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Synergistic pro-apoptotic effects of xanthocillin X dimethylether (XDME) with targeted BH3 mimetics Navitoclax and Venetoclax in acute myeloid leukemia

항생물질유도체 Xanthocillin X dimethylether (XDME)와 BH3 유사체 Navitoclax 와 Venetoclax 의 급성 골수성 백혈병세포의 세포사멸 동반상승효과 연구

2016 년 07 월

서울대학교 대학원 약학과 의약생명과학전공

임 지윤

1
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지도교수 Marc Diederich
이 논문을 약학석사학위논문으로 제출함

2016 년 6 월

서울대학교 대학원
약학과 의약생명과학전공
임지윤

임지윤 석사학위논문을 인준함

2016 년 6 월

위원장 ______________ (인)
부위원장 ______________ (인)
위원 ______________ (인)
Abstract

Synergistic pro-apoptotic effects of xanthocillin X dimethylether (XDME) with targeted BH3 mimetics Navitocinax and Venetoclax in acute myeloid leukemia

Jiyun Rhim
College of Pharmacy
The graduate School
Seoul National University

Xanthocillin X derivatives were originally isolated from cultures of various fungi for their anti-viral activities. Selected derivatives are known to induce cell cycle arrest but with considerable toxicity. Interestingly, xanthocillin X dimethylether (XDME), extracted from the marine microorganism Penicillium commune, showed a strong anti-proliferative effect on acute myeloid leukemia (AML) cell lines without toxicity. As AML treatment did not change over the last 25 years and as novel treatment strategies are needed in order to reduce lethality of this disease, which remains high at about 70%, we further investigated the effect of XDME on AML cells alone or in combination with clinically relevant BH3 mimetics Venetoclax and Navitocinax.

First, we investigated cell cycle arrest by flow cytometry and showed an accumulation of cells in G1 with a reduced S phase as early as 24h and further increasing after 48h. Interestingly, XDME did not significantly reduce viability of cells as assessed by Trypan blue staining, thus demonstrating cytostatic without cytotoxic effects. Absence of cytotoxicity was further confirmed by zebrafish

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toxicity assays. In order to assess the molecular mechanisms linked to cell cycle arrest, we detected decreased expression levels of S-phase marker proliferating cell nuclear antigen (PCNA), of cyclin D1, essential for G1 to S phase transition and of c-myc by immunoblot.

At a cellular level, we observed morphological changes in XDME-treated cells by GIEMSA staining. U937 cells presented significant cytoplasmic vesicle formation after 24h at 5µM. Next, we investigated the effect of XDME time- and concentration-dependently on both endoplasmic reticulum (ER) stress and autophagy, known to induce such vesicle formation. Our results show that XDME (5µM) decreased the expression of glucose regulated protein (GRP)78 after 24 hours, followed by an increase after 48 and 72h, witnessing ER stress response. In addition, both phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and expression of transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP) were increased in a time- and dose-dependent manner. Moreover, we did not observe splicing of X-box protein (XBP)-1. In parallel to increased CHOP expression, we observed conversion of microtubule-associated protein light chain (LC) 3-I to -II by immunoblot. As the amount of LC3-II is known to be correlated to the number of autophagosomes, we concluded that autophagic flux was induced in parallel to adaptive unfolded protein response (UPR), triggered by ER stress.

From a translational point of view and to generalize our findings with U937, XDME concentration-dependently inhibited 3D colony formation of various cell lines representing essential AML subtypes (HL60, THP-1, MOLT-3 and KG1) at concentrations between 100nM and 1µM. Furthermore, XDME sensitized U937 cells against two BH3 mimetics currently under clinical investigation, namely ABT199 (Venetoclax) and ABT263 (Navitoclax), inducing synergistic apoptotic cell death, quantified by Hoechst/prodium iodide (PI)-fluorescence microscopy, caspase-3/7 immunoblots and activity assays as well as by poly (ADP-ribose) polymerase (PARP)-1 cleavage. Synergistic inhibition of colony formation with U937 further confirmed the pharmacological relevance of a combination of XDME with such BH3 mimetics.
To further generalize our findings, we validated XDME as an inhibitor of other myeloproliferative diseases including chronic myeloid leukemia (CML). Our preliminary data show inhibition of proliferation of K562 cells already at 1µM concomitant with cell death induction as shown by flow cytometry, induction of ER stress witnessed by modulation of CHOP expression, autophagy induction shown by time- and dose-dependent LC3-I to -II conversion proceeded by a decrease of oncogenic breakpoint cluster region (Bcr)-Abelson (Abl) kinase phosphorylation at Tyr245.

Altogether, our results provide a detailed insight into the potent cytostatic effect induced by the non-toxic marine natural compound XDME at nanomolar concentrations. Results further encourage validation of its anti-leukemic capacity in animal or patient-derived xenograft models followed by clinical trials alone or in combination with targeted therapeutic agents such as BH3 mimetics (AML) or Imatinib (CML).

Keywords
Leukemia; ER stress; autophagy; BH-3 mimetic; synergy

Student number: 2014-22980
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ATF6 α</td>
<td>Activating transcription factor-6α</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related gene</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma extra large</td>
</tr>
<tr>
<td>Bcr-Abl</td>
<td>Breakpoint Cluster Region-Abelson</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 Homology domain</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>CF</td>
<td>Colony Formation</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT-enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose-Regulated Protein 78 kDa</td>
</tr>
<tr>
<td>HPF</td>
<td>Hours Post Fertilization</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Inositol-requiring protein 1α</td>
</tr>
<tr>
<td>LC-3B</td>
<td>Microtubule-associated protein light chain 3B</td>
</tr>
<tr>
<td>MPER</td>
<td>Mammalian Protein Extraction Reagent</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>PARP-1</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein Kinase RNA-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Institute Park Memorial Institute</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>T-CLL</td>
<td>T cell lymphoblastic leukemia</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>WHO</td>
<td>The World Health Organization</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>XDME</td>
<td>Xanthocillin X Dimethylether</td>
</tr>
</tbody>
</table>
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1. Introduction

1.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a most common form of leukemia in older compared younger patients and it takes up to 75% of all acute leukemia. The survival rate of AML patients is 30% [1]. According to the American Cancer Society, nearly 20,000 patients were diagnosed for leukemia in the USA, and about 10,000 will die in 2016.

