ANTICANCER RESEARCH *39*: 4179-4184 (2019)
doi:10.21873/anticanres.13577

Wedelolactone Targets EZH2-mediated Histone H3K27 Methylation in Mantle Cell Lymphoma

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Abstract. *Background/Aim: Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2), possesses histone N-methyltransferase (HMT) activity and plays an essential role in cancer initiation and development. The aim of the present study was to investigate the potential of Wedelolactone (WL) to inhibit the methylation activity of EZH2. Materials and Methods: The mantle cell lymphoma (MCL) cell line, Mino, was treated with WL, while untreated cells were used as control. HMT activity and EZH2 amount were measured in nuclear extracts from WL-treated and control Mino cells. Results: WL was found to target EZH2-mediated histone H3K27 methylation. Along with the inhibition of H3K27 methylation in vitro (IC50=0.3 μM), WL suppressed HMT activity in Mino cells with an IC50 value of 3.2 μM. We detected a reduced amount of EZH2 in Mino cells treated with WL, compared to untreated control cells. Conclusion: This is the first study to show that WL induces inhibition of H3K27 methylation via EZH2 modulation and decreases cell proliferation in MCL, in vitro. WL is proposed as a promising agent and a novel epigenetic approach in MCL investigation and treatment.*

Recent investigations have revealed the involvement of multifunctional protein complexes in the regulation of cell malignancies. In the last few years much attention was paid to association of polycomb repressive complex 2 (PRC2) with cancer progress (1-3). EZH2, the enzymatic unit of the PRC2 complex, catalyzes the transfer of 1-3 methyl groups from S-adenozyl-L-methionine to the lysine residues of histone H3 (4). The tri-methylated lysine 27 of histone H3 (H3K27) has been shown as a stable facultative heterochromatin mark which promotes the recruitment of polycomb group proteins for promoter-targeted transcriptional gene silencing (5). Increased histone H3K27 methylation has been found to be involved in some pathological processes, especially cancer progress.

It has been shown that EZH2 is frequently overexpressed in many cancer types including lymphomas, breast, prostate, lung, brain, and liver cancer, and moreover, it is critical for cancer cell proliferation (6-10). Few reports have proposed mechanisms by which the PRC2 complex controlled methylation level. For example, it was shown that embryonic ectoderm development (EED) component of PRC2 is required for the histone H3 lysine methylation (11-13). Several natural compounds, such as curcumin, gambogic acid, triptolide, wedelolactone, and ursolic acid, have been shown to serve as EZH2 modulators (14), but their mechanism of action is poorly studied. Chen and co-authors demonstrated that wedelolactone (WL) binds with a high affinity to the EED and blocks the EZH2-EED interaction *in vitro*, inducing the degradation of PRC2 core components (15). WL has been used as an inhibitor of IκB kinase, which is critical for the activation of NF-κB survival pathway (16), and was shown to inhibit proliferation of prostate cancer cells *in vitro* and *in vivo* (17). In addition, WL has been found to reduce the production of interleukin-6 in pancreatic cancer cells, which is responsible for their survival and proliferation (18). Moreover, WL induced apoptosis and prevented osteolytic bone metastasis in patients with bone metastasis of breast cancer origin (19, 20).

Mantle cell lymphoma (MCL) is a well-defined and aggressive type of B cell non-Hodgkin's lymphoma. Although drugs such as ibrutinib have shown promising therapeutic effect in clinical trials, MCL patients often acquired drug resistance (21). Therefore, new therapeutic approaches for MCL treatment are required. In this study we focused on the effects of WL on histone methylation mediated by the catalytic subunit EZH2 of PRC2 complex, in MCL cells.

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Key Words: EZH2, H3K27 methylation, mantle cell lymphoma, wedelolactone, PRC2.

Materials and Methods

Cell lines and cell culture. The MCL (non-Hodgkin's B-cell lymphoma) cell line, Mino, was obtained from American Type Cell

Culture Collection (ATCC, CRL-3000; Manassas, VA, USA). Mino cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 15% fetal bovine serum (all from Sigma-Aldrich, St. Louis, MO, USA), at 37˚C in a 5% CO_2 humidified atmosphere.

Cell treatment with WL. WL was purchased from TargetMol Corp. (Boston, MA, USA) and was dissolved in dimethyl sulfoxide as 100 mM stock solution. Mino cells were treated with the appropriate concentration of WL for indicated time periods, while untreated cells were used as control.

