, in part, via p38 MAPK-dependent down-regulation of BIRC5 expression.

Materials and methods

Cells and culture conditions

Blood from patients with CLL was obtained under a protocol approved by The Ohio State University hospital internal review board. All patients had understood and signed the informed consent in accordance with the Declaration of Helsinki. B-CLL cells were isolated from freshly collected whole blood using Rosette-Sep kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Human B-lymphocyte cell lines MEC-1, Raji, Ramos, 697 and isolated primary B-CLL cells were incubated using procedures previously described (Liu *et al*, 2008). MEC-1 cell line was obtained from the German cell line bank (Braunschweig, Germany) and Raji, 697 and Ramos cell lines were from American Type Culture Collection (ATCC, Manassas, VA).

Reagents

OSU-DY7 was prepared from *D*-tyrosine, of which the synthetic procedure will be published elsewhere. The identity and purity were confirmed by nuclear magnetic resonance and mass spectrometry. The chemical structure of OSU-DY7 is shown in Fig 1A. The pharmacological agents were purchased from the respective vendors: *D*-tyrosine (Sigma-Aldrich; Saint Louis, MO), p38 MAPK inhibitor SB202190 and

MAP kinase kinase (MEK) inhibitor PD98059 (Calbiochem, Gibbstown, NJ); Z-VAD-FMK (BIOMOL, Plymouth, PA); caspase-3 substrate (Ac-DMQD)2-Rh110 (AnaSpec, San Jose, CA); TRIzol reagent (Invitrogen, Carlsbad, CA); MG132 (Cayman Chemical, Ann Arbor, MI).

Cell viability and apoptosis assay

The cell viability was assessed by dual staining with annexin V conjugated to flourescein isothiocyanate (FITC) and propidium iodide (PI). Cells (1×10^6) were stained by annextin V-FITC (BD Pharmingen, San Diego, CA) and PI (BD Pharmingen) using a procedure previously published (Liu *et al*, 2008). Cells were analyzed by a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Annexin V-FITC and/or PI positive cells were identified as apoptotic cells. Viable cells were those with both annexin V-FITC negative and PI negative staining. The viable cells in each sample were expressed as % by normalizing annexin V^-/PI^- cells to untreated control.

Analysis of caspase-3 activity

Caspase-3 activity was determined by using (Ac-DMQD)2-Rh110 (AnaSpec) as the fluorogenic substrate for caspase-3 using a procedure previously described (Hung *et*

al, 2008).

MTS assay

Measurement of cell growth was performed using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit purchased from Promega (Madison, WI). Cells (0.25×10^6/mL for cell lines and 1×10^6/mL for primary B-CLL) were placed in 200 μL volume in 96-well microtiter plates with indicated reagent and incubated in 37°C. MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium] and PMS (phenazine methoxulfate) solution were mixed in 20:1 in volume. The colorimetric measurements were performed 4 hours later at 490-nm wavelength by a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The cell viability was expressed as a percentage of absorbance value in treated sample compared to that observed in control vehicle treated sample.

Western blotting

Cell lysates were prepared using RIPA buffer (150 mM NaCl, 50 mM Tris PH 8.0, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor (Sigma) and phosphatase inhibitor cocktail (Calbiochem).

Antibodies against various proteins were obtained from the following sources: poly-ADP-ribose polymerase (PARP), Akt, p-Akt (Ser473), ERK1/2, p-ERK1/2 (pThr202Tyr204), JNK, p-JNK (Thr183Tyr185), p38 mitogen-activated protein kinase (p38 MAPK), p-p38 MAPK (Thr180Tyr182), MAPKAPK2, p-MAPKAPK2 (Thr334) (Cell Signaling, Danvers, MA); BIRC5 (R&D, Minneapolis, MN), tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); actin (MP Biomedicals, Solon, OH). The goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA).

Reverse transcription and polymerase chain reaction

Cells (5×10^6) were washed with PBS twice and mRNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription to cDNA was performed using BioRad iScript cDNA Synthesis Kit (BIO-RAD Laboratories, Hercules, CA). Briefly, 1 μg of mRNA, 4 μL of 5 X iScript-Reaction Mix and 1 μL of iScript Reverse Transcriptase were mixed in nuclease-free water with final volume of 20 μL. The condition for reverse transcription was: 25^{o}C for 5 min, 42^{o}C for 30 min and 85^{o}C for 5 min. The real-time PCR for *BIRC5* was performed using Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) using RN18S1 as an internal control.