AML is classified by the French-American-British (FAB) system of the World Health Organization (WHO). The FAB system subdivides AML into 7 subtypes based on the morphology of the cell (M0-M7). M0 to M5 subtypes represent immature forms of white blood cells. M6 corresponds to immature forms of red blood cells, whereas M7 AML correspond to platelets. The WHO classification uses not only morphological differences, but includes also information including genetic, immune-phenotypic, biologic, and clinical features to define specific disease entities [2].

AML is mainly treated by induction chemotherapy, which is a combination drug treatment to destroy as many abnormal leukemia cells as possible. Patients are treated with a combination of daunorubicin and cytarabine. Anthracyclines such as daunorubicin, doxorubicin or aclarubicin inhibit the access of topoisomerase II to the DNA and epipodophyllotoxins, such as etoposide and teniposide bind to the enzyme [3]. Both inhibitors block topoisomerase II activity after inducing DNA double-strand breaks and finally induce cell death [4]. However, numerous cell models developed resistance against these treatments. Therefore, overcoming the resistance of cells will be essential to further development of AML treatment.
After induction therapy, intensification (consolidation) therapy is used to eliminate remaining leukemia cells thus preventing relapse with high or intermediate doses of cytarabine [1, 5]. However, this therapy is not recommended for AML patients with comorbidities [6].

As AML is not a single, but a complex disease with many subtypes, it is a challenge to develop novel therapeutic approaches. [4]. Indeed, the therapeutic approach for AML has not much changed for 25 years [7] so that it is essential to find more effective and less toxic combination chemotherapies for older patients or with comorbidities [8].

1.2. Endoplasmic reticulum stress as a therapeutic target

Based on our initial results, we hypothesized that endoplasmic reticulum (ER) stress could be an important sensitizing mechanism in AML. ER stress triggers the unfolded protein response (UPR) to maintain ER homeostasis by activating three different transmembrane proteins; protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6α (ATF6α) [9]. Under physiological conditions, IRE1α, PERK and ATF6α are maintained in an inactive state by interaction with the chaperone protein glucose-regulated protein (GRP)78/binding immunoglobulin protein (BiP) [10]. Once unfolded proteins accumulate in the ER lumen, UPR activates an adaptive pathway. IRE1α and PERK are released from GRP78/BiP, dimerize, and auto-phosphorylate. Then phosphorylated IRE1α and PERK activate the splicing of X-box protein 1 (XBP1) and phosphorylate eukaryotic translation initiator factor 2α (eIF2α) respectively to activate transcription of genes related to cell survival and to slow
down general protein translation to reduce ER protein Bulk. Similarly, ATF6α becomes active by releasing from the BiP proteins, and then cleaved and translocated to the Golgi and cleavage to induce the expression of target genes. However, when the stress gets more severe, then a maladaptive UPR pathway is activated to eliminate the defective cells. Recently, the ER got more attention as an essential organelle in deciding cell survival and death [11, 12]. ER stress is closely associated with activation of both UPR and induction of autophagy. Upregulation of UPR could also activate autophagic mechanisms to eliminate misfolded or abnormal proteins by autophagosomes derived from the ER membrane [13]. Among the mammalian transmembrane proteins in UPR pathway, especially, PERK/eIF2α and IRE1α have been suggested as mediators of autophagy in response to other stresses that activate PERK-related kinases [14].
**Figure 1: Scheme of endoplasmic reticulum (ER) stress.** Accumulation of unfolded or misfolded proteins in the ER lumen activates ER stress. ER stress activates the unfolded protein response (UPR) pathway. The UPR signals increase the expression of ER chaperon protein, glucose-regulated protein 78 kDa / binding immunoglobulin protein (GRP78/BiP). Elevated GRP78/BiP then released from the three transmembrane proteins in the ER lumen; ATF6 α, IRE1 α and PERK. These three different axis then activate downstream signaling then finally transcript cell survival or cell death related genes like CCAAT-enhancer-binding protein homologous protein (CHOP).
1.3. Bcl-2 family proteins as therapeutic target

B-cell lymphoma 2 (Bcl-2) family was first discovered in follicular lymphoma and played an important role in regulation of apoptosis. Bcl-2 belongs to the pro-survival or anti-apoptotic proteins of the Bcl-2 family (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1/A1). Anti-apoptotic Bcl-2 family members contain four conserved Bcl-2 homology (BH) domains. They bind to pro-apoptotic proteins family members (Bax, Bak) to inactivate their activity. Some pro-apoptotic Bcl-2 family proteins only have a BH3 domain [15, 16], they are called BH-3 only proteins. These BH3 only proteins can be subdivided to activator (BID, BIM) and sensitizer (BAD, BIK, NOXA, BMF, PUMA) [17]. Pro-apoptotic proteins induce the release of apoptotic factors like cytochrome c from mitochondria to the cytosol and the subsequent activation of the caspase cascade. BH-3 only proteins like Bid link intrinsic and extrinsic cell death pathway after truncation.

