Role of Valdien in proliferation and apoptosis of human non-Hodgkin’s lymphoma cells in vitro

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Abstract: The aim of this study was to investigate the apoptosis of human non-Hodgkin lymphoma Namalwa cells induced by Valdien (N1, N3-bis (3-methoxysalicylidene) diethylenetriamine) and possible underlying mechanisms. Namalwa cells were cultured in vitro. MTT assay was used to observe the proliferation of Namalwa cells induced on Valdien. Annexin V-FITC/PE staining was used to quantify the percentages of apoptosis in the total cell population. Protein expression of cleaved caspase-3, caspase-9, Bcl-2, and Bax was investigated by Western blotting. Phosphoinositide 3 kinase (PI3K)/Akt signaling pathways were also examined. PI3K inhibitor LY294002 was used to examine the involvement of PI3K/Akt signaling pathways in this apoptosis-inducing effect. Results showed that Valdien (2.5-40 µg/mL) markedly inhibited the proliferation of Namalwa cells and induced apoptosis in vitro. Valdien significantly increased the protein expression of cleaved caspase-9, caspase-3, and Bax, whereas it inhibited protein expression of Bcl-2 in the Valdien treatment group. In addition, Valdien significantly elevated levels of cytochrome c in cytosol. Further analysis demonstrated that Valdien-induced apoptosis was related to the inhibition of PI3K expression, which inactivated the phosphorylation of Akt. This phenomenon could be inhibited by the PI3K inhibitor LY294002. Thus, Valdien induced apoptosis in Namalwa cells through mitochondria-mediated and caspase-dependent pathways. Moreover, Valdien may induce the growth arrest and apoptosis of Namalwa cells through inhibiting PI3K/Akt signaling pathways.

Keywords: Valdien, Namalwa cells, apoptosis, mitochondria, caspase, phosphoinositide 3 kinase/Akt

Introduction

Lymphoma, including both Hodgkin’s and non-Hodgkin’s lymphoma (NHL), is a common hematological malignancy. The overall cure rate remains unsatisfactory and new therapeutic strategies are urgently needed [1, 2]. Human malignant NHL represents a heterogeneous group of tumors which vary in their biological aggressiveness and clinical course [3]. The standard chemotherapy regimen of NHL is cyclophosphamide, doxorubicin, vincristine, and prednisone, which play an important role in the treatment of NHL. Rituximab, an anti-CD20 antibody, when used alone or in combination with systemic chemotherapy, has expanded the therapeutic options for patients with B-cell lymphoma [4]. New monoclonal antibodies, such as galiximab, an anti-CD80 antibody, have shown significant anti-tumor activity as a single agent or in combination with rituximab against various B-cell lymphoma cell lines in vitro and in vivo [5]. Proteasome inhibitors and immunomodulatory drugs, such as bortezomib and lenalidomide, have demonstrated clinical potential for NHL [6]. Unfortunately, a considerable number of patients undergoing cyclophosphamide, doxorubicin, vincristine, and prednisone have relapsed thereafter or suffered from dose-related side effects and complications. Furthermore, stem cell transplantation, as a treatment of curing NHL, is too expensive for many patients. Thus, curative treatment can only be achieved in a minority of NHL patients [7]. Therefore, novel therapeutic strategies and new anticancer agents are urgently needed to improve palliative treatment, prolong life expectancy, and improve quality of life in patients with lymphoma.

Schiff base is an important ligand which could coordinate with most metals to form com-
plexes. Schiff base and its metal complexes play potential roles as antibacterial, antiviral, and anticancer agents. In recent years, the antitumor activity of some Schiff bases has been widely reported [8, 9]. Valdien (N1, N3-bis (3-methoxysalicylidene) diethylenetriamine, Figure 1), which belongs to one of the Schiff bases [10, 11], is more likely to be a potential antitumor agent. In view of this reason, the antitumor effects of Valdien were tested and proven by preliminary experiments in vitro. Results showed that Valdien clearly inhibited the proliferation of HCT-116 cells and induced apoptosis in vitro and in vivo [12]. However, anticancer effects of Valdien in human non-Hodgkin’s lymphoma cells have not been reported. Since Namalwa cells (a human B cell Burkitt’s lymphoma cells) provide a useful system for studying cellular and molecular events involved in apoptosis by chemical agents, the present study used Namalwa cells to investigate the mechanisms of Valdien-induced apoptosis and the effects of Valdien against human non-Hodgkin’s lymphoma.