Briefly, 1 μL of cDNA, 12.5 μL of Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 1.25 μL *BIRC5* probe (Hs 00977611_g1, Applied Biosystems) or RN18S1 control (Hs 03003631_g1, Applied Biosystems) were mixed to DNAse-free water to final volume of 25 μL. The real-time PCR reaction was carried on 7900HT Real-Time PCR System (Applied Biosystems).

Statistical analysis

All statistical analyses were performed by biostatisticians in the Center for Biostatistics at the Ohio State University. Nonlinear mixed models were used to obtain IC_{50}. For comparisons, linear mixed models were used for modeling treatment effect and patient random effect. Holm's method was applied to adjust for multiplicity and control the overall Type I error rate at α=0.05. SAS software (version 9.1, SAS Institute, Inc., Cary, NC) was used for all statistical analyses.

Results

OSU-DY7 mediates cytotoxicity of B-lymphocytic cell lines and primary B cells from CLL patients

Lymphoid cell lines representative of CLL (MEC-1), ALL (697), Burkitt lymphoma (Raji and Ramos) were cultured with 0, 1, 2, 4, 6, 8, 10, 12, and 15 μM concentrations

of OSU-DY7. The OSU-DY7 induced dose and time-dependent decrease in cell survival (Fig 1B). The IC_{50} value for each of the cell lines is shown in Table 1.

In order to determine the cytotoxic efficacy of OSY-DY7 in B-CLL cells, freshly isolated CD19+ B-CLL cells were treated with OSU-DY7 (ranging from 0, 1, 2, 4, 6, 8, and 10 μM) and the cell viability was evaluated by annexin-V/PI staining analysis at 24 or 48 hours. The IC_{50} for 16 patients were 3.58 μM (95% CI: 2.60 4.57) and 3.26 μM (95% CI: 2.20 4.32) for 24 hours and 48 hours, respectively (Fig 1C, Table 1) demonstrating maximal apoptosis was observed at 24 hours with no advantage for extended exposure beyond this time period.

OSU-DY7 induces caspase-3 activation and PARP cleavage in B-lymphocytic cell lines and primary B-CLL cells

To investigate the relationship of OSU-DY7-mediated cytotoxicity and activation of caspase 3, cells were treated with OSU-DY7 0 μM, 4 μM and 8 μM for 24 hours. There was a significant linear increase in caspase-3 activity as concentration of OSU-DY7 increased (Fig 2A, *$p<0.0001$ in Raji cells and *$p=0.0048$ in primary B-CLL cells).

In order to determine if caspase activation results in PARP cleavage, Raji cells were incubated with OSU-DY7 at 0, 2, 4, or 8 μM for 24 hours. MEC-1 cells were

incubated with OSU-DY7 at 0, 0.5, 1, and 2 μM for 24 hours. Lower concentrations of OSU-DY7 were chosen for MEC-1 cells due to their relatively higher sensitivity compared to Raji cells (see Table 1). The results showed OSU-DY7 induced PARP cleavage in Raji and MEC-1 cell lines in a dose-dependent manner as evidenced by appearance of the cleaved 89 Kd band (Fig 2B, left panels). Similar to the results obtained in cell lines, OSU-DY7 lead to PARP cleavage in primary B-CLL cells in 6 independent patient samples. Representative results from two patients are shown in Fig 2B.