As previously described, chemo-resistance is a major problem during treatment. Anti-apoptotic Bcl-2 family protein overexpression is correlated with resistance in hematopoietic malignancies such as follicular non-Hodgkin’s lymphoma and solid tumors, such as small-cell lung cancer (SCLC) [18, 19]. Navitoclax (ABT-263), a small molecule Bcl-2 inhibitor that binds to Bcl-2, Bcl-xL, and Bcl-w but not to Mcl-1 or Bfl-1/A1 [20] was designed to target cancer types addicted to overexpression of these anti-apoptotic proteins by interacting with the BH-3 only domain of these anti-apoptotic Bcl-2 family members. By targeting Bcl-xL, Navitoclax triggered severe on-target side effects including thrombocytopenia. Accordingly, AbbVie designed a modified variant of Navitoclax devoid of any affinity for Bcl-xL. The resulting drug, Venetoclax (ABT-199), acts similar to Navitoclax, without leading to bleeding of
the patients but with potent anti-cancer activities leading to canonical apoptosis with cytochrome c release from the mitochondria [21].
Figure 2: Bcl-2 family of cell death regulators. The Bcl-2 family can be categorized into three sub-families sharing sequence homology in 4 regions called Bcl-2 homology domain (BH)1 to BH4; anti-apoptotic proteins, pro-apoptotic proteins and BH3-only proteins.
1.4. Xanthocillin X dimethylether (XDME)

Organic molecules derived from plants and microbes have been developed as an anti-cancer agent over the last decades. Natural products played an essential role as established anti-cancer chemotherapeutic agents, either in unmodified or modified forms. Indeed, from all anticancer drugs approved by the Food and Drug Administration (FDA) approximately 50% were classified as derived from natural products. Many plant, animal, or microorganisms were investigated to discover potential anti-cancer drugs [22]. Many drugs in the clinics used against AML in particular originate from natural products, for example, daunorubicin is isolated from *Streptomyces peucetius* and doxorubicin is a biosynthetic compound produced originally by various strains of *Streptomyces* [23].

For our project we used a compound from the xanthocillin family provided by Prof. Gabriele König (University of Bonn, Germany). Xanthocillin was first isolated from *Penicillium notatum* in 1950s and possesses a wide antibiotic spectrum against gram-positive and gram-negative. Xanthocillin has two subtypes: X and Y. Both are yellow compounds and have similar inhibitory effects on cell proliferation with considerable toxicity [24]. The compound we used, xanthocillin X dimethylether (XDME) was originally isolated from *Penicillium commune*.

![Figure 3: Structure of Xanthocillin X Dimethylether (XDME).](image)
2. Hypothesis and aims

2.1. General hypothesis

It is our hypothesis that XDME generates ER stress in leukemia cells under non-toxic concentrations leading to cell cycle inhibition and sensitization against BH3 mimetics with synergistic induction of apoptosis.

2.2. Specific aims

1. It will be our first aim to demonstrate that XDME reduces the growth of leukemia cells in culture. We will choose the AML cell line U937 as a model to determine the effect on proliferation and viability by using the trypan blue staining method.

2. Our second aim will be to investigate how XDME generates ER stress in U937 AML cells. Here, we will analyze the morphological changes by Diff-Quik staining. We will also conduct immunoblots to quantify the expression levels of ER stress-related proteins like GRP78/BiP, PERK, eIF2α, and CHOP. We will use RT-PCR to validate XBP1 splicing. We will then investigate the effect of XDME on autophagy by ER stress. We will use immunoblotting to assess LC3B conversion in a time- and concentration-dependent manner in XDME-treated U937 cells.

3. Our final aim will be to investigate how XDME sensitizes U937 AML cells to clinically used drugs like Navitoclax and Venetoclax by fluorescence microscopy using Hoechst/propidium iodide (PI) staining, caspase 3/7 activity
assays and immunoblots of caspase 3/7 activation as well as poly (ADP- ribose) polymerase (PARP)-1 cleavage.
3. Material and Methods

3.1. Cell culture and compounds

U937 (human histiocytic lymphoma), HL60 (human promyelocytic leukemia), THP1 (human monocytic leukemia), MOLT-3 (human acute T lymphoblastic leukemia) cells were cultured in Roswell Institute Park Memorial Institute (RPMI) 1640 medium (Bio-Whittaker, Lonza, USA) containing 1% (v/v) with an antibiotic/antimycotic mixture of penicillin 100U/ml, streptomycin 100µg/ml, amphotericin B 0.25µg/ml (Bio-Whittaker, Lonza, USA) at 37°C and 5% of CO₂ in a humidified atmosphere. Cells were regularly tested against mycoplasma infection (Mycoalert™, Lonza USA) according to the manufacturer’s instructions.

XDME was isolated from a strain of *Penicillium commune* with a molecular mass of 316.3g mol⁻¹. The compound was received as a powder and solubilized in DMSO (Sigma-Aldrich, USA) and further diluted to get a final concentration at 5µM. Stocks and aliquots are stored at -20°C and protected against light.

ABT199 and ABT263 were purchased from Selleckchem (USA) and dissolved at 50mM in 100% DMSO.

3.2. Cell proliferation assay

The amount of viable cells was assessed by counting trypan blue (0.2% Trypan blue, Lonza, USA)-excluded cells with a Malassez cell counting chamber. At time 0h, cells were seeded in 24 well plates (200,000 cells/ml).
3.3. **Cell cycle analysis**

Cells were washed twice in PBS-1X and then fixed and permeabilized with 70% of cold ethanol in Phosphate Buffered Saline (PBS)-1X (Sobolewski et al., 2011). Then, cell cycle distribution was analyzed by flow cytometry (FACS Calibur™, Becton Dickinson Biosciences, Korea) by DNA staining with propidium iodide (PI) (1 µg/ml, Sigma-Aldrich) and RNase A (100µg/ml; Sigma-Aldrich, Korea) in PBS. Events were recorded (10,000 events/sample) using the Cell Quest Software. Data were analyzed with the FlowJo 7.6.5 software.