Materials and methods

Chemicals and reagents

N1, N3-bis (3-methoxysalicylidene) diethylenetriamine (Valdien) was kindly provided by Professor Wei Dou (Lanzhou University, China). The purity of the compound was 98%. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), the fluorescent dye DCFH-DA (2,7-dichlorofluorescin diacetate), and cell culture medium RPMI-1640 were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Antibodies against cytochrome c, cleaved caspase-3, caspase-9, Bcl-2, Bax, phosphoinositide 3 kinase (PI3K), phosphorylated Akt (p-Akt, Ser 473), and total Akt were purchased from Cell signaling Technology (Beverly, Mass, USA). PI3K inhibitor LY294002 was obtained from Sigma-Aldrich, Inc. (St Louis, USA). Goat anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, CA, USA) was used as the secondary antibody.

Cell culture

Human non-Hodgkin lymphoma cells line Namalwa and the human acute myeloid leukemia (AML) cell lines NB4 and U937 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells of Namalwa, NB4, and U937 were all cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO, USA), 100 μg/mL of streptomycin, and 100 U/mL of penicillin at 37°C in a humidified atmosphere containing 5% CO₂. Cells in all experiments were gathered when they were in the exponential growth phase.

Cell proliferation and MTT viability assay test

Inhibition effects of Valdien on the proliferation of Namalwa, NB4, and U937 cells were measured by MTT assay. Namalwa cells were seeded in 96-well plates at a density of 10⁵ cells/well and then were treated with Valdien (0, 2.5, 5, 10, 20 and 40 μg/mL) for 24, 48 and 72 hours. After incubation, MTT was dissolved in PBS at 5 mg/mL and added to the culture media at a final concentration of 0.5 mg/mL. After incubation at 37°C for 4 hours, the media were removed and 100 μL DMSO were added to each well to dissolve purple crystals of formazan. The plate was shaken for 10 minutes to allow for complete solubilization. Absorbance was read at 570 nm with a microplate reader (Molecular Devices, Sunnyvale, USA). Based on these results, IC₅₀ values of the compound were tested. Moreover, morphological changes of Namalwa cells were examined in cell smears using light microscopy of cytospin preparation stained with May-Grunwald-Giemsa solution (Merck, Darmstadt, Germany) [13].

Assay for cell apoptosis

After incubation with various concentrations of Valdien (2.5, 5, 10, 20 and 40 μg/mL) for 48 hours, Namalwa cells were harvested and washed twice with cold PBS. The cells were resuspended in 100 μL of binding buffer and
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**Figure 2.** Valdien inhibits proliferation of Namalwa cells. A. Valdien inhibits proliferation of Namalwa, NB4, and U937 cells in a concentration-dependent manner. Cells were cultured in 96-well plate and treated with different concentrations of Valdien for 48 hours. B. Valdien inhibits proliferation of Namalwa cells in a concentration-dependent manner and a time-dependent manner. Cells were cultured in 96-well plates and treated with different concentrations of Valdien (2.5-40 μg/mL) for 24, 48, and 72 hours, respectively. MTT assay was used to evaluate the proliferation rate. Each datum indicated mean ± SD of three independent experiments. **P < 0.01 vs. control.

**Western blotting analysis**

Namalwa cells were incubated for 48 hours in the presence of indicated concentrations of Valdien. After incubation, the cells were harvested and washed with PBS solution. Mitochondrial proteins and cytosolic proteins were isolated using Mitochondrial Fractionation Kit (Active Motif, Carlsbad, Calif, USA), according to manufacturer instructions [14]. Protein concentrations were determined by BCA assay (Applygen Technologies Inc., Beijing, China). Cell lysates were denatured in SDS-loading buffer and boiled for 5 minutes. For Western blotting analysis, thirty micrograms of proteins from each sample were separated by 10-12% SDS-PAGE and transferred to nitrocellulose membranes. These membranes were blocked with 0.5% BSA in TBST (pH 8.0) for 1.5 hours and then incubated overnight at 4°C with suitably diluted primary antibodies against cytochrome c, cleaved caspase-3, cleaved caspase-9, Bcl-2, Bax, PI3K, p-Akt and Akt. Expression of β-actin was used to show equal protein loading. The blots were detected using enhanced chemiluminescence (ECL) reaction. Quantification of protein bands was achieved by densitometric analysis using Image-Pro Plus® software (Media Cybernetics, Inc., USA). Western blotting analyses were carried out at least three times.