In order to determine the relevance of caspase activation in OSU-DY7 mediated cytotoxicity, we tested the effect of OSU-DY7 on cells pretreated with pancaspase inhibitor Z-VAD-FMK. As shown in Fig 2C, OSU-DY7-mediated cytotoxicity was partially yet significantly rescued by Z-VAD-FMK, in both cell lines and primary B-CLL cells. In MEC-1 cells, 100 μM Z-VAD-FMK pretreatment rescued the OSU-DY7-mediated cytotoxicity as evidenced by the significant increase in viable CLL cells from 21.3% to 44.5%. Thus, Z-VAD-FMK significantly decreased OSU-DY7-mediated MEC-1 cell killing by 52% (95% CI: 48.3% 55.7%, n=4, *$p<0.0001$). Similar rescue effect of Z-VAD-FMK was also observed in Raji cells where OSU-DY7-mediated cytotoxicity was partially rescued with the increased viable cells from 34.1% to 44.5%. Thus, Z-VAD-FMK significantly decreased

OSU-DY7-mediated Raji cell killing by 23% (95% CI: 21.0 25.6%, n=4, *p<0.0001) (Fig 2C). Consistent with the cell line data, Z-VAD-FMK also rescued OSU-DY7-mediated cytotoxicity in primary B-CLL cells (Fig 2D). Without Z-VAD-FMK, the ratio in survival between OSU-DY7 and DMSO was 62.1% (95% CI: 55.3% 69.7%), with the Z-VAD-FMK, the ratio was 74.3% (95% CI: 60.6% 91.0%). The Z-VAD-FMK significantly decreased the cell killing by OSU-DY7 by 16.5% (95% CI: 3.9% 27.4%, n=5, *p=0.0298).

OSU-DY7 induces phosphorylation of p38 MAPK in lymphoid cell lines and primary CLL cells

To investigate the possible mechanisms involved in OSU-DY7-mediated cytotoxicity, pathways of Akt, ERK, p38 MAPK, JNK, NF-κB were examined. No differences in levels of p-JNK (Thr183Tyr185), JNK and NF-κB between vehicle and OSU-DY7 treated groups were noted (data not shown). However, OSU-DY7 induced significant increase of phosphorylation of p38 MAPK in a dose-dependent manner in Raji (n=4, *p<0.0001) and MEC-1 (n=4, *p=0.0325) when comparing OSU-DY7-treated group with DMSO (Fig 3A). In Raji cells, the ratio of p-p38 MAPK (Thr180Tyr182) versus p38 MAPK after OSU-DY7 at 2, 4 and 8 μM concentrations increased 2.2 fold (1 3.8), 4.7 fold (1.2 11.8) and 11 fold (2.9 31.2) compared with control group. Similar

increase in p-p38 MAPK was also observed in MEC-1 cells. The ratio after treatment with OSU-DY7 0.5, 1 and 2 μM for 24 hours increased 1.1 fold (1 1.3), 1.9 fold (1.2 2.8) and 2.9 fold (1.4 5.5) respectively compared with control group. The phosphorylated MAPKAPK2 (Thr334), a downstream target of p38 MAPK, was also increased after OSU-DY7 treatment.

Consistent with the findings in cell lines, OSU-DY7 induced phosphorylation of p38 MAPK and MAPKAPK2 in primary B-CLL cells (Fig 3B). The ratio of p-p38 MAPK and p38 MAPK increased 1.3 fold (1 1.8), 2.3 fold (1.2 3.4) and 90 fold (1 412) in cells from six CLL patients after treatment with OSU-DY7 2, 4 and 8 μM for 24 hours (n=6, *p=0.0004 when comparing OSU-DY7-treated group with DMSO vehicle control).

The time course analysis of OSU-DY7-induced phosphorylation of p38 MAPK in Raji cells revealed increased phosphorylation of p38 MAPK as early as 2 hours post treatment with the progressive increase leading to ~ 44 fold increased phosphorylation by 24 hours. The difference in phosphorylation of p38 MAPK between the trends of OSU-DY7 and the negative control was significant (p=0.0022). (Fig 3C).