3.4. **Differential Quick Histochemical analysis**

Cells (7x10⁴) were washed with phosphate buffer solution (PBS) and then re-suspended in PBS and fixed on a slide using the cytocentrifuge system (Cytopro™, Wescor, USA). The slides were sequentially applied in Diff-Quik staining solution (Siemens Diff Quik Colour Kit, Cruinn, USA). All slides were washed in water and observed with an optical microscope (Olympus bx51. Korea).

3.5. **Cell lysates preparation and Western blot analysis**

Total protein lysates were obtained by re-suspending the cells in Mammlian Protein Extraction Reagent (MPER, Pierce) containing protease inhibitors (Complete, Roche, USA), phenylmethylsulphonyl fluoride (PMSF) 1mM (Roche, USA), Sodium orthovanadate 1mM (Sigma, USA), and Phosphostop inhibitor 100µl/ml (Roche, USA).
Cells were treated with XDME in both dose- and time-dependent manner and then centrifuged at 4°C, 350g for 7 minutes. After removal of supernatants, pellets were directly stored at -80°C until use. Depending on the pellet size, 300µl of reagent were used for the control, and 100µl for treated pellets. Dissolved pellets were mixed gently at 4°C for 25 minutes, then centrifuged for 15 mins, 4°C at 15.7rcf. Aliquots were stored at -80°C.

Protein concentrations were calculated using Bradford method [25]. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% or 10%) using 20-40μg of total protein extract. Then, proteins were transferred to PVDF membranes and blocked for 1 hour with 5% non-fat milk, or 5% bovine serum albumin (BSA) in PBS-Tween 20 (PBS-T). Membranes were incubated with 0.5-1 μg/ml of the primary antibodies for each conditions. After hybridization, membranes were washed three times with PBS-T, and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. (Table I) The different antibodies were diluted in a PBS-T solution containing 5% of skim milk or 5% Bovine Serum Albumin (BSA) (Bovostar, Korea). The protein bands were detected with ECL Plus Western Blotting Detection System Kit (GE Healthcare, South Korea). β-actin was used as a loading control.
<table>
<thead>
<tr>
<th>Target protein</th>
<th>Company</th>
<th>Primary antibody condition</th>
<th>Secondary antibody condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78/BiP</td>
<td>Cell signaling</td>
<td>1:1000, PBS-T 5% milk</td>
<td>1:4000 PBS-T 5% milk</td>
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<td></td>
<td></td>
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<td>Room Temperature, 1 hour</td>
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<td>LC3B</td>
<td>Cell signaling</td>
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<td></td>
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<td>4°C, Overnight</td>
<td>Room Temperature, 1 hour</td>
</tr>
<tr>
<td>PERK (C33E10)</td>
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<tr>
<td>p-eIF2alpha (ser51)</td>
<td>Signalway antibody</td>
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3.6. Zebrafish toxicity assay

Zebrafish (Danio rerio) were obtained from the Zebrafish International Resource Center (ZIRC, OR). Zebrafish were grown at 28.5°C in a standard fish aquarium with 14:10h light/dark cycle and fed 3 times/day. 14h before the toxicity assay, embryos were treated with 0.003% of phenylthiourea in order to remove pigmentation. 2h before the assay, the embryo’s shell was eliminated and then treated for up to 24h of XDME (0-30µM) in 24 well plates. Viability and abnormal development were assessed after 24h of treatment under light microscopy (Carl Zeiss Stereo microscope DV4, Seoul, Korea). Pictures were taken by fixing zebrafish embryos onto a glass slide with 3% methyl-cellulose (Sigma Aldrich, Korea).

We thank Professor Kyu-Won Kim (SNU-Harvard Neurovascular Protection Center, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Korea) for the permission to use his zebrafish facility.

3.7. Analysis and quantification of apoptosis/necrosis

2x10^5/ml cells were seeded in 24 well plates prior to XDME treatment. 300µl of treated cells were transferred into another 24 well plate for staining with DNA-specific dye Hoechst 33342 (Sigma, USA) at 1µl/mg for 20 minutes of incubation and propidium iodide (PI) (Sigma, USA) at 1.5µl/mg for 10 minutes of incubation. The percentage of apoptotic cells was evaluated by counting the number of cells displaying apoptotic features, such as fragmented and condensed nuclei. At least 100
cells in three random fields were counted by fluorescent microscopy (Olympus bx51, Korea).

3.8. Caspases 3/7 assay

The activity of caspases-3/-7 was assessed with a luminescent- based assay (Caspase-3/-7 Glo assay, Promega, Korea). Briefly, cells were seeded in 24 well plates at a density of 200,000 cells/well. After treatment, cells were harvested according to the manufacturer’s protocol. The activity of caspases was revealed by luminescence using a Centro LB 960 Microplate Luminometer (Berthold, USA).

3.9. Colony Formation assay

U937, HL60, THP1, and MOLT3 (1000 cells per well), and KG1 cells (2000 cells per well) were grown in semi-solid methylcellulose medium (MethoCult H4230, StemCell Technologies Inc., Vancouver, Canada) supplemented with the XDME (0.5 –10µM), ABT199 (100nM), and ABT263 (100nM). Colonies were detected after 10 days of culture by adding 5 mg/ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) reagent (Sigma, Korea) and were scored by Image J software (U.S. National Institute of Health, Bethesda, MD, USA). Photos were taken with a LAS4000 (GE Health, Korea).
3.10. Statistical Analysis

Data are presented as mean of at least three independent experiments with standard deviations. Statistical analysis was performed using the Graphpad Prism 6.0 software. One-way and two-way ANOVA followed by Holm-Sidak multiple comparison tests were used for statistical comparisons. P-values below 0.05 were considered as statistically significant.
4. Results