**Statistical analysis**

Statistical analyses were performed using SPSS software. Results are expressed as mean
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± SD. Data were statistically evaluated by one-way ANOVA, followed by Dunnett’s test between the control group and multiple dose groups. Differences are considered to be significant when \( P < 0.05 \).

Results

Valdien concentration-dependently and time-dependently inhibited proliferation of Namalwa cells

The cytotoxic activities of Valdien were evaluated using the MTT method. Exponentially growing cells were treated with various concentrations of Valdien (2.5-40 µg/mL) for 48 hours. As shown in Figure 2A, Valdien significantly reduced the proliferation of Namalwa, NB4, and U937 cells in a dose-dependent manner. Results also showed that human non-Hodgkin’s lymphoma cell line Namalwa was most sensitive to Valdien. Treatment with Valdien (2.5-40 µg/mL) resulted in dose-dependent and time-dependent inhibition of Namalwa cell proliferation. This effect was more pronounced at 24, 48, and 72 hours post-treatment, compared with that of pre-treatment (Figure 2B). IC_{50} values of Valdien for the Namalwa cells at 24, 48,

Figure 3. Effects of Valdien on morphology of Namalwa cells demonstrated by light microscopy. Cells were treated without (A) or with (B) 10 µg/mL, (C) 20 µg/mL, (D) 40 µg/mL Valdien for 48 hours. Cell smears were stained with May-Grunwald-Giemsa solution.

Figure 4. Valdien promotes apoptosis of Namalwa cells. Namalwa cells were seeded in 12-well-plates and then treated with different concentrations of Valdien (0-40 µg/mL) for 48 hours. Annexin-V/PE stain was used to measure apoptosis rates. Apoptosis rates were measured by flow cytometry. Results are representative of three separate experiments.
Valdien induced apoptosis in Namalwa cells

Apoptosis is a cellular self-destruction mechanism that is crucial in the anti-tumor process [15]. As shown in Figure 3, treatment with Valdien for 48 hours induced morphological changes typical of apoptosis, such as nuclear shrinkage, chromatin condensation, and nuclear fragmentation in Namalwa cells. There were no changes in control group cells. Annexin-V/PE double staining assays were used to determine whether Valdien could induce apoptosis in Namalwa cells. Annexin-V positive cells were considered as early and late apoptosis populations. As shown in Figure 4, treatment of Valdien induced apoptosis of Namalwa cells in a dose-dependent manner.

Valdien induced cytochrome c release from mitochondria to cytosol

To confirm whether Valdien induces apoptosis through mitochondrial pathways, Namalwa cells were seeded into 12-well-plates and Valdien was added into each well. Mitochondria and cytosol proteins were prepared from cells treated with Valdien, which were subjected to Western blotting for measuring cytochrome c in mitochondria and cytosol. Parallel blotting was performed with β-actin antibody. *P < 0.05, **P < 0.01 vs. control.

Valdien influenced expression of apoptosis-related proteins in Namalwa cells

Caspase-3 has been shown to be a key component of the apoptotic machinery. It is activated in apoptotic cells and cleaves to several cellular proteins. To further address the apoptotic effects of Valdien on Namalwa cells, this study examined cleaved caspase-3 and caspase-9 by Western blotting. As shown in Figure 6, cleaved caspase-3 and caspase-9 were increased significantly in cells treated with 5, 10, 20 and 40 µg/mL Valdien for 48 hours.