OSU-DY7-mediated cytotoxicity is dependent on phosphorylation of p38 MAPK

To further examine the role of p38 MAPK pathway in OSU-DY7-mediated cytotoxicity, p38 MAPK specific inhibitor SB202190 was used. OSU-DY7 induced increased phosphorylation of MAPKAPK2 and PARP cleavage in Raji cells. SB202190 reversed the phosphorylated MAPKAPK2 and reduced the degree of PARP cleavage (Fig 4A). Meanwhile, SB202190 rescued OSU-DY7-mediated cytotoxicity in Raji cells partially either at 24 or 48 hours (Fig 4B). At 24 hours, without SB202190, the ratio in survival between OSU-DY7 and DMSO was 34.1% (95% CI: 32.7% 35.6%, n=4); with SB202190, the ratio was 53.9% (95% CI: 51.7% 56.2%, n=4). The SB202109 significantly decreased OSU-DY7-mediated Raji cell killing by 36.7 % (95% CI: 33.9% 39.4%, *p<0.0001 when comparing SB202190 and OSU-DY7 interaction). Similarly, at 48 hours treatment, there was significant interaction between SB202190 and OSU-DY7 (Fig 4B). Without SB202190, the ratio in survival between OSU-DY7 and DMSO was 24.3% (95% CI: 22.7% 26.1%, n=4), with the SB202190, the ratio was 48.2% (95% CI: 51.7% 56.2%, n=4). The SB202109 significantly decreased OSU-DY7-mediated Raji cell killing by 49.5 % (95% CI: 45.0% 53.6%, *p<0.0001 when comparing SB202190 and OSU-DY7 interaction).

We then checked the salvage effect of SB202190 for OSU-DY7 in primary CLL cells (Fig 4C). Briefly, without SB202190, the ratio in survival between OSU-DY7 and DMSO was 58.4% (95% CI: 52.2% 65.4%, n=4) at 24 hours. With SB202190, the

ratio was 77.2% (95% CI: 68.5% 81.7%, n=4). The SB202109 significantly decreased OSU-DY7-mediated cell killing by 24.3 % (95% CI: 14.1% 33.3%, *p=0.002 when comparing SB202190 and OSU-DY7 interaction).

OSU-DY7 down regulates BIRC5 via p38 MAPK activation

The underlying molecular mechanism by which p38 MAPK causes cell apoptosis is not completely understood. We investigated pro-apoptotic and anti-apoptotic proteins in response to OSU-DY7 treatment in Raji cells. Compared with DMSO group, there was no significant change in expression of Bcl-2, Bcl-xl and cIAP2 proteins as detected by immunoblot analysis following OSU-DY7 treatment (data not shown). However, significant reduction in expression of BIRC5 protein was observed in OSU-DY7-treated group. The down-regulation of BIRC5 level could be partially reversed by SB202190 (Fig 5A, left panel). Without P38 MAPK inhibitor SB202190, OSU-DY7 significantly decreased BIRC5 expression (24% of DMSO, 95%CI: 21.5% 25.7%, p<0.0001, n=3). The pretreatment with 10 μM of SB202190 partially rescued BIRC5 expression level by 1.9 fold (1.5 2.1, *p<0.0001, n=3) (Fig 5A, right panel). To further delineate the cause of down-regulation of BIRC5 protein, RT-PCR analysis of *BIRC5* transcripts was analyzed. As shown in Fig 5B, OSU-DY7 significantly decreased *BIRC5* expression (p=0.0002, n=3). Pretreatment with 10 μM

of SB202190 significantly prevented OSU-DY7 induced *BIRC5* mRNA expression by 2.4 fold (Fig 5B, 95% CI: 1.56 3.84 fold, *p=0.0026 when comparing SB202190 and OSU-DY7 interaction, n=3). This implied that OSU-DY7 activated p38 MAPK that suppressed BIRC5 expression by transcriptional inhibition. To investigate if the down-regulation of BIRC5 protein level was related to increased proteasome activity, Raji cells were treated with DMSO or 4 μM OSU-DY7 for 12 hours, followed by DMSO or 10 μM of MG132, a proteasome inhibitor, for 12 hours. In contrast to OSU-DY7 induced Mcl-1 protein that was partially rescued, MG132, failed to modulate the BIRC5 protein level (Fig 5C). Collectively these results suggest that OSU-DY7 induces activation of p38 MAPK that leads to down regulation of BIRC5 protein expression through transcriptional mechanism.

Discussion

We have described here development of a novel *D*-tyrosinol derivative, OSU-DY7 that mediates cytotoxicity in primary CLL B cells and B cell lines representing CLL (MEC-1), ALL (697), and Burkitt lymphoma (Raji and Ramos) cell lines. The cytotoxic effect of OSU-DY7 is dependent on activation of caspase and downstream PARP cleavage. Z-VAD-FMK at concentration that inhibited the activation of caspases prevented OSU-DY7 mediated apoptosis. The partial inhibitory effect of Z-VAD-FMK suggested potential additional mechanism in OSY-DY7 mediated cytotoxicity. Consistent with this hypothesis, the OSU-DY7 induced activation of p38 MAPK in B-CLL cells and B cells lines.