4.1. XDME reduces growth of leukemic cell lines.

The effect of XDME (0, 0.1, 0.3, 0.5, 1, 5, and 10µM) on the viability and proliferation (of U937 cells was assessed by trypan blue staining assays for up to 72h of treatment. Our results showed that XDME did not induce significant toxicity over the range of concentrations tested (Figure 4A). Interestingly, XDME reduced the growth of U937 cells in a time-dependent manner at low micromolar concentrations as proliferation decreased from 0.5µM of XDME (Figure 4B) with an IC50 of 2µM at 48h and 0.6µM at 72h. To further evaluate the toxicity of XDME on a healthy model, we used zebrafish embryos which were treated with XDME (0-30µM), and viability and morphology were assessed after 24 hours. Our data indicated that for up to 30µM, XDME had only a reduced effect on the viability of zebrafish, with 70-90% of viable fish after 24 hours of treatment up to 30µM far beyond the range of concentrations used for the cell treatments. No significant changes of the morphology were observed at any concentration (Figure 4C).
Figure 4: XDME decreases the growth of U937 human leukemia cell lines without cytotoxicity. U937 cell were seeded at 2 x 10^5 cells/ml and then treated with 0, 0.1, 0.3, 0.5, 1, 5, and 10µM of XDME for up to 72 hours. (A) Cell viability and (B) cell concentration was estimated by trypan blue exclusion assay. The IC50 values were calculated by Graphpad Prism. Data are the mean of SD ± of three independent cultures. (C) Toxicity assessment of XDME on zebrafish model. Zebrafish embryos (24 hours post fertilization, hpf) were treated for 24h with XDME (0, 0.5, 1, 5, 10, and 30µM). The viability and morphology analysis of zebrafishes were assessed under light microscopy. The data are representative of three independent experiments with 10 embryos/condition in each condition. The pictures and the mean values for the percentage of viability are presented in graph. *P < 0.05, **P< 0.01, ***P < 0.001 compared with controls.
4.2. XDME induces cell cycle alteration at the G1/S transition.

As we observed an inhibition of proliferation at low micromolar concentrations after XDME treatment, we decided to investigate underlying molecular mechanisms. To investigate potential cell cycle alterations, we analyzed cell cycle distribution of U937 cells treated by XDME up to 72 hours by FACS. Data showed that XDME induced cell cycle arrest with G1 phase accumulation by 30% and reduction of S phase up to 24% from 1µM of treatment after 24 hours (Figure 5A). This effect further increased at 5µM after 48 hours of treatment (Figure 5A and B). We compared our results to another inhibitor of U937 known to block the cells in G1, celecoxib (40µM), a clinically used anti-inflammatory agent [26]. Whereas this compound also induces cell cycle arrest, it appears more toxic with 20% of cells in the subG1 phase compared to XDME-treated samples with 9.7% of cells in subG1 after 72 hours of treatment at 5µM.

Next, we assessed the expression of cell cycle regulators involved in the G1/S transition. In XDME-treated U937 cells, cyclin D1, proliferating cell nuclear antigen (PCNA) and c-myc expression levels were decreased in a time-dependent manner by 82, 32 and 71% respectively (Figure 5C). Altogether, our results revealed that XDME blocked cell proliferation by inducing G1 accumulation with a reduction of the S phase in U937 AML cells.
Figure 5 (see next page): XDME induces cell cycle arrest by accumulation in G1 phase and reduction of S phase. (A) The impact of XDME (0, 0.5, 1, and 5µM) and Celecoxib (40µM) cell cycle was analyzed by FACS for up to 72 hours of treatment in U937 cells. (B) The quantification of cell cycle analysis by FACS. (C) U937 cells were treated with 5µM of XDME in a time-dependent manner. The expression of cyclin D1, PCNA, and c-myc was decreased in a time-dependent treatment. The results are representative of three independent experiments (± S.D). *P < 0.05, **P < 0.01, ***P < 0.001 compared with controls.
4.3. XDME induces endoplasmic reticulum stress in U937

In parallel to the investigation of the cell cycle, we performed a morphological analysis of XDME-treated U937 cells by Diff-Quik staining. U937 cells were treated with 5µM XDME in a time-dependent manner. After 24h of treatment, we observed extensive formation of cytoplasmic vesicles in 98% of the cells at 24h, further increasing to 99% after 48h and 72 hours (Figure 6A). Formation of such vesicles is characteristic for cells undergoing stress which might lead to UPR or autophagic response [27].

First, we analyzed the expression of ER stress-related proteins. We detected a significant downregulation of GRP78/BiP expression at 24h by 82.2%, followed by a gradual increase after 48h and 72h to reach control levels (Figure 6B). As GRP78/BiP is a well-accepted ER stress marker, we hypothesized that XDME could modulate ER stress in U937 cells and decided to further investigate this pathway. In agreement with our hypothesis, we observed induction of ER-stress-related transcription factor CHOP expression between 24 and 72hours (Figure 6B).

We then investigated the expression of ER stress-related proteins at 24 hours in a concentration-dependent manner. Our results showed that XDME reduced GRP78/BiP at concentrations of 0.5µM and abrogated its expression at 1µM. Similarly, XDME induced phosphorylation of PERK at the same concentrations. Finally, XDME did not further increase phosphorylation levels of eIF2α, which were already at constitutively elevated levels in U937 cells. Finally, we did not observe splicing of XBP-1 in XDME-treated U937 cells (Figure 6D). To validate our results, we used the Ca^{2+} depleter thapsigargin (300nM) as a bona fide ER-stress inducer (Figure 6C).
As vesicle formation could also correspond to an onset of autophagic flux, we investigated microtubule-associated protein light chain (LC)3B conversion in a time dependent manner. Interestingly, LC3 conversion occurred after XDME treatment from 24h (Figure 7A) whereas CHOP was already expressed at 24 hours (Figure 6B). We concluded here that autophagy triggered by XDME autophagy was subsequent to UPR induction and potentially triggered by ER stress.
Figure 6: XDME induces ER stress and ER-related autophagic flux in U937 cells. (A) U937 cells were treated XDME (5µM) for up to 72h. Then, the percentage of cytoplasmic vesicles cells was evaluated by Diff-Quik staining. The number of vesicles was estimated by counting cells with vesicles in the cytoplasmic region. (B) The modulation of ER stress marker, GRP78/BiP, and CHOP were investigated by immunoblots in time dependent manner. (C) Inhibition of GRP78/BiP expression and activation of ER stress related downstream proteins, phosphorylation of PERK and eIF2α and CHOP were assessed by immunoblots in a dose-dependent manner at 24h. (D) No changes in XBP1 splicing was observed by RT-PCR at 24h of XDME concentration-dependent treatment. (E) LC3B conversion occurred in a time- and concentration-dependent manner. The data present the mean of three independent experiments (± S.D). *P < 0.05, **P< 0.01, ***P < 0.001 compared with controls.
4.4. XDME alone reduced the colony formation ability in AML cell lines.