Bcl-2 family proteins play critical roles in mitochondrial mediated apoptosis. The Bax/Bcl-2 ratios drew interest due to their significance in the mitochondrial mediated event. To investigate the molecular events involved in Valdien-induced apoptosis, expression of the anti-apoptotic Bcl-2 and pro-apoptotic Bax was assessed. Results showed that expression of Bcl-2 was decreased in Valdien treatment groups, whereas expression of Bax protein was increased.
Inhibition of PI3K/Akt pathways associated with Valdien mediated apoptosis

After treating Namalwa cells with Valdien (5, 10, 20 and 40 µg/mL) for 48 hours, there was a dose-dependent decrease in levels of PI3K and p-Akt, while total Akt remained unchanged (Figures 7A and 7B). To further verify the roles of PI3K/Akt pathways in Valdien-induced apoptosis, Namalwa cells were treated with Valdien (20 µg/mL) in the presence or absence of PI3K inhibitor LY294002 (20 µM). Valdien combined with LY294002 significantly inhibited expression of p-Akt (Figures 7C and 7D).

Discussion

Non-Hodgkin lymphomas (NHL) are malignant neoplasms of lymphoid cells, the predominant cells of the immune system [16, 17]. At present, chemotherapy is widely applied for clinical lymphoma treatment. However, clinical responses of NHL patients to chemotherapy vary greatly and even some patients are generally or particularly resistant to chemotherapy, leading to different curative effects for Non-Hodgkin’s lymphoma. With the improvement of various treatment methods, treatment of malignant lymphoma, prognosis, and disease-free survival rates have been greatly improved. However, there remains many people suffering from malignant lymphoma deaths yearly. Thus, exploring the mechanisms of malignant lymphoma and developing new antitumor drugs is urgent. Previous studies have indicated that Valdien exhibits antitumor effects in colon cancer [12]. However, anticancer effects of Valdien in human non-Hodgkin’s lymphoma have not yet been assessed. The present study examined the effects of Valdien on proliferation and apoptosis of a human non-Hodgkin’s lymphoma line Namalwa in vitro. Though the inhibition of proliferation by Valdien, a concentration-dependent action was shown in all three kinds of cell lines used. This finding suggests that Valdien may have a relatively wide spectrum of antitumor growth effects. As Namalwa cells showed the most significant inhibitory rates, these cells were chosen for the present study.

Normal cell function and tissue homeostasis are maintained by a balance between proliferation and apoptosis. Cancer is a typical disorder in which clones of malignant cells escape such balance and proliferate inappropriately without compensatory apoptosis [18, 19]. Blockade of proliferation or induction of apoptosis has been recognized as a rational approach to eliminate genetically damaged or preneoplastic cells before any malignancy manifests [20-23]. This
study examined the effects of Valdien on proliferation and apoptosis of a human non-Hodgkin lymphoma line Namalwa in vitro. Growth inhibition of Valdien on Namalwa cells was observed by MTT assay. Results showed that Valdien inhibited Namalwa cell proliferation in a time-dependent and dose-dependent manner. Apoptosis is an active process of cell death that takes place under a variety of conditions. It is important in inducing tumor destruction. It is characterized by distinct morphologic changes and regulated by a series of biochemical events that lead to cell death. Annexin V-PE staining was used to study the apoptosis rates of cells treated with Valdien. Results confirmed that Valdien can strongly inhibit the proliferation of human non-Hodgkin lymphoma line Namalwa in vitro by inducing apoptosis.

Apoptosis can be initiated by a death receptor-mediated pathway or by mitochondria mediated pathway [24-26]. In the mitochondria-mediated pathway, cytochrome c is released from mitochondria into cytosol due to mitochondrial membrane permeability transition. To further discuss the exact mechanisms of apoptosis induced by Valdien, this study detected the changes on expression of cytochrome c in mitochondria and in cytosol by Western blotting, respectively. Results showed that Valdien can induce release of cytochrome c from mitochondria to cytosol in Namalwa cells, indicating involvement of apoptosis via the mitochondria route. Caspases play an important role in the execution phase of apoptosis.