Three lines of evidences suggested a potential role for p38 MAPK phosphorylation in OSU-DY7 mediated apoptosis. First, concentrations of OSU-DY7 that induced apoptosis resulted in time dependent phosphorylation of p38 MAPK on Thr180 and Tyr182 residues that has been shown to promote apoptosis (Wada & Penninger, 2004; Seo *et al*, 2007). Second, SB201190 that resulted in inhibition of p38 MAPK activity also resulted in inhibition of OSU-DY7 induced apoptosis. Third, the OSU-DY7 resulted in phosphorylation of MAPKAPK2, a downstream target of p38 MAPK that is implicated in apoptosis (Ono & Han, 2000; Dolado *et al*, 2007). Concentrations of SB201190 that inhibited MAPKAP2 phosphorylation prevented the

OSU-DY7 mediated apoptosis in B cell lines and B cells from CLL patients.

Our finding indicating OSU-DY7 induced activation of p38 MAPK leading to cytotoxicity of CLL cells is consistent with the potential role for p38 MAPK in tumor suppressive effect (Nebreda & Porras, 2000; Ono & Han, 2000; Dolado *et al*, 2007; Han & Sun, 2007; Hui *et al*, 2007b; Hui *et al*, 2007a; Kennedy *et al*, 2007). Interestingly a negative role for p38 MAPK in cell survival has been documented suggesting the complex role for p38 MAPK activation in cell growth and apoptosis (Juretic *et al*, 2001; Park *et al*, 2002; Wada & Penninger, 2004). Multiple key cell cycle controls are known to be targets of p38 MAPK. Lavoie and colleagues demonstrated that p38 MAPK could inhibit cyclin D1 expression that was reversed by p38 MAPK inhibitor (Lavoie *et al*, 1996). Bulavin DV found that inactivated p38 MAPK in vivo would expedite tumor formation by suppressing p53 activation (Bulavin *et al*, 2002). The same group further found p38 MAPK activation could suppress tumor appearance by modulating the *CDKN2A* tumor-suppressor gene (Bulavin *et al*, 2004). Importantly, a downstream signal of p38 MAPK pathway, MAPKAPK-2, has been found to be a member of the cell cycle checkpoint kinases, exhibiting it's activity via phophorylation of Cdc25 protein phosphatase (Manke *et al*, 2005). Several evidences suggest a correlation between p38 MAPK activation and apoptosis induction. For example, withdrawal of nerve growth factor from Rat PC-12

pheochromocytoma cell was found to cause sustained activation of p38 MAPK and JNK, as well as induction of apoptosis (Xia *et al*, 1995). Knockout studies further demonstrated a decreased cell survival in cells lacking MKK6, p38αMAPK and MAPKAPK2 (Nebreda & Porras, 2000).

Despite the extensive description, the precise molecular mechanisms by which p38 MAPK causes cell apoptosis are not completely understood. A potential role for regulation of Bcl-2 family and mitochondrial pathway has been implicated (Kennedy *et al*, 2007; Seo *et al*, 2007; Cai & Xia, 2008). Down-regulation of cIAP-1/2, XIAP and BIRC5, as well as accumulation of p53, Bax and Bak in mitochondria were noted in sulindac-induced p38 MAPK activation and cell apoptosis (Seo *et al*, 2007). OSU-DY7 induced down regulation of BIRC5 is consistent with the observations in human lung carcinoma cells where down-regulation of BIRC5 protein expression was caused by activation of p38 MAPK pathway (Chao *et al*, 2004). Similar down modulation of BIRC5 by activation of p38 MAPK and JNK pathways was also observed in response to arsenic trioxide in lung adenocarcinoma cells (Cheng *et al*, 2006), and vitamin D_3-mediated cell growth inhibition and apoptosis (Li *et al*, 2005). Together, these data suggest that p38 MAPK activation plays a role in tumor suppression. Although untreated primary CLL cells have low expression of BIRC5 compared with ALL or follicular lymphoma cells (de Graaf *et al*, 2005; Hui *et al*, 2006),

up-regulation of BIRC5 is found in post-chemotherapy CLL cells, which maybe one of the mechanisms by which CLL becomes chemoresistant (Hui *et al*, 2006).