WST-8 cell proliferation assay. To evaluate WL cytotoxicity, WST-8 assays (Cayman Chemicals, Ann Arbor, MI, USA) were performed according to manufacturer's protocol, as it was already described (22). All data are given as the mean of three independent experiments±standard deviation (SD).

Nuclear proteins extraction. The kit from Abcam (Cambridge, UK) was used for extracting nuclear proteins from Mino cells, according to manufacturer's protocol. Mino cells (5×10^6/ml) were treated with/without the appropriate concentration of WL, for indicated time periods. The concentration of nuclear proteins was measured at optical density 595 nm (OD_{595}) using Bradford reagent (Abcam, Cambridge, UK), according manufacturer's protocol and was calculated using bovine serum albumin standard curve. All data are given as the mean of three independent experiments±SD.

Histone H3K27 methyltransferase activity assay. The effect of WL on HMT activity was investigated by two ways. First, HMT activity was measured in NEs from untreated Mino cells, after direct exposure to WL for 1 h at concentrations of 0.1 μM, 1.0 μM and 10.0 μM. Second, HMT activity was measured in NEs from cells treated for 24 h with WL at concentrations of 0.1 μM, 1.0 μM, 3.0 μM and 7.5 μM. In both experiments, NE from untreated cells, not further exposed to WL was used as control (C). The Histone H3 (K27) Methyltransferase Activity Quantification Assay kit (Abcam, Cambridge, UK) was used according to the manufacturer's instructions. Briefly, in a 96-well plate coated with histone H3 substrate, nuclear extracts (NEs) were added in each well. H3K27 histone methyltransferase (HMT) activity was detected with an anti-trimethyl-H3K27 specific antibody followed by a HRP-conjugated secondary antibody. Color development was measured at OD_{450} and the HMT activity was quantified in *WL*-treated/untreated cells using the formula: $OD/h/mg=(treated/untreated\ NE\ OD_{450} - blank\ OD_{450})*1000/NE\ (\mu g)\times time\ (h)$. HMT activity in untreated cells (C) was considered as 100% activity. All data are given as the mean of four independent experiments±SD.

H3K27 methylation activity of EZH2 in the PRC2 complex, in vitro. Experiments were performed using the EZH2 Chemiluminescent Assay Kit (BPS Bioscience, San Diego, CA, USA) according to manufacturer's protocol, which measures the methylation activity of the PRC2 complex (EZH2/EED/SUZ12/RbAp48/AEBP) or reduced (EZH2/EED/SUZ12) PRC2 complex. The 96-well plate was precoated with H3K27 substrate, S-adenosyl- methionine was incubated with EZH2 (10 ng/μl) with WL or without WL in control. Primary antibody against methylated H3K27 and HRP-labeled secondary antibody followed by addition substrate to produce chemiluminescence were used. Luminescence in untreated wells was taken as 100% activity. All data are given as the mean of four independent experiments±SD.

Table I. *Histone methyltransferase (HMT) activity specific for H3K27 methylation and cell proliferation, were measured in Mino cells treated with Wedelolactone for 24 h compared to untreated cells in the control (C). The data are presented as mean±SD.*

Wedelolactone (μM)	HMT activity		Alive cells %
	OD/h/mg	%	
C	71.8±1.7	100.0±2.4	100.0±4.6
0.1	62.5±1.4	87.1±3.2	91.0±7.2
1.0	49.2±0.6	68.5±1.7	84.1±4.7
3.0	37.4±0.6	52.1±2.1	58.7±3.5
7.5	39.8±0.5	55.5±1.9	34.8±5.6

EZH2 quantification by ELISA assay. Quantitative detection of EZH2 amount in nuclear extracts of Mino cells was done by enzyme-linked immune sorbent assay (ELISA) using the Human EZH2 (Enhancer of zeste homolog 2) kit (XpressBio, Frederick, MD, USA). Anti-EZH2 antibody was pre-coated onto 96-well plates by the manufacturer. Standard concentrations of human EZH2 (2 μg/ml-31.25 pg/ml), or 10 μg of NE were added into each well. For the detection of EZH, a biotin-conjugated anti-EZH2 antibody was used. The concentration of EZH2 was calculated in pg/ml from the standard curve measured at OD_{450}. The half maximal inhibitory concentration (IC_{50}) was obtained from linear regression analysis of the concentration-response curves plotted. All data are given as the mean of four independent experiments±SD.