Figure 7. Effects of Valdien on the PI3K/Akt signaling pathway. A. Namalwa cells were treated with 5-40 μg/mL Valdien for 48 hours. Valdien significantly reduced the expression of PI3K as well as p-Akt, but not of total Akt. B. Columns represent the ratios of PI3K, p-Akt, and total Akt to β-actin. *P < 0.05 or **P < 0.01 vs. control. C. Namalwa cells were pre-incubated with 30 μmol/L LY294002 for 1 hour before the treatment of 20 μg/mL Valdien for 48 h. Valdien combined with LY294002 significantly inhibited expression of PI3K and p-Akt. D. Columns represent the ratios of PI3K and p-Akt to β-actin after treatment with Valdien and LY294002. **P < 0.01 vs. untreated control; ΔΔP < 0.01 vs. LY294002-treatment group. Data are representative of three separate experiments.
apoptosis. “Initiator” caspases, which have long pro domains, such as caspase-8 and caspase-9, either directly or indirectly activate “effector” caspases, such as caspase-3 and caspase-7. Cytochrome c, once released, forms an apoptosome with Apaf-1 and procaspase-9 in the presence of dATP, resulting in the activation of caspase-9 [27]. The active subunit of caspase-9 further activates downstream procaspase-3. Caspase-3 can cleave to several cellular proteins, initiating a cascade of events that eventually leads to apoptosis [28]. In the present study, treatment of Namalwa cells with Valdien resulted in cleavage/activation of caspase-9 and caspase-3.

The Bcl-2 family can be broadly divided into those members that inhibit (Bcl-2) or promote cell death (Bax). Bcl-2 family members primarily function at the mitochondria where they control the permeability of the outer mitochondrial membrane, resulting in cytochrome c release and caspase activation. Many cancers have demonstrated defects in apoptotic pathways. Results from Western blotting showed that, after treatment with Valdien, Bcl-2 expression was dramatically decreased and Bax expression was slightly increased at protein levels. The value of Bax/Bcl-2 increased, thus, apoptosis occurred. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome. The cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and PARP [29]. This study shows that Valdien could downregulate the expression of Bcl-2 while upregulating expression of Bax, change the morphological structures and stability of mitochondria, increase the release of Cyt c from mitochondria to cytoplasm, and activate caspase-3. Thus, Valdien induces apoptosis of Namalwa cells through the mitochondrial pathway.

Phosphatidylinositol 3-kinase (PI3K) has an important signaling role in cell proliferation and survival. Its specific inhibitors have been considered to be promising anti-tumor drug candidates [30]. Akt mediates various downstream effects of PI3K by regulating many biological processes, such as proliferation, apoptosis, and cell growth [31]. PI3K/Akt activation is present in many NHL cases. It has been suggested that constitutively activated PI3K-AKT pathways provide a survival advantage to lymphoma cells and are involved in carcinogenesis through inhibition of apoptosis [32]. Akt might also regulate the activity of numerous downstream apoptosis-related proteins, such as caspase-9 and some Bcl-2 family members that interfere with apoptosis [33]. PI3K/Akt activation has been associated with malignant transformation and may contribute to accelerated tumor growth, angiogenesis, metastasis, and resistance to chemotherapy [34]. Moreover, activated Akt decreases sensitivity of tumor cells to chemotherapy and radiotherapy by increasing the threshold for cell death induction [35]. For this reason, the PI3K/Akt signaling pathways represent a promising target for therapeutic intervention [34]. Present data showed that Valdien inhibited the activation of Akt in Namalwa cells through inhibiting expression of PI3K. The blockade of Akt signaling by the PI3K inhibitor LY294002 resulted in the inhibition of Akt phosphorylation in Namalwa cells. When Valdien was combined with LY294002, the ability of Valdien to induce apoptosis of Namalwa cells was potentiated. Phosphorylation of Akt was significantly down-regulated, which indicated the inactivation of PI3K/Akt signaling pathways.

Overall, the present study has demonstrated the antitumor effects of Valdien. Mechanisms underlying the effects mainly involve the induction of apoptosis through activating caspase-3 via mitochondrial pathways and inhibiting the PI3K/Akt signaling pathways. To the best of our knowledge, this is the first study to examine the function of Valdien in human non-Hodgkin’s lymphoma Namalwa cells. Present findings may be helpful in understanding the properties of Valdien as a candidate antitumor drug.

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Disclosure of conflict of interest
None.

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