The pro-apoptotic effect of p38 MAPK pathway has also been revealed in CLL. Pedersen and colleagues demonstrated that rituximab, besides its activity to induce antibody-dependent cellular toxicity, could induce apoptosis in B-CLL through a p38 MAPK activation-dependent pathway, and inhibition of p38 MAPK reduced the degree of anti-CD20-induce apoptosis (Pedersen *et al*, 2002). In another study, a vitamin D3 analog EB1089 was reported to induce apoptosis in B-CLL cells via a mechanism involving p38 MAPK activation and ERK suppression (Pepper *et al*, 2003b). Interestingly OSU-DY7 also induces activation of p38 MAPK with concomitant down regulation of ERK1/2 phosphorylation (data not shown) suggesting a potential reciprocal regulation of these two signaling pathways. Pepper C el al also showed that flavopiridol-induced apoptosis in B-CLL was associated with activation of p38 MAPK and suppression of ERK activity (Pepper *et al*, 2003a). Altering the balance between these two pathways could provide a rationale for the p53-independent nature of flavopiridol-induced apoptosis.

In conclusion, these studies describe a newly synthesized *D*-tyrosinol derivative, OSU-DY7, that is active for B lymphocytic cell lines representing ALL, Burkitt lymphoma and CLL and primary B cells from CLL patients. p38 MAPK activation

dependent mechanisms involving down modulation of BIRC5 protein and mRNA and caspase dependent apoptosis reveal OSU-DY7 as an attractive alternative therapy targeting the p38 MAPK pathway for CLL and other lymphocytic malignancies. Further studies are warranted to validate OSU-DY7 for clinical development for CLL and other B cell malignancies.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure Legend

Fig 1. Chemical structure of OSU-DY7 and cytotoxicity study in B-lymphocytic cell lines and primary B CLL cells. (A) Chemical structure of OSU-DY7 with a molecular weight of 265.39. (B) MEC-1, 697, Raji, and Ramos cells (0.25×10^6 cells/mL) were incubated with indicated concentrations of OSU-DY7 or DMSO vehicle for 24 hours (□) or 48 hours (■). The cells were analysed with MTS assay, as described in "MTS assay in Methods" (n=3). (C) purified B-CLL cells (1×10^6 cells/mL) were incubated with OSU-DY7 or DMSO for 24 hours (□) or 48 hours (■). The cells were stained with annexin V-FITC and PI for accessing cell viability (n=16).

Fig 2. OSU-DY7-mediated cytotoxicity is dependent on caspase activation and apoptosis. (A) Raji cells (0.25×10^6 cells/mL) and primary B-CLL cells (1×10^6 cells/mL) were incubated with OSU-DY7 or DMSO for 24 hours. Cells (1×10^6) were analyzed for caspase-3 activity as described in "Analysis of caspase-3 activity in Methods". The numbers in each graph represent the percentage and the range of caspase-3 positive cells (n=3). (B) OSU-DY7 induces PARP cleavage in Raji cell, MEC-1 cell and primary B-cell CLL in 24 hours. Raji cells (0.25×10^6 cells/mL), MEC-1 cells (0.25×10^6 cells/mL) and primary B-CLL cells (1×10^6 cells/mL) were incubated with OSU-DY7 for 24 hours.

Total cell lysates (10 μg) were used for western blot. The data represents results from 2 of 6 patient samples with similar observation. (C) OSU-DY7-induced cytotoxicity can be rescued in part by Z-VAD-FMK in MEC-1 and Raji cells. In MEC-1 study, cells (0.25×10^6 cells/mL) were pretreated with medium or 100 μM Z-VAD-FMK, followed by incubation with DMSO or 2 μM OSU-DY7 for 24 hours. In Raji study, cells (0.25×10^6 cells/mL) were pretreated with medium or 50 μM Z-VAD-FMK, followed by incubation with DMSO or 4 μM OSU-DY7 for 24 hours. The viability of cells was checked with MTS assay (n=4). (D) OSU-DY7-induced cytotoxicity can be rescued in part by Z-VAD-FMK in primary CLL cells. Primary B-CLL cells (1×10^6 cells/mL) were pretreated with medium or 100 μM Z-VAD-FMK, followed by incubation with DMSO or 4 μM OSU-DY7 for 24 hours. At 24 hours, the cells were stained with annexin V-FITC and PI for accessing cell viability (n=5).