Statistical analysis. Student's *t*-test was used to analyze the difference between control and WL-treated samples. Statistical significance was determined at $p<0.05$.

Results

WL exhibited cytotoxicity against Mino cell line. Mino cells were treated with WL for 24 h and cell proliferation was measured by WST-8 assay. WL displayed a dose- dependent inhibition of cell viability with IC_{50} value of 5.6 μM ($p<0.05$) in comparison to untreated cells in WST-8 proliferation assay.

WL inhibited histone H3K27 methyltransferase activity in NEs from Mino cells. HMT activity was measured in NEs from Mino cells, which were exposed to WL at concentrations ranging from 0.1 μM to 10 μM in reaction well for 1 h. Results revealed that WL inhibited HMT activity for H3K27 methylation, in a dose-dependent manner (Figure 1) with an IC_{50} value ≥10 μM (no statistical significance was found).

WL inhibited histone H3K27 methyltrasferase activity in Mino cells. HMT activity was measured in NEs isolated from Mino cells, which were grown for 24 h with WL at a range of concentrations from 0.1 μM to 7.5 μM (Figure 2). WL treatment inhibited HMT activity targeted to H3K27 in

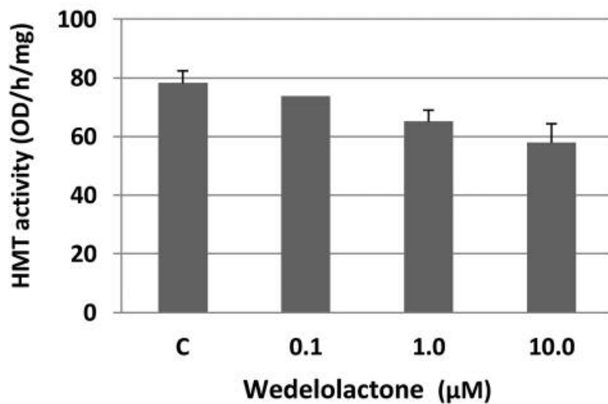

Figure 1. *Histone methyltransferase (HMT) activity for methylation of histone H3 at lysine 27 in nuclear extracts (NE) from Mino cells exposed in reaction with different concentrations of Wedelolactone, compared to untreated NE (C). The data are presented as mean±SD. No statistical significance was found.*

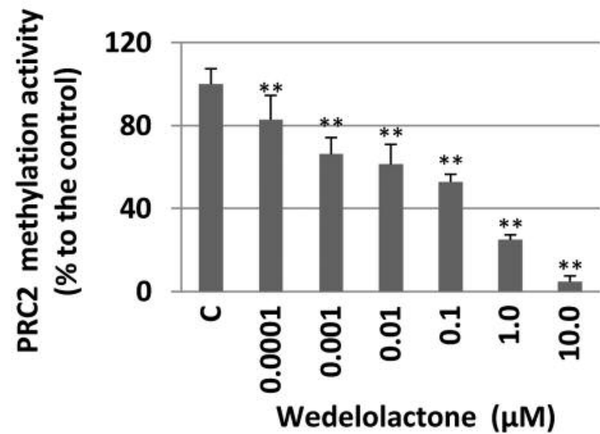

Figure 3. *Percentage of histone 3 lysine 27 (H3K27) methylation activity of the PRC2 complex (EZH2/EED/SUZ12/RbAp48/AEBP) exposed in reaction with or without Wedelolactone in control (C). The data are presented as meanσ±SD. **p<0.01 (compared to control).*

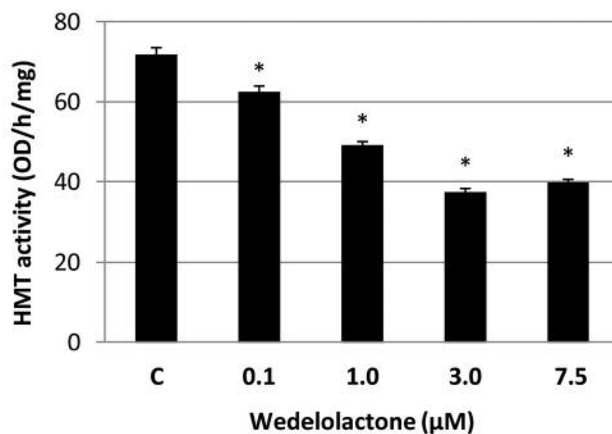

Figure 2. *Histone methyltransferase (HMT) activity for methylation of histone H3 at lysine 27 in nuclear extracts (NE) from Mino cells cultivated with different concentrations of Wedelolactone for 24 h compared to untreated NE (C). The data are presented as mean±SD. *p<0.05 (compared to control).*