In a more translational approach we wanted to investigate the capacity of XDME to reduce the colony formation (CF) capacity of various AML cell lines which also allowed us to generalize previous data. For our CF assays, we used U937, HL60, THP1 and KG1 representing different forms of AML. For each cell line, XDME significantly reduced colony formation from 1µM for U937, 0.5µM for THP1, HL60 and KG-1 cells (Figure 7A). Moreover, we generalized our findings towards other leukemia types including K562 chronic myeloid leukemia and MOLT-3 acute lymphoblastic leukemia where XDME inhibited CF significantly at 100nM (Figure 7B). We concluded that XDME had an inhibitory effect on 2D cell culture as well as in 3D colony formation assays concentrations between 100nM and 1µM.
Figure 7: XDME alone inhibits colony formation both in AML cell lines and CML cell line. (A) U937, HL60, THP1, and KG1 cells were seeded in methylcellulose with XDME (0 – 10µM). XDME has capacity to reduce colony formation on AML cell lines. (B) K562 and MOLT-3 cells were seeded in methylcellulose with XDME (0 – 5µM). XDME inhibited colony formation at concentration between 100nM to 1µM. The data present the mean of three or four independent experiments (± S.D). *P < 0.05, **P< 0.01, ***P < 0.001 compared with controls.
4.5. **XDME sensitized U937 cells against BH3 mimetics**

Previously published data showed that AML cells are particularly sensitive against Bcl-2 family inhibitors. As XDME was able to potently interfere with AML cell proliferation, we then hypothesized that XDME would efficiently synergize with BH3 mimetics of the ABT family. We treated U937 cells with XDME and BH3 mimetics ABT1-99 (Venetoclax) and ABT263 (Navitoclax) at sub-toxic concentrations for 18h, alone or in combination. Hoechst/PI staining revealed that combination treatments induced cell death up to 40% for both combinations of both XDME with ABT-199 or ABT-263 (Figure 8A and B). We validated our results by caspase-3/7 luminescent assays with or without pan-caspase inhibitor z-vad (Figure 8C) as well as caspase-3/7 and PARP-1 cleavage immunoblotting (Figure 8D). Results obtained by caspase assays validated the induction of caspase-dependent cell death as z-vad efficiently inhibited XDME/ABT-induced caspase activity as well as cell death induction.

As we previously observed an optimal growth inhibition by XDME only after 48 hours, we considered that the synergistic induction of cell death could even be exacerbated by such an XDME pretreatment followed by 18 hours of treatment with ABT compounds. Our results showed that this treatment schedule induced cell death up to 60% (Figure 8E and F), confirmed by caspase-3/7 and PARP-1 cleavage immunoblots (Figure 8G). We concluded that that XDME sensitized U937 cells so that the BH3 mimetics induced cell death even at sub-toxic concentrations. Finally, we confirmed the synergistic cell death induction also by colony formation assays validating previous combination treatments (Figure 8H).
Figure 8: XDME sensitizes U937 cells by a combination treatment with BH3 mimetics. U937 cells were treated with XDME 5µM and ABT-199 or ABT-263 100nM for 18h and 66h. (A) For 18h of combination treatment, Hoechst/PI staining by microscopy of XDME showed induction of apoptotic cell death. Red cells represented late apoptotic cells. (B) The quantification results showed that XDME induced cell death up to 40% in 18h of combination treatment. The results are representative of three independent experiments (± S.D). (C) Caspase-3/7 activity assay with and without pan-caspase inhibitor, z-vad (100µM, 4h) revealed that combination of XDME and ABT-199 or ABT-263 increase caspase-dependent cell death. (D) Weak activation of caspase-3/7 and PARP-1 cleavage was observed by
immunoblots. (E-F) For the 66h of treatment, U937 cells were pre-treated with XDME (5µM) then treated ABT-199 or ABT-263 (100nM). By Hoechst/PI staining under microscopy, the combination treatment of XDME and ABT compounds induced cell death up to 60%. Red cells represented late apoptotic cells. These data represented quantification of three independent experiments. (G) Cleavage of caspase-3/7 and PARP-1 were assessed after a combination treatment with XDME and ABT-199 or ABT-263 by immunoblots. (H) Combination of XDME and ABT compounds inhibited cell growth synergistically by colony formation assays. *P < 0.05, **P < 0.01, ***P < 0.001 compared with controls.
5. Discussion

Here we demonstrated that XDME isolated from *Penicillium commune*, initially described to be active against micro-organisms [28], is able to interfere with proliferation in hematologic cancer cells. In our study, we first focused on cell cycle arrest in AML U937 cells to define the effect of XDME. Comparing XDME and celecoxib, clinically used as an anti-inflammatory agent, we found that XDME induced cell cycle arrest without toxicity whereas celecoxib inhibited cell growth with considerable rate of cell death. In addition to that, we used colony formation assay using various AML cell lines like HL60, THP1, and KG1, widely used to study the proliferation and differentiation pattern [29] to generalize our findings and validated the inhibitory effect on proliferation in AML cell lines. Interestingly, we found that XDME reduced cell growth of U937 AML cell lines after 24h of treatment with 30% of accumulation in G1 phase compared with control cells. G1/S transition is tightly regulated by cyclin dependent kinases (CDK)s and cyclins [30], especially activation of cyclin D1 is essential for the entry into the S phase. Also c-myc is a key protein essential for the S phase [31]. We found that XDME induced inhibition of cell proliferation at the G1 phase by downregulating cyclin D1, c-myc and PCNA proteins. In our study, we concluded that XDME blocked cell proliferation with accumulation in G1 and reduction of S phase of U937 AML cell lines.