Fig 3. OSU-DY7 induces phosphorylation of p38 MAPK. (A) Raji cells and MEC-1 cells (0.25×10^6 cells/mL) were incubated with OSU-DY7 or DMSO for 24 hours. Cell lysates of 15 μg were used for western blot analysis. The ratio of p-p38 MAPK versus p38 MAPK in indicated concentrations of OSU-DY7 was compared with that in DMSO. ▬ represents average of the values from 4 different experiments. * p=0.0325 for Raji cells and p=0.0325 for MEC-1 cells when comparing OSU-DY7-treated group with

DMSO control. (B) Primary B-CLL cells (1×10^6 cells/mL) were incubated with OSU-DY7 or DMSO for 24 hours. Cell lysates of 15 μg at each point were used for western blot analysis. The ratio of p-p38 MAPK versus p38 in indicated concentrations of OSU-DY7 was compared with that in DMSO. ▬ represents average of the values from 6 patients. * p=0.0004 when comparing OSU-DY7-treated group with DMSO control. (C) OSU-DY7 induces phosphorylation of p38 MAPK in Raji cells in a time-dependent manner. Cell lysates of 15 μg at each point were used for western blot analysis. The data shown here is a representative of two independent experiments with similar results.

Fig 4. OSU-DY7-induced cytotoxicity is dependent on p38 MAPK activation. (A) OSU-DY7 induces upregulation of phosphorylation of MAPKAPK2 that is reversed by p38 MAPK inhibitor SB202190 in Raji cells. Cells (0.25×10^6 cells/mL) were pretreated with medium or 10 μM SB202190 for 2 hours, followed by incubation with DMSO or 4 μM OSU-DY7 for 24 hours. Cell lysates of 15 μg at each point were used for western blot analysis. (B) OSU-DY7-induced cytotoxicity is partially rescued by SB202190 in Raji cells. Cells (0.25×10^6 cells/mL) were pretreated with medium or 10 μM SB202190 for 2 hours, followed by incubation with DMSO or 4 μM OSU-DY7. The viability of cells was checked with MTS assay (n=4). (C) OSU-DY7-induced

cytotoxicity is partially rescued by SB202190 in primary CLL cells. B-CLL cells (1×10^6 cells/mL) were pretreated with medium or 10 μM SB202190 for 2 hours, followed by incubation with DMSO or 4 μM OSU-DY7 for 24 hours. The cells were stained with annexin V-FITC and PI to assess cell viability (n=4).

Fig 5. OSU-DY7-induced down regulation of BIRC5 protein and mRNA transcription in p38 MAPK activation dependent manner. (A) Raji cells (0.25×10^6 cells/mL) were pretreated with medium or 10 μM SB202190 for 2 hours, followed by DMSO or 4 μM OSU-DY7 for 24 hours. Cell lysates of 15 μg at each point were used for western blot (left panel). The ratio of BIRC5 versus actin protein was compared between OSU-DY7 alone and OSU-DY7 plus SB202190 (right panel, n=3). (B) Raji cells (0.25×10^6 cells/mL) were pretreated with DMSO or 10 μM SB202190 for 2 hours, followed by DMSO or 4 μM OSU-DY7 for 24 hours. mRNA was reverse transcribed to cDNA which was further compared for BIRC5 expression using real-time PCR. The data shown here represents relative mRNA level of *BIRC5* compared with DMSO-treated cells. (C) Raji cells (0.25×10^6 cells/mL) were pretreated with DMSO or 4 μM OSU-DY7 for 12 hours, followed by DMSO or 10 μM MG132 for 12 hours. Cell lysates of 15 μg at each point were used for western blot.