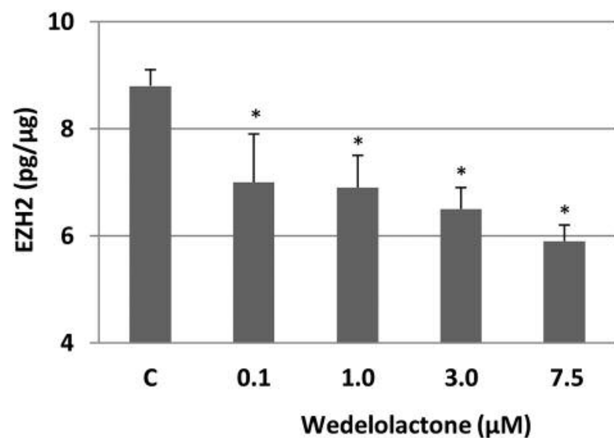

Figure 4. *EZH2 amount measured in nuclear extracts from Mino cells treated with Wedelolactone (0.1, 1.0, 3.0, and 7.5 μM) or untreated (C). The data are presented as mean±SD. *p<0.05 (compared to control).*

a dose-dependent manner, with an IC_{50} value of 3.2 μM (p<0.05) in Mino cells. Thus, WL inhibitory effect showed a 3-fold increase in NE from treated cells in comparison to the experiments described above, in which NEs were exposed to WL. This effect could be explained by the suppressive impact of WL on EZH2 expression in Mino cells, which resulted in dose-dependent inhibition of histone H3K27 methylation and Mino cell death (Table I).

WL suppressed H3K27 methylation activity of PRC2 complex in vitro. The H3K27 methylation activity of EZH2, in the context of complete EZH2/EED/SUZ12/RbAp48/AEBP or reduced EZH2/EED/SUZ12 PRC2 complex, was measured in Mino cells with/ without WL treatment, *in vitro*. Our results revealed that WL, at a range of WL concentrations between 1 nM and 10 μM, inhibited H3K27 methylation activity of PRC2 complex (Figure 3), with an IC_{50} value of 0.3 μM (p<0.01). The inhibitory effect of WL on the H3K27-targeted methylation activity of the reduced PRC2 complex was detected with an IC_{50} of 1.4 μM (p<0.05). Therefore, WL suppressed EZH2 activity in the complete PRC2 complex 3-fold more efficient than in the reduced

PRC2 complex. Thus, removal of cofactor proteins RbAp48/AEBP from PRC2 complex decreased WL activity towards inhibition of histone H3K27 methylation.

WL down-regulated the expression of EZH2 in Mino cells. Together with the HMT activity we measured the EZH2 amount in NEs from Mino cells treated with WL at concentrations of 0.1, 1, 3, and 7.5 μM, for 24 h. ELISA assay showed that WL decreased EZH2 quantity in a dose-dependent manner in Mino cells (Figure 4). More specifically, maximum EZH2 amount was decreased from 8.8±0.3 pg/μg in untreated Mino cells to 5.9±0.3 pg/μg (p<0.05) in WL- treated cells with 7.5 μM concentration.

Discussion

Natural products targeting EZH2 are considered as promising agents for the prevention and treatment of cancer. Several natural products, such as gambogic acid, triptolide, curcumin, and ursolic acid, have been shown to serve as EZH2 modulators (14). There are still no data about the epigenetic regulation of EZH2-mediated catalysis of H3K27 methylation in MCL. The present study was focused on the effects of WL, a plant coumestan that occurs in *Wedelia calendulacea*, on H3K27 methylation in the MCL cell line, Mino.

A summary of the effects of WL on the EZH2 amount, HMT activity, and cell proliferation in Mino cells, is presented in Figure 5. Specifically, WL inhibited the proliferation of Mino cells. Moreover, down-regulated HMT activity was detected in NEs directly exposed to WL, as well as in NEs from MCL cultivated with WL. We supposed that down-regulated expression of EZH2 in WL-treated Mino cells could entail suppression of HMT activity. Indeed, EZH2 amount was decreased in WL-treated cells. Thus, we showed two different plausible mechanisms of WL impact on MCL. First, WL inhibited the proliferation of Mino cells (Figure 5). Second, WL suppressed methylation of histone 3 at lysine 27 through down-regulation of EZH2 amount and subsequent reduction of HMT activity. To determine the exact mechanism of WL action additional experimental studies would be required.

PRC2 is an important epigenetic regulator. It is involved in the transcriptional regulation of target genes, it is responsible for the H3K27 methylation, and plays an important role in tumorigenesis by silencing tumor suppressor genes (23). However, natural compounds targeting the EZH2 to disable PRC2 complex are scarcely described. It was recently shown that WL disrupts the interaction of EZH2 and EED components *in vitro* and induces the degradation of PRC2 complex, resulting in cancer cell growth arrest (15). Moreover, it was shown that WL acts as an anti-cancer agent for breast and prostate carcinomas, *in vitro* and *in vivo* (24). Herein, it was demonstrated that absence of the RbAp48 and AEBP proteins in the core enzymatic subunit EZH2/EED/SUZ12 of

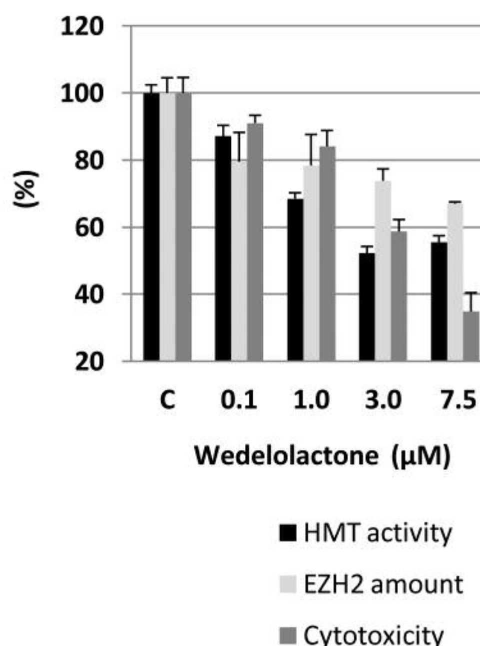

Figure 5. *Summary of the effects of Wedelolactone treatment on histone 3 lysine 27 (H3K27) histone methyltransferase (HMT) activity, EZH2 amount, and cytotoxicity (in percent) in Mino cells treated with 0.1, 1.0, 3.0, and 7.5 μM of Wedelolactone in comparison to untreated cells (C). The data are presented as mean±SD.*

the PRC2 complex decreased of the inhibitory activity of WL on H3K27 methylation. Thus, we proposed that WL inhibited EZH2 methylation activity in MCL in the case of complete PRC2 complex. The functions of the PRC2 complex cofactors are not still completely clear. Ciferri and co-workers have revealed that AEBP protein interacts with EZH2 and SUZ12 in the PRC2 complex to enhance EZH2 enzymatic activity (25). Although they are not required for the EZH2-modulated HMT activity, the RbAp46/48proteins have been shown to possess an important PRC2-related function such as chaperone proteins that bind to histone H3–H4 and plays a pivotal role in the assembly of nucleosomes (26, 27).

In conclusion, our results suggested WL as a promising agent for the treatment of MCL and provided a plausible mechanism for the presumable anticancer effects. Taken together, down-regulation of EZH2 expression in Mino cells treated with WL was combined with the inhibition of cell proliferation and suppression of histone methyltransferase activity *in vitro* and WL-treated cells. Thus, WL could serve as a candidate for the therapy of MCL.

Authors' Contributions

Romanchikova N. designed and performed experiments, analyzed data, wrote the paper and provided final approval of the version to

publish. Trapencieris P. generated ideas, made literature analysis, and contributed to the manuscript editing.

Conflicts of Interest

The Authors declare that there is no conflict of interests.

Acknowledgements

This project was supported by IG-2018-12 grant of Latvian Institute of Organic Synthesis.

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Received May 24, 2019
Revised June 20, 2019
Accepted June 24, 2019