The ER plays an essential role in various intra-cellular progresses leading to UPR triggering ER stress pathways via three main axes: PERK, IRE1α, and ATF6α activate the expression of genes related cell death and survival. For this reason, ER stress recently drew attention as a potential therapeutic target. The IRE1α/XBP1 axis is an important survival factor in multiple myeloma and accordingly inhibitors
targeting XBP1 mRNA splicing show anti-myeloma activity [32, 33]. Moreover, overexpression of GRP78/BiP is considered as a crucial factor in breast cancer survival. Expression of this protein can be inhibited to reduce its cell-protective effects [34]. In that sense, investigation of new therapeutic agents acting through ER stress-related gene expression mechanisms and UPR could be of interest to further personalize cancer treatment [35]. In a first step, we observed that XDME generated ER stress through downregulation of GRP78/BiP after 24h of treatment followed by an increase at 48h and 72h. Even though the expression of GRP78/BiP protein reached again control levels after 48h of XDME treatment, the downstream signaling pathway remained activated by phosphorylation of PERK and eIF2α. Thus XDME triggered ER stress at 24h and constitutively affected UPR pathway by activating the PERK-eIF2α UPR pathway. As a consequence, the expression of transcription factor CHOP also increased in a time- and dose-dependent manner. However, splicing of XBP1 did not occur in XDME-treated U937 cells, suggesting that XDME induces ER stress by the PERK branch of the UPR pathway, but not the IRE1 branch. A morphological analysis by Diff-Quik staining clearly revealed vesicle formation in XDME-treated U937 cells witnessing a stress response which could be due to ER stress or autophagy. Moreover, both mechanisms were shown to interact so that a transition from ER stress to autophagy could be hypothesized. Moreover, CHOP is a transcription factor downstream of UPR signaling. The expression of CHOP is known to arrest cell growth and to act as a mediator of ER stress-induced apoptosis [36]. Interestingly, it has also been involved in pro-survival autophagic processes [37] by activating autophagy-associated genes through the cooperation with ATF4 and/or CCAAT/enhancer-binding protein (C/EBP)β [38]. Indeed, this mechanism remains largely to be explored but it is already well known that CHOP could
influence the balance between autophagy and apoptosis [39]. In agreement with these published data, we observed the expression of CHOP in a time- and concentration-dependent manner followed by the conversion of LC3-I to LC3-II. Taken together, XDME induced ER stress by activating one of the UPR signaling pathways, PERK/eIF2α axis, and subsequent autophagic flux was triggered by ER stress.

Recent studies showed that Navitoclax, a clinically used BH3 mimetic, has side effects including thrombocytopenia, lymphocytopenia and neutropenia in a phase 1 study [40]. Indeed, Venetoclax is derived from Navitoclax to have only high affinity for Bcl-2 [41]. Even though Venetoclax reduced side effects compared to Navitoclax, treatments with high or intensive doses of drugs lead to resistance mechanisms, including overexpression of other Bcl-2 family proteins like Mcl-1. Accordingly, a combination treatment with a sensitization agent targeting a completely different mechanism, could potentially allow to apply lower concentrations of the BH-3 mimetics.

Re-gaining of apoptosis ability in AML cells is essential to prevent leukemia cell resistance [42]. Previous studies already showed that AML cells are highly sensitive to proteasome inhibitors prior to activating apoptotic pathways [43-45]. Furthermore, the use of BH3 mimetics as AML therapeutic agents could be improved when combined with other drugs [46, 47]. Here we focused on an ER stress-related cell cycle arrest so that eventually AML cells became sensitized against BH3 mimetics to exacerbate cell death induction. In our study, we observed significant apoptotic cell death after a combination treatment of XDME and ABT-199 or ABT-263 at sub-toxic concentrations for 18h. Moreover, pretreatment of XDME for 48 hours leading to a complete cell cycle arrest further sensitizes U937
AML. Our results show that the treatment of ABT compounds showed a synergistic effect on the induction of apoptotic cell death that we confirmed by caspase-3/7 cleavage immunoblots and combinatory colony formation assays. Altogether, our findings revealed that the combination treatment of XDME and BH3 mimetics, ABT199 or ABT263 synergistically triggered caspase-dependent cell death at sub-toxic concentrations. Finally, we generalized the inhibitory potential of XDME by colony formation capacity using K562 a chronic myeloid leukemia (CML) cell line and MOLT-3 a T cell lymphoblastic leukemia (T-CLL) validating a more general effect of XDME.
6. Conclusions and Perspectives

In summary, our study provides new insights about the effect of XDME as an anti-cancer drug on AML leukemia by induction of ER stress, autophagy and cell cycle arrest. XDME sensitized U937 AML cell lines and showed synergistic cell death effects with drugs such as BH3 mimetics at sub-toxic concentrations. Figure 9 presents an overview of the investigated effects of XDME:

However, additional mechanisms remain to be elucidated for both AML and CML cell lines.

- **For AML**, we plan to further investigate ER stress and autophagy triggered by ER stress using bafilomycin A1, an inhibitor of the early phase of autophagy, and small interfering RNA (siRNA) against CHOP, beclin-1, autophagy-related gene (ATG) regulators of both autophagy and cell death.

- We also intend to use transmission electron microscopy (TEM) to verify more details of morphological changes in AML. Considering our combination results, we supposed to further validate ER stress and cell death pathway markers like Mcl-1 to explore the pathway triggered by the combination treatment of XDME and Navitoclax or Venetoclax. We will quantify the synergistic induction of cell death by using the Compusyn software according to Chou-Talalay.

- As transplantation of human cancer cell lines into zebrafish models have been set up recently in our lab and provide an important mechanistic approach in cancer research [48], we will use this approach. We intent to develop a xenograft-zebrafish model of injecting XDME-treated cell lines with and
without ABT compounds using CM-DIL-stained AML U937, HL60, THP-1 and KG1 cell lines. Also, we expect to conduct patient-derived xenografts in zebrafish to prove the effect of XDME.

- Moreover, our results showed that XDME had similar inhibitory effects of cell proliferation not only in AMLs, but also in other leukemia cell lines like K562 and MOLT-3. We further implement to generalize our ideas with KBM5 and MEG01 CML cell lines including stably transfected K562 cells expressing a red fluorescent protein marker.

- For CMLs, we need to verify the synergistic effects of XDME and Imatinib, a clinically used drug for CML, in CML cells and Imatinib-resistant K562 available at the Leukemia Bank of Korea.

- Besides we intend to perform the cell line derived zebrafish xenograft model of XDME-treated RFP-K562 cells with and without Imatinib and patient derived xenografts.

Overall, natural compound XDME is a potent promising agent potentially able to enhance novel targeted anti-cancer therapeutic approaches especially in hematologic malignancies.
Figure 9: Scheme representing the mechanism action of XDME. Altogether, XDME acts as a potent anti-cancer agent at a low micromolar concentration, which leads to arrest cell cycle at G1 phase by inducing ER stress. At a consequence, autophagic flux was triggered. Moreover, a combination with BH3 mimetics induced apoptotic cell death.
7. References


42. Suh WS, Kim YS, Schimmer AD, Kitada S, Minden M, Andreeff M, et al. Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving


요약 (국문초록)

Xanthocillin은 항바이러스작용을 가진 다양한 곰팡이에서 추출되고, 다양한 유도체들은 세포의 성장을 억제하지만 독성으로 인한 세포사멸을 일으킨다고 알려져 있습니다. *Penicillium notatum*에서 추출한 Xanthocillin X Dimethylether (XDME)는 독성을 갖지 않고 급성 골수성 백혈병(AML)세포의 성장을 억제하는 것을 확인할 수 있었습니다. 임상연구에 의하면 현재 AML의 생존율은 30%에 불과하며, 지난 25년 동안 치료법이 많이 바뀌지 않아 새로운 치료법의 개발이 필요한 실정입니다. 본 연구를 통해서 XDME가 백혈병세포에 대해 가는 효과와, XDME와 임상연구단계에 있는 BH3 유사체인 Navitoclax, Venetoclax와의 combination treatment을 통해 AML에 어떤 효과가 있는지 규명하고자 했습니다. XDME는 AML세포의 성장을 현저히 저하시키며 불구하고 세포 사멸이 발생하지 않는 것을 Trypan blue assay로 확인할 수 있었고, Zebrafish을 사용한 독성테스트에서 독성이 없는 것을 확인하였습니다. 또한 western blot을 통해 XDME가 cell cycle 조절 인자인 PCNA, cyclin D1, c-myc의 발현이 감소되는 것을 증명하였습니다. 따라서 XDME는 AML에서 세포사멸을 유발시키지 않으면서 세포의 성장을 효과적으로 억제시킨다는 사실을 밝혔습니다.

XDME가 AML에 미치는 영향을 세포형태학적인 분석을 수행한 결과, XDME에 의해 vesicle형성되는 것을 확인하였고, 이로써 ER stress 및 Autophagy와 관련이 있다고 추측할 수 있었습니다. 이는 ER stress와 관련된 GRP/Bip 단백질의 감소와 autophagy를 유발하는 CHOP인자의 증가, LC3B conversion을 통해 증명하였습니다. 이에 따라 XDME는 ER stress를 유발하여 autophagy를 초래한다는 것을 확인할 수 있었습니다. 또한, U937 cell을 비롯한 다른 AML cell line에서도 XDME는 100nM내지 1µM의 낮은 농도로 colony 형성을 억제하는 효과를 입증하였습니다. 더욱이 현재 임상연구단계에 있는 ABT199(Venetoclax), ABT263(Navitoclax)와 XDME를
함께 AML에 처리했을 때, 세포를 sensitize시켜 기존 농도보다 현저히 낮은 농도로 AML 세포사멸을 유도하는 사실을 규명하였습니다. 이는 Colony formation assay, Hoechst/PI staining, Caspase 3/7 assay에 의해서도 와 상응함을 확인하였습니다.

XDME는 AML세포이외에도 만성 골수성 백혈병(CML)에서 BCR-ABL의 인산화를 감소시킴으로써, ER stress를 유발해 Autophagy를 활성화시켜 세포 성장을 억제하는 것을 확인 할 수 있었고, 유세포분석을 통해서 세포사멸의 효과를 지닌다는 사실을 증명할 수 있었습니다.

결론적으로 항생물질유도체인 XDME 는 nano 단위의 적은 농도에서 독성을 가지지 않고 cytostatic 효능이 있음을 규명함으로써, 본 화합물을 임상연구에 적용함에 있어 부작용을 최소화하고 암세포활성을 특이적으로 억제할 수 있는 유력한 물질임을 연구를 통하여 입증하였습니다.

주요어
급성골수성백혈병, ER 스트레스, autophagy, BH-3 유사체, 동반상승효